polymorphism for monitoring of both disease susceptibility and therapeutic response variability. Further studies clarifying the reason for an association of this polymorphism with altered GR function could provide additional insight into the variability of the *GR* locus and the role of the *Bcl*I polymorphism.

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Free Thyroxine Measured by Equilibrium Dialysis and Nine Immunoassays in Sera with Various Serum Thyroxine-binding Capacities, *Rémy Sapin[1*] and Michèle d'Herbomez[2]* [[1] Laboratoire Universitaire de Biophysique, Unité d'Analyses Endocriniennes, Université Louis Pasteur (ULP)/Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche (UMR) 7004, Faculté de Médecine, 67085 Strasbourg Cedex, France; [2] Service Central de Médecine Nucléaire, Hôpital Salengro, Centre Hospitalo-Universitaire Regional (CHRU), 59037 Lille Cedex, France; * address correspondence to this author at: Institut de Physique Biologique, Faculté de Médecine, F-67085 Strasbourg Cedex, France; fax 33-3-90-24-40-57, e-mail sapin@ipb.u-strasbg.fr]

Despite the predominant role of thyrotropin measurements in the assessment of thyroid status, free thyroxine (FT_4) measurements remain useful either when thyrotropin determination is not conclusive or when a diagnosis of thyroid disease must be confirmed *(1)*. Because it represents only a minute fraction (0.02%) of total T_4 (TT_4), FT_4 is more difficult to measure *(2)*. Direct equilibrium dialysis (ED) methods are considered analytically accurate *(3)* and are the methods against which others are compared *(4)*. Compared with ED, other FT_4 immunoassays may show significant biases related to protein-bound T_4 or to the serum T_4-binding capacity (sBC: concentration $\times$ affinity of binding proteins) *(4–6)*. We assume assays are calibrated to have roughly the same euthyroid range in samples with normal sBC, and we expect that markedly negative biases may be observed in samples with low sBC and that smaller positive biases may be observed in samples with high sBC *(7)*. The aim of our study was to determine, in clinical samples from euthyroid patients classified into three groups as a function of their low, normal, or high sBC, the bias between FT_4 measured with ED and that measured with nine frequently used immunoassays. We also studied the specificity of each assay method and the concordance of immunoassays with ED.

FT_4 was determined with the Nichols ED/RIA assay (Nichols Institute Diagnostics) and the following nine immunoassays: Elecsys (EL) from Roche Diagnostics, VIDAS (VD) from bioMérieux, Vitros ECi (VT) from Ortho-Clinical Diagnostics, GammaCoat 2-step RIA (GC) from DiaSorin, Immulite (IM) from Diagnostic Products Corporation (DPC), Nichols Advantage (AD), AxSYM (AX) from Abbott Diagnostic, ACS (AC) from Bayer Diagnostics, and AIA (AI) from Tosoh Bioscience. All assays were performed in compliance with the manufacturers' instructions. The sBC was calculated by dividing the TT_4 concentration determined with the EL assay by the FT_4

concentration determined by ED *(8)*. The sBC was further assessed by measuring the concentration of thyroxine-binding globulin (TBG), the main T_4-carrier protein, with the Ria-gnost RIA from CIS bio international, and the EL T-Uptake (TU), which estimates the number of unoccupied serum protein binding sites. TU results are normalized and should be directly related to the sBC.

To study a wide range of sBCs, we selected sera from pregnant women in the last 3 months of pregnancy (n = 29) and hospitalized patients (n = 42). All patients were euthyroid, and none had been known to have patent thyroid dysfunction in the past. Except for one patient treated with heparin, none received a treatment known to interfere with the thyroid function or T_4 measurements. The sera were classified into three groups, high-, normal-, or low-sBC, depending on whether sBC results (TT_4/ED FT_4), were above, within, or below the reference interval determined in ambulatory patients (4.2–8.5 nmol/pmol) *(8)*. All of the procedures that we followed were in accordance with the Helsinki Declaration of 1975 and the subsequent 1996 amendments. The patient sera were collected for routine analysis in tubes without anticoagulant, were kept frozen at −20 °C, and were analyzed shortly after thawing.

