Spurious Conclusions on Analog Free Thyroxine Assay Performance

To the Editor:

I read with misgivings the recent communication by Fritz et al. (1) alleging that because under some circumstances an analog free thyroxine (FT$_4$) immunoassay correlates total T$_4$ and FT$_4$ values, it does not measure FT$_4$ but something akin to T$_4$. I have shown repeatedly through mass action analyses (2–4) that many experiments, including the one reported by Fritz et al. (1) and others described in earlier papers (5, 6), cannot meaningfully address the working of any of these assays. Allegations of shortcomings cannot be substantiated if they depend on experiments using artificial T$_4$ solutions in which serum T$_4$-binding proteins are either lacking or are present at concentrations insufficient to prevent overlarge T$_4$ abstraction by the assay antibody (conditions strictly invalid for FT$_4$ measurements).

The amount of antibody that can be used in analog FT$_4$ assays having a radioactive probe is limited (3). Constraints on the stability of molecules radiolabeled with carrier-free $^{125}$I atoms dictate the minimum usable amount of labeled probe for adequate tracer detection in the assay (3). To enable measurement, the minimum amount of stripping antibody must remove at least 1% of the total T$_4$ from a normal serum sample (2–4). Although this amount far exceeds the actual number of FT$_4$ molecules present in serum (approximately 0.02% of the total), the procedure remains valid because the serum-bound free equilibrium for FT$_4$ bound T$_4$ is negligibly disturbed, and the measured FT$_4$ concentrations (obtained through calibration of standards) are not significantly affected (3–5). Sequestrations of more than approximately 3% invalidate any results. Because the FT$_4$ concentration in ultrafiltrates or dialysates remains equivalent to only 0.02% of the total T$_4$ (i.e., the ambient, undiluted FT$_4$ concentration), any assay designed to sample 1% of T$_4$ cannot detect this small amount, and total T$_4$ assays similarly fail.

Results from varying serum (and therefore serum T$_4$-binding protein) concentrations can also be explained quite differently. I understand that this early analog immunoassay retained a “TBG effect”. That is, residual binding of the labeled analog to T$_4$-binding globulin (TBG; and probably other proteins) was large enough to correlate FT$_4$ values with TBG (partly but not completely mitigated by a T$_4$ blocking agent). Thus, when serum amounts are altered by dilution or concentration, 2 connected events ensue. First, a more concentrated serum will have a higher concentration of TBG relative to antibody probe and act as a high-TBG serum, resulting in increased FT$_4$. Conversely a diluted sample will mimic a low-TBG serum giving a low result. Second, intrinsic effects of dilution will occur. If in undiluted serum the assay samples 1%–2% of the total T$_4$, in a 4-fold diluted serum, it will sample 4%–8%, an interval that lies well outside the window of validity of the assay, insofar as too much T$_4$ is sequestered by the antibody probe to maintain equilibrium between FT$_4$ and the T$_4$ bound to the serum proteins. The assay essentially “collapses” into one more closely related to T$_4$, and will do so in all cases in which too much T$_4$ is sampled by the antibody.

Much of the work of Nelson et al. (5, 6) aimed at probing the working of analog FT$_4$ assays is not applicable to the topic under discussion, but these investigators seem to suggest that in regard to these FT$_4$ assays any experiment should be meaningful in any situation (whether physiologic or not). The cardinal property of direct 1-step assays (analog or labeled antibody) is that, although they are designed for all physiologic circumstances (i.e., they work with all sera regardless of serum T$_4$-binding protein concentrations), they cannot be used with artificial solutions outside the assay’s limits of validity. This point has been argued elsewhere (2–4).

Another questionable implication (5, 6) is that analyses of the performance of this particular assay may apply generally to all such assays. On the contrary, FT$_4$ assays are individual with regard to serum protein effects, assay ingredients, robustness to dilution, and percentage sampling by the antibody probe. General conclusions on the class of tests cannot be drawn by examining a single assay (3). Assays with residual analog binding to serum proteins cannot be easily compared with newer examples that lack this characteristic.

Finally, although ultrafiltration, equilibrium dialysis, and direct analog assays have the same aims, in regard to sampling and dilution these different assays represent opposite ends of a spectrum of valid methods. Experiments performed by one group of methods do not always apply to the other, because ultrafiltration and dialysis measure FT$_4$ in the absence of serum proteins and bound TBG whereas analog assays accommodate their presence.

References:


John E.M. Midgley
North Lakes Clinical
6 High Wheatley
Ilkley LS29 8RX, United Kingdom

DOI: 10.1373/clinchem.2007.090878

Editor’s Note: Dr. Fritz declined to reply for publication.
Presence of the Hemochromatosis S65C Mutation Leads to Failure of Amplification in a Multiplex C282Y/H63D PCR

To the Editor:

Hemochromatosis is an autosomal recessive disorder of iron metabolism affecting 0.2%–0.5% of white populations. Approximately 90% of affected individuals are homozygous for the C282Y mutation, but the H63D and S65C mutations are also of interest.

