

# Variability among TSH Measurements Can Be Reduced by Combining a Glycoengineered Calibrator to Epitope-Defined Immunoassays

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## Key Words

Thyroid-stimulating hormone measurements · Immunoassays · Glycosylation · Recombinant thyroid-stimulating hormone · Harmonization

## Abstract

**Objectives:** Measuring protein markers with variable glycosylation, such as thyroid-stimulating hormone (TSH), with high accuracy is not an easy task. Despite highly sensitive third-generation tests, discrepancies among TSH assays still remain unsolved and are the focus of important standardization efforts. Earlier work from our group showed that a lack of similarity in epitope expression between standards and samples may account for discordant hormone measurements. In this study, we aimed at producing a glycoengineered TSH with serum-type glycosylation and compared its immunological behavior to that of the international standards. **Study Design:** Recombinant glycoengineered TSH

(rgTSH) was produced in glycoengineered Chinese hamster ovary cells to express a highly sialylated TSH and tested in newly designed assays. Two groups of assays targeting defined epitopes were constructed and TSH levels were estimated in a panel of 84 clinical samples (2.1–22.4 mIU/l) based on the use of the current 3rd IS 81/565, the 1st IRP 94/674 and rgTSH calibrations. **Results:** Calibration based on rgTSH was found to significantly reduce the percentage difference means of assays compared to the pituitary standard. We also found that a switch from a mIU/l (3rd IS 81/565) to ng/l (rgTSH) basis can be established within the normal as well as in the mid to upper normal range of TSH levels. Of interest, TSH assays targeting the main immunogenic region displayed variable TSH values, indicating that, in this region, epitopes should be defined for assays to deliver similar values. **Conclusions:** A glycoengineered TSH with serum-type glycosylation proved to be a new calibrator efficient in harmonizing TSH values.

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## Introduction

Over the past years substantial variability among thyroid-stimulating hormone (TSH) measurements has been extensively described, largely influencing the critical discrimination between normal and diseased TSH levels [1]. To overcome this limitation and meet the expectations of regulatory bodies, an important international effort has been developed towards a standardization of TSH immunoassays [2].

Several issues have been consistently debated by the laboratory medicine community to account for such a situation [3], and a lack of structural similarity between standard and serum TSH remains a key limitation [4]. Also, the measurement of bioactive TSH has never been approached or documented. Previously, we demonstrated that changes in TSH glycosylation, especially sialylation [5], significantly alter antibody recognition [6–8]. TSH is an N-glycosylated protein hormone for which glycosylation is essential for hormone folding, activity and duration in the blood [9]. The current international standard, namely the 3rd IS 81/565 extracted from the pituitary (pitTSH), is composed of a heterogeneous mixture of predominantly N-acetylgalactosamine (GalNAc)-sulfated biantennary glycoforms [5, 10]. Such TSH is short lived because it is specifically cleared from the circulation by a liver GalNAc-sulfate receptor [11]. In contrast, circulating TSH is essentially composed of sialylated glycans [12–14] which escape hepatic clearance [11, 15] and is long-lived [16]. Since the sialylation of TSH increases as hypothyroidism develops [12–14], we hypothesized that assays may differentially bind TSH variants in an extractive standard and in serum samples, and thus deliver incorrect TSH values.

Very early on, a preparation of recombinant TSH (recTSH) was produced in mammalian cells to replace extractive standards [17] but the preparation did not meet this expectation [18]. Even though expression systems may synthesize complex glycans of a mammalian type, they often lack the  $\alpha$ 2,6-sialic acid [19, 20] typical of human serum glycoproteins and also found in hypothyroid TSH [21]. So far, biotechnological processes have not been sufficient to provide fully sialylated products [22] and recombinant preparations still differ from native glycoproteins. Recently, our group was able to design a panel of minigenes to produce sialyltransferases of enhanced activity and perform efficient serum-type sialylation [22–24]. In this study, we engineered cells with such a variant of the human  $\alpha$ 2,6-sialyltransferase [25] and produced a TSH calibrator with a high content in

sialic acid designed herein as recombinant glycoengineered TSH (rgTSH).

To solve discordances among assays, we postulated that only antibodies targeting regions equally shared by all TSHs will achieve the necessary accuracy. We therefore constructed about 100 assays targeting the 2 same antigenic regions of TSH and compared their ability to measure TSH levels in 84 clinical samples (2.1–22.4 mIU/l) based on various calibrators. We identified 2 groups of assays for which calibration with rgTSH proved to reduce variability among TSH measurements and achieve conversion from international to mass units.

