Screening for Hemoglobin Variants with Abnormal Oxygen Affinity

To the Editor:
Rai et al. (1) recently described an elegant method for detecting hemoglobin (Hb) variants that cause erythrocytosis, using electrospray mass spectrometry. Many of these variants are not detectable with conventional electrophoretic techniques. The authors also stated that “functional studies of oxygen saturation curves would be the ideal method to screen for Hb variants that cause erythrocytosis, but such methods are not generally available. . . .”

On the contrary, the determination of $P_{50}$ (partial pressure of $O_2$ at which Hb is half-saturated) is easily done in any laboratory with a blood gas analyzer and a multiwavelength oximeter. The $P_{50}$ when normalized to pH 7.40 and a $P_{co_2}$ of 5.33 kPa (40 mmHg), is a direct (inverse) reflection of Hb oxygen affinity. A one-point method and calculation have been described in an IFCC Guideline (2), and these or equivalent calculations have been included in the software of many blood gas analyzers and can be performed automatically. Heparinized venous blood is used.

One advantage of electrospray mass spectrometry is that specimens do not need to be analyzed immediately, but can be stored and shipped to a laboratory where the electrospray mass spectrometry method is available. On the other hand, heparinized whole blood specimens for $P_{50}$ determination are stable for at least 2–3 h at room temperature, and minimal change is observed after 24 h when specimens are held at 4°C (3).

We have routinely used $P_{50}$ measurements with instruments in the laboratory for many years to screen for Hb variants in patients with unexplained anemia or erythrocytosis.

References

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Standardization: Comparability and Traceability of Laboratory Results

To the Editor:
The opinion article by Stenman, entitled “Immunoassay Standardization: Is It Possible, Who Is Responsible, Who Is Capable?” (1), is one of many appearing at present on this subject (2,3). The requirements set by the EC Directive on in vitro diagnostic (IVD) medical devices (4) appear to be a driving force behind this increase in published comments on standardization. In particular, the requirement of the traceability clause in the Directive calls for attention: it is the subject of a European Standard mandated by the EC and developed by the Comité Européen de Normalisation (CEN) in collaboration with the International Standardization Organization (ISO) (5).

The implementation of the EN/ISO Standard is addressed indirectly by Stenman (1), and it concerns us all: the IVD industry, the medical laboratory, and the patients. The final aim is to achieve trueness and, where this is not yet possible, to improve comparability of results obtained for patient samples by medical laboratories within a region, a country, and eventually worldwide.

For a more precise understanding of the term ‘standardization’, in particular in relation to immunoprocessed, a few points from the Standard are given here for the reader’s attention.

The first point is that two classes of analytes in laboratory medicine [see the introduction of the Standard (5)] are to be distinguished, i.e., those analytes whose measurement results are traceable to SI units (for convenience called class A analytes) and those whose measurement results are not traceable to SI units but to arbitrary units, e.g., mass units or WHO International Units (for convenience called class B analytes). The latter class includes all (glyco)proteins, which usually are measured by means of immunoprocesses.

It is obvious that standardization of the measurements of the class A analytes may still pose some problems, but in principle, it should and could be readily done. However, class B analytes pose far greater problems. The class B analytes are, for the most part, highly complex and heterogeneous mixtures in the biological fluids in which they are measured. In the immunoprocessure measurement, the “analyte” (analyte mixture; many protein and/or glyco forms) in the reference material and in the biological fluids are definitely nonidentical, which consequently invalidates the basic rule of immunoprocessures: to compare “like with like”.

For the past 30 years, the flaw of nonvalidity has been taken for granted, with claims that we should remain practical. One of the results is an ever-increasing flow of publications on discrepancies between results obtained in national, regional, or worldwide external quality assessment schemes.

The second point is that one of the crucial remarks in the EN/ISO Standard (6) is that, when establishing traceability, “insufficient definition of the analyte in the human samples” is regarded as one of the major pitfalls. This holds true for class B analytes, which means that before developing an immunoprocessure, one has to establish “what do we want to measure?” and “is it clinically relevant?”. The following motto thus emerges: “define the analyte in the biological fluid that has the best clinical relevance”.

There is an encouraging example of this approach: the IFCC working group on standardization of mea-
measurement of hemoglobin $\text{A}_{1c}$. They started off with the identification of glycohemoglobin forms in blood of diabetic patients; thus, they defined their analyte first and then proceeded with development of a reference material and a reference measurement procedure (7). Future work by IFCC along this approach includes identification of “thyroid-stimulating hormone” forms in the blood of patients suffering from thyroid disease.

The opinion article by Stenman (1) does not explicitly deal with these two points. Because the issue of traceability will be discussed further during the coming years, I believe it to be essential that the two points above are clearly addressed and explicitly mentioned.

On the question posed by Stenman (1), “who is responsible”, the EN/ISO Standard gives an appropriate answer: in the upper tier of Figs. 1–5 of the Standard it is mentioned that responsibility lies with international and worldwide organizations, such as the professional scientific organization IFCC, the international metrology organization Bureau International des Poids et Mesures (BIPM), and the professional medical organization WHO, together with the IVD industry and others. The idea is that a truly global working party consisting of the stakeholders should address these problems.

It will be counterproductive when organizations, even when they claim to cover a worldwide audience, e.g., in the fields of endocrinology or oncobiology, embark by themselves on standardization of analytes in their field of interest in their own manner without taking into account requirements demanded in the EN/ISO Standard (5). A global agreement is especially important for the IVD industry because the establishment of national or regional reference materials and/or reference measurement procedures should be avoided at all costs (8). The IVD market is a transnational one. Truly global consensus is the way forward.