Various molecular diagnostic methods for detection of these mutations have been described, including a multiplex PCR from Stott et al. (1) that simultaneously detects the C282Y and H63D mutations. In this method, PCR-mediated site-directed mutagenesis is used to create a BbrPI restriction site in the wild-type C282Y and H63D products. To detect the C282Y mutation and the H63D mutation, respectively, 1 specific mismatch in the forward 282mut primer and 2 specific mismatches in the reverse 63mut primer are incorporated. The presence of either mutation abolishes the restriction site.

Comparing the method of Stott et al. (1) with a reverse hybridization line-probe assay (LiPA) (2) we found a discrepant result. A compound heterozygous H63D/S65C sample was characterized as H63D heterozygote by the LiPA method, and as H63D homozygote by the method of Stott et al. (1). The S65C mutation introduces an additional 3rd mismatch between primer and target, because the S65C mutation is also located in the binding site of the reverse 63mut primer. We therefore hypothesize that only the H63D mutant allele is amplified in the multiplex PCR in the case of a H63D/S65C compound heterozygous genotype, resulting in a H63D homozygous genotype.

Starczynski et al. (3) found the same discrepant results in compound heterozygous H63D/S65C samples with the method of Stott et al. (1). Starczynski et al. (3) hypothesized, however, that the presence of the S65C-mutation leads to a failure of digestion and not to a failure of amplification because an undigested PCR product was produced in all S65C/H63D compound heterozygous samples. The failure of digestion occurred only in samples from S65C-heterozygous patients who were also heterozygous for H63D. In patients who were heterozygous for S65C and did not have H63D, complete digestion at the BbrPI site did occur. Although the S65C mutation is outside the BbrPI restriction site and is therefore not expected to interfere with the digestion, Starczynski et al. (3) theorized that methylation occurs and prevents digestion.

Recently we found a sample that produced only the C282Y PCR product and failed to produce the H63D PCR product. DNA sequencing results confirmed that this sample was S65C homozygous and H63D wild-type. This S65C homozygous genotype provides evidence that presence of the hemochromatosis S65C mutation leads to failure of amplification and not to failure of digestion.

Because it is assumed that the H63D and S65C mutations do not occur on the same chromosome (4), the risk for overestimation of H63D homozygotes should be taken into account when using the method of Stott et al. (1), which may lead to error in up to 2% of the samples (3). Either an alternative method can be considered, or every sample found to be H63D homozygous or "no-product" should be genotyped separately for the S65C mutation to exclude the presence of this mutation.

Grant funding/support: None declared. Financial disclosures: None declared.

References

2 error sources are quite different. This confusion seems to have taken place during the preparation of the report, in which the result of a proficiency survey was compared to the simulation. Because neither controls nor pooled samples are used in a proficiency survey, random patient interferences cannot be estimated. Thus, conclusions drawn from this comparison are suspect. Moreover, their alternative way to describe creatinine assay performance goals clearly leaves out random patient interferences [Table 1 in Myers et al. (1)].

The following is suggested as a more useful way to model assay performance:

\[ Y = \text{bias} + \text{total imprecision} + \text{random patient interferences} \]  

(1)

The only difference between Eq. 2 and Eq. 1 is that total imprecision and random patient interferences have been separated. In a previous letter published in Clinical Chemistry (2), we added a 4th term, random biases unrelated to interferences from a specific patient sample. It is simpler, however, to lump this term into total imprecision, which gives the same model described by Lawton et al. (3).

Assay performance goals should account for all 3 terms in Eq. 2. In setting these goals, average bias can be set at a low amount because, as stated by Myers et al. (1), manufacturers have a way of achieving low average bias through standardization. Thus, the majority of error can be allocated between imprecision and random patient interferences. Random patient interferences are also a known factor—their expected value would be zero for an assay with perfect analytical specificity—because Myers et al. (1) discuss analytical nonspecificity problems for several types of creatinine assays and recommend improvement, yet do not specify the magnitude of improvement needed. The specific magnitude of improvement needed could be calculated with the 3-term model described above.

Estimates for each of the 3 error sources can be generated by comparing field and reference creatinine measurements with a series of patient samples. Analysis is simplified because the concentration range of interest for glomerular filtration rate estimation is narrow. The average difference and the SD of differences (for which the difference is between the field and reference method for each patient sample) gives the 2 quantities in Eq. 1, with total imprecision limited to the time interval of the method comparison experiment. (To ensure that differences are largely due to the field method, the reference method should be replicated to minimize imprecision). If an independent estimate of total imprecision for the field creatinine assay for this time interval is available, the imprecision term from Eq. 1 can be separated into the 2 components expressed in Eq. 2.

Grant/funding support: None declared.  
Financial disclosures: None declared.

References


Jan S. Krouwer
Krouwer Consulting  
26 Parks Drive, Sherborn, MA 01770  
Fax 1-508-647-9380  
E-mail jan.krouwer@comcast.net

DOI: 10.1373/clinchem.2007.089763

The authors of the article cited above respond:

To the Editor:

Krouwer (1) makes a valid point that total error for a measurement procedure must include analytical nonspecificity influences (referred to as “random patient interferences” by Krouwer) as well as mean calibration bias and imprecision components. Fig. 3 in the report from the Laboratory Working Group (LWG) of the National Kidney Disease Education Program showed boundaries for combinations of bias and imprecision that would contribute no more than a 10% change in root mean square error for estimates of glomerular filtration rate calculated from a serum creatinine measurement (2). The emphasis in that report was on reducing the calibration bias and imprecision of creatinine measurement procedures to reduce the variability in estimates of glomerular filtration rate.