The 29 sera from pregnant women were classified into the high-sBC group [mean (SD) sBC, 13.1 (2.5) nmol/pmol], 29 sera from hospitalized patients into the normal-sBC group [5.6 (1.0) nmol/pmol], and 13 from hospitalized patients into the low-sBC group [2.7 (0.9) nmol/pmol]. Compared with the results in the normal-sBC group [TBG, 22.2 (5.0) mg/L; TU, 0.97 (0.09)], TBG and TU were significantly decreased (P <0.01, Mann–Whitney U-test) in the low-sBC group [16.4 (4.3) mg/L and 0.80 (0.13), respectively] and were increased in the high-sBC group [59.2 (10.2) mg/L and 1.39 (0.08), respectively].

The biases determined between each immunoassay and ED as a function of sBC are reported in Fig. 1. In the normal-sBC group, we found no significant bias with the EL (mean, −1.1 pmol/L), AI (−0.9 pmol/L), VT (0.8 pmol/L), and GC (1.0 pmol/L) immunoassays (P >0.05, Wilcoxon test) and a modest but significant (P <0.01) negative bias with the AC (−2.5 pmol/L), IM (−2.9 pmol/L), AX (−3.2 pmol/L), VD (−3.3 pmol/L), and AD (−3.7 pmol/L) immunoassays. The bias was more marked when the sBC was in the lower part of the reference interval (4.2–5.0 nmol/pmol). In the high-sBC group, except with the VT and AC assays, which showed no bias, we observed significant but very modest biases, negative for AX (−0.8 pmol/L) and AD (−1.1 pmol/L), and positive for the EL (1.0 pmol/L), VD (1.1 pmol/L), GC (1.4 pmol/L), AI (1.5 pmol/L), and IM (1.7 pmol/L) assays. A moderate positive bias can be related to the high sBCs of these samples, but a negative bias was unexpected. In the low-sBC group, all methods showed a significant and marked negative bias increasing in the following order: VT (−8.0 pmol/L), GC (−14.9 pmol/L), AI (−15.8 pmol/L), AC (−17.2 pmol/L), EL (−17.4 pmol/L), AD (−17.5 pmol/L), AX (−20.4 pmol/L), VD (−20.7 pmol/L), and IM (−21.3 pmol/L).

All patients of this study were considered as euthyroid, and their FT_4 results were expected to be within the reference interval. For each assay, the FT_4 range, number of decreased or increased results, and concordance with ED are reported in Table 1. In the normal-sBC group, concordance with ED ranged between 59% (IM) and 96% (VT). GC, AI, and EL assays, for which no significant bias was evidenced, showed a poorer concordance than did AD and AC assays, which were significantly biased. These findings can be explained in terms of different calibrations. Except for the VT assay, all of the immunoassays yielded decreased FT_4 values for some of these sera despite sBCs within the reference interval. In the high-sBC group, with all immunoassays as well as with ED, irrespective of the bias, we found a high, method-dependent number of decreased results. In the low-sBC group, assays other than ED, VT, and AD yielded some decreased results.

In the normal-sBC group, the biases, when significant, were modest. This was expected because FT_4 assays are calibrated against the ED method, using sera samples from ambulatory patients with normal sBC. Except for the VT assay, however, we observed some decreased values, which lowered the concordance with ED. Therefore, the sera of not severely ill hospitalized patients with normal sBC should be included in the panel used to establish the reference interval. Alternatively, specific reference intervals for ambulatory and hospitalized individuals could be better suited. This applies in particular to methods yielding a not inconsiderable number of decreased values (i.e., IM, EL, VD, AI, and AX).

In the high-sBC group, the biases, when significant, were also modest. Contrary to previous reports *(10, 11)*, and yet in agreement with other observations *(6, 8, 12–15)*, subnormal values, whether measured with ED or any other immunoassay FT_4 method, are not uncommon in the last months of pregnancy. Moreover, the maximum value was clearly and systematically below the upper limit found in nonpregnant women. These findings underline the absolute necessity to consider assay-specific FT_4 reference intervals for women in the last months of pregnancy, to be in a position to diagnose not only hypothyroidism but also hyperthyroidism in a reliable way and to adjust an appropriate T_4 treatment.

