Haemolysis index for the screening of intravascular haemolysis: a novel diagnostic opportunity?

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Abstract
The diagnostic approach to patients with intravascular haemolysis remains challenging, since no first-line laboratory test seems to be entirely suitable for the screening of this condition. Recent evidence shows that an enhanced cell-free haemoglobin (fHb) concentration in serum or plasma is a reliable marker of red blood cell injury, and may also predict clinical outcomes in patients with different forms of haemolytic anaemias. However, the routine use of the haemiglobincyanide assay, the current reference method for measuring fHb, seems unsuitable for a timely diagnosis of intravascular haemolysis, for many safety and practical reasons. The spectrophotometric assessment of fHb by means of the so-called haemolysis-index (H-index) has now become available in most clinical chemistry analysers. This measure allows an accurate, rapid and inexpensive assessment of fHb in a large number of serum or plasma samples, and its use has already proven to be useful for identifying some forms of haemolytic anaemias. Therefore, the aim of this article is to provide an update and a personal opinion about the potential clinical use of the H-index for screening patients with suspected intravascular haemolysis.

Keywords: haemolysis, intravascular haemolysis, haemolytic anaemia, haemoglobin, haemolysis index.

Introduction
In vivo (intravascular) haemolysis, frequently known also as haemolytic anaemia, is a life-threatening condition characterised by premature destruction of red blood cells (RBC) that can be sustained by a kaleidoscope of primary or secondary disorders1,2. Haemolysis may result from diverse pathologies that are intrinsic or extrinsic to the erythrocytes. The most frequent disorders associated with haemolytic anaemia include immune and autoimmune disorders, certain types of infections (i.e. cytomegalovirus, Epstein-Barr virus, hepatitis viruses, Mycoplasma pneumoniae, malaria), reactions to drugs, toxic compounds or blood transfusions (i.e. ABO mismatch transfusion), hypersplenism, burns, massive trauma or strenuous exercise (i.e. footstrike haemolysis), blood cancers (e.g. chronic lymphocytic leukaemia, lymphomas), extracorporeal circulation, prosthetic cardiac valves, disseminated intravascular coagulation (DIC), haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TPP). While these conditions are typically acquired, other diseases associated with haemolysis are inherited; these include sickle cell disease, thalassaemias, spherocytosis, paroxysmal nocturnal haemoglobinuria (PNH), and deficiency of glucose-6-phosphate dehydrogenase or pyruvate kinase. Most intrinsic causes of haemolysis are inherited, while the extrinsic causes are typically acquired. The unique exception is represented by PNH; although it is an acquired defect, PNH RBCs have an intrinsic defect1,2.

Whatever the underlying cause or trigger, and although haemolytic anaemia is a relatively rare condition (1:10,000/100,000), the potential complications can be many and severe, mostly triggered by haemoglobin-nitric oxide scavenging reactions and reactive oxygen species (ROS) generation. These typically include jaundice, hepatosplenomegaly, tachycardia, myocardial ischaemia, respiratory and renal failure, and, ultimately, multiorgan dysfunction and death. The mortality rate can be as high as 10%3-5.

Diagnosis of haemolytic anaemia
The current diagnostic approach to patients with suspected intravascular haemolysis remains rather challenging. A vast array of laboratory tests is available to guide the diagnostic reasoning. Some of these analyses are prevalently used for the diagnosis (i.e. screening tests, including the complete blood cell count, reticulocyte counts, peripheral blood smear revision, total and unconjugated bilirubin, lactate dehydrogenase, haptoglobin, ferritin, urinalysis), whilst others, conventionally called second-line tests, are most frequently used to reach a presumptive or definitive etiopathogenetic diagnosis (e.g. Coombs’ test, serological testing, enzymatic testing, osmotic fragility test, haemoglobin analysis, genetic testing, etc.)1,6,7.
major drawback in the conventional diagnostic workup of patients with suspect intravascular haemolysis is that no single first-line (screening) test has such a high diagnostic efficiency (i.e. 1.00 negative predictive value) to safely rule out the condition in all patients. Haptoglobin testing is a paradoxical example. Although this test is commonly advocated as “diagnostic”, there are several lines of evidence showing that the frequency of false negative results may be higher than 10% even when the concentration of haptoglobin in serum or plasma falls below the reference range8,9.

