Letters to the Editor

methodological issues concerning design and conduct of test-outcome studies. Comprehensive discussions of this complex and important area are available (1–4), and in the textbook Evidence-Based Laboratory Medicine the chapter on assessment of outcomes is especially relevant (5).

Our purpose is to highlight the importance of moving from diagnostic accuracy studies to evaluations of the effects of test results on clinical decision-making and subsequent health outcomes. In agreement with other investigators (1–5), we support efforts to increasing the use of outcome studies to enhance the effectiveness of health-care policy and decision-making.

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### References


### Electrospray Ionization Mass Spectrometric Analysis of the Globin Chains in Hemoglobin Heterozygotes Can Detect the Variants HbC, D, and E

**To the Editor:**

We would like to point out that 2 recent articles in this journal about human hemoglobin (Hb) analysis (1, 2) give the false impression that variant globin chains with <6 Da mass difference from normal cannot be detected in heterozygotes by electrospray ionization mass spectrometry (ESI-MS). Kleinert et al. (1) state: “Two important drawbacks of the MS methods should be mentioned. First, its insufficient resolution prevents the detection of Hb mutations with small mass differences of the globin chains. The precision of normal low-resolution mass measurements was insufficient to distinguish the wild-type β-chain from several β-chain variants such as HbC, D, or E”. Brennan (2) comments similarly by stating that whereas traditional methods readily detect the majority of common variants, such as HbC, HbD, or HbE, “the substitutions involved in these, and similar charge variants (Glu→Lys, Glu→Gln, Asp→Asn, and Lys→Gln) involve mass changes of 1 Da or less, and are not detectable by mass spectrometry.” Kleinert et al. (1) also state: “Second, MS as described here is only a qualitative technique, and in particular, minor Hb fractions such as HbA1c or HbA2, which are important for diagnosis of diabetes mellitus or thalassemias, respectively, cannot be quantified.”

While we agree that ESI-MS cannot detect the zero mass change mutations (Lys→Gln and Leu→Ile), we maintain it is not necessary to resolve the variant and normal globin chains in heterozygotes to detect variants that differ in mass from normal by ±1 Da (Glu→Lys, Glu→Gln, Asp→Asn, Asn→Ile). In 2003, Rai et al. (3) showed that variants differing by 1 Da from normal can be detected if present at >10% abundance. In that report, the normal β-chain mass was determined with a precision of 0.05 Da SD, which resulted in mass changes of ±0 Da being detectable with 95% confidence. We routinely analyze Hb on a quadrupole instrument and, owing to improved performance since 2003, generally achieve ±0.03 Da SD on the normal β-chain when using the α-chain for internal calibration. For example, 50 normal blood samples analyzed over the last 4 months gave a mean β-chain mass of 15 867.255 Da (0.026 Da SD).

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On 22 D-Punjab (β121Glu→Gln) heterozygotes, the mean β-chain mass was 15 866.832 Da (0.039 Da SD), which is 0.423 Da lower than that determined for the normal β-chain. This mass difference is readily detected and even allows the mean abundance of the variant β-chain to be estimated as 43.0%, which agrees with the mean abundance determined from the HPLC data associated with each sample (41.0%, 3.2% SD, n = 22). The above SD values imply that mass changes down to between 0.1 and 0.2 Da can be reliably detected. A −0.2 Da mass change would suggest a heterozygous −1 Da mutation (Glu→Lys, Glu→Gln, Asp→Asn, or Asn→Ile) at 20% abundance or heterozygous Lepore-Boston-Washington (normal β − 2 Da) at 10% abundance.

Of course, although ESI-MS of the globin chains can detect ± 1 Da mutations, precise identification requires enzymatic digestion. Hemoglobins C, D-Punjab, E, and O-Arab can all be positively identified directly from the mass spectra of 30-min digests with trypsin (4). It is still necessary to initially detect zero mass change mutations by electrophoretic or chromatographic means, however. Nevertheless, it is useful to know that if HPLC has detected a >20% abundant variant with a significant polarity change and ESI-MS has not detected a mass change, then Gln→Lys mutations should be considered.

We also point out that the minor components, HbA1c and HbA2, are readily quantified by ESI-MS. HbA1c can be determined from the concentrations of gly-cated α- and β-chains after calibration with standards (5). Furthermore, we routinely determine HbA2 (approximately 3%) and find it useful for distinguishing homozygous HbE and HbE/β-thalassemia (HbA2 and HbE coelute by HPLC).

The methods we employ for analyzing Hb have been described in detail (3–5). To reliably achieve the mass measurement precision mentioned above, however, we stress that the data must be acquired over a limited m/z range (we currently scan m/z 930–1210) with a minimum of 32 data points per m/z unit. Also, each m/z spectrum must be internally calibrated, generally using the multiply protonated α-chain ions present from most samples. In this way, in-accuracies due to uncertainties in the atomic weights of the elements are largely eliminated, since the latter are likely to be the same in all proteins in a given sample. We deconvolute m/z 980–1180 to produce mass spectra using the maximum entropy (MaxEnt)-based software supplied with the mass spectrometer.

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References

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Macromolecular Cystatin C Can Be a Caveat for Estimating Glomerular Filtration Rate in Biliary Obstruction

To the Editor:

Cystatin C is the most commonly used marker for the estimation of glomerular filtration rate (GFR) in routine clinical use (1), but serum concentrations of low molecular mass proteins can provide an interesting alternative for estimating renal function. Serum concentrations of low molecular weight proteins are primarily determined by GFR, and an ideal marker should have a constant production rate and not vary in concentration during an acute-phase reaction. Cystatin C (13 kDa) and β-trace protein (23–29 kDa) share these properties (1–3). Cystatin C appears to be superior to creatinine for estimation of GFR, especially in the so-called creatinine-blind range. As a screening test for detecting decreased GFR, cystatin C has fewer inherent limitations than serum creatinine. Moreover, unlike creatinine, cystatin C reflects renal function independent of age, sex, height, and body composition (1). Formulae have been developed allowing estimation of GFR based on cystatin C (1). However, standardization for cystatin C assays is still lacking. Recently, several caveats have been reported:

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