Development of a dried whole-blood spot thyroglobulin assay and its evaluation as an indicator of thyroid status in goitrous children receiving iodized salt[1–3]

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ABSTRACT

Background: Serum thyroglobulin appears to be a sensitive marker of thyroid dysfunction in endemic goiter. However, its value as an indicator of thyroid status in children after the introduction of iodized salt has not been tested.

Objective: The objective was to optimize and validate a thyroglobulin assay on dried whole blood spots and to evaluate thyroglobulin as an indicator of thyroid response to iodized salt.

Design: A standardized, commercially available, sandwich fluoroimmunometric serum thyroglobulin assay was adapted for use on blood spots and validated in Swiss children. In a 1-y prospective study in 377 goitrous Moroccan children aged 6–15 y, the assay was used to measure thyroglobulin before and after the introduction of iodized salt. Urinary iodine, thyroid volume, thyrotropin, and thyroxine were measured, and regression was done with thyroglobulin as the dependent variable.

Results: Correlation between the blood spot and serum assays was excellent ($r = 0.98$). The SD of the difference between the blood spot and serum assays was 3.8 µg/L; the median CVs for the blood spot assay in controls and samples were 6.3% and 14.4%, respectively. Median thyroglobulin was 24.5 (range: 0–328.8) µg/L at baseline and fell significantly after the introduction of iodized salt to 6.2 (0–83.1) and 4.4 (0–47.1) µg/L at 5 and 12 mo, respectively ($P < 0.0001$). Regression of urinary iodine and thyroid volume on thyroglobulin was highly significant at baseline and at 5 mo ($P < 0.001$).

Conclusion: Thyroglobulin, measured in dried whole blood spots, may be a valuable indicator of improving thyroid function in children after supplementation with iodized salt.   *Am J Clin Nutr* 2003;77:1453–8.

KEY WORDS   Iodine, thyroid, goiter, blood spots, thyroglobulin, iodized salt, iodine-deficiency disorders, children, Morocco

INTRODUCTION

Iodine deficiency is the single most important preventable cause of brain damage worldwide (1). Universal salt iodization is the recommended approach for the control of iodine deficiency disorder (IDD) (1). The major biological indicators of response to salt iodization are urinary iodine, goiter prevalence, and thyrotropin (1). However, all of these indicators have limitations. Urinary iodine is a sensitive indicator of recent iodine intake, but not of thyroid function. Because thyroid size decreases only slowly after iodine repletion, goiter in children is a poor IDD indicator for several years after the introduction of iodized salt (2). Thyrotropin is a sensitive IDD indicator in the newborn period only (1). An additional indicator of thyroid status that is sensitive to recent changes in iodine nutrition and is applicable in children would be valuable in IDD monitoring.

Thyroglobulin, the most abundant thyroid protein, is a key precursor in the production of thyroid hormone and is thyroid specific (3). It is a large dimer glycoprotein of 660 kDa with no known physiologic role outside the thyroid (4). Transcytosis of thyroglobulin-containing endosomes across the thyrocyte results in small amounts of thyroglobulin being released into the blood; secretion is stimulated by thyrotropin (5). Once secreted, thyroglobulin is cleared mainly by the liver, with a half-life of 3–65 h (3). In the absence of thyroid damage, the major determinants of serum thyroglobulin are thyroid cell mass and thyrotropin stimulation (6). In areas of endemic goiter, elevated serum thyroglobulin reflects thyrotropin hyperstimulation and thyroid hyperplasia. Thyroglobulin has been proposed as a potential IDD indicator (1) and may have particular value in detecting short-term changes in thyroid function in response to salt iodization.

However, data on thyroglobulin as an IDD indicator are scarce. Cross-sectional studies have reported a negative correlation between thyroglobulin and urinary iodine and a positive correlation between thyroglobulin and thyroid volume and thyrotropin (7–9). Intervention studies have suggested that thyroglobulin is a more sensitive indicator than is thyrotropin or thyroxine in measuring the response to iodized oil (7, 10, 11) and potassium iodide (12). Salt iodization is the most widely used method for controlling IDD, but we are aware of no published studies that evaluated serum thyroglobulin as an IDD response indicator after

TABLE 1
Comparison of assays for measurement of thyroglobulin in serum and dried whole blood spots

