published own observations); and (*c*) 6-TGNs are considered the active thiopurine metabolites, whereas the role of 6-methyl-mercaptopurine for therapeutic drug monitoring is still a matter of debate (except for compliance).

We agree that oxidation of the thiopurine thiol moiety is also a critical point in the analysis of thionucleotide metabolites. This point certainly requires more attention when method harmonization is intended. Although we concentrated on the hydrolysis step, we addressed the question of oxidation in our investigation by including a set of experiments on the effect of increasing concentrations of the antioxidant dithiothreitol on 6-thio-guaninc (6-TG) recovery. Higher dithiothreitol concentrations were associated with enhanced 6-TG recovery *(1)*.

The aim of our publication was to draw attention to method-dependent therapeutic ranges that preclude a comparison of results from clinical studies based on different methods. A considerable number of methods for the measurement of 6-TGNs have been published, and new methods or modifications of existing assays are still being generated. As discussed in our report, these methods differ in several analytical steps, including the choice of matrix, the analytical procedure, and the way results are reported. However, these new or modified methods have usually not been compared with an established procedure. In addition, there is unfortunately no external quality-control system available that could provide information about comparability of results. The major problems for a real standardization include the lack of a reference method and an approved and appropriate reference material such as a 6-TGN standard. We also agree that the matter is complicated by the fact that the product of drug metabolism in vivo is a mixture of mono-, di-, and triphosphates. However, according to our experience, many physicians involved in the care of patients receiving thiopurine drugs, as well as laboratories involved in therapeutic drug monitoring of these patients, do not have sufficient information about the method dependency of the reported 6-TGN concentrations and may be misled when comparing individual values with therapeutic ranges derived from the literature. To improve the comparability between the different methods, it is therefore important that efforts be made to find a consensus on the analytical conditions as well as the format of result reporting.

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Natriuretic Peptides (NPs): Automated Electrochemiluminescent Immunoassay for N-Terminal pro-BNP Compared with IRMAs for ANP and BNP in Heart Failure Patients and Healthy Individuals

To the Editor:

We evaluated the performance and diagnostic accuracy of an electrochemiluminescence immunoassay (ECLIA) method for N-terminal pro-B-type natriuretic peptide (NT-proBNP) in healthy persons and in patients with cardiac disease, and then compared the results obtained with the ECLIA method with results from IRMA methods for BNP and atrial natriuretic peptide (ANP).

We studied 58 healthy individuals [mean (SD) age, 58 (8) years; 19 women and 39 men] and 148 consecutive patients [mean age, 64 (13) years; range, 20–80 years; 47 women and 101 men] with cardiomyopathy, admitted to the Department of Cardiovascular Medicine of our Institute. The study was done from November 2001 to October 2002. All healthy participants were nonobese, normotensive, and free from acute diseases, and all denied the use of any drug during the 4 weeks before the study. All had normal values for the main plasma indices and nonpathologic erythrocyte and leukocyte counts and urine analysis. In all of the participants, a complete cardiologic examination, including electrocardiogram and echocardiographic investigation (left ventricular ejection fraction >55%), was performed; in patients >50 years of age, an effort stress test was performed to exclude asymptomatic heart disease. Cardiac morphology and function were assessed in all patients by Doppler echocardiography, radionuclide ventriculography, or cardiac catheterization, when needed. Primary dilated cardiomyopathy was found in 95 patients, whereas the other 53 patients suffered from secondary cardiomyopathy; of these, 38 had ischemic cardiomyopathy. A total of 22 patients were in functional New York Heart Association (NYHA) class I, 72 in class II, and 54 in class III-IV; the mean (SD) left ventricular ejection fraction was 31.4 (9.6)%.

NT-proBNP was measured by a fully automated "sandwich" ECLIA method using an Elecsys® 2010 analyzer (Roche Diagnostics). This ECLIA is based on two polyclonal antibodies: a biotinylated antibody and a ruthenium derivative-labeled antibody. Total duration of assay was 18 min. Plasma ANP and BNP

were measured with two-site IRMA methods, previously set up in our laboratory, as described elsewhere in detail *(1, 2)*. We assayed blood samples (10 mL), collected into ice-chilled disposable polypropylene tubes containing aprotinin (500 kIU/mL of plasma) and EDTA (1 g/L of plasma), in such a way as to use the same samples for all three assays, even if the addition of plasma protease inhibitors (such as aprotinin) was not necessary for NT-proBNP assay. Plasma samples were rapidly separated by centrifugation for 15 min at 4 °C, and then were frozen and stored at −20 °C in 1-mL aliquots in polypropylene tubes until assay.

