

Statement of Concern about a Commercial Assay Used to Measure Soluble Hemojuvelin in Humans

Orlando M. Gutiérrez^a Chia Chi Sun^b Wenjie Chen^b Jodie L. Babitt^b Herbert Y. Lin^b

^aDivision of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Ala., and ^bProgram in Anemia Signaling Research, Division of Nephrology, Program in Membrane Biology, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, Mass., USA

Dear Editor,

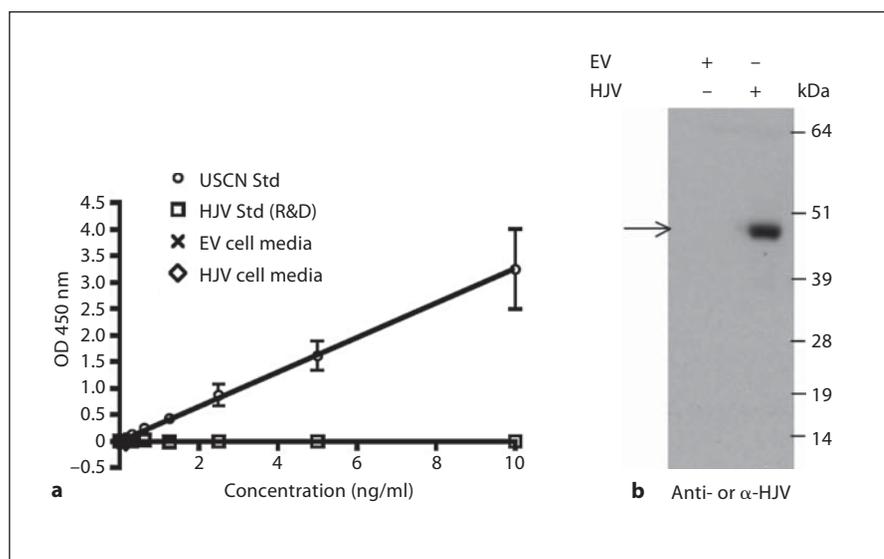
Dr. Rumjon and colleagues recently reported the measurement of soluble hemojuvelin (sHJV) in serum samples obtained from 93 individuals with chronic kidney disease (CKD) [1]. The sHJV concentrations were measured using a commercially available two-site antibody enzyme-linked immunosorbent assay (ELISA) (E91995Hu; USCN Life Science Inc., Wuhan, China). In order to assess the performance of this ELISA, we acquired this kit from the same com-

pany to detect and measure recombinant human HJV protein and sHJV in cell-conditioned media from HJV-transfected cells.

We examined the ability of the USCN ELISA to detect known quantities of full-length, purified recombinant human HJV protein (hHJV.his; R&D Systems, Minneapolis, Minn., USA), diluted in the buffer solution provided by the kit using twofold serial dilutions. As shown in figure 1a, the OD 450 nm readings from this experiment

were close to zero (open boxes), indicating that the assay does not recognize full-length, purified hHJV.his. This purified protein is used widely in the field and was also used to generate a standard curve in the competitive HJV ELISA published by Brasse-Lagnel et al. [2]. The ELISA was able to detect its own kit standard, producing a linear calibration curve with OD 450 nm readings from 3.24 ± 1.07 to 0.07 ± 0.07 (fig. 1a).

Fig. 1. USCN HJV ELISA kit cannot detect purified recombinant human HJV protein (R&D Systems) or sHJV in HJV-transfected Hep3B cell media. **a** The USCN HJV ELISA kit (E91995Hu) detected its own standard in a linear fashion (open circles and straight line). However, the USCN kit did not detect recombinant human HJV protein (R&D Systems; 3720-RG; open squares) diluted in diluent buffer (from USCN kit). Conditioned media samples from HJV (open diamond) or Empty Vector (EV, X)-transfected Hep3B cells were also undetectable with the USCN kit. Data shown from two separate experiments \pm SEM values. **b** Western blot using anti-HJV antibody (R&D Systems; AF3720, 0.1 μ g/ml) detected a \sim 45 kDa band in conditioned media from HJV- but not EV-transfected Hep3B cells.