The LWG report reviewed information on specificity limitations of routine creatinine measurement procedures and recommended that in vitro diagnostics manufacturers address and reduce the influence of interfering substances in patient samples. No specific recommendations were provided, however, because no consensus was reached on what those recommendations should be, primarily because there was no recent literature describing specificity characteristics of current versions of routine measurement procedures for creatinine.

Evaluation of sample-specific interferences is challenging because it is necessary to measure creatinine with both routine and high-level reference measurement procedures for relatively large numbers of samples from both healthy volunteers and patients with disease. Gas-chromatography–isotope dilution mass spectrometry procedures have limited throughput; however, the availability of newer, higher throughput liquid chromatography–isotope dilution mass spectrometry procedures may facilitate evaluation of sample specific interferences.

The total error boundaries represented in Fig. 3 of the LWG report remain useful criteria for measurement procedure performance. Nonspecificity of the measurement proce-
Attributes

dure for the measurand cause a sample-specific bias. If a sample-specific bias exists, it would be incremental to any existing calibration bias, and if clinically significant, would cause the error for a particular sample to exceed the total error budget. Therefore, the same total-error budget as defined in the LWG report (2) can be applied to evaluate the performance of a serum creatinine measurement procedure when challenged with samples that contain known quantities of potentially interfering substances. Characterization of the specificity performance of routine serum creatinine measurement procedures relative to the total error goal will allow objective assessment of performance and will establish a basis for recommendations on improvement of specificity performance.

References


W. Greg Miller1*
Gary L. Myers2
John H. Eckfeldt3

1 Department of Pathology
Virginia Commonwealth University
Richmond, VA
2 Division of Laboratory Sciences
National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA
3 Department of Laboratory Medicine and Pathology, University of Minnesota
Minneapolis, MN

* Address correspondence to this author at: Department of Pathology, Virginia Commonwealth University, PO Box 980286, Richmond, VA 23298-0286. Fax 804-828-0375; e-mail gmiller@vcu.edu.

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

DOI: 10.1373/clinchem.2007.093567

Measurements of Free Hemoglobin and Hemolysis Index: EDTA- or Lithium-Heparinate Plasma?

There is no agreement as to the optimal sample type to be used for measurements of free hemoglobin (fHb) and the Hemolysis Index (HI). For comparison with the HI, we used the 2-wavelength method of Golf et al. (4) to measure fHb and not the more commonly used method of Harboe (5), because measurements at multiple wavelengths are not available for users on the Modular. Briefly, fHb was determined by using the difference of absorbances at 540 and 600 nm. A hemoglobin solution, prepared as described by Fairbanks et al. (2), was used as a calibrator at 1250 mg/L. The method detection limit was approximately 50 mg/L as determined by analysis of replicate measurements (n = 10) of calibrator dilutions and the 0 calibrator. At concentrations of 90, 300, 1250 and 9500 mg/L, intraassay imprecision values (CVs) for fHb were 9%, 5.6%, 1.3%, and 0.5%, respectively, and for HI were 15%, 3.6%, 0.6%, and 0.3%. Interassay CVs, determined by replicate measurements during 10 days at 90 and 1250 mg/L fHb concentrations, were 9.4% and 3.5% for fHb and 12.4% and 0.6% for HI, respectively. The values for fHb in our patients ranged from <50 to 794 mg/L in EDTA plasma and from <50 to 1120 mg/L in Li-heparinate plasma. The corresponding values for HI ranged from <50 to 760 (EDTA plasma) and <50 to 1070 mg/L (Li-heparinate plasma), respectively. In univariate ANOVA, no significant differences in mean (SD) fHb values were observed between specimens collected in EDTA [232 (179) mg/L] or heparin [254 (226) mg/L]. Similarly, mean (SD) HI did not differ between the 2 materials [153 (173) for EDTA and 173 (225) for Li-heparinate]. Correlation coefficients, R, between fHb and HI were 0.939 in EDTA plasma and 0.967 in Li-heparinate plasma. Difference plots (fHb – HI) for both plasma collections showed no correlation of difference with the mean of fHb and HI. Correlations between fHb and HI in EDTA plasma were substantiated in 200 additional samples (R = 0.900). In these samples, values for fHb and HI ranged from <50 to 6300 mg/L and <50 to 7030 mg/L, respectively. In samples with fHb values <900 mg/L, the correlation remained high (0.860). We therefore could not confirm with these sample collection tubes the statement that fHb may be 20-fold higher in EDTA plasma than in heparin plasma (1).