Serum assay	Blood spot assay
Wash plate once with 300 μL wash solution	
Add sample of 50 μL serum and 50 μL buffer solution	Add whole blood spots (4.75-mm diameter) and 50 μL buffer solution
Incubate for 1 h; fast shaking at room temperature	Incubate for 24 h at 5 °C
Wash plate once with 300 μL wash solution; add 200 μL tracer (tracer stock solution and buffer solution at a ratio of 1:100)	
Incubate tracer-bound antibody reaction; 1 h slow shaking at room temperature	
Wash unbound tracer from plate 6 times with 300 μL wash solution	
Add 200 μL enhancement solution; incubate for 5 min; slow shaking	
Count	

introduction of iodized salt. Although thyroglobulin antibodies may be common in children from IDD-affected areas (13) and are often a source of thyroglobulin assay error (3), previous studies reporting thyroglobulin response to iodine repletion did not measure thyroglobulin antibodies (7, 10–12).

Commercially available assays measure serum thyroglobulin, which requires venipuncture, centrifugation, and the transport of frozen samples, which may be difficult in remote IDD-affected areas. Therefore, we adapted a widely used sandwich fluoroimmunometric serum thyroglobulin assay for use on dried whole blood spots. The study aims were *1*) to optimize and validate this assay to measure thyroglobulin in dried whole blood spots and *2*) to use this assay to evaluate thyroglobulin as an indicator of thyroid status in schoolchildren before and after the introduction of iodized salt.

SUBJECTS AND METHODS

Adaptation and validation of the thyroglobulin assay on dried whole blood spots

We adapted a 2-site Delfia (dissociation enhanced lanthanide fluorescent immunoassay) serum thyroglobulin assay (Perkin-Elmer Life Sciences, Wallac, Turku, Finland). An advantage of 2-site thyroglobulin assays is their low cross-reactivity and improved specificity compared with 1-site assays (3). To prepare the blood spot standards for calibration of the assay, whole blood obtained from the local blood bank was centrifuged at $1529 \times g$ for 10 min at 18 °C (Rotanta; Hettich Zentrifugen, Tuttlingen, Germany), the serum was removed, 0.9% NaCl was added, the solution was rotated in a blood mixer until homogeneous and then recentrifuged at $1529 \times g$ for 10 min at 18 °C, and the supernatant fluid was removed. This step was repeated 3 times. Calibration and control solutions from the serum thyroglobulin assay kit were then added to the washed erythrocytes, rotated in a blood mixer for 10 min, dropped onto filter paper (grade 903; Schleicher & Schuell, Einbeck, Germany), and air-dried for 24 h at 20 °C. The calibration curve was constructed by using duplicate measurements at 0, 1, 10, 100, 500, and 1000 μg/L and smoothed by fitting a third-order polynomial with the use of an automatically smoothed spline technique (Multicalc Program; Perkin-Elmer Life Sciences).

To adapt and optimize the reagents and incubation times for the small amount of serum ($\approx$2–5 μL) in the dried blood spot sample, different calibration curves and control concentrations were tested with several protocol variations. Assay buffer, wash concentrate, enhancement solution, coated microtiter plate, standards, and control solutions were all from the serum thyroglobulin assay kit. Because of the high molecular weight of thyroglobulin and the small amount of serum in the blood spots, the first incubation step was critical for assay specificity and sensitivity. A 24-h incubation provided the highest sensitivity and specificity. A 2-h incubation with fast shaking yielded comparable results but slightly reduced sensitivity. Other protocols, including the addition of additional buffer to extract material from the spot or a greater dilution of tracer gave lower counts and more variable responses. The protocol with the sharpest response and lowest variability is shown in **Table 1**. Mean minimal detectable concentrations for the dried whole-blood spot thyroglobulin assay were calculated by measuring 30 samples without antigen (0 standard).

The validation of the assay was done by comparing serum thyroglobulin and blood spot thyroglobulin in Swiss children ($n = 29$) with no known thyroid disease undergoing venipuncture for other reasons. Oral informed consent to measure thyroglobulin was obtained from the parents of the children. Blood was collected in EDTA-containing tubes, and a portion was spotted onto filter paper (14). The dried blood spots were stored at 5 °C. The remaining blood was centrifuged at $1500 \times g$ for 3 min at room temperature, and the serum was stored at -20 °C. Serum samples were centrifuged with a microfuge (Beckman Coulter, Krefeld, Germany) for 3 min at 3000 rpm and -20 °C after defrosting, and serum and blood spot thyroglobulin were measured as shown in Table 1. The precision of the blood spot assay was evaluated in duplicate measurements and with day-to-day comparisons. Because of large interassay variability in thyroglobulin measurement, even with the use of Community Bureau of Reference standards (6), we did not attempt to determine the accuracy of the blood spot assay by using reference material. Thyroglobulin antibodies were measured in all samples with a radioimmunoassay (RIA TgAb; RSR, Cardiff, United Kingdom).

