Standardized immunophenotypic analysis of myeloperoxidase in acute leukaemia

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Supplement
**Supplement 1 – Manual Analysis**

**Acute Leukemia Orientation Tube (ALOT)**

| Dye     | PB     | PO     | FITC   | PE     | PerCP-Cy5.5 | PE-Cy7 | APC   | APC-H7 |
|---------|--------|--------|--------|--------|-------------|--------|-------|--------|
| Marker  | CyCD3  | SmCD45 | CyMPO  | CyCD79a| SmCD34      | SmCD19 | SmCD7 | SmCD3  |
| Clone   | UCHT1  | HI30   | MPO-7  | HM57   | 8G12        | J3-119 | 124-1D1| SK7    |
| Manufacturer | Becton Dickinson | Invitrogen | Dako | Dako | Becton Dickinson | Beckman Coulter | eBioscience | Becton Dickinson |

**Gating Strategy**

Samples were manually analysed in Infinicyt Software (Cytognos, Salamanca, Spain), by experienced cytometrists. Each analysis was verified, by another experienced cytometrist, from another EuroFlow center. First, debris and doublets were removed. Second, normal lymphoid cells were gated (i.e. the B cells, T cells and NK cells). Third, normal myeloid cells were gated (i.e. the nucleated red blood cells, neutrophils, monocytes and eosinophils). Only populations with completely distinct and absolutely normal immunophenotypes were considered to be normal. Finally, the leukemic cells were gated, which was generally the remainder of cells. Arbitrary gates on MPO were avoided. An exemplary AML case is shown below:

**Step 1 – Doublets; based on scatter characteristics (FSC-A and FSC-H):**
Step 2 – Debris; based on scatter (FSC-A and SSC-A):

Step 3 – Lymphocytes; based on scatter and CD45 expression.
Step 4 – Lymphocyte subsets (i.e. B-cells, T-cells and NK-cells); based on CD19, CD3 and CD7.

The normal myeloid cell populations were gated in order of distinctness, which varied among the samples. Thus, in each following step, the normal myeloid cell population with the most distinct immunophenotype was gated. Notably, the APS plots show the principal component analysis (PCA), where APS1 essentially shows PC1 vs. PC2, and APS2 essentially shows PC1 vs. PC3, which gives a good impression of distinctness.

Step 5 – In this case, firstly the eosinophils; fully distinguishable based on scatter.
Step 6 – In this case, secondly the NRCB; fully distuingishable based on CD45, CD34 and cyMPO.

Step 6 – In this case, thirdly the neuutrophils; fully distuingishable based on MPO and CD34 expression
Step 8 – In this case, fourty the monocytes; fully disquinsisable based on CD34 and cyMPO expression.

Step 9 – In this case, the remainder only consisted of abnormal myeloid cells.
Step 9 – In case necessary, obvious debris and/or doublets were removed from individual populations.

![Graphs showing data analysis](image)

**Detailed Information**

It should be emphasized that contamination of AML cells cannot be ruled-out. Fortunately, the effect of potential contamination on the MPO status seems negligible. Essentially, four types of contamination can be defined:

- **MPO-negative** AML with **MPO-positive** normal cells
- **MPO-positive** AML with **MPO-negative** normal cells
- **MPO-negative** AML with **MPO-negative** normal cells
- **MPO-positive** AML with **MPO-positive** normal cells

The “first two types” seem rather unlikely, as MPO itself generally allows for discrimination, or in the worst case, at least gives a clear indication of contamination (i.e. bimodal MPO expression should trigger the cytometrists, e.g. to perform additional immunophenotyping to assess the cause of modality, as was also done in this study, by referring to the EuroFlow AML/MDS panel, as routinely acquired along with the ALOT itself). The “third type of contamination also seems unlikely, as normal MPO-negative cells (e.g. nucleated red blood cells and lymphocytes) can be easily separated from the AML cells, given their rather distinct immunophenotype. The “fourth type” of contamination seems most likely, as the immunophenotype of normal neutrophils may potentially match the immunophenotype of AML cells (given the eight ALOT markers). However, the MPO status is presumably never negatively influenced by such contamination, as any AML that reaches such MPO intensities (i.e. causing complete overlap with normal neutrophils) should be considered MPO-positive anyway.
To our best knowledge, within this study, the contamination of AML cells was low, essentially for three reasons: Firstly, in case of doubt (e.g. bimodal MPO histogram), the EuroFlow AML/MDS panel (containing 32 markers) was used to assess the immunophenotype of the AML cells (e.g. confirm the existence of subclones). Secondly, for every case that “appeared to have neutrophils” the neutrophils could be gated without using arbitrary gates, as shown in the subsequent examples. Thirdly, the vast majority of AML cases (≈90%) featured continuous MPO expression patterns ([Sup. 4]), thus these cases are extremely unlikely to be affected by substantial contamination. For the other of cases, the AML/MDS panel was always consulted in case no neutrophils could be gated.