An effect of EDTA became apparent when we determined fHb and HI in blood from healthy volunteers collected with increasing EDTA concentrations. At EDTA concentrations 3 times the usual, both fHb and HI were >50% higher than values in EDTA plasma collected under standard conditions. In contrast, similar experiments with Li-heparinate plasma showed no dependence of fHb or HI on Li-heparinate concentrations in the range of 16–48 KIE/L whole blood. Thus EDTA influences fHb and HI in a concentration-dependent manner, but the effect can...
be disregarded when collection tubes are correctly filled.

We conclude that fHb and HI can be measured in blood collected either with heparin or EDTA provided that EDTA tubes are adequately filled. Because of the excellent agreement of values for fHb measurements and HI in the P-module of the Modular, HI may be sufficient in many clinical settings.

Financial disclosures: None declared.

References

Hemoglobin Hagley Park: A Novel (α82Ala→Thr) Substitution Identified in an Infant with Severe Hemolytic Anemia

To the Editor:
The identification of new hemoglobin mutations and the correlation of structural changes with potential pathology continue to provide insights into how normal structure and function are preserved in the tetrameric molecule.

Hematological investigation of a 6-week-old infant with severe hemolytic anemia revealed a decreased hemoglobin concentration of 53 g/L, a decreased hematocrit of 0.15, and a decreased erythrocyte count of \( \frac{1.8 \times 10^{12}}{L} \). In addition the infant had an increase in reticulocytes (\( \frac{322 \times 10^{12}}{L} \)) and bilirubin (96 μmol/L). Blood films showed a normochromic picture with irregularly shaped cells, elliptocytes, and erythrocyte fragments suggestive of an erythrocyte membrane defect. G6PD and pyruvate kinase concentrations were within reference intervals, and a presumptive diagnosis of hereditary elliptocytosis was made.

Cellulose acetate and citrate agar electrophoresis results were both within reference intervals, as was cation exchange chromatography on the Bio-Rad Variant β-thalassemia system. Examination of whole lysate by electrospray ionization mass spectrometry on a VG Platform (1) showed that the complement of β (15 867 Da), γ\( ^c \) (15 995 Da), and γ\( ^A \) (16 010 Da) chains were within reference intervals for the age of the infant. However, the valley between the Na and K adducts of the α chain (15 126 Da) contained a new component with a mass increase of 30–33 Da over the normal α chain (Fig. 1A). Reversed-phase HPLC on a C-4 Jupiter column (2) failed to separate out the new component, so DNA sequencing was used to identify the putative mutation.

The entire coding regions of both the hemoglobin alpha 1 and alpha 2 genes were individually amplified from genomic DNA using the primer pairs 5′-TGG AGG GTG GAG ACG TCC TG-3′ with 5′-CCA TGC TGGCAC GTT TCT GA-3′, and 5′-TGG AGG GTG GAG ACG TCC TG-3′ with 5′-CCA TTG TTG GCA CAT TCC GG-3′, respectively, and sequenced on an ABI 3130xl genetic analyzer with Big Dye Terminator v3.1 cycle sequencing chemistry according to the manufacturer’s recommendations. This process revealed heterozygosity for a GCC→ACC transition at codon 82 of the α1 gene, and we have named this new α82Ala→Thr variant Hb Hagley Park.

Unstable hemoglobinopathies are a well-recognized cause of hemolytic anemia, and the identification of this novel substitution raises the question as to whether it contributed to the hemolytic condition. This question could be answered by stability tests; however, insufficient blood (50 μL) was available for analysis and, in any case, the presence of substantial amounts of HbF, which registers a positive result in these tests, would confound the results. Family studies would also be expected to be informative; however, neither parent was available for analysis.

There are no published reports of any other mutation at position α82, the 3rd residue of the F helix. This short amphipathic helix contains the proximal histidine, and its inner hydrophobic surface makes direct contact with the heme plate. Mutations along this surface result in molecular instability and can cause mild hemolytic anemia (1–3). The Ala82 side chain is positioned between the inner and outer hydrophilic surface of the helix, and introduction of an additional CH\( _2 \)OH moiety could potentially destabilize the structure. However, alignment of 439 α-globin sequences from different species shows that residue α82 is only loosely conserved as alanine, and some 18 species, including mouse and rabbit, have a threonine residue at position α82. Threonine is also found at the F3 position of the human β chain, supporting the notion that its occurrence in the α chain should not cause any major distortion, at least of the helix.
We received a 2nd blood sample when the infant was 18 months old, and again this sample showed evidence of significant hemolysis, with decreased hemoglobin (60 g/L), hematocrit (0.18), and erythrocyte count \(2.4 \times 10^{12}\). The normochromic blood film showed pencil cells, some elliptocytes, and erythrocyte fragments. Mass spectrometry confirmed a complete switch to an adult pattern of hemoglobin synthesis, with no significant amount of \(\gamma\) chains detected; the abnormal 15157-Da \(\alpha\) chain was still present at a level of approximately 18\% (Fig. 1).

Isopropanol stability tests showed no precipitation at 30 min, and although a small amount of precipitate formed after the incubation was extended to 45 min, electrospray analysis showed no enrichment of the variant chain. This normal stability suggests that the mutation is benign; making it unlikely that it contributes to the hemolytic condition.

