Author’s response to reviews

Title: Comparative measurement of CNP and NT-proCNP in human blood samples: a methodological evaluation.

Authors:

Andreas Kuehnl (kuehnl@gmx.de)
Jaroslav Pelisek (j.pelisek@lrz.tum.de)
Martin Bruckmeier (martin.bruckmeier@t-online.de)
Wajima Safi (WajimaSafi@aol.com)
Hans-Henning Eckstein (hheckstein@web.de)

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Author’s response to reviews: see over
Dear Professor Olsen,

we are very happy to hear from you that our manuscript #1739690838809163, “Comparative measurement of CNP and NT-proCNP in human blood samples: a methodological evaluation” might be considered for publication in the Journal of Negative Results in Biomedicine after revision.

As requested, we have provided a point-by-point response to all of the very valuable referee comments. We considered them carefully in the revised manuscript. All changes to manuscript have been highlighted according to the author’s guidelines for resubmitting a manuscript.

We hope that you and the referees would consider the revised manuscript for publication in the Journal of Negative Results in Biomedicine.

Sincerely,

Prof. Dr.med. H.-H. Eckstein
Point-by-point response to Referee comments:

Referee 1:

This is a brief manuscript comparing blood-processing methods in the evaluation of CNP and NTproCNP levels. Twelve young men had blood drawn into three tubes, a clotting activator tube, a serum citrate tube and an EDTA tube. For baseline readings, tubes were immediately centrifuged and serum, plasma (Na citrate) and plasma (K EDTA) obtained and assayed. Tubes also had delayed processing to assess stability. Serum and plasma (Na citrate) were obtained and held at room temperature for 30 minutes and 2 hours. The EDTA tubes were held for the stated time, then centrifuged and plasma obtained.

Samples were assayed for NTproCNP and CNP by a commercial assay. Results showed NTproCNP levels are not different between processing methods and stable after 30 min 2 hours at room temp. The CNP results are problematic. Levels were twice as high in the plasma (sodium citrate) than in serum or plasma (K EDTA). The absolute levels are roughly 1,000 fold higher than previously published ranges, done with RIAs. The inter-sample variability is very high with coefficients of variation approaching 50% (compared to about 10% in the reports using RIAs). The authors suggest the difference between the plasma values are likely due to the presence of Na citrate or possibly differences in pH affecting the assay. The authors try to identify the source of the differences between the CNP RIA and ELISA, but do not show a head-to-head comparison. External standards gave expected results in the ELISA. No conclusion is reached as to the difference.

The authors include a list of studies and reported ranges. They do not mention the 2-fold difference in values for NTproCNP between the Biometric ELISA and the RIA. A head-to-head comparison can be found in Olney RC, Permuy JW, Prickett TCR, Han JC, Espiner EA. 2012 Amino-terminal propeptide of C-type natriuretic peptide (NTproCNP) predicts height velocity in healthy children. Clin Endocrinol. (Oxf). 77(3):416-22 and a more comprehensive adult reference range can be found in Prickett TCR, Olney RC, Cameron VA, Ellis MJ, Richards AM, Espiner EA. Impact of age, phenotype and cardio-renal function on plasma C-type & B-type natriuretic peptide forms in healthy adults. Clin Endocrinol (Oxf). 2012 Sep 11 [Epub ahead of print].

The study is useful only in showing the stability of NTproCNP in processing. Until
these questions regarding the CNP ELISA are answered, results using this assay are suspect.
We fully agree with the referee. As also pointed out in detail by referee #3 these remarkable differences between ELISA and RIA assays are important methodological issues and have already been described for some other natriuretic peptides. “This large difference in results depends on the specificity of monoclonal or polyclonal antibodies used, the design of immunoassay system (competitive versus non-competitive assay), the analytical matrix (serum versus EDTA or heparinized plasma) used, and the huge number of circulating forms of CNP, as previously reported for ANP and BNP immunoassays”. Therefore, we added a paragraph discussing this very important point concerning methodology. (redline version, page 7, para 3 and page 9, para 1)