Evaluation of the dried whole-blood spot thyroglobulin assay in Moroccan children in response to iodized salt

The study was conducted in rural villages in the Rif mountains of northern Morocco. This is a region of long-standing endemic goiter and severe IDD, where the goiter rate among schoolchildren is 53–72% (15). The subjects were 6–15-y-old children from 2 neighboring primary schools in the villages. All children in the schools were invited to participate in the study; all accepted and were enrolled ($n = 377$). Informed oral consent was obtained from
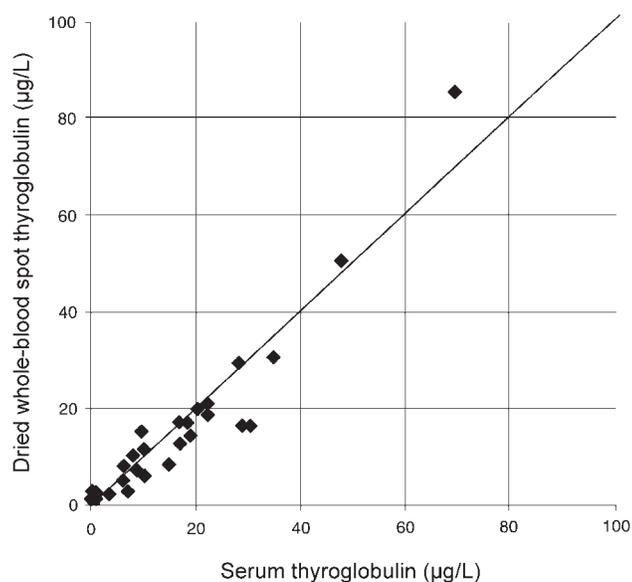
FIGURE 1. Correlation between thyroglobulin concentrations in dried whole blood spots and serum in Swiss children (*n* = 29) with no known thyroid disease and negative for thyroglobulin antibodies.

the school directors and the parents of the children. The University Children's Hospital in Zürich and the Ministry of Health in Morocco gave ethical approval for the study. At baseline, weight and height were measured and a casual spot urine sample was collected for measurement of iodine. Thyroid volume was measured with a portable Aloka SSD-500 Echocamera (Aloka, Mure, Japan) with a high-resolution, 7.5-MHz linear transducer. Whole blood was spotted onto filter paper for measurement of total thyroxine, thyrotropin, thyroglobulin, and thyroglobulin antibodies.

The salt for this region is supplied by a small local cooperative. Its native iodine content is <2 ppm (15). Reagent-grade potassium iodide (Sigma & Aldrich, Buchs, Switzerland) was dry mixed into the local salt by using a rotating drum mixer (Engelsmann, Ludwigshafen, Germany) at a concentration of 25 μg I/g salt. Each household with children enrolled in the study was provided 2 kg iodized salt at the beginning of each month for 1 y. The salt was dispensed directly to the head of the household from a central supply at the local health center. At 5 and 12 mo, the weight and height of the children were remeasured, and spot urine samples were collected for measurement of iodine. Thyroid volume was measured by ultrasound. Whole blood was spotted onto filter paper for determination of thyroxine, thyrotropin, thyroglobulin, and thyroglobulin antibodies. Urine samples were portioned and frozen at −20°C until analyzed. Urinary iodine was measured by using a modification of the Sandell-Kolthoff reaction (16). Dried blood spots on filter paper were analyzed for whole blood thyrotropin and serum thyroxine with an immunoassay (17). Normal reference values are 0.2–3.7 mU/L for whole blood thyrotropin and 65–165 nmol/L for serum thyroxine. Thyroglobulin antibodies were measured by radioimmunoassay (RIA TgAb; RSR); values >10 U/mL were considered elevated. Thyroid volume was calculated by using the method of Brunn et al (18).