This case highlights the importance of isolating the potential pathological effect of novel hemoglobin mutations when they are identified in association with changes in hemoglobin concentrations. In this case it appears the hemolysis is due to the accompanying, but unrelated, erythrocyte membrane defect.

Grant support/funding: None declared.
Financial disclosures: None declared.
Acknowledgments: We gratefully acknowledge the assistance of Jaine Duncan and Vanessa Buchan.

References

Stephen O. Brennan1,2*
Tim Chan1
Michael Beard1
1 Canterbury Health Laboratories, Christchurch, New Zealand
2 Pathology Department Christchurch School of Medicine University of Otago Christchurch, New Zealand

* Address correspondence to this author at: PO Box 151, Canterbury Health Laboratories, Christchurch, New Zealand. Fax 64-3-3640545; e-mail steve.brennan@chmeds.ac.nz.

DOI: 10.1373/clinchem.2007.092262

Comparison of the Diagnostic Accuracy of BNP and NT-proBNP in Acute and Chronic Heart Failure

To the Editor:
I read with great interest the paper by Clerico et al. (1), which described a comparable diagnostic accuracy of brain natriuretic peptide (BNP) and the N-terminal part of the propeptide of BNP (NT-proBNP) in patients with heart failure. The authors are to be congratulated for addressing this important issue.

There are two details that concern me that might benefit from additional explanations from the authors. First, the paper cites an important study that compared both markers in elderly patients only (2). This patient subset is of major importance because diagnostic uncertainty seems to be highest in elderly patients (3). BNP was found to have significantly higher diagnostic accuracy than NT-proBNP (area under the curve 0.85 vs 0.80) in elderly patients. Unfortunately, in Table 2 and in Fig. 3, these data seem to be incorrectly displayed, indicating the opposite result (superiority of NT-proBNP). The authors may wish to correct this presentation of their data and recalculate their metaanalysis accordingly. Second, the expression “chronic heart failure” does not seem appropriate for the second part of the analysis, because the studies summarized in Table 1 and Fig. 2 tested both peptides in the detection of left ventricular systolic and/or diastolic dysfunction. Please note that chronic
heart failure must not be used interchangeably as a term for these conditions.

Grant/funding support: None declared.
Financial disclosures: I have received research support from Biosite, Brahms, Abbott, and Roche, and speaker’s honoraria from Abbott, Bayer, Biosite, Brahms, Dade Behring, and Roche.

References

Christian Mueller
Department of Internal Medicine
University Hospital Basel
Petersgraben 4, CH-4031
Basel, Switzerland
Fax 0041-61-2655353
E-mail chmueller@uhbs.ch.
DOI: 10.1373/clinchem.2007.091876

The authors of the article cited above respond:

To the Editor:
We are grateful to Dr. Christian Mueller for his constructive suggestions regarding our review (1). Actually, area under the curve (AUC) values of brain natriuretic peptide (BNP) and the N-terminal part of the propeptide of BNP (NT-proBNP) (0.85 and 0.80, respectively) assays related to the study by Ray et al. (2) were erroneously inverted, whereas sensitivity and specificity were correctly indicated in Table 2 of our review (1). Nevertheless, we confirm that the conclusions of our review were correct; they were based on the diagnostic odds ratios, which were derived from sensitivity and specificity values.

As suggested by Dr. Mueller, we recalculated the pooled AUC values by using the random-effects model according to the DerSimonian-Laird method, and we report the Forest plots for BNP and NT-proBNP assays in Fig. 1, A and B, respectively. Indeed, the calculated AUC values for NT-proBNP [0.8615 (0.8144–0.9007)] and BNP [0.8477 (0.7909–0.9045)] assays were very similar to those reported in our review (1), and statistical reappraisal confirmed that the AUCs are not significantly different between BNP and NT-proBNP assays for diagnosis of acute heart failure.

With regards to Mueller’s challenge of our definition of “chronic heart failure” regarding the studies reported in Table 1 and Fig. 2 of our review (1), we note that all of these studies aimed to detect structural myocardial impairment leading to
left ventricular systolic and/or diastolic dysfunction either in asymptomatic or symptomatic patients, that is, stage B or C of the definition of heart failure, according to the classification of the American Heart Association/American College of Cardiology task force for the diagnosis and management of chronic heart failure (3).

One important finding of our metaanalysis (1) is represented by the heterogeneity of data published on the comparison of diagnostic accuracies of BNP and NT-proBNP, especially for studies concerning chronic heart failure. Evidently, the large variability of these data may be decreased if statistical analyses are performed separately for specific clinical conditions (e.g., systolic or diastolic dysfunction), as suggested by Mueller. However, this approach may not be feasible at this time, considering the small number of studies comparing the diagnostic accuracies of BNP and NT-proBNP. In conclusion, further studies are needed to evaluate differences in diagnostic accuracy of BNP and NT-proBNP assay in patients with heart failure.

Grant/funding support: None declared. Financial disclosures: None declared.