We then examined the ability of the assay to detect sHJV in conditioned media from Hep3B cells transfected with human HJV plasmid cDNA. To confirm the presence of sHJV in conditioned media from transfected cells, we performed Western blot analysis using goat polyclonal anti-HJV antibodies (R&D Systems; AF3720, 0.1 µg/ml), and detected a band at ~45 kDa corresponding to sHJV in conditioned media from cells transfected with HJV plasmid but not from cells transfected with empty vector (EV) plasmid, confirming the presence of sHJV in cell media (fig. 1b). We then used the USCN ELISA to quantify sHJV in the same conditioned media samples and did not obtain detectable OD 450 nm readings (EV cell media (-0.01 + 0.01 ng/ml), X; HJV cell media (-0.01 + 0.01 ng/ml), open diamond;

fig. 1a), indicating that the ELISA was unable to detect sHJV in media from Hep3B cells transfected with human HJV plasmid cDNA.

These results raise serious concerns about the ability of the USCN HJV ELISA to detect sHJV in humans. Adding to these concerns is the performance of a separate ELISA for measuring mouse HJV produced by the same company. Krijt et al. [3] reported that while this assay generated a linear calibration curve using its own standard, there were no differences in detectable sHJV plasma concentrations from *Hjv*^{+/+} mice as compared to *Hjv*^{-/-} mice, despite a nearly complete absence of *Hjv* mRNA in tissues of the knockout animals. This result indicates that the USCN mouse HJV ELISA detects cir-

culating sHJV in animals that should have undetectable plasma concentrations. These results collectively suggest that the USCN HJV ELISA does not detect human or mouse HJV, but instead is recognizing some other protein(s). As a result, we believe that the performance of the USCN HJV ELISA needs to be independently validated prior to being used in future studies. In addition, we believe it is incumbent on the company to indicate how it developed the standards provided in these kits, and provide evidence verifying that these standards are actually HJV. Until such information is provided, we believe that investigators should interpret data generated from these assay kits with extreme caution and wait until validation data become available prior to performing further experiments using these kits.

References

- 1 Rumjon A, Sarafidis P, Brincat S, Musto R, Malyszko J, Bansal SS, Macdougall IC: Serum hemojuvelin and hepcidin levels in chronic kidney disease. *Am J Nephrol* 2012; 35:295–304.
- 2 Brasse-Lagnel C, Poli M, Lesueur C, Grandchamp B, Lavoigne A, Beaumont C, Bekri S: Immunoassay for human serum hemojuvelin. *Haematologica* 2010;95:2031–2037.
- 3 Krijt J, Frydlova J, Kukackova L, Fujikura Y, Prikryl P, Vokurka M, Necas E: Effect of iron overload and iron deficiency on liver hemojuvelin protein. *PLoS One* 2012;7:e37391.

Reply

Adam Rumjon^a Iain C. Macdougall^a Jolanta Malyszko^b

^aDepartment of Renal Medicine, King's College Hospital, London, UK, and ^bDepartment of Nephrology and Transplantology, Medical University, Bialystok, Poland

Dear Editor,

We thank Dr. Gutiérrez and colleagues for their useful comments on our recent paper, and also yourself for allowing us the opportunity to respond. Having reviewed Dr. Gutiérrez's experimental data, there seems little doubt that the commercially available soluble hemojuvelin (sHJV) assay that we used for our analysis is problematic.

Unlike Dr. Gutiérrez, we did not have the luxury of having developed our own in-house assay for sHJV, but when we became aware of this commercially available assay for sHJV, and the increasing recognition that sHJV has on the regulation of hepcidin production, we were interested in analysing the same serum samples that we already had hepcidin measurements on, to

see if there was any relationship between the two analytes.

The striking finding of our analysis was not the presence of a correlation between hepcidin and hemojuvelin, but the tight correlation between ferritin and hemojuvelin. It is now clear that the antibody used in the commercially available assay was not detecting hemojuvelin, and it remains uncertain what exactly it was detecting. The measurements of both ferritin and hemojuvelin in our investigation were conducted in two separate institutions that were both blinded to each other's data, and it is extremely unlikely that a correlation could have happened by chance, unless the sHJV assay was detecting something that is inherently linked to ferritin measurement.

We are concerned to have learned that this assay is being sold commercially without having undergone the rigour of scientific evaluation normally required, and perhaps the main message of our paper now is to warn clinical scientists not to rely on a commercially available assay without carefully scrutinising its validity and credentials. The fact that we did not receive replies from the manufacturers in relation to a couple of e-mails about their assay should perhaps have alerted us that something was not quite right.

We thank Dr. Gutiérrez and colleagues once again for their helpful comments.