

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

References

1. Clerico A, Iervasi G, Del Chicca MG, Maffei S, Berti S, Sabatino L, et al. Analytical performance and clinical usefulness of a commercially available IRMA kit for the measurement of atrial natriuretic peptide in patients with heart failure. *Clin Chem* 1996;42:1627-33.
2. Del Ry S, Clerico A, Giannesi D, Andreassi MG, Caprioli R, Iacone MR, et al. Measurement of brain natriuretic peptide in plasma samples and cardiac tissue extracts by means of an IRMA method. *Scand J Clin Lab Invest* 2000;60:81-90.
3. National Committee for Clinical Laboratory Standards. NCCLS Guideline EP5-A. Evaluation of precision performance of clinical chemistry devices; approved guideline. NCCLS, 1999;19:1-50.
4. Vasan RS, Benjamin EJ, Larson MG, Leip EP, Wang TJ, Wilson PW, et al. Plasma natriuretic peptides for community screening for left ventricular hypertrophy and systolic dysfunction. *JAMA* 2002;288:1252-9.
5. Bluestein BI, Belenky A, Lin S, Despres N, Vajdi M, Armstrong G. Development of an automated test for B-type natriuretic peptide (BNP) as an aid in the diagnosis and evaluation of congestive heart failure on the Bayer ADVIA Centaur chemiluminescent system [Abstract]. *Clin Chem* 2002;48(Suppl 6):A85.
6. Kelly PM, Gaston S, Mackay R, Arthur K, Taylor V, Shih J, et al. A novel assay for the measurement of plasma B-type natriuretic peptide by an AxSYM microparticle based immunoassay with use of stable liquid calibrators [Abstract]. *Clin Chem* 2002;48(Suppl 6):A94.
7. Hammerer-Lercher A, Neubauer E, Muller S, Pachinger O, Puschendorf B, Mair J. Head-to-head comparison of N-terminal pro-brain natriuretic peptide, brain natriuretic peptide and N-terminal pro-atrial natriuretic peptide in diagnosing left ventricular dysfunction. *Clin Chim Acta* 2001;310:193-7.

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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 3000g 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 $\pm 10\%$ 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.

Group	Mean, pmol/L	CV_A , %	CV_I , %	CV_G , %	II ^a	Desirable quality specifications			CD, %	No. of specimens
						Imprecision, %	Bias, %	Total error, %		
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.

able to the lower CV_I in the men. The quality specifications for assay imprecision ($CV_A < 0.5CV_I$) were widely fulfilled. The individuality index was close to 0.6, indicating that an individual's results are more useful as reference values than are population-based data when the results are used in monitoring. Finally, serial results for pro-BNP must change by $>26\%$ before significance can be claimed.

References

1. Clerico A. Pathophysiological and clinical relevance of circulating levels of cardiac natriuretic hormones: are they merely markers of cardiac disease? *Clin Chem Lab Med* 2002;40:752–60.
2. Krishnaswamy P, Lubien E, Clopton P, Koon J, Kazanegra R, Wanner E, et al. Utility of B-natriuretic peptide levels in identifying patients with left ventricular systolic or diastolic dysfunction. *Am J Med* 2001;111:274–9.
3. Tabbibizar R, Maisel A. The impact of B-type natriuretic peptide levels on the diagnoses and management of congestive heart failure [Review]. *Curr Opin Cardiol* 2002;17:340–5.
4. Fraser CG. Biological variation: from principles to practice. Washington: AACCC Press, 2001: 151pp.
5. Hunt PJ, Richards AM, Nicholls MG, Yandle TG, Doughty RN, Espiner EA. Immunoreactive amino-terminal pro-brain natriuretic peptide (NT-PROBNP): a new marker of cardiac impairment. *Clin Endocrinol* 1997;47:287–96.