## Materials and Methods

### *TSH Preparations*

The international standards were obtained from the National Institute for Biological Standards and Controls (South Mimms, UK), recTSH from ThermoFischer Scientific (Courtaboeuf, France) and pitTSH from Aalto Bio Reagents (Dublin, Ireland). To produce rgTSH, we transfected  $\alpha$ - and  $\beta$ -TSH genes (Uniprot P01215 and P01222, respectively) in Chinese hamster ovary (CHO) cells stably engineered with a tagged  $\alpha$ 2,6-sialyltransferase minigene [25]. Clone selection was performed in Ham's F12 Medium (Lonza, Basel, Switzerland) and production occurred in a chemically defined medium (Dominique Dutscher, Brumath, France). TSH was collected and stored at  $-20^{\circ}\text{C}$ .

### *Immunostaining*

Cells were fixed in 10% formalin (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and saturated with 5% goat serum.  $\alpha$ 2,6-sialylation was detected with biotinylated *Sambucus nigra* agglutinin (SNA; Vector Laboratories, Burlingame, Calif., USA) and streptavidin-TRITC (ThermoFisher Scientific, Courtaboeuf, France). After permeabilization with 0.05% Triton (Sigma-Aldrich), labeling of the transferase was performed with an anti-Flag monoclonal antibody (mAb; Sigma-Aldrich) and FITC-conjugated antibodies (ThermoFisher Scientific). The cells were finally fixed in DAPI-containing mountant (ThermoFisher Scientific) and analyzed with a confocal microscope (Zeiss, Marly-le-Roi, France).

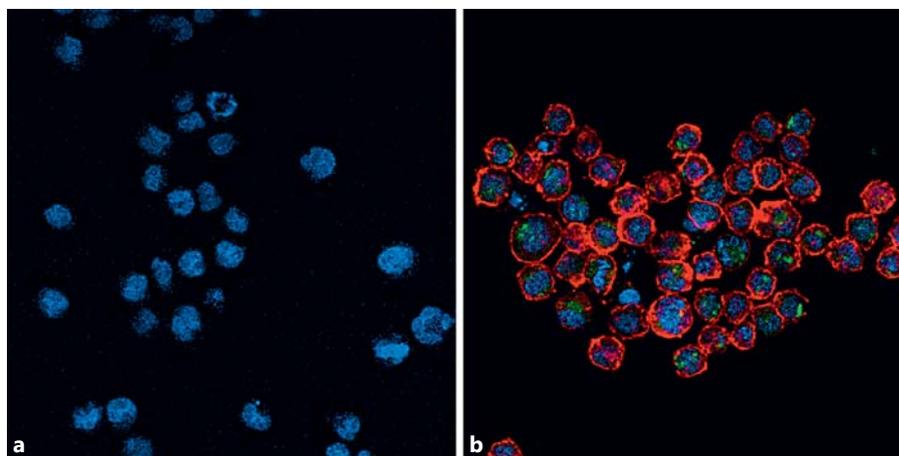
### *TSH Glycoprofiling*

Lectins (Vector Laboratories) were coated on 96-well plates and the binding of TSH was revealed using anti-TSH mAb (DIA-Source ImmunoAssays, Louvain-la-Neuve, Belgium) and horseradish peroxidase (HRP)-conjugated antibodies (ThermoFisher Scientific). Antibody glycosylation was controlled not to interfere with lectin binding. Detection was performed with UltraTMB (ThermoFisher Scientific). Optical density was measured at 450 nm (BioTek Instruments, Colmar, France).

### *TSH Bioactivity*

CHO cells expressing the recombinant human TSH-receptor were exposed to the same amounts (mIU) of various TSH preparations for 2 h in modified hypotonic medium [26] supplemented with 10 mM HEPES (ThermoFisher Scientific, Illkirch, France),

**Fig. 1.** Expression of TSH in CHO cells stably transfected with an engineered  $\alpha$ 2,6-sialyltransferase enzyme. Cells were stably expressing TSH (**a**) or TSH and engineered ST6Gal (**b**). Nuclei were colored with DAPI (in blue; colors refer to the online version only), the enzyme was detected with anti-Flag antibodies/FITC-conjugated anti-mouse antibodies (in green) and  $\alpha$ 2,6-sialylated proteins were labelled with biotin-SNA/streptavidin-TRITC (in red).



0.25 mM of isobutylmethylxanthine (Sigma-Aldrich) and 0.75% bovine serum albumin (BSA; Sigma-Aldrich), pH 7.4. Cyclic AMP released from the cells was measured by RIA (Immunotech, Marseille, France). The negative sera of pooled TSH-receptor antibodies (normal sera) were used to measure cAMP basal production. Results were expressed as the ratio of secreted cAMP (nM) to TSH (mIU/l).