Within CD34-positive AML cases, the AML cells (red) were easily separated from neutrophils (pink):
Within most CD34-negative AML cases, the AML cells (red) were also easily separated from neutrophils (pink):
Within some CD34-negative AML cases, the AML cells (red) could not be distinguished from neutrophils (pink) based on CD34 vs. MPO, however, they could be separated based on FSC-A vs. SSC-A and/or based on the APS view (i.e. the principal component analysis, with PC1 vs. PC2). Seven exemplaric cases (one per row):
Supplement 2 – Stability

On top, MPO expression is shown for each lymphocyte population (left), each neutrophil population (middle), each BCP/T-ALL population (right), and ordered by median MPO expression. Horizontal grey bars represent the 98% interval for each population. Red lines connect the medians, and thereby visualize the ECDF. Black lines represent the 90% interval for each set of medians. At the bottom, the corresponding Q-Q plots, with logicle transformed median MPO expression levels (y-axis), and the theoretical T distribution (x-axis). The dashed grey lines represent the 95% envelope. Each case was within the projected envelope. Altogether, each set of controls (lymphocytes, neutrophils, and BCP/T-ALL cells) followed a t distribution (a heavy-tailed variant of the normal distribution), and was clearly unimodal in nature (confirming their suitability as control).

In addition, the median MPO expression levels were grouped by centre and year, resulting in sixteen groups; “EMC 2010” up to “EMC 2018” and “DCOG 2010” up to “DCOG 2016”. No differences were found by ANOVA (p=0.5860 for lymphocytes, p=0.5486 for BCP/T-ALL cells, and p=0.1417 for neutrophils) and Kruskall-Wallis (p=0.5583, p=0.9998 and p=0.1417 respectively). Thus, the ALOT was stable in terms of absolute FIU for MPO (over time and across centres).
Supplement 3 – Reproducibility of Manual Analysis

All samples from the study cohort (n=1180) were analysed by one experienced flow cytometrist (from the EMC). Each analysis was reviewed by another experienced flow cytometrist (from one of the other participating centres). The resulting analysis, referred to as the “cytometrist 1” analysis, formed the basis of this study.

In order to evaluate reproducibility of the manual analysis, in total 182 samples were randomly selected from the study cohort. These 182 samples were anonymized, and analysed by another (less-experienced) flow cytometrist (also from the EMC). The resulting analysis, referred to as the “cytometrist 2” analysis, was compared to the “cytometrist 1” analysis, in terms of the MPO.MEDIAN values, as reported for each population (see next page).

The cytometrists (i.e. “cytometrist 1” and “cytometrist 2”) were instructed to follow the aforementioned gating strategy (Sup. 1), and were instructed to avoid arbitrary gates at all times (i.e. only populations with truly distinct and normal immunophenotypes were to be considered normal, and the remainder of cells were to be considered abnormal (details in Sup. 1)).

An exemplary sample, as analysed by both cytometrists:
The MPO.MEDIAN values, as reported by two independent cytometrists, for 182 samples, for nine populations, showed strong correlations, proving reproducibility across “cytometrist 1” and “cytometrist 2”.

Importantly, the MPO.MEDIAN values for lymphocytes, monocytes and neutrophils showed strong correlations, thus the controls as used for stability checks, cut-off determinations, and/or thresholds determinations were barely influenced by inter-expert variation. The MPO.MEDIAN values for acute leukaemia cells also showed strong correlations, thus the MPO status itself was barely influenced by inter-expert variation. Notably, the outlier in the bottom right panel was caused by “cytometrist 2” who accidentally assigned one completely normal population to the “acute leukaemia node” in the population tree (of note; these “nodes” were next to each other in the tree).
Supplement 4 - Heterogeneity

For each AML case (n=519), the nature of the MPO histogram was characterized manually. Overview:

- Unimodal and Normal 14.7% (76/519)
- Unimodal and Skewed 76.1% (395/519)
- Bimodal 5.2% (27/519)
- Ambiguous 4.0% (21/519)

Note: the vertical red line visualized the later established positivity threshold of 780 FIU.