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PCR-based Detection of CYP21 Deletions

To the Editor:

We read with interest the Technical Brief by Lee et al. (1), in which the authors describe a novel method to detect *C4-CYP21* deletions in patients with steroid 21-hydroxylase deficiency. Such deletions result from an unequal crossover in the RCCX module (*RP-C4-CYP21-TNX*) on chromosome 6. In most cases, chromosome 6 carries two RCCX modules, one with a *CYP21P* (*CYP21A1P*) pseudogene and a truncated *XA* pseudogene, and one with a functional *CYP21* (*CYP21A2*) gene (encoding steroid 21-hydroxylase) and a functional *TNXB* gene (encoding tenascin-X). Meiotic misalignment and recombination may occur at several locations and create a chromosome with a single chimeric RCCX module. The PCR described by Lee et al. uses one primer in the 5' flanking sequence of *CYP21* and *CYP21P* (2), whereas the other primer is positioned in a 120-bp sequence of *TNXB* that is not present in the *XA* pseudogene (3). Although this PCR is indeed suitable for the detection of chimeric *CYP21P/CYP21* genes, it would fail to detect any RCCX chimera in which the pseudogene-like region includes the 120-bp deletion of *XA* (4), as illustrated in Fig. 1.

Lee et al. (1) successfully characterized 18 patients by this method, finding three categories of *CYP21P/CYP21* chimeras. Therefore, *XA/TNXB* chimeras may be rare in the Chinese population they studied. In The Netherlands, however, such hybrids are common (5, 6): in our patient group, the PCR would have yielded no product in four of nine chimeric RCCX modules on bimodular chromosomes (6) as well as in a recently described de novo deletion (7). Thus, this method fails to detect all *CYP21* deletions.

To amend this problem, we recommend that the *TNXB*-specific primer be positioned beyond the RCCX duplication boundary, in the nonduplicated area of *TNXB* (see Fig. 1). This will produce three additional *TaqI*

fragments, but these are smaller than 1 kb and should not interfere with the agarose gel separation shown in Fig. 1D of the Technical Brief by Lee et al. (1). In addition, *CYP21P/CYP21* chimeras would then produce a 2.5-kb *TaqI* fragment and could be readily distinguished from *XA/TNXB* chimeras, which would produce a 2.4-kb *TaqI* fragment.

Southern blotting remains the established approach for comprehensive analysis of this highly complex and variable region of the human genome. Genomic *TaqI* digestion coupled with cohybridization with *CYP21*, *TNX*, and *C4* probes provides direct information about these three genes and alerts the investigator to uncommon configurations that require further analysis by long-range restriction mapping (8, 9). Although we recognize the benefits of rapid nonradioactive detection methods, especially for diagnostic purposes, careful evaluation of such methods is necessary, notably if the method is recommended for general use and not limited to the population for which it was originally designed.

References

1. Lee HH, Chang SF, Lee YJ, Raskin S, Lin SJ, Chao MC, et al. Deletion of the *C4-CYP21* repeat module leading to the formation of a chimeric *CYP21P/CYP21* gene in a 9.3-kb fragment as a cause of steroid 21-hydroxylase deficiency. *Clin Chem* 2003;49:319–22.
2. Higashi Y, Yoshioka H, Yamane M, Gotoh O, Fujii-Kuriyama Y. Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. *Proc Natl Acad Sci U S A* 1986;83:2841–5.
3. Bristow J, Tee MK, Gitelman SE, Mellon SH, Miller WL. Tenascin-X: a novel extracellular matrix protein encoded by the human *XB* gene overlapping P450c21B. *J Cell Biol* 1993;122:265–78.
4. Burch GH, Gong Y, Liu W, Dettman RW, Curry CJ, Smith L, et al. Tenascin-X deficiency is associated with Ehlers-Danlos syndrome. *Nat Genet* 1997;17:104–8.
5. Schalkwijk J, Zweers MC, Steijlen PM, Dean WB, Taylor G, van Vlijmen IM, et al. A recessive form of the Ehlers-Danlos syndrome caused by tenascin-X deficiency. *N Engl J Med* 2001;345:1167–75.
6. Koppens PFJ, Hoogenboezem T, Degenhart HJ. Carriership of a defective tenascin-X gene in steroid 21-hydroxylase deficiency patients: *TNXB-TNXA* hybrids in apparent large-scale gene conversions. *Hum Mol Genet* 2002;11:2581–90.
7. Koppens PFJ, Smeets HJM, de Wijs IJ, Degenhart HJ. Mapping of a de novo unequal crossover causing a deletion of the steroid 21-hydroxylase (*CYP21A2*) gene and a non-functional hybrid