Statistical analysis

Data processing and statistical analyses were done by using PRISM3 (GraphPad, San Diego), S-PLUS 2000 (Mathsoft, Seattle),

and Excel 97 (Microsoft, Seattle). For validation of the thyroglobulin assay, Pearson correlation coefficients were calculated and the CIs were estimated for measurement differences between the methods (19). To calculate mean minimal detectable concentrations with the dried blood spot assay, the following formula was used:

$$MDC = S_0 + t_s \sqrt{1/n_0} + 1/n \qquad (1)$$

where S_0 is signal without antigen, t_s is Student's t test (95%), n_0 is the number of replicates for the zero concentration, and n is the number of replicates in the assay for standards and samples (20). In the Moroccan children, one-way analysis of variance was done to compare changes in urinary iodine, thyrotropin, thyroxine, thyroglobulin, thyroglobulin antibodies, and thyroid volume. Tukey's test was used for post hoc comparisons. Variables not normally distributed were logarithmically transformed before analysis. For thyroglobulin, because the variance in groups was not identical and the distribution was not entirely normalized after log transformation, rank tests were also done. To evaluate the relation between thyroglobulin and other IDD indicators, Pearson's correlation coefficients were calculated and linear regression models were calculated with thyroglobulin as the dependent variable. For the regression, to reduce the influence of age and sex on the variable of thyroid volume, the volumes were standardized by using current reference values (21) with the following equation:

$$\text{Standardized thyroid volume (Tvol}_s)$$
$$= \log(\text{Tvol}) - \log(\text{P50Tvol}_s)/\text{SD} \qquad (2)$$

where P50Tvol$_s$ is the mean reference volume for a certain body surface area. Significance was set at $P < 0.05$.

RESULTS

Adaptation and validation of the dried whole-blood spot thyroglobulin assay

All samples from the Swiss children used for validation tested negative for thyroglobulin antibodies. The correlation between serum thyroglobulin and thyroglobulin in dried blood spots is shown in **Figure 1**. The correlation coefficient between the 2 methods was $r = 0.98$ ($P < 0.0001$). The SD of the difference between blood spot and serum thyroglobulin was 3.8 μg/L, and the limits of agreement at the 95% significance level were −8 and 11 μg/L. The quality-assessment data for the thyroglobulin assays are shown in **Table 2**. Intraassay imprecision (CV) in the controls ($n = 36$) was <10%, and the day-to-day CV was <20% over the range of the calibration curve. The median CV was 6.3%. As shown in Table 2, a higher variation was observed in sample duplicates than in control duplicates.

Evaluation of the dried whole-blood spot thyroglobulin assay in Moroccan children in response to iodized salt

The mean (±SD) age and body mass index (kg/m^2) of the children in the test group were 10.2 ± 2.4 y and 16.1 ± 1.8, respectively. Forty-seven percent of the subjects were female. The children were severely iodine deficient, with a median urinary iodine concentration of 17 μg/L and a goiter rate of 72% (15). The changes in urinary iodine, thyroid volume, thyrotropin, thyroxine, and thyroglobulin antibodies during the study are shown in **Table 3**. The children's blood spot thyroglobulin concentrations over the course of the study are shown in **Figure 2**. The median thyroglobulin concentration decreased rapidly from a baseline of

TABLE 2
Performance indicators for the serum and dried whole-blood spot thyroglobulin assays

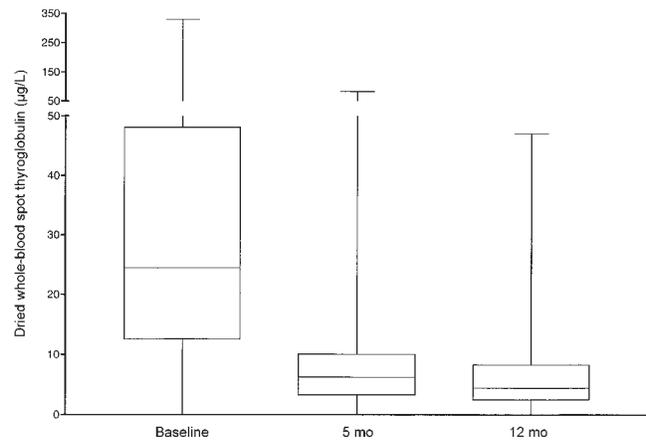|	Value
Serum (*n* = 29)	
Median CV (%)	2.6
Blood spot (%)	
Median CV in controls (*n* = 36)	6.3
Median CV in duplicate samples (*n* = 489)	14.4
<10 µg/L	16.7
≥10 µg/L	13.4
Minimal detectable concentration (µg/L)	1.42
Agreement between serum and blood spot assays	
Pearson's correlation coefficient	0.98
95% CI for the lower limit (µg/L)	−8
95% CI for the upper limit (µg/L)	11.9

FIGURE 2. Thyroglobulin concentrations in dried whole blood spots in Moroccan schoolchildren (*n* = 377) at baseline and 5 and 12 mo after the introduction of iodized salt. The boxes contain data between the 25% and 75% percentiles with medians; whiskers represent the range.