**Haemoglobin measurement**

There is, therefore, little doubt that the measurement of an increased concentration of cell-free haemoglobin (fHb) in serum or plasma should be considered as the most reliable marker of RBC injury and breakdown, both in vitro and in vivo9. Moreover, recent evidence also suggests that the concentration of fHb is a strong and independent predictor of death in patients undergoing extracorporeal membrane oxygenation (ECMO) procedures who develop intravascular haemolysis11. An increased (i.e. abnormal) concentration of fHb is usually defined as that exceeding 0.25 g/L in serum and 0.13 g/L in plasma, respectively12. Therefore, whenever fHb values exceeding these limits are encountered in clinical practice, and spurious (i.e. in vitro) haemolysis has been definitely ruled out, a virtually unquestionable diagnosis of haemolytic (in vivo) anaemia should be made. Although the haemoglobinicyanide (HiCN) assay (formerly known as Drabkin’s method) is still regarded as the reference technique and gold standard for haemoglobin assessment13,14, it is not convenient to use in clinical laboratories for many safety (i.e. toxicity and practical (manual assay, long turnaround time, high imprecision) reasons. To overcome these limitations, many spectrophotometric techniques have been developed, such as the Fairbanks (1 and 2 assays), Golf, Harboe, Kahn and Noe methods15,16. Although these techniques may be considered a reliable and practical alternative to the reference HiCN assay, some technical issues mean that their use in routine practice is not straightforward. This has led to the emergence of an attractive alternative. The novel generation of clinical chemistry analysers is equipped with the so-called HIL (Haemoglobin, Icterus, Lipaemia/Turbidity) indices, which can estimate the presence of fHb, bilirubin and turbidity in samples17. Briefly, HIL indices are calculated according to absorbance measurements at different wavelengths which correspond to the specific absorbance spectra of haemoglobin (i.e. between 340-440 nm and between 540-580 nm), bilirubin (i.e. 460 nm), and lipaemia/turbidity (i.e. below 400 nm)18. The absorbance measures are then resolved by specific equations, and the final concentration of these substances is reported in arbitrary units, which can then be converted into more conventional units of measurement (e.g. g/L of haemoglobin for H-index, mmol/L of triglycerides for the L-index, μmol/L of bilirubin for the I-index). Although HIL indices have mostly been used to check sample quality, phlebotomy performance19, and usability of blood products before transfusion20, there is increasing evidence that these measures may also generate clinically useful information, especially the values of the H-index21,22. The advantages of the routine use of the H-index include full-automation, rapid turnaround time (i.e. it only takes a few minutes to perform), low sample volume (i.e. generally between 2 to 35 μL), and no additional costs (i.e. test procedures typically entail a simple dilution of test samples with water, saline or Tris buffer)23-25. Unlike direct spectrophotometric techniques used for fHb assessment, the H-index is hence virtually insensitive to other endogenous interfering substances26-29.

**Analytical and clinical performance of the H-index**

A number of studies have provided firm evidence that the H-index may reliably reflect the concentration of fHb in serum or plasma (Table I)26-32. Unger et al. measured the H-index on Modular System P using routine clinical samples and compared data with those obtained with the 2-wavelength method of Golf26, concluding that the two measures were highly correlated (r=0.990). Moon-Massat et al. spiked plasma samples with a haemoglobin-based oxygen carrier (HBOC) to obtain 192 aliquots with gradually increasing values of fHb27. The comparison of Modular System H-index vs the actual HBOC concentration yielded an excellent correlation (r=0.99). Lippi et al. performed a multicentre study on H-index performance and found a perfect agreement between fHb values measured with Roche Modular System H-index and with the reference HiCN method (r=1.00)28. In a subsequent study, Petrova et al. compared Roche Modular system H-index measurements with two other 2-wavelength assays (i.e. Harboe and Fairbanks) using 100 random samples with varying degrees of haemolysis collected from inpatients29. Interestingly, an excellent correlation was found between fHb values obtained with H-index and both the Harboe (r=0.982) and Fairbanks (r=0.969) methods. Fernandez et al. prepared 6 aliquots from the same clinical sample with increasing concentrations of fHb and compared values obtained with the HiCN method and 7 different clinical chemistry platforms (Roche Cobas c511, c711 and Modular System P; Beckman Coulter 5400 and Synchront LXi725; Siemens Advia 2400 and Vista)30. An overall good agreement
was found between H-index and the reference methods (κ comprised between 0.821 and 0.982). Lee et al. measured the H-index on Modular System P using 6 aliquots of the same clinical sample with different concentrations of fHb; an excellent correlation was found between the theoretical and measured values of fHb (r²=0.999).

More recently, Gabaj et al. carried out an extensive study on the H-index by carefully analysing the performance of this measure with two different clinical chemistry platforms (i.e. Roche Cobas c501 and Abbott Architect c8000)32. The H-index was measured in 7 samples, with increasing amounts of haemolysis (i.e. 0.312-20 g/L) and results were then plotted against theoretical fHb values. A good agreement was found among the theoretical and measured values, especially for Roche Cobas 6000 (slope of regression 1.01; intercept 0.03), while a less impressive but still acceptable agreement was observed with Abbott Architect c8000 (slope of regression 1.07; intercept 0.02). Even more importantly, a bias of slightly over 10% was only observed when measuring sample aliquots with the lowest (and likely clinically insignificant) fHb concentration (i.e. 0.312 g/L). Another important aspect that emerged from this study is that the lack of precision of the H-index on the two clinical chemistry analysers was still definitely acceptable: 0.7-1.7% (intra-assay) and 0.7-2.1% (inter-assay), respectively. Notably, there was a non-clinically significant interference with the H-index from high lipaemia and bilirubin concentrations, suggesting that this measure may be robust and clinically accurate even in samples with high values of other interfering substances33.