For 14.7% of the AML cases (76/519), the MPO histogram was unimodal and normal:
For 76.1% of the AML cases (395/519), the MPO histogram was unimodal and skewed (first 36 cases shown):

For 5.2% of the AML cases (27/519), the MPO histogram was bimodal:

For 4.0% of the AML cases (21/519), the MPO histogram was ambiguous: (i.e. either a skewed unimodal distribution, or bimodal distribution)
Two negative controls were evaluated, namely lymphocytes (B/T/NK together, from 1175 individual samples) and ALL cells (BCP/T-ALL together, from 661 individual samples). A. For each lymphocyte population, the 98th percentile of MPO expression was derived, and subsequently, the 98th percentile of all 98th percentiles (=780 FIU) was used as threshold for the MPO.PPC calculation. B. The same procedure was repeated based on ALL cells, resulting in a threshold of 1503 FIU.
Supplement 6 – ROC and AUC

Two distinct positivity thresholds were established (780 and 1503 FIU), based on two negative controls (Sup. 5). The threshold of choice (i.e. 780 FIU vs. 1503 FIU) clearly influenced the MPO.PPC values (Sup. 5). Presumably, the threshold of choice also influences the diagnostic performance, which can be quantified in terms of the area under the curve (AUC) on the receiver operator curve (ROC). For the following ROC analysis, the WHO-NEG cases were merged (and used as negative control), and the WHO-POS cases were merged (and used as positive control):

By creating one ROC based on the MPO.PPC.780 values (the MPO.PPC values based on the 780 FIU threshold), and one ROC based on the MPO.PPC.1503 values, the diagnostic performance (in terms of AUC values) can be easily evaluated, by taking the WHO-NEG as negative control, and the WHO-POS as positive control. In this case, the MPO.PPC.780 values resulted in a slightly higher AUC, compared to the MPO.PPC.1503 values:
Obviously, the same analysis can be performed for any arbitrary positivity threshold. The following figure shows the MPO.PPC values as calculated based on six arbitrary thresholds (i.e. 300, 500, 700, 900, 1100, and 1300 FIU), and the corresponding ROC with AUC values (i.e. 0.913, 0.935, 0.943, 0.946, 0.945 and 0.043 respectively):

Within “the AUC overview” figure (Fig. 1.D) the blue components visualize the AUC values for one pair of controls (i.e. “WHO-POS vs. WHO-NEG”) and purple components visualize the AUC values for another pair of controls (i.e. BCP/T-ALL cells vs. monocytes). The solid blue line visualizes the AUC values, as were derived by repeating the aforementioned evaluation for any positivity threshold within the 250 up to 1500 FIU range. For clarity, the AUC values as shown in the six ROC above, were highlighted in red. Essentially, any positivity threshold between 780 and 1105 FIU seems to results near-optimal AUC values (i.e. for “WHO-POS vs. WHO-NEG”).
Essentially, the same analysis can be performed based on the MPO.MEAN and MPO.MEDIAN values as well:

Within “the AUC overview” figure (Fig. 1.D), for reference, the AUC value as calculated based on the MPO.MEAN values is visualized by a dotted line, and the AUC value as calculated based on the MPO.MEDIAN values is visualized by a dashed line. For clarity, the AUC values as shown in the two ROC above, were highlighted in red. Notably, the MPO.MEAN and MPO.MEAN do not depend on any positivity threshold, explaining the horizontal lines. The MPO.PPC always resulted in superior AUC values (even regardless of the positivity threshold of choice).