#### TSH Immunoassays

Mass estimation of rgTSH was carried out by amino acid analysis (Alphalyse, Odense, Denmark) of a purified recTSH followed by immunological assessment. Coated anti-TSH mAbs (antibodies-online, DIASource ImmunoAssays and ThermoFisher Scientific) were incubated with TSH samples supplemented or not with a 50 mM of phosphate buffer, pH 7.5, containing 5% BSA and a human anti-mouse antibody-blocking reagent (Fitzgerald Industries International, Acton, Mass., USA) to avoid interferences with circulating antibodies [27]. Bound TSH was revealed with in-house biotinylated (Roche, Meylan, France) anti-TSH mAbs and HRP-streptavidin (ThermoFisher Scientific). Detection was performed with ABTS (Sigma-Aldrich) or UltraTMB. Optical density was measured at 405 or 450 nm, respectively (Biochrom, Cambridge, UK).

#### Forward-Phase Protein Microarray – Infrared

Anti-TSH mAbs were spotted on microarrays and TSH (1,000 pg/ml) was allowed to bind before adding infrared-labeled anti-TSH mAbs. TSH binding was analyzed at 670 nm (Innopsys, Carbone, France).

#### Serum Samples

Serum samples were collected anonymously and stored at  $-20^{\circ}\text{C}$ . TSH levels were measured using the same immunoradiometric assay (IM3712-IM3713; Beckman-Coulter, Villepinte, France) and calibrator vials provided by the manufacturer. The procedures were approved by the local institution's responsible committee.

#### Statistical Analysis

Results are expressed as the mean  $\pm$  SD. Statistical analysis was conducted using the one-way ANOVA test. Calculations were performed using Analysis ToolPak (Microsoft Excel add-in program).

## Results

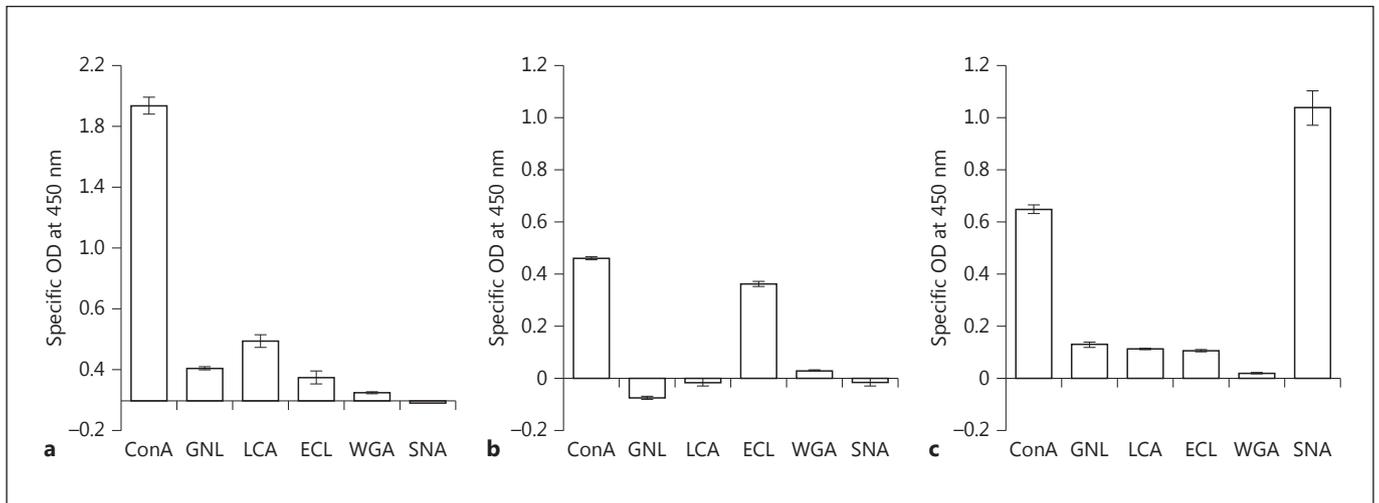
### Production and Characterization of rgTSH