References

Aldo Clerico1,2,*
Marianna Fontana1
Luc Zyw1
Claudio Passino1,2
Michele Emdin1
1 Consiglio Nazionale delle Ricerche Institute of Clinical Physiology Cardiovascular Medicine Department
Cardiovascular Endocrinology Laboratory
Pisa, Italy
2 Scuola Superiore S. Anna
Pisa, Italy

*Address correspondence to this author at: Consiglio Nazionale delle Ricerche Institute of Clinical Physiology Via Trieste 41, 56126 Pisa, Italy. Fax 011-39-6985; e-mail clerico@cnr.it.

DOI: 10.1373/clinchem.2007.092924

Genetic Factors for Warfarin Dose Prediction

To the Editor:
Warfarin, a commonly prescribed anticoagulant drug used to prevent thrombosis, has a narrow therapeutic range, and small dose variations may result in hemorrhagic or thrombotic complications. The 2 key enzymes in the metabolism of warfarin are cytochrome P450 (CYP) 2C9 (CYP2C9 gene) and the C1 subunit of the vitamin K 2,3 epoxide reductase complex (VKORC1 gene). CYP2C9 accounts for up to 85% of the metabolism of the pharmacologically more potent S-warfarin enantiomer, and VKORC1, the 2nd key enzyme in warfarin metabolism, is responsible for recycling reduced vitamin K. In their recent article, Zhu et al. (1) concluded that genotyping both VKORC1 and CYP2C9, in conjunction with patient physical characteristics, facilitated more precise estimation of warfarin dose and thus improved the efficiency of the dosage titration process. This conclusion was supported by the evidence that VKORC1 and CYP2C9 genotypes, age, sex, and body weight accounted for 61% of the variance in warfarin daily maintenance dose. The findings of Zhu et al. (1) are consistent with earlier findings of Caldwell et al. (2), which demonstrated that CYP2C9 and VKORC1 each contribute substantially to dose variability and, together with clinical factors, explain 56% of the individual variability in stable warfarin dose. Taken together, these studies support the hypothesis that the formulation of dense genetic maps on the basis of single-nucleotide polymorphisms is an important approach to elucidating polygenic traits of drug response and, in combination with appropriate nongenetic factors, might help to define a warfarin dose-response phenotype.

We wish to point out additional aspects in the challenging endeavor to individualize warfarin therapy. First, the single contribution of the VKORC1 and CYP2C9 gene polymorphisms accounts for approximately 27% and 22% of the variability of maintenance dosage, respectively (1). Therefore, the aggregate variability of warfarin dosage explained by these 2 genes approaches 50% provided that other nongenetic factors are maintained fairly steadily throughout the titration period. Second, synthetic preservative substances, such as benzethonium chloride, are potent inhibitors of CYP2C9 activity in vitro, producing unpredictable effects of warfarin therapy (3). Third, the effectiveness of therapy is affected by numerous variables including drug interactions, illnesses, dietary or gastrointestinal features that alter the bioavailability of vitamin K, and physiologic variables that modify the synthetic or metabolic fate of the vitamin K-dependent coagulation factors. Thus, genetic algorithms might be efficient only when all these variables are stable (4). Finally, CYP2C9 genotyping may not be useful in African-Americans or as a marker of long-term over-anticoagulation once a stable dose is reached (5).

Although individualization of therapy based on genetic factors has great potential to improve efficiency and safety of the dosage titration process, genetic variability explains only a large fraction, not all, of the interindi
individual variation in warfarin dosage. Prospective studies that incorporate both gene testing and a variety of ethnic, clinical, pharmacological, and environmental variables, along with age, sex, and body weight, will be required to demonstrate the real safety, cost-effectiveness, and feasibility of individualized dosing regimens according to the statistical models for warfarin dose calculation.

Grant/funding support: None declared. Financial disclosures: None declared.

References
2. Caldwell MD, Berg RL, Zhang KQ, Glurich I, Brandin H, Myrberg O, Rundlof T, Arvidsson AK, Thor at: Istituto di Chimica e Microscopica Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università degli Studi di Verona, Ospedale Policlinico Verona, Italy. Fax 0039-045-8201889; e-mail G.B. Rossi, Piazzale Scuro, 10, 37134 Verona, Italy. Fax 0039-045-8201889; e-mail gianluca.salvagno@univr.it.

Giuseppe Lippi*  
Gian Luca Salvagno  
Gian Cesare Guidi

Sezione di Chimica Clinica  
Dipartimento di Scienze Morfologico-Biomediche  
Università degli Studi di Verona  
Verona, Italy

*Address correspondence to this author at: Istituto di Chimica e Microscopica Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università degli Studi di Verona, Ospedale Policlinico G.B. Rossi, Piazzale Scuro, 10, 37134 Verona, Italy. Fax 0039-045-8201889; e-mail ulippi@tin.it.

Evaluation of Analytical Performance of the Siemens ADVIA TnI Ultra Immunoassay

To the Editor:
In light of recommendations on the quality (1) and clinical use (2) of troponin assays, we evaluated the analytical performance of the ADVIA Centaur and ADVIA CP® platforms (TnI-Ultra, Siemens Medical Solutions Diagnostics SrL) for measurement of cardiac troponin I (cTnI). The chemiluminescent TnI-Ultra method uses 2 monoclonal capture antibodies directed to epitopes at amino acids 41–49 and 87–91 and a tracer polyclonal goat antibody labeled with acridinium ester, directed against amino acids 27–40 (1, 3, 4).