**References**


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Dr. Stenman responds:

To the Editor:

The intent of my recent opinion article (1) in *Clinical Chemistry* was to stimulate discussion on the problems of immunoassay standardization. I am therefore grateful for the letter by Rudolf Lequin, which addresses some aspects that I did not deal with, or on which he is of a different opinion.

The EC Directive on in vitro diagnostic medical devices (IVD Directive) is an important document, but it was not the reason that I wrote the opinion article, the thoughts for which have emerged during several years (2). The IVD Directive requires diagnostic companies to use reference measurement procedures for calibration of their assays. However, it does not require any change from current practice unless reference methods are available.

The division of analytes into two classes is a crude oversimplification of the real world. As I tried to point out, it is possible to define the molar concentrations of many protein and peptide antigens, including glycoproteins, in a satisfactory way (1). Even if proteins are heterogeneous, it is in many cases possible to define both the standard and the analyte in the sample sufficiently well to calibrate immunoassays on the basis of substance (molar) concentrations. One of the aims of the Working Group on human chorionic gonadotropin (hCG) has been to test this approach, which if successful could be used as a model for other protein immunoassays. This has not yet been demonstrated, but it should not be considered impossible until tested.

The problem of “insufficient definition of the analyte in the human sample” is certainly important, and hemoglobin (Hb) $\text{A}_{1c}$ is an example of how this can be solved. However, the concentrations of Hb$\text{A}_{1c}$ in blood are more than 1 million-fold higher than those of most of the analytes that we need to standardize. Therefore, the same approach is not easily applied to other immunoassays. In spite of this, we know enough about which forms of hCG and related molecules in plasma are clinically relevant to establish satisfactory standardization. Assays for another complex analyte, prostate-specific antigen (PSA), can now be acceptably standardized (3), although PSA exists in plasma both free and complexed to various protease inhibitors (4). It is obvious that many other analytes still pose problems, some of which may seem insurmountable. However, that should not keep us from establishing reference methods for less complex “class B” analytes.

The question of responsibility described by Rudolf Lequin reveals the problem. Too many organizations are formally responsible, but none of them has the capacity to alone handle the task, and only a few of them have actually attempted to do it. I have suggested that one organization
should take the main responsibility (1), and I still think that this is necessary.

The warning against initiatives by regional or specialized groups may seem justified, and I certainly agree that standardization is an international issue (1, 2). I have been involved in projects organized by the International Society for Oncodevelopmental Biology and Medicine, which apparently is what Lequin considers a “specialized group”, aimed at mapping the epitopes of PSA and hCG. Well-characterized antibodies and standards are necessary for establishment of reference methods, but much work remains for the international organizations mentioned by Lequin before such methods can be established. I think that we need more projects of this kind rather than more restrictions.

References

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Presence of Anti-Tissue Transglutaminase Antibodies as a Sign of Tissue Lesion

To the Editor:
We read with interest the report (1) on IgG antibodies against tissue transglutaminase (tTG) in patients positive for anti-double-stranded DNA with systemic lupus erythematosus and in patients positive for anti-SSA/SSB. We agree with the authors’ hypothesis that in some autoimmune disorders, such as systemic lupus erythematosus, the content of apoptotic bodies (including tTG) could come into contact with the immune system, leading to an autoimmune response. Nevertheless, some aspects of this study require comments.

tTG, an intracellular enzyme, has recently been proposed as the major autoantigen of anti-endomysial antibodies (EMAs), and measurements of IgA anti-tTG antibodies are used in the diagnostic evaluation of celiac disease (CD) (2–4). Furthermore, the presence of IgG anti-tTG antibodies has been associated with IgG1 EMA positivity in CD patients either with or without selective IgA deficiency (5, 6). To exclude CD, we believe that determination of serum IgG1 EMA could be useful in the above-mentioned antinuclear-positive patients (1).

We have recently demonstrated that, in the occurrence of CD, tTG is released by fibroblasts into the extracellular matrix compartment (7). We also found IgA anti-tTG antibodies in EMA-negative patients with Crohn disease and ulcerative colitis, suggesting that anti-tTG induction could be attributable to tTG released into the extracellular matrix of tissue lesions rather than to the autoimmune component of CD (7). In support of this thesis, another study demonstrated the presence of IgA anti-tTG antibodies in EMA-negative patients with non-Hodgkin lymphoma (8).

These observations, together with the interesting data in the report by van der Sluijs and Vermes (1), lead us to hypothesize that tTG is not the only antigen for EMAs and that further studies are necessary to clarify the issue.

References

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The authors of the article cited above respond:

To the Editor:
In the report cited above, we examined the diagnosis of celiac disease in our patients (none of whom had an IgA deficiency) by determination of IgA anti-gliadin and IgA anti-tissue transglutaminase (tTG). Drs. Di Tola et al. indicate that IgG1 anti-endomysial antibody determinations can also be useful for this purpose, which would support their theory that transglutaminase is not the only an-
tigen of the endomysial antibodies. Another possible explanation of their findings concerns the nature of the antigen used in the anti-tTG assays. Our results in autoimmune patients could be found only with guinea pig liver tTG as the antigen in the assay. With recombinant tTG, at least the one prepared in a baculovirus system, the positivity of the anti-DNA and SSA/SSB samples for IgG anti-tTG disappeared (whereas the IgA anti-tTG in the celiac disease patients remained intact). From this finding (unpublished data), we concluded that the in vivo antigen from the apoptotic bodies differs from the “pure” tTG. This can be the result of different posttranslational changes in the tTG molecule or of neoantigens that occur only in tTG substrate complexes. Thus, in discussions about positivity of anti-tTG assays, the nature of the antigen used should always be specified. This holds as well for the antigen released in vivo, e.g., by fibroblasts.

It is a question of semantics, then, to state that tTG is not the only antigen of the endomysial antibodies.

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