Figure 1 shows that the  $\alpha$ 2,6-sialyltransferase mini-gene was correctly expressed in glycoengineered cells and the enzyme was fully active, delivering sialylated proteins intensely labeled at the cell surface. The glycosylation pattern of rgTSH was compared to those of primary standards. Commercial pitTSH had to be substituted for the 3rd IS 81/565 because of the presence of lactose that inhibits lectin binding. All TSHs showed different profiles. pitTSH showed a higher ConA signal, most probably due to a high content in biantennary structures [5, 28–30], and elevated core fucosylation (LCA), but very low level of galactose (ECL),  $\alpha$ 2,3- (WGA) and  $\alpha$ 2,6-sialic acid (SNA) because its glycans terminate in sulfated-GalNAc [5, 10] (fig. 2a). recTSH was also poorly sialylated (fig. 2b), while rgTSH contained a high level of  $\alpha$ 2,6-linked sialic acid (fig. 2c). Both recombinant preparations exhibited a low content in core fucose. Such a glycosylation profile for rgTSH was reproducible over several runs of production (data not shown).

When tested for activity, rgTSH was found to be more than 3-fold more active than recTSH in activating its receptor compared with the commercial recombinant product ( $2.35 \pm 0.18$  vs.  $0.74 \pm 0.05$  nmol/IU;  $p < 0.05$ ).

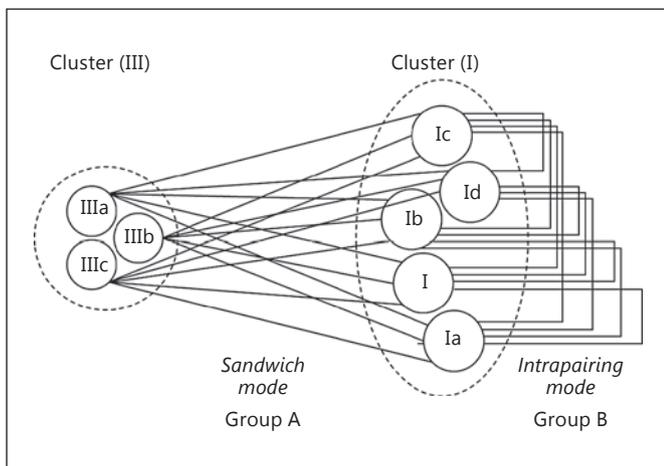
### Epitope-Defined Immunoassays

Figure 3 shows that the panel of antibodies used in this study shared the same specificity and targeted the same 2 main antigenic regions: cluster (I) is designated as the main immunogenic region (MIR) and cluster (III) as a remote cluster. Both of them contain several determinants and have been shown to be targeted in laboratory



**Fig. 2.** Characterization of TSH glycosylation. Lectin binding was performed on commercially available pitTSH (**a**), TSH produced in CHO cells (recTSH; **b**) and TSH produced in ST6Gal-engineered CHO cells (rgTSH; **c**). Concanavalin A (ConA) binds core-trimannose of complex, hybrid and high-mannose glycans [28], *Galanthus nivalis* lectin (GNL) binds hybrid and high-mannose

glycans [29], *Lens culinaris* agglutinin (LCA) recognizes preferentially core- $\alpha$ 1,6-fucosylated glycans [30], *Erythrina cristagalli* lectin (ECL) binds terminal  $\beta$ 1,4-galactose of complex glycans [30], wheat germ agglutinin (WGA) binds  $\alpha$ 2,3-sialic acids [30], and SNA binds  $\alpha$ 2,6-sialic acids [30]. Error bars indicate standard deviation.



**Fig. 3.** Schematic representation of the epitope-defined strategy based on the MIR of TSH.

medicine [5]. It is worth noting that in cluster (I), epitopes are distributed within a large area and thereby allow 2 anti-MIR antibodies to bind. Conversely, cluster (III) is more limited and does not allow the intrapairing of antibodies. As a result, targeting each of the epitopes in these 2 regions allowed the construction of about 100 different sandwich assays which all bind TSH in the same 2 regions, albeit through different modes.

Combining cluster (III) to the MIR (I) allowed the design of group A assays with 19 combinations effective in the serum matrix. A1, A2 and A3 assays were selected as they displayed the best analytical limits of detection [31] of 23.6, 2.4 and 2.4 pg/ml, respectively. Additionally, 79 other combinations were able to additionally bind epitopes within the MIR in an intrapairing mode (group B assays). Seven such assays showed higher TSH binding than group A assays. We selected 4 of them (fig. 4a–d), B1, B2, B3 and B4, with analytical limits of detection of 30.1, 8.8, 3.7 and 22.5 pg/ml, respectively. They also showed enhanced TSH binding in sera from hypothyroid patients (fig. 4e–h). These 7 assays thus displayed enhanced recognition of TSH compared to group A by targeting different epitopes in the same regions (table 1).