Within-run and total imprecisions were tested following the guideline approved by NCCLS *(3)* by repeatedly measuring two plasma samples on 20 different working days with NT-proBNP concentrations in the upper part of the reference interval (e.g., 103.8 ng/L) and the lower part of abnormal values (e.g., 601.7 ng/L; typical of patients with only moderate heart failure), in such a way as to better evaluate the imprecision in the discrimination zone between healthy individuals and patients with cardiac disease. Within-run imprecisions (CV) were 1.7% and 1.8%, and total imprecisions were 4.0% and 3.8%, for the two samples, respectively. These data indicate that ECLIA for NT-proBNP measures peptide concentrations in the discrimination zone between healthy participants and patients with cardiac disease with an imprecision better than that of ANP and BNP assays *(1, 2)*.

Peptide concentrations of patients with cardiomyopathy were much higher than those of healthy participants and progressively increased with disease severity (as measured: in healthy participants, 51.6 (34.6) ng/L; in NYHA class I, 367.0 (349.9) ng/L; NYHA class II, 1376.4 (1590.4) ng/L; NYHA class III, 5297.9 (6373.6) ng/L; NYHA class IV, 8421.1 (9231.1) ng/L). Moreover, we tested and compared the different degrees of diagnostic accuracy for ANP, BNP, and NT-proBNP assays in discriminating between the group of healthy participants and the group of patients with heart failure by ROC analysis using the "bootstrap percentile method" with 1000 bootstrap replications (Table 1). Our data indicate that NT-proBNP assay performance is better than that of both ANP and BNP assays in discriminating diseased from healthy individuals. In particular, the better performance of NT-proBNP ECLIA is more evident when patients with only mild heart failure (NYHA class I and II) are considered in the ROC analysis (Table 1). Of course, this finding is of great clinical relevance when considering that NT-proBNP could be used as a screening test for the diagnosis of chronic heart failure. However, it is important to note that a recent report *(4)* indicated pitfalls in community screening for left ventricular dysfunction when IRMA methods were used for the assay of BNP and NT-proANP in asymptomatic individuals, particularly in women.

Our findings cannot indicate whether the improved clinical performance of NT-proBNP compared with the two other cardiac natriuretic hormone (CNH) assays is a result of its better assay precision or a true larger separation of NT-proBNP distribution values in healthy and patient populations than those of ANP and BNP. To address this important issue, we need to compare the performance of the NT-proBNP ECLIA method with that of a new generation of BNP assays *(5, 6)*. In this regard, a previous study *(7)*, which used a different immunoassay for NT-proBNP, showed no significant

Table 1. ROC analysis.[a]

	AUC[b]	95% CI AUC	*P*	Optimal cutoff, ng/L	Sensitivity (at cutoff), %	Specificity (at cutoff), %
Healthy individuals (n = 58) vs all patients with heart failure (n = 148)[c]						
ANP	0.862	0.809–0.908	≪0.001	27.53	74	96
BNP	0.922	0.884–0.954	≪0.001	71.15	77	96
NT-proBNP	0.957	0.925–0.981	≪0.001	2425.7	91	95
Healthy individuals (n = 58) vs patients with mild heart failure (NYHA class I–II; n = 94)[d]						
ANP	0.789	0.708–0.859	≪0.001	20.28	63	96
BNP	0.881	0.819–0.935	≪0.001	116.24	74	90
NT-proBNP	0.933	0.88–0.971	≪0.001	641.17	88	93
Healthy individuals (n = 58) vs patients with severe heart failure (NYHA class III–IV; n = 54)[e]						
ANP	0.989	0.974–0.998	≪0.001	15.90	92	93
BNP	0.922	0.974–1.000	≪0.001	36.92	96	98
NT-proBNP	0.957	0.993–1.000	≪0.001	618.81	96	98

[a] Ref. *(10)*.

[b] AUC, area under the curve; CI, confidence interval.