24.5 µg/L (range: 0–328.8 µg/L) to 6.2 µg/L (0–83.1 µg/L) and 4.4 µg/L (0–47.1 µg/L) at 5 and 12 mo, respectively (*P* < 0.0001). The correlations between the major response variables during the study are shown in **Table 4**. At baseline and at 5 and 12 mo, thyroglobulin showed a significant negative correlation with urinary iodine and a significant positive correlation with thyroid volume. Thyroglobulin was significantly correlated with thyrotropin and thyroxine at baseline only. The regression of thyroglobulin on urinary iodine, thyrotropin, thyroxine, and thyroid volume was done at each time point. The regression of urinary iodine and thyroid volume on thyroglobulin was significant (*P* < 0.0001) at baseline and at 5 mo (*P* < 0.01).

DISCUSSION

These data indicate that a widely available standardized serum thyroglobulin assay with high sensitivity and specificity can be adapted for use with dried whole blood spots. In IDD monitoring, the use of a blood spot assay makes acquisition and transport of samples practical, even in remote areas. The blood spot thyroglobulin assay showed acceptable precision and high sensitivity. The assay correlated closely with the serum thyroglobulin assay (*r* = 0.98), but more informative are the limits of agreement (mean ± 2 SD) between the 2 methods. On the basis of the mean differences between serum and blood spot thyroglobulin and calculated upper and lower limits of agreement, 95% of the blood spot measurements will have a difference from serum measurements of between −8 and 11.9 µg/L. Thyroglobulin concentrations in our Swiss and Moroccan children were mainly distributed between 5 and 40 µg/L, so the inherent measurement error (ie, SD of the difference with serum thyroglobulin = 3.8 µg/L) was high for thyroglobulin concentrations at the lower end of the measured range. Missler et al (7) previously measured thyroglobulin in dried whole blood spots by using an in-house immunofluorimetric assay. Missler et al's blood spot assay showed better precision (CV = 7.9–9.1%) than did our blood spot assay but a lower correlation with a serum thyroglobulin assay; the limits of agreement with the serum thyroglobulin assay were not reported. Using our blood spot assay, there was a higher variation in sample duplicates than in control duplicates, indicating that sampling of whole blood spots can be a source of measurement bias.

A major limitation to the use of thyroglobulin in IDD monitoring is large interassay variability and poor reproducibility between laboratories, even with the use of Community Bureau of Reference

TABLE 3
Changes in urinary iodine concentration, in thyroid volume by ultrasound, and in thyrotropin, thyroxine, and thyroglobulin antibodies in Moroccan schoolchildren in response to the provision of iodized salt[1]

	Baseline	5 mo	12 mo
Urinary iodine (µg/L)[2,3]	17 (0–143)[a]	181 (22–529)[b]	165 (22–439)[b]
Thyroid volume (mL)[3,4]	9.0 ± 3.5[a]	7.9 ± 3.1[b]	5.9 ± 2.5[c]
Thyrotropin (mU/L)[2,5]	0.9 (0.4–27.0)[a]	1.1 (0.3–8.6)[b]	0.7 (0.3–2.3)[c]
Total thyroxine (nmol/L)[4,5]	82.9 ± 18.4[a]	98.4 ± 20.5[b]	93.3 ± 18.8[c]
Thyroglobulin antibodies (U/mL)[2]	0.21 (0.01–0.88)	0.26 (0.04–3.10)	0.29 (0.29–4.58)
No. of children with >0.3 U/mL	32	69	34
No. of children with >10 U/mL	0	0	0

[1] *n* = 377. For urinary iodine, samples below the limits of detection were assigned a value of 0. Values in the same row with different superscript letters are significantly different, *P* < 0.05 (Tukey's test for post hoc comparisons).
[2] Median; range in parentheses.
[3] *P* < 0.0001 (ANOVA).
[4] $\bar{x}$ ± SD.
[5] *P* < 0.001 (ANOVA).