#### Quantitative Antibody Binding

As shown in figure 5, assays from both groups displayed 2- to 5-fold higher quantitative binding of rgTSH than pitTSH, possibly because the IS preparation contains a significant amount of denatured/nonimmunoreactive TSH due to its prolonged storage [32]. Again, most group B assays showed quite a superior binding capacity, with the highest signals being observed with B2 and B3 for both calibrations.

**Table 1.** Composition of epitope-defined assays

	Name	Targeted epitopes
Group A	A1	Ia, III
	A2	Ib, III
	A3	Ic, III
Group B	B1	Ia, Ic, III
	B2	I, Ic, III
	B3	Ia, Ib, III
	B4	Ia, Ib, III

**Table 2.** Correlation between rgTSH and the 2 international standards

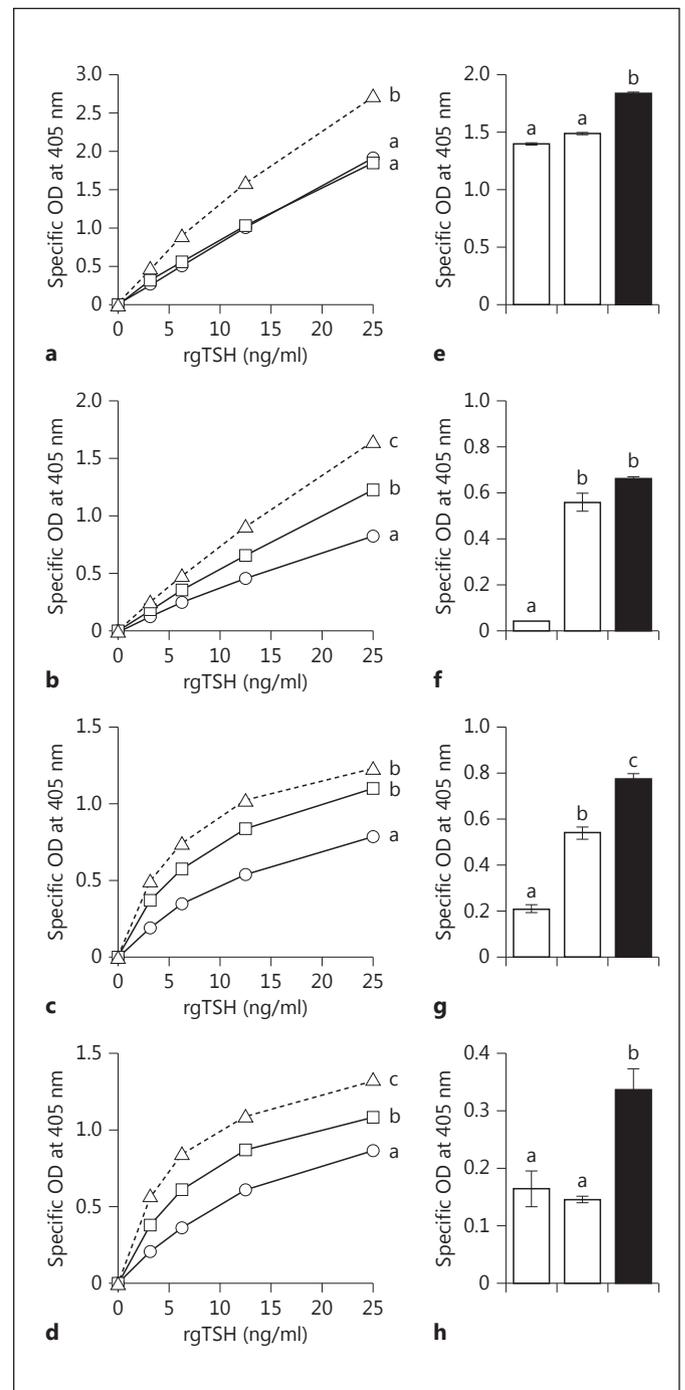
		Assay	Slope	R <sup>2</sup>
3rd IS pitTSH 81/565	Group A	A1	47.2	0.999
		A2	30.0	0.998
		A3	32.6	0.999
	Group B	B1	46.5	0.895
		B2	40.1	0.994
		B3	42.0	0.995
		B4	32.3	0.997
1st IRP recTSH 94/674	Group A	A1	13.9	0.998
		A2	11.9	0.994
		A3	13.2	0.991
	Group B	B1	16.2	0.939
		B2	15.1	0.996
		B3	15.4	0.987
		B4	11.7	0.997

Measurements obtained with the international standards (x) were compared to those obtained with rgTSH calibration (y). Slopes represent the ng/mIU factor of conversion.