In the low-sBC group, we found a significant bias with all immunoassays, VT included, contrary to what has been reported previously *(8)*. This finding may be related to some very high ED results (up to 65.9 pmol/L). Extremely low doses of heparin release lipase activity into the plasma and can thereby cause artifactual increases of serum FT_4 concentrations as measured by ED *(16–19)*. None of the patients in this group was known to be treated with heparin. In hospitalized patients, however, heparin is frequently used for multiple blood samplings. Except for the VT and AD FT_4 immunoassays, decreased values were also observed. Albumin, added to the assay ingredients to buffer the effects of increased amounts of nonesterified fatty acids that develop in serum in vitro, may have induced a negative bias *(4, 20)*. Contrary to VT

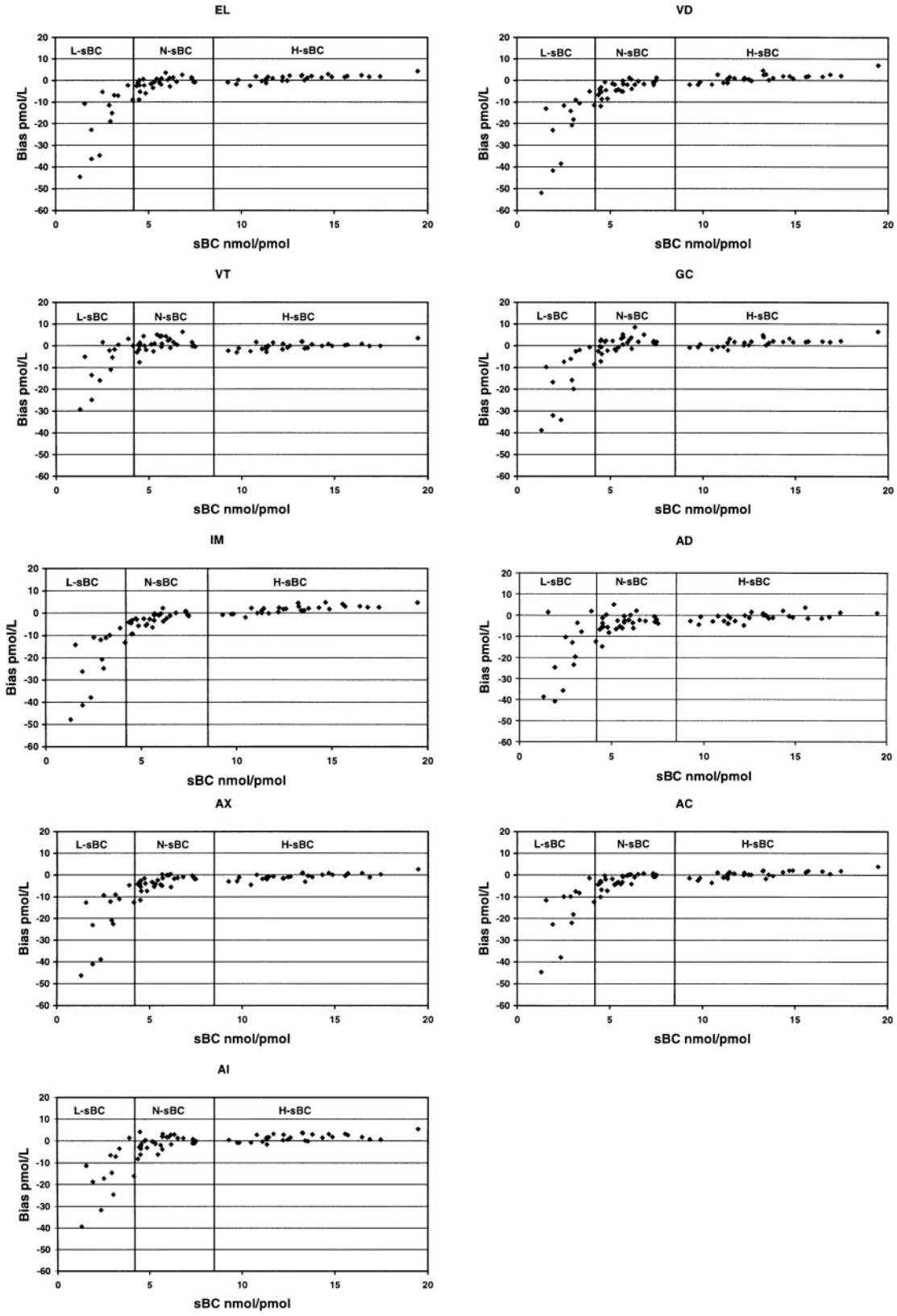

Fig. 1. Bias between immunoassay and ED FT_4 results vs sBC determined as the TT_4/ED FT_4 ratio.