Preliminary evidence published by Ko et al.,33 Yasar et al.,34 and Said et al.35 shows that systematic assessment of H-index on all routine samples may be an attractive strategy for both identifying patients with intravascular haemolysis due to different clinical conditions and for monitoring therapeutic effectiveness, while this measure was also found to be more useful than haptoglobin for monitoring the risk of foot-strike haemolysis in ultramarathon runners36.

### Conclusions

The results published in the available scientific literature also show that results of fHb generated using the H-index are highly correlated with those obtained with more widely validated haemoglobin assays (Table I) which were, in turn, proven reliable for not only measuring fHb in patients with some forms of haemolytic anaemia, but also for predicting these patients’ clinical outcomes30,37,38. Notably, there is no obvious reason to suspect that the analytical performance of the H-index may differ according to the haemolysis trigger, as well as using in vitro or in vivo haemolysed blood, as proven in some independent studies32,39,40.

Unlike the reference HiCN method, the H-index does not entail the use of toxic compounds, it is accurate, rapid, cheap, suitable for total automation, and is less vulnerable to the typical inter-observer

<table>
<thead>
<tr>
<th>Authors</th>
<th>Samples</th>
<th>H-index method</th>
<th>Comparison assay</th>
<th>Agreement</th>
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</thead>
<tbody>
<tr>
<td>Unger et al., 2007</td>
<td>200 clinical samples</td>
<td>Modular System P</td>
<td>Golf (2-wavelength) spectrophotometric assay</td>
<td>r=0.99</td>
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<td>Moon-Massat et al., 2008</td>
<td>192 plasma aliquots spiked with HBOC</td>
<td>Modular System P</td>
<td>Actual calculated HBOC concentration</td>
<td>r²=0.99</td>
</tr>
<tr>
<td>Lippi et al., 2009</td>
<td>5 clinical samples</td>
<td>Modular System P</td>
<td>HiCN spectrophotometric assay</td>
<td>r=1.00</td>
</tr>
<tr>
<td>Petrova et al., 2013</td>
<td>100 clinical samples</td>
<td>Modular System P</td>
<td>Harboe (3-wavelength) and Fairbanks (3-wavelength) spectrophotometric assays</td>
<td>r=0.982 (Harboe) and r=0.969 (Fairbanks)</td>
</tr>
<tr>
<td>Fernandez et al., 2014</td>
<td>6 aliquots of the same sample</td>
<td>Roche Cobas c511, c711 and Modular System P; Beckman Coulter 5400 and Synchron LX725; Siemens Advia 2400 and Vista</td>
<td>HiCN spectrophotometric assay</td>
<td>Roche Cobas c511, c711 and Modular System P; κ= 0.973 Beckman Coulter AU 5400; κ= 0.833 Beckman Coulter Synchron LX725; κ= 0.790 Siemens Advia 2400; κ= 0.982 Siemens Vista; κ=0.821</td>
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<tr>
<td>Lee et al., 2016</td>
<td>6 aliquots of the same sample</td>
<td>Modular System P</td>
<td>Actual calculated fHb concentration</td>
<td>r²=0.999</td>
</tr>
<tr>
<td>Nikolae Gabaj et al., 2018</td>
<td>7 aliquots of the same sample</td>
<td>Roche Cobas c501 and Abbott Architect c8000</td>
<td>Actual calculated fHb concentration</td>
<td>Roche Cobas c501; slope of regression 1.01; intercept 0.03 Abbott Architect c8000; slope of regression 1.07; intercept 0.02</td>
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Fhb: cell-free haemoglobin; HBOC: haemoglobin-based oxygen carrier; HiCN: haemiglobincyanide.

References:
variability which plagues the visual identification of haemolysed serum or plasma samples. We can, therefore, propose a tentative algorithm with which to use this measure for screening patients with suspected intravascular haemolysis (Figure 1) based on sequential steps entailing H-index assessment in serum or plasma, conversion of the arbitrary and instrument-dependent H-index values into g/L of fHb, exclusion of potential sources of in vivo haemolysis (i.e. by requesting another sample or troubleshooting potential problems that may have occurred during sample collection, transportation or storage), followed by release of data to the clinicians when the fHb concentration is above the upper reference limit (i.e. typically ≥0.25 g/L in serum or ≥0.13 g/L in plasma, respectively). Provided that this algorithm could be validated in clinical studies, the H-index could be used for rapid and inexpensive screening of serum or plasma samples collected from patients with clinical suspicion of intravascular haemolysis. This may be especially useful in subjects with those inherited (e.g. sickle cell anaemia, spherocytosis) or acquired (disseminated intravascular coagulation, haemolytic uremic syndrome, immune thrombocytopenia) conditions which are quite frequently associated with major RBC injury and breakdown, and which would benefit most from measurement and serial monitoring of fHb for predicting clinical outcome.

One final consideration is that although H-Index is now available on all clinical chemistry analysers, this measure is not currently intended or validated for diagnostic purposes. Nevertheless, this hurdle can easily be overcome by implementing a local quality assurance programme for serum indices, as recently advocated by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM).

The Authors declare no conflicts of interest.

References