#### TSH Measurements of Serum Samples

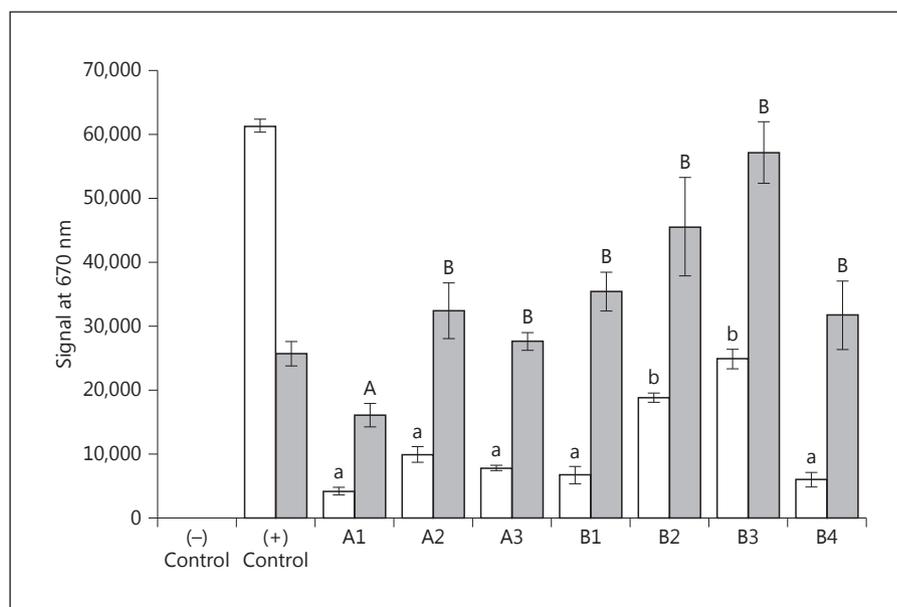
Group A and B assays were further compared within a panel of 84 human sera covering the range from euthyroid subjects to hypothyroid patients (2.1–22.4 mIU/l). These were based on 3 calibrations: rgTSH (pg/ml) as well as the 3rd IS pitTSH 81/565 and the 1st IRP recTSH 94/674 (mIU/l).

Table 2 shows that the comparison of TSH measurements (x, 3rd IS81/565; y, rgTSH) delivered a linear relationship with a high correlation factor ( $R^2 > 0.94$ ) for both groups A and B. Similar results ( $R^2 > 0.99$ ) were obtained with the 1st IRP 94/674 (x). The slopes represent the conversion factor (ng/mIU) relevant under our experimental conditions, and their variations indicate that assays such as A1 or B1 with elevated slopes bind rgTSH more effi-



**Fig. 4.** Comparative binding of group A and group B assays: B1 (**a, e**), B2 (**b, f**), B3 (**c, g**) and B4 (**d, h**). **a-d** Dose-dependent curves of rgTSH. Group A assays are represented with squares and continuous lines, group B assays are represented with triangles and dashed lines. **e-h** Results obtained with 10 ng/ml of a pool of hypothyroid sera (50–200 mIU/l) diluted in human serum with the TSH level <0.05 mIU/l. Group A assays are represented with white bars and group B assays with black bars. Error bars indicate standard deviation. Values overwritten with different letters (a–c) are significantly different from each other ( $p < 0.05$ ).

**Fig. 5.** Quantitative antibody binding based on forward-phase protein microarray – infrared. Group A assays (A1 to A3) and group B assays (B1 to B4) were tested for their binding to the 3rd IS pitTSH 81/565 (white bars) and to rgTSH (gray bars). The current IS preparation was spotted on a mass basis of 1 ng/ml (5.75  $\mu$ IU/ng as indicated on the product data sheet) to be similar to the amount of rgTSH determined by amino acid analysis (see Materials and Methods). The positive and negative controls were IR-labeled protein and 0.05% BSA, respectively. Error bars indicate  $\pm$ SD. Values overwritten with different letters [(a, b) and (A, B)] are significantly different from each other ( $p < 0.05$ ).



ciently than the current IS when compared to A2 or B4 assays.