TABLE 4
Pearson's correlation coefficients for thyroglobulin and other indicators of iodine status and thyroid function in Moroccan schoolchildren before (baseline) and 5 and 12 mo after the introduction of iodized salt[1]

	Dried blood spot thyroglobulin (µg/L)		
	Baseline	5 mo	12 mo
Thyrotropin (mU/L)	0.31[2]	0.08	0.09
Total thyroxine (nmol/L)	−0.44[2]	−0.12	−0.14
Urinary iodine (µg/L)	−0.38[2]	−0.17[3]	−0.16[3]
Thyroid volume (mL)	0.52[2]	0.23[4]	0.19[3]

[1] $n = 377$.
[2–4] Linear regression: [2] $P < 0.001$, [3] $P < 0.05$, [4] $P < 0.01$.

standardization (6). This has made it difficult to establish normal ranges and cutoffs to distinguish the severity of iodine deficiency. Serum thyroglobulin is high at birth and declines steadily during childhood and adolescence to reach adult concentrations (22). Vanderschueren-Lodeweyckx (23) proposed normal mean values and ranges for serum thyroglobulin in children aged 1–10 and 11–20 y of 35 µg/L (range: 2–65 µg/L) and 18 µg/L (2–36 µg/L), respectively. In 1994 the World Health Organization proposed that a median thyroglobulin concentration of <10 µg/L in both children and adults indicates iodine sufficiency (24). In the present study, 6–15-y-old children with severe, long-standing IDD had a median thyroglobulin concentration at baseline of 24.7 µg/L, which is within the normal range proposed by Vanderschueren-Lodeweyckx (23). Previous studies that measured serum thyroglobulin in children from IDD-deficient areas reported median values ranging from 27 to 214 µg/L (7, 10–12). Along with different severities of IDD, interassay variation likely contributed to these large reported differences in thyroglobulin among goitrous children.

Another potential question regarding thyroglobulin assays for IDD monitoring is the need for concurrent measurement of thyroglobulin antibodies to avoid potential underestimation of thyroglobulin. Although this is a common source of thyroglobulin assay error in adults followed for thyroid cancer (3), it is unclear what the prevalence of thyroglobulin antibodies is in iodine-deficient children and whether the antibodies are precipitated by iodine prophylaxis (25). Several studies have reported high prevalences, ranging from 7% to 69%, in children from IDD-affected areas and during iodine prophylaxis (13, 26). In contrast, our data and others (27, 28) suggest that elevated thyroglobulin antibodies in IDD-affected children are rare (0–2% prevalence).

Intervention studies examining the potential of thyroglobulin as an indicator of the response of IDD to iodized oil (7, 10, 11) and potassium iodide (12) have shown that thyroglobulin decreases rapidly with iodine repletion and that thyroglobulin is a more sensitive indicator of iodine repletion than is thyrotropin or thyroxine. Because transient iodine block of the thyroid from large doses of iodine may increase serum thyroglobulin (3), thyroglobulin data from the studies that gave large doses of iodized oil should be interpreted cautiously. Our study is the first to show a similar thyroglobulin response to a more gradual increase in iodine intake from iodized salt. Median thyroglobulin decreased rapidly, and concentrations were at or below normal ranges described for children and adults after 5 and 12 mo of iodized salt intake (3, 23, 24). According to cutoffs of the World Health Organization/International Council for the Control of Iodine Deficiency Disorders,

a median thyroglobulin concentration <10 µg/L at 5 and 12 mo indicates normalization of iodine status (24). Moreover, both correlation coefficients and regression indicated highly significant associations between thyroglobulin and thyroid volume and urinary iodine, the 2 other major indicators of IDD response, at baseline and 5 mo.

The data from the current study suggest that thyroglobulin concentrations, used in conjunction with urinary iodine concentrations to measure recent iodine intake and with thyroid volume to assess long-term anatomic responses, may be a useful biological indicator for monitoring thyroid function in children after the introduction of iodized salt. The development of a sensitive and specific thyroglobulin assay for use with whole blood spots may facilitate its use in IDD monitoring. However, further research is needed to improve the interassay precision of the assay to allow establishment of normal ranges for thyroglobulin in children and to address the question of whether simultaneous measurement of thyroglobulin antibodies is necessary.   ❧

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