Two clinical laboratories participated in the study: the CNR Institute of Physiology in Pisa and the San Bor tolo Hospital in Vicenza.

The limit of detection (limit of the blank) for the TnI-Ultra method was calculated as the concentration corresponding to a signal of 3 SD above the mean of 60 replicates (obtained in 4 different runs and pooled together) for the calibrator in which cTnI was absent; a mean cTnI concentration of 0.015 (0.018) µg/L, median 0.012 µg/L, range 0–0.196 µg/L, n = 204 for men; 0.009 (0.014) µg/L, 0.008 µg/L, 0–0.130 µg/L, n = 214 for women; P < 0.0001 by Mann–Whitney U-test). We found that both sex (as a dummy independent variable with F = 1 and M = 2) and age (as a continuous independent variable) independently contributed to the regression with cTnI (as a dependent variable after log transformation of original values) by using a stepwise multiple regression analysis (log cTnI = −3.164 + 0.456 sex + 0.007 age; P < 0.0001, F-value = 71.962, R = 0.508, n = 416).

A close linear relationship was found between cTnI values measured by ADVIA Tnl-Ultra with the Centaur CP® platform and the Access AccuTnI® method on the UniCell® DxI 800 platform (Beckman Coulter) in 318 plasma samples of 155 apparently healthy individuals and 163 cardiac patients (ADVIA = 0.016 ± 1.272 Access; R = 0.936). The Tnl-Ultra method showed higher cTnI values than the Access AccuTnI
method (on average by 22.0%; \( P <0.0001 \) by Wilcoxon signed-rank test) and based on the 99th percentile values for each assay, 9 discords were found between assays for values within the reference interval vs increased values.

The ADVIA TnI-Ultra method showed no interference from dilutions with plasma samples that contained high concentration of triglycerides (6.6 g/L, final dilution 1:128; \( y = -0.044 + 0.14x, n = 8, R = 0.99 \)) or hemoglobin (1.47 g/L, final dilution 1:4996; \( y = 0.04 + 0.060x, n = 13, R = 0.99 \)). No apparent negative interference was seen in 58 patients with symptomatic rheumatoid arthritis [10 men and 48 women, mean (SD) age 60.8 (10.2) years] with a mean concentration of rheumatoid factor of 189.6 kIU/L (range 40–1280 kIU/L), because the mean (SD) cTnI concentration of rheumatoid arthritis 

The present study indicates that the ADVIA TnI-Ultra method meets the quality specifications recommended by NACB and IFCC Committee for the Standardization of Cardiac Damage (5).

Grant/funding support: None declared. Financial disclosures: None declared.

References


Midtrimester Amniotic Fluid Adiponectin in Normal Pregnancy

To the Editor:

Adiponectin is an adipose tissue-derived protein with important metabolic effects and a strong correlation with insulin sensitivity. In pregnancy there is a progressive increase of insulin resistance, whereas plasma adiponectin concentrations decrease in the 2nd half of gestation (1). In contrast, cord plasma adiponectin concentrations increase throughout gestation (2). Nothing is known about the concentration, origin, or role of amniotic fluid adiponectin, particularly in relation to amniotic insulin. Therefore we evaluated adiponectin and insulin concentrations in the midtrimester amniotic fluid of women with normal pregnancies.

Beginning January 1, 2006, we selected the first 50 pregnant women who underwent a midtrimester amniocentesis for prenatal diagnosis (15–18 weeks gestation) and were found to have a normal pregnancy, defined as an uncomplicated pregnancy with full-term delivery of an infant of adequate size for gestational age. The study was approved by the institutional review board, and all women gave written informed consent.

Amniotic fluid samples were obtained by transabdominal amniocentesis and collected in 15 mL dry tubes. All samples were free of blood contamination, as estimated by microscopic inspection. The samples were immediately centrifuged for 10 min at 3000 g and stored at –70 °C.

The intra- and interassay imprecision (CVs) for adiponectin at a concentration of 15.0 \( \mu \)g/L were 3.3% and 5.5%, respectively. The intra- and interassay imprecision values (CVs) for insulin at a concentration of 4.0 \( \mu \)IU/L were 3.3% and 5.6%, respectively.

Amniotic and plasma adiponectin and amniotic insulin concentrations are presented as the median and the 25th–75th percentile range; all other variables are presented as the mean (SD). The Mann-Whitney U-test was used to compare continuous variables between the 2 groups. Univariate correlations between amniotic fluid adiponectin and all the other variables were assessed using the Spearman test. The statistical analysis was performed using SPSS 13.0 (SPSS Inc.). All tests were 2-sided; a \( P \) value <0.05 was considered statistically significant.