We also analyzed the mean bias ( $y = \text{mean} - \text{assay} / \text{mean} \times 100$ ) relative to the mean sample ( $x$ ) (fig. 6). As anticipated, the distribution of TSH values was clearly dependent on the calibration. Group A assays calibrated with the 3rd IS 81/565 delivered TSH values that were significantly different from each other ( $p < 0.05$ ), with A2 significantly different from A1 and A3 ( $p < 0.05$ ). In striking contrast, the same assays delivered comparable TSH values with rgTSH calibration ( $p > 0.05$ ) centered on the origin axis. The 1st IRP 94/674 behaved as an intermediate situation (fig. 6a–c). A1 and A2 performances have to be correlated with the most elevated and the low conversion factor respectively obtained with pituitary calibration (table 2). Instead, changes in calibration did not much affect measurements of group B assays: all assays gave a significantly comparable TSH dataset ( $p > 0.05$ ; fig. 6d–f). Of note, 3 out of 84 individual samples showed increased difference means in the high TSH levels with assays B1 and B2, and therefore may not be representative of the cohort.

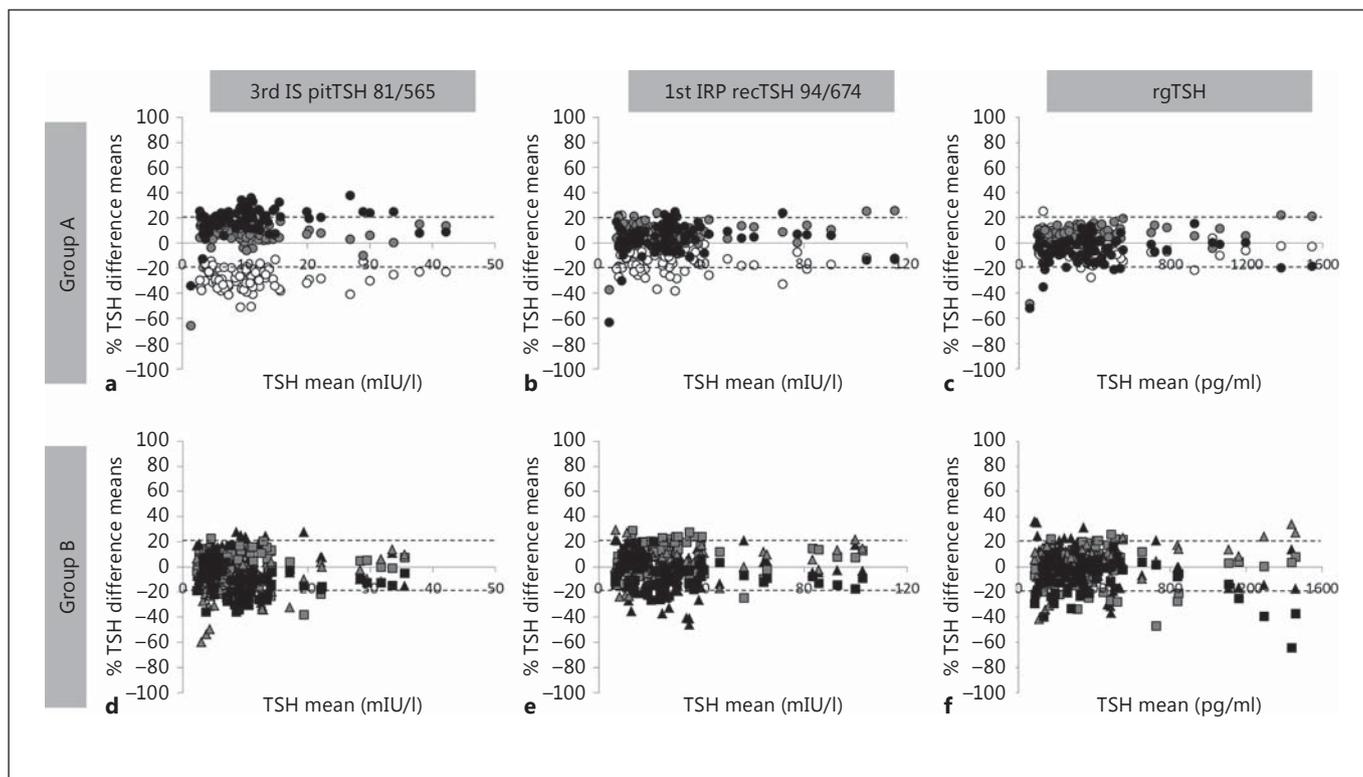
## Discussion

By definition, if immunoreactive molecules present in standard and samples are not similar, an immunoassay is decreed to be invalid and the results have no

significance [33]. Structural identity is not optimally achieved in TSH assays currently used in laboratory medicine because a variable amount of long-lived TSHs have to be measured, which are low in standard and high in serum [34]. Since TSH sialylation is progressively enhanced along with thyroid deficiency [12–14], a lack of similarity is thus increasing between standards and samples, thereby introducing variability between measurements [1]. This is probably also affecting the accuracy in the decision limit. In this study, we have produced a glycoengineered TSH to provide unlimited supply of a highly sialylated hormone which can mimic serum TSH. It appears that this material is active in stimulating the TSH receptor and elicits signal transduction. Based on these 2 innovative findings, we elaborated a strategy to achieve similar antibody recognition in both standard and samples, and ultimately assessed rgTSH as a potential calibrator and measure of the TSH level in a functional way.