EL, Elecsys (Roche Diagnostics); *VD,* VIDAS (bioMérieux); *VT,* Vitros ECi (Ortho-Clinical Diagnostics); *GC,* GammaCoat (DiaSorin); *IM,* Immulite (DPC); *AD,* Nichols Advantage; *AX,* AxSYM (Abbott); *AC,* ACS:180 (Bayer Diagnostics); *AI,* AAI (Tosoh Bioscience). The *vertical lines* define the three sBC zones: low (*L-sBC*; sBC <4.2 nmol/pmol), normal (*N-sBC*; 4.2 nmol/pmol <sBC <8.5 nmol/pmol), and high (*H-sBC*; sBC >8.5 nmol/pmol).

Table 1. FT_4 measured with ED and nine immunoassays in sera with normal sBC (4.2 nmol/pmol <sBC <8.5 nmol/pmol), high sBC (>8.5 nmol/pmol), or low sBC (<4.2 nmol/pmol).

	ED	EL	VD	VT	GC	IM	AD	AX	AC	AI
Reference interval, pmol/L	10.3–34.7[a]	12.8–23.4[b]	10.3–21.3[b]	10.2–28.5[b]	12.2–24.5[b]	11.6–23.4[b]	8.1–21.5[b]	10.3–20.1[b]	10.3–20.5[b]	10.7–25.8[b]
Normal sBC (SD), 5.6 (1.0) nmol/pmol (n = 29)										
Interval,[c] pmol/L	8.6–33.6	10.0–24.6	5.1–21.7	10.2–26.0	9.0–26.4	6.3–24.3	6.4–20.4	7.1–22.0	7.1–23.6	7.4–27.4
n decreased[d]/n increased[e]	1/0	8/1	7/1	0/0	5/1	12/1	3/0	6/1	4/1	7/1
Concordance with ED,[f] %		72	76	96	83	59	93	79	86	76
High sBC (SD), 13.1 (2.5) nmol/pmol (n = 29)										
Interval,[c] pmol/L	7.5–16.7	9.4–14.5	8.9–17.2	7.4–14.1	9.5–16.0	10–15.6	6.9–14.2	7.8–12.2	8.7–13.4	8.2–16
n decreased[d]/n increased[e]	10/0	18/0	4/0	12/0	13/0	6/0	7/0	14/0	5/0	7/0
Concordance with ED,[f] %		66	83	79	76	83	76	72	83	90
Low sBC (SD), 2.7 (0.9) nmol/pmol (n = 13)										
Interval,[c] pmol/L	13.6–65.9	7.8–24.5	5.5–22.0	13.5–40.8	8.8–27.1	4.4–20.3	12.1–27.2	5.9–20.9	7.1–21.2	7.2–26.6
n decreased[d]/n increased[e]	0/5	3/1	3/1	0/5	1/3	5/0	0/1	3/1	1/2	3/1
Concordance with ED,[f] %		31	31	69	62	31	69	31	58	50

[a] From the assay package insert and Ref. *(4)*, range observed in 263 nonpregnant healthy adults with one outlier deleted at each end.
[b] Determined in a multicenter study from the results of 152 control sera of ambulatory euthyroid patients (2.5th–97.5th percentile) *(9)*.
[c] Interval, minimum and maximum FT_4 values with each method.
[d] n decreased, number of results below the lower limit of the reference interval of each method.
[e] n increased, number of results above the upper limit of the reference interval of each method.
[f] Concordance between ED and each immunoassay expressed as the percentage of concordant results.

reagents *(21)*, EL *(19)* and AX *(4)* reagents probably contain albumin. Our results confirm the great variability in FT_4 measurements in hospitalized patient sera with low sBC values *(11, 19, 22)*. As with ED, increased results may be observed, but contrary to ED, decreased values were yielded by most immunoassays. In these sera, some methods (ED, VT, GC) yielded preferentially increased results; others, such as IM and to a lesser extent VD, AX, EL, and AI, preferentially decreased results.