The clinical characteristics of pregnant women are reported in Table 1. Median adiponectin amniotic fluid values were 26.8 (13.9–37.3) \( \mu \)g/L, but when we dichotomized for sex, there was a significant difference (\( P = 0.01 \)) between female 34.8 (18.2–48.7) \( \mu \)g/L and male fetuses 18.2 (13.4–26.8) \( \mu \)g/L. Univariate analysis

Concetta Prontera
Antonio Fortunato
Simona Storti
Antonella Mercuri
Giovanni Longombardo
Gian Carlo Zucchelli
Michele Emdin
Aldo Clerico

1 CNR Institute of Clinical Physiology of Pisa
Pisa, Italy

2 Clinical Chemistry Laboratory
San Bortolo Hospital
Vicenza, Italy

3 Clinical Immunology Unit
Department of Internal Medicine
University of Pisa
Pisa, Italy

4 Scuola Superiore Sant’Anna,
Pisa, Italy

* Address correspondence to this author at: Laboratory of Cardiovascular Endocrinology and Cell Biology, C.N.R. Institute of Clinical Physiology, Via Trieste 41, 56126 Pisa, Italy. Fax 39-0585-493601; e-mail clerico@ifc.cnr.it.

DOI: 10.1373/clinchem.2007.089995
showed a positive correlation between amniotic adiponectin and insulin \( (r = 0.47, P = 0.001) \) and also with gestational age at amniocentesis \( (r = 0.31, P = 0.03) \); in contrast amniotic fluid adiponectin did not correlate with plasma adiponectin \( (r = 0.07, P = 0.6) \), with maternal age \( (r = 0.01, P = 0.9) \), with maternal body mass index (BMI) \( (r = -0.07, P = 0.5) \), with gestational age at delivery \( (r = -0.15, P = 0.1) \), or with birth weight \( (r = 0.05, P = 0.7) \).

To our knowledge, this is the first study that deals with amniotic fluid concentrations in normal pregnancy at the time of midtrimester amniocentesis. Our results suggest that amniotic adiponectin might be of fetal origin. Maternal origin seems unlikely because there was no correlation between plasma and amniotic adiponectin or between amniotic adiponectin and maternal age or BMI. In addition, Corbetta et al. \( (3) \) found no adiponectin in placental tissue. Thus, a fetal origin of amniotic adiponectin appears likely; insulin production by the human fetus from 11 weeks of gestational age has been demonstrated in an experimental study \( (4) \), and we have shown a strong correlation between amniotic adiponectin and amniotic insulin. Furthermore, the statistically significant difference between female and male fetuses supports the theory that amniotic adiponectin is of fetal origin. Clinical and experimental studies have demonstrated that the sex dimorphism of adiponectin concentrations might be caused by testosterone-induced inhibition of its secretion from adipocytes \( (5) \).

In conclusion, based on our study of 50 normal pregnancies at the time of midtrimester amniocentesis, we suggest that adiponectin found in amniotic fluid is of fetal origin.

**References**


Giovanni Baviera Francesco Corrado Corrado Dugo Maria L. Cannata Silvia Russo D’Anna Rosario

Department of Gynecology, Obstetrics and Reproductive Medicine
University of Messina
Messina, Italy

* Address correspondence to this author at: University of Messina, via setajoli n. 15, 98121 Messina, Italy. Fax 30 90 692021; e-mail rosariodanna@tin.it.

DOI: 10.1373/clinchem.2007.088542

**Correction**

In the article entitled “Diagnosis of α1-Antitrypsin Deficiency: An Algorithm of Genotyping, Phenotyping, and Quantitation” by Melissa R. Snyder, Jerry A. Katzmann, Malinda L. Butz, Ping Yang, D. Brian Dawson, Kevin C. Halling, W. Edward Highsmith, and Stephen N. Thibodeau (Clin Chem 2006;52:2236–42; DOI: 10.1373/clinchem.2006.072991), the name of one of the coauthors of the study was inadvertently omitted from the author list. The correct author list should read: Melissa R. Snyder, Jerry A. Katzmann, Malinda L. Butz, Carmen Wiley, Ping Yang, D. Brian Dawson, Kevin C. Halling, W. Edward Highsmith, and Stephen N. Thibodeau.

Dr. Carmen Wiley’s current affiliation is Division of Laboratory Medicine, Marshfield Clinic, Marshfield, WI 54449.

The authors regret the oversight. Dr. Wiley’s contributions to the manuscript occurred at the initiation of the study and included participation in study design and assay development.

Dr. Wiley has read and agrees with the content and conclusions in the published manuscript.

DOI: 20.1373/clinchem.2007.092346

**Table 1. Clinical characteristics of the study group.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age</td>
<td>32.5 (3.1)</td>
</tr>
<tr>
<td>Maternal BMI</td>
<td>23.7 (3.4)</td>
</tr>
<tr>
<td>Gestational age at amniocentesis (days)</td>
<td>120 (6)</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>38.8 (1.5)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3199 (317)</td>
</tr>
<tr>
<td>Amniotic adiponectin (μg/L)</td>
<td>26.8 (13.9–37.3)</td>
</tr>
<tr>
<td>Plasma adiponectin (mg/L)</td>
<td>12.7 (6.6–16.1)</td>
</tr>
<tr>
<td>Amniotic insulin (mIU/L)</td>
<td>3.8 (1.6–6.8)</td>
</tr>
</tbody>
</table>

**Grant/funding support:** This study has been funded by the University of Messina, Italy.