### TSH Standards

Over the past 50 years, international standards have been extracted from pituitaries and their immunological activity expressed in arbitrary international units. The first standard 63/14 set up in 1963 was assessed for bioactivity and the others followed by successive immunological assignments, including the 3rd IS 81/565 established 10 years ago [32, 35]. Meanwhile, the assessment of the 1st IRP 94/674 as a potential recombinant standard [18]



**Fig. 6.** Percentage difference plots of assays as a function of their calibration and construction. Calibrations were the 3rd IS pitTSH 81/565 (**a, d**), the 1st IRP recTSH 94/674 (**b, e**) and rgTSH (**c, f**) calibrations; group A assays (**a–c**) and group B assays (**d–f**) are

represented separately. Assay symbols: A1, black circles; A2, white circles; A3, gray circles; B1, black squares; B2, gray squares; B3, black triangles; B4, gray triangles.

indicated that replacement of extractive materials by a recombinant preparation may not be without problems [35]. The production of recombinant TSH with a high content in  $\alpha 2,6$ -sialylation indeed requires complex glycoengineering, because such glycosylation is absent in most if not all expression systems, including human cell lines [36]. It is worth noting that hypersialylated rgTSH proved to be biologically active and displayed an immunological behavior similar to serum TSH. Furthermore, a conversion factor could be determined between rgTSH and the 3rd IS 81/565, suggesting that shifting from international to mass units is technically feasible and applicable to routine assays in laboratory medicine. rgTSH can thus be satisfactorily used as the first calibrator to measure bioactive TSH.

#### *Epitope-Defined Strategy*

Since most assays used in laboratory medicine display variable recognition of TSH [5, 8] and deliver discordant values [1], we wanted to identify which epitopes should

be best targeted to measure serum TSH with the highest accuracy. rgTSH was found to essentially share 2 main regions with pitTSH [5], in full agreement with previous studies [37, 38], including antibodies kindly provided by in vitro diagnostic manufacturers [5]. We thus compared assays which bind these 2 clusters but through different epitopes and sandwich modes. Quantitative binding analysis revealed that such assays indeed differently quantitated a given amount of TSH. We further observed that group A assays behaved differently when calibrated with the 3rd IS 81/565 and with rgTSH, suggesting that the MIR targeted in all of these assays must play a pivotal role in the binding capacity of each assay. It may very well be that all the MIR epitopes are not present in all these TSHs. We therefore concluded that epitope expression in this large region may vary between standards and samples and introduce significant discrepancies among measurements. Alternatively, group B was found to display similar TSH values, suggesting assays need to combine several epitopes to equally bind all TSHs and de-

liver harmonized TSH values. It is tempting to speculate that group A assays, particularly A1 and A2, differentially bind TSH glycoforms present in the pituitary standard and serum, thereby leading to the over- or underestimation of the TSH level, while group A as well as group B assays equally measure TSH because in rgTSH calibration they found the full array of glycoforms commonly found in serum.

#### Harmonization of TSH Measurements

While this work was ongoing, the Committee for Standardization of Thyroid Function Tests studied the validation of a mathematical recalibration as a method for harmonizing TSH measurements [39, 40]. So far, the gain of recalibration has appeared rather minimal, especially in the upper normal range (>5 mIU/l) [40], indicating that results are not necessarily improved and the concentration of decision limit still remains unsolved. Alternatively, we demonstrated herein that changing the secondary calibrator to rgTSH can substantially reduce variability in TSH measurements over the whole clinical range.

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#### Conclusions

Introducing rgTSH as a new calibrator may provide a practical approach to the harmonization of TSH measurements. It may help in solving current limitations with regulatory authorities and establish a reliable basis for traceability because a bioproduct is virtually unlimited in supply and its quality control is easily monitored. Meanwhile, measuring bioactive TSH should also benefit assays in increasing diagnostic performances and ultimately achieve an optimal clinical outcome.

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#### Disclosure Statement

The authors declare no conflict of interest.

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