[c] AUC comparisons: ANP vs BNP, $P = 0.037$; ANP vs proBNP, $P \ll 0.0001$; BNP vs proBNP, $P \ll 0.0001$.

[d] AUC comparisons: ANP vs BNP, $P = 0.028$; ANP vs proBNP, $P \ll 0.0001$; BNP vs proBNP, $P \ll 0.0001$.

[e] AUC comparisons: ANP vs BNP, $P = 0.72$ (not significant); ANP vs proBNP, $P = 0.078$ (not significant); BNP vs proBNP, $P = 0.04$.

differences from the clinical results respectively obtained with NT-proBNP and BNP assays, thus suggesting that the performance of the immunoassays used may a very crucial point in determining the results of a clinical study comparing different CNH assays.

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Biological Variation of N-Terminal Pro-Brain Natriuretic Peptide in Healthy Individuals

To the Editor:

Brain natriuretic peptide (BNP) and its N-terminal prohormone (NT-proBNP) fragment have been shown to be effective in diagnosing left ventricular dysfunction *(1, 2)*, and in particular, they have a strong negative predictive value *(3)*. NT-proBNP and the hormone are secreted on an equimolar basis, but NT-proBNP lacks a clearance receptor. It therefore has a longer half-life in serum than the active hormone does, and its circulating concentration is believed to be less influenced by the conditions under which the blood sample is taken.

Information on the biological variation of NT-proBNP is not available; this is limiting because the clinical utility of laboratory data can be affected by physiologic variation *(4)*. Here we report the results of a study to determine the biological variability of NT-proBNP.

Five blood specimens were collected from each of 16 apparently healthy laboratory workers (5 men and 11 women; age range, 43–62 years) twice a week (Tuesdays and Fridays) over a 17-day period. None of the workers smoked, took any medication, or consumed substantial quantities of alcohol. In accordance with Helsinki Declaration II, the design and execution of the experiment were explained thoroughly to the participants, and informed consent was obtained. Blood was collected under standardized conditions to minimize sources of preanalytic variation. After an overnight fast, a blood specimen was taken by conventional venipuncture between 0800 and 0900 with the volunteers in the sitting position, avoiding venous stasis. All samples were drawn by the same phlebotomist, allowed to clot, and then centrifuged at 3000*g* for 15 min at room temperature within 1 h of collection. Sera were separated and stored at −70 °C until analysis. It has been documented that the N-terminal peptide can be safely stored frozen at −20 and −80 °C for at least 3 months *(5)*.

At the end of the collection period, all frozen samples were thawed, mixed, and centrifuged for analysis in a single run in duplicate. NT-proBNP concentrations were determined by an electrochemiluminescence sandwich immunoassay (Roche Diagnostics). The assay was performed on an Elecsys System 2010 by the same analyst, who followed the assay manufacturer's recommendations. After exclusion of one outlier and logarithmic transformation of the data (required because of the skewed distributions of the NT-proBNP data), the analytical (CV_A) and intra- (CV_I) and interindividual (CV_G) components of variation were calculated by nested ANOVA. We also calculated the critical difference for significant changes in serial results ($P < 0.05$), the index of individuality, the number of specimens required to estimate the homeostatic setpoint of an individual (within ± 10% with a confidence of 95%), and the desirable quality specifications for imprecision (I), bias (B), and total error (TE), which were calculated using the formulas: $I < 0.5CV_I$; $B < 0.25\ (CV_I^2 + CV_G^2)^{1/2}$; and $TE < 1.65\ I + B$ ($\alpha < 0.05$). The results are reported in Table 1.

Minor, not statistically significant differences ($P = 0.87$), were observed between genders and were attribut-

Table 1. Mean values; estimated mean analytical (CV_A), intraindividual (CV_I), and interindividual (CV_G) variation; and derived indices for serum NT-proBNP.

						Desirable quality specifications				
Group	Mean, pmol/L	CV_A, %	CV_I, %	CV_G, %	II[a]	Imprecision, %	Bias, %	Total error, %	CD, %	No. of specimens
All	8.37	2.7	9.1	14	0.64	4.6	4.22	11.72	26.33	3
Men	9.42	1.1	6.5	16	0.41	3.2	4.29	9.65	18.18	2
Women	7.98	3.1	10	14	0.71	5.0	4.32	12.57	29.04	4

[a] II, index of individuality; CD, critical difference.