In conclusion, although methodologies have somewhat improved, as far as commonly used immunoassays are concerned, it remains absolutely necessary to consider the assay method to correctly interpret FT_4 results in pregnant women when these are decreased or lie in the upper zone of the reference interval for nonpregnant women. The same goes for hospitalized patients, regardless of whether increased or decreased results have been obtained. This implication is particularly important for sera with low sBC (severely ill patients) but also seems valid, at least as far as some immunoassays are concerned, for sera from hospitalized patients whose sBC lies in the lower part of reference interval. Clinicians should be aware of the method used to determine FT_4 because the evaluation of the effect of pregnancy, as well as of severe or even mild non-thyroidal illness, on FT_4 results varies as a function of the various immunoassays.

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High Mobility Group Protein 1 (HMGB1) Quantified by ELISA with a Monoclonal Antibody That Does Not Cross-React with HMGB2, *Shingo Yamada,[1*] Keiichi Inoue,[1] Keiko Yakabe,[1] Hitoshi Imaizumi,[2] and Ikuro Maruyama[3]* ([1] Central Institute, Shino-Test Corporation, Sagamihara, Kanagawa 229-0011, Japan; [2] Department of Traumatology and CCM, School of Medicine, Sapporo Medical University, Sapporo 060-8543, Japan; [3] Department of Laboratory and Molecular Medicine, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520, Japan; [*] author for correspondence: fax 81-0427-86-8553, e-mail Shingo.Yamada@shino-test.co.jp)

High mobility group protein 1 (HMGB1) has been implicated in diverse cellular functions, including determination of nucleosomal structure and stability and binding of transcription factors to their cognate DNA sequences *(1–4)*. HMGB1 is also present in a membrane-associated form, termed amphoterin, that mediates neurite outgrowth *(5)*. Amphoterin can interact with macrophage cell surface receptors for advanced glycation end products to enhance expression of tissue-type plasminogen activator *(6)*.

Recently, HMGB1 was identified as a late mediator of endotoxin lethality *(7)*. Mice had increased serum HMGB1 concentrations after exposure to endotoxin, and sepsis patients who succumbed to infection also had increased serum HMGB1. It would therefore be useful to develop an easy and highly sensitive method to measure serum HMGB1. However, this study revealed that HMGB1 and HMGB2, with extremely high homology (81%) to HMGB1, coexist in the serum. We report an ELISA method we have developed that measures only HMGB1 without simultaneous determination of HMGB2.

To prepare an anti-peptide monoclonal antibody reacting only with HMGB1, we selected a peptide sequence (peptide 1; GKGDPKKPRGK) with high antigenicity and different from that of HMGB2. The monoclonal anti-calf HMGB1 antibody was prepared against calf thymus-derived HMGB1, which was 98% homologous to human HMGB1. Each protein sample used for the analysis was prepared as follows.

The peptides were purified and separated by HPLC. The peptide synthesized was added to maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co.)-labeled keyhole limpet hemocyanin (Calbiochem) or maleimidobenzoyl-*N*-hydroxysuccinimide ester-bovine serum albumin (BSA; Sigma). Calf HMGB1 and HMGB2 were obtained from calf thymus by the method of Sanders *(8)*. Human HMGB1 and HMGB2 were purified from HL60 cells *(9)*. As a result, the anti-peptide 1 monoclonal antibody (no. 77) reacted with human HMGB1 but not with human HMGB2. The anti-calf thymus HMGB1 monoclonal antibody (no. 03E5) reacted with both human HMGB1 and HMGB2 (Fig. 1A).

We next detected HMGB1 in the serum of sepsis patients and ulcerative colitis patients by Western blotting. Serum was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were transferred electrophoretically to nitrocellulose membranes (Millipore). After the membranes were blocked, the monoclonal antibody to HMGB1 (no. 77 or 03E5) was dissolved in phosphate-buffered saline (PBS), and the membrane was soaked in the solution at room temperature overnight. After the membrane was washed, it was incubated at room temperature for 2 h in a solution of peroxidase-labeled anti-mouse immunoglobulin polyclonal antibody (Dako) diluted 30 000-fold with PBS containing 30 g/L BSA. After another washing step, the membrane was soaked in ECL reaction reagent (Pharmacia) according to the manufacturer's instructions.

Chemiluminescently labeled bands were visualized after 10-min exposure to Xomat XAR5 film (Eastman Kodak Co.). HMGB1 was determined by immunoblot analysis and quantified from the blots by measurement of the absorbance of the band (NIH 1.59 software; NIH) and comparison with a calibration curve constructed with purified human HMGB1 serially diluted in normal human serum. Western blotting results for serum samples