Specific comments:
Methods: an n of only 12 is barely adequate when concluding there is no difference between difference sample groups. A larger n improves confidence that there really is no difference.
We completely agree with the referee that a sample size of 12 is small to conclude that there is no difference at all. However, from a statistical point of view, it is important to specify how large the difference must be to be of medical relevance. To our knowledge, the latter is not yet clearly defined. Therefore, the raw 95%-confidence intervals of all measures were given in the figure to enable every reader to draw his own interpretation from the data as well. In addition, a paragraph “Limitations of the study” discussing this issue was added to the manuscript. (redline version, page 8/9, para 3/1)

Discussion, last sentence: I am not certain what is meant by “disrupting substance.”
We thank the referee for pointing out this mistake. Key message of this sentence is expressed already by the formulation: “…NT-proCNP being only a by-product of the active peptide”. For more clarity, the last subordinate clause was deleted.
Referee 2:

General comments:
This manuscript reports a study of the effects of collection medium (plasma, serum and whole blood) and storage time on the concentrations of CNP and NTproCNP in humans. The study is well designed and well-written. The results of this study will be informative and useful for researchers working in the field of natriuretic peptide research. I recommend that the manuscript be accepted in its current form.

Major Compulsory Revisions:
None

Minor Essential Revisions:
The author can be trusted to make these. For example, missing labels on figures, the wrong use of a term, spelling mistakes.
1. Please change the word “level” to “concentration” on the figure and throughout the manuscript.
We thank the referee for pointing out this mistake. The manuscript and figure were changed as proposed.

2. The manuscript would benefit from proof-reading by a native English speaker.
As recommended, the complete manuscript was proofread by a native speaker.
General Considerations

The aim of this study was to investigate whether there are some differences of CNP and NT-proCNP levels between serum and plasma samples. In particular, this study was focused on the stability of CNP and NT-proCNP in full blood samples stored at room temperature. Authors found that levels of CNP and NT-proCNP are stable for at least two hours, even when sample processing is delayed or blood probes are stored at room temperature. Furthermore, NT-proCNP assay demonstrated more consistent and reliable data than CNP assay. As a result, Authors suggest that NT-proCNP assay should be preferably used in clinical applications.

The manuscript is concise and well written. The results are interesting, although the data reported, in particular those regarding the stability, are strictly method dependent. Authors are troubled about the great difference in results among different RIA and EIA methods (Discussion, page 6), but this discrepancy is theoretically conceivable, and so largely expected. It is well known that similar results have been reported also for the immunoassays of other natriuretic peptides, such as BNP and ANP related peptides (for a review on this important topic see: Clerico A. et al. Clin Chem 2000; 46: 1529-34; Clerico A. et al. Clin Chem 2004; 50: 33-50; Clerico A. et al. Clin Chim Acta 2012; 414: 112-9; Clerico A. et al. Adv Clin Chem 2012; 58: 31-44). For this reason a standardization/harmonization for ANP and BNP immunoassays is recommended since the dawn of this century (Clerico A. et al. Clin Chem 2000). This large difference in results depends on the specificity of monoclonal or polyclonal antibodies used, the design of immunoassay system (competitive versus non-competitive assay), the analytical matrix (serum versus EDTA or heparinized plasma) used, and the huge number of circulating forms of CNP, as previously reported for ANP and BNP immunoassays (Clerico A. et al. Clin Chem 2000; Clerico A. et al. Adv Clin Chem 2012; 58: 31-44; Emdin M. et al. J Am Coll Cardiol 2011; 57: 1396-8). Authors should better discuss this important point in the revised version (this point should be considered as a minor essential revision).

We thank the referee very much for highlighting this important background information. Discussion section of the manuscript was amended by a paragraph discussing the above-mentioned evidence. (redline version, page 7, para 3)