VALIDATION OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA DETECTION BY FLOW CYTOMETRY

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Background:
Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare and acquired disease due to a mutation, in hemopoietic stem cells, of phosphatidylinositol glycan complementation class A (PIG-A) gene leading to a partial or total deficiency of the enzyme involved in the synthesis of glycosylphosphatidylinositol (GPI). This prevents the binding of many proteins linked to the cell membrane of erythrocytes and leucocytes, such as CD 55 and CD 59. Without these 2 proteins on the red blood cells (RBC) surface, RBC are more sensitive to the lytic action of the complement system, which leads to intravascular haemolysis.

Diagnostic of PNH is carried out by flow cytometry (BD FACSCANTO II). In this study, we have followed the guidelines established by Borowitz et al. (Cytometry B, 78B: 211, 2010). The guidelines include a first panel of antibodies to identify neutrophils and monocytes and a second panel directed against GPI-linked proteins (CD 54 and CD 14). Besides antibodies, the protocol uses an invariant variant of aerolysin (FLAER) which is directly linked to GPI anchor. When GPI-deficient leucocytes are detected, an additional panel is used to quantify GPI-deficient RBC, using glycophrin A gating and CD59. The validation of PNH detection was undertaken in accordance to ISO15189 standard and included the following parameters: intra-assay precision, bias, uncertainty of measurement, limits of detection and quantification (sensitivity), interference analysis, overall linearity of flow-cytometric measures and sample stability.

Method:
The panel used by Borowitz et al. includes Fluor AF488: CD45 PerCP - CD33 PC7, CD15 AF67 - CD 24PE - CD 14 AF7 provides us a sequence of analysis that discriminates neutrophils from monocytes and highlights GPI-deficient cells with 2 parameters:
- Plot 4: GPI-deficient neutrophils are in the lower left corner (CD 24 - Flaer -)
- Plot 5: GPI-deficient monocytes are in the lower left corner (CD 14 - Flaer -)

Results:
- Sensitivity (N = 30 normal samples)
  \[ D = \text{Limit of detection} \%
  \[ Q = \text{Limit of quantification} \%
  \]
The background of 30 normal samples allowed us to define the sensitivity of the method. As shown in the table, the method can detect GPI deficient clones as low as 0,01 %.

- Interferences by eosinophils and immatures granulocytes

Samples with eosinophils or immature granulocytes were tested (N=30 for each category)

- Intra-assay precision

<table>
<thead>
<tr>
<th>GPI deficient events (%)</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>10,59</td>
<td>9,75</td>
<td>11,24</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0,15</td>
<td>0,42</td>
<td>0,11</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>1,38</td>
<td>4,28</td>
<td>1,02</td>
</tr>
</tbody>
</table>

- Overall linearity of cytometric measures

High range: dilution of CD3 + T cells

Low range: dilution of CD24+

- Combined uncertainty of measurement \( u \) (neutrophil analysis): based on 15 external quality controls (UKNEQAS), and computed from our average bias and the group dispersion with regards to the median.

- Stability

Typing must be done within 2 days for WBC analysis and within 7 days for RBC, to avoid antigen alteration. Samples must be stored at 4°C. Stained samples must be analyzed at 1 to 3 hours after fixation.

Conclusion:
The detection of PNH by flow cytometry may be validated according to ISO15189 standard, when using published international guidelines. The estimation of linearity should be completed with dilutions of a sample with a large PNH clone.

References: