We did not publish these data originally, however, because our follow-up for both studies combined was only 4 years and because our calculation of the regression dilution factor was based on values obtained 10 years apart. Thus, although our uncorrected hazard ratios were somewhat underestimated, the corrected hazard ratios given above likely are overestimates of the actual hazard ratios (4).

We also agree that it would be interesting to see data on the separate associations of iron concentration and transferrin concentration with total-mortality risk. We therefore have constructed a new Fig. 1, which shows total-mortality risks separately for increasing plasma transferrin saturation, iron concentration, and transferrin concentration. Each stratum contains the same number of individuals in ascending order of value corresponding to the strata for transferrin saturation (thus some intervals overlap in concentration); however, the strata do not necessarily contain the same individuals. Fig. 1 shows that the increase in total-mortality risk with increasing iron concentration is similar to that for increasing transferrin saturation, whereas the association is less clear for increasing transferrin concentration.

In summary, we have shown that the increased total mortality associated with increased transferrin saturation was not driven primarily by haemochromatosis genotypes, that hazard ratios in our setting corrected for regression dilution bias likely are overestimated (whereas uncorrected hazard ratios are underestimated), and that the increase in total-mortality risk with increasing transferrin saturation also is seen with increasing iron concentration but not to the same extent with increasing transferrin concentration.

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In Vitro Molecular Structure of N-Terminal B-Type Natriuretic Peptide: Monomer or Oligomer?

To the Editor:

In the circulation, the A-type natriuretic peptide and the B-type natriuretic peptide (BNP) regulate cardiovascular homeostasis. Each hormone is derived from biosynthetic precursors that are processed to the active C-terminal hormones and the N-terminal fragments (1). Measurements of both BNP and N-terminal proBNP in plasma are recommended as diagnostic tools for heart failure. The peptides are measured by immunoassays that use antibodies directed against specific epitopes. A basic understanding of the native peptide structure in terms of posttranslational modifications is thus paramount, because changes in post-translational processing will affect assay measurement and clinical interpretation drastically.

A recent report in Clinical Chemistry addressed the possibility of natriuretic peptide oligomeriza-
tion in a study that used a recombinant N-terminal proBNP peptide (possessing a glycine-serine N-terminal extension) spiked into 3 matrices (2). Buffer, plasma, or serum was incubated for various times and temperatures; the material was then applied to a reversed-phase column, a gel-filtration column, and a reversed-phase column, respectively. All 3 columns were eluted with acidic acetonitrile. Antibodies directed against linear epitopes (residues 1–22, 10–29, and 57–76) were used to assay fractions for N-terminal proBNP immunoreactivity with the intent of delineating the peptide sequences. We note that the immunoreactivity of endogenous N-terminal proBNP from unspiked plasma does not yield the same response with the different assays [see Fig. 1A of (2)], suggesting that the antibodies possess different affinities for full-length N-terminal proBNP. We believe this possibility is worth considering, given that immunoreactivity differences are a primary means the authors used to infer analyte structure. In other words, how are the different affinities taken into account in the elution profiles? An experiment showing the relative affinities for full-length N-terminal proBNP in buffer alone seems warranted. The authors also note from Fig. 1 in their report that chemical deglycosylation decreases the immunoreactivity for all of the studied epitopes, as well as for N-terminal pro–A-type natriuretic peptide. We suggest this result is due to N- to O-acyl migration in the presence of the harsh acid they used, which likely could be reversed by treatment with base. Alternatively, enzymatic deglycosylation may mitigate these deleterious effects.

The matrix-incubation experiments were used to evaluate the effect of protease activity on N-terminal proBNP. It is, however, unclear whether stable fragments could simply be a consequence of either the absence of protease-sensitive sites or the presence of sites that might require proteases not found in the matrix of study. Our major concern is that the authors conclude that a region of N-terminal proBNP oligomerizes and that this region contains the stable domain deduced from the matrix-incubation experiments. This conclusion is derived from the immunoactivity results from the columns, including mass spectrometry data for the fractions from the third column. When gel-filtration columns are eluted with 300 to 500 mL/L acetonitrile, the ensuing denaturation destroys noncovalent protein–protein interactions (3). Thus, the eluted material described in the authors’ Fig. 3B is extremely unlikely to retain quaternary structure. We have tested the effect of denaturing conditions on the elution of synthetic proBNP 1–76 (Fig. 1). Elution under benign conditions produces an aberrant elution pattern that is sometimes interpreted as oligomerization but is more likely due to a highly unfolded, disordered structure (4, 5). The addition of 6 mol/L guanidine in this case apparently produced a slightly more compact structure and thus a later elution. Furthermore, rigorous biophysical measurements have revealed only monomeric N-terminal proBNP for solid-phase synthesized 1–76 N-terminal proBNP (4) and methionine-1–76 N-terminal proBNP (5). We note that each peptide gave a single band on a protein-stained denaturing gel (data not shown), in contrast to the authors’ antibody-stained immunoblot of recombinant N-terminal proBNP (Fig. 4C in (2)). It would be informative to perform an additional Western blotting experiment with the other antibodies they used.

By their very nature, immunoassays are defined by the choice of antibodies. Our ability to extract detailed structural information has improved dramatically, owing primarily to the widespread use of mass spectrometry. This increased knowledge is not without consequences, because such modifications may have diagnostic relevance if the antibodies used are sensitive to these modifications. We are, therefore, in agreement with Ala-Kopsala et al. that it is imperative to determine the structure of the endogenous analyte of interest. In this way, robust immunoassays providing clinicians the best possible diagnostic tools can be developed.

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**References**


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In Reply

We thank Drs. Crimmins and Goetze for their valuable comments. We would like to clarify a couple of points. As to the question of whether the antibodies against different epitopes of N-terminal pro–B-type natriuretic peptide (NT-proBNP) possess different affinities against NT-proBNP\(_{1-76}\), they certainly do; however, we use competitive assays that employ NT-proBNP\(_{1-76}\) as a calibrator and tracer, as we have detailed in our previous publications (1, 2). Any synthetic or recombinant peptide containing the specific epitope sequence cross-reacts fully, regardless of its length. The differences in affinity are reflected in the analytical sensitivities of the assays, which do vary from assay to assay, but that does not affect the quantitative results.

With regard to oligomerization as the explanation for our results, we discussed various possibilities and found that oligomerization, which is not our original idea (3), appeared to be the one most consistent with our results (4). We are open to other explanations. Because NT-proBNP seems to lack any biological effects or receptors of its own and currently has only diagnostic value, our statement that “we found that NT-proBNP is susceptible in blood to modifications that interfere with immunoassays” contains the important conclusion of our study (4) from a pragmatic point of view.

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