The same analysis was performed based on ALL cells (as negative control) and monocytes (as positive control), and is visualized by the purple components in “the AUC overview” figure (Fig. 1.D). For completeness, the most important underlying ROC are shown below (for MPO.MEAN, MPO.MEADIAN and MPO.PPC.780):
Supplement 7 – MPO-positive BCP/T-ALL cases

| Case | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| BCP/T-ALL | T  | BCP | BCP | BCP | BCP | BCP | BCP | BCP | BCP | BCP |
| MPO^flow | 47.29 | 99.76 | 33.97 | 27.13 | 26.19 | 22.28 | 21.80 | 30.19 | 39.17 | 20.99 |
| MPO^cyto | NT | 0 | NT | NT | NT | NT | NT | NT | NT | 0 |
| CD13 | neg | neg | dim | neg | neg | neg | neg | neg | neg | neg |
| CD33 | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| CD117 | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| CD34 | pos | part | pos | pos | pos | pos | pos | pos | pos | part |
| TdT | neg | neg | pos | pos | pos | pos | pos | pos | pos | pos |
| CD10 | neg | pos | pos | pos | pos | pos | pos | pos | pos | pos |
| CD19 | neg | pos | pos | pos | pos | pos | pos | pos | pos | dim |
| CD22 | NT | neg | pos | pos | pos | pos | pos | pos | pos | pos |
| CD20 | NT | pos | neg | part | neg | pos | pos | neg | neg | pos |
| cyCD79a | neg | pos | pos | dim | dim | pos | pos | pos | pos | dim |
| CD66c | NT | neg | pos | pos | pos | pos | pos | part | neg | dim |
| cyCD3 | pos | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| CD2 | neg | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| CD5 | pos | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| CD7 | pos | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| CD4 | neg | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| CD8 | pos | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| CD1a | neg | NT | NT | NT | NT | NT | NT | NT | NT | NT |

Based on the established definition; ten BCP/T-ALL cases were considered MPO-positive (seven originated from the study cohort and three originated from the validation cohort). For these cases, expression of various markers is shown, in terms of being negative (“neg”), positive (“pos”), partially expressed (“part”), dimly expressed (“dim”) or not tested (“NT”). The MPO histograms are shown on the subsequent page, including the established threshold (=780 FIU, vertical bar). Except for MPO-positivity, there is no other evidence for myeloid involvement; therefore, we would recommend being cautious in considering these cases as MPAL instead of BCP/T-ALL. The percentage of MPO-positive MPAL cells is given, based on flow cytometry (MPO^flow, using the here established positivity threshold of 780 FIU), and based on cytomorphology (MPO^cyto, using the Sudan Black B staining).
MPO histograms for MPO-positive BCP/T-ALL cases (established threshold shown by vertical bar, 780 FIU).

Case 1
MPO.PPC = 47.29

Case 2
MPO.PPC = 99.76

Case 3
MPO.PPC = 33.97

Case 4
MPO.PPC = 27.13

Case 5
MPO.PPC = 26.19

Case 6
MPO.PPC = 22.28

Case 7
MPO.PPC = 21.80

Case 8
MPO.PPC = 30.19

Case 9
MPO.PPC = 39.17

Case 10
MPO.PPC = 20.99
### Supplement 8 – MPO-negative MPAL cases

| Case | Lineage | Treatment | Relapse | MPO<sup>flow</sup> | MPO<sup>cyto</sup> | CD13 | CD33 | CD117 | CD36 | CD14 | CD64 | CD34 | DR | TdT | CD10 | CD19 | CD22 | cyCD79a | cyCD3 | CD2 | CD5 | CD7 | CD4 | CD8 | CD1a |
|------|---------|-----------|---------|-------------------|-------------------|------|------|------|------|------|------|------|-----|-----|------|------|------|--------|--------|-----|-----|-----|-----|-----|------|
|      | M/T     | ALL-based | no      | 18.81             | 5                 | pos  | neg  | pos  | neg  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | pos  | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |
|      | M/T     | ALL-based | no      | 15.03             | 5                 | pos  | neg  | pos  | neg  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | pos  | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |
|      | M/T     | ALL-based | no      | 14.55             | 11                | pos  | neg  | part | pos  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | part | part | part | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |
|      | M/T     | ALL-based | no      | 13.97             | 22                | pos  | neg  | pos  | neg  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | part | part | part | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |
|      | M/T     | ALL-based | refractory | 6.62              | NT                | pos  | neg  | pos  | neg  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | part | part | part | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |
|      | M/B     | ALL-based | no      | 4.52              | NT                | pos  | neg  | pos  | neg  | neg  | neg  | neg  | pos | pos  | neg  | neg  | neg  | neg  | dim  | neg  | part | part | part | neg  | neg  | neg  | neg  | neg  | neg  | neg  | neg  |

Twenty-nine MPAL cases with myeloid involvement (originally classified as such based on the same ALOT files) were re-evaluated based on the here-established definition for MPO-positivity, resulting in 23 MPO-positive and six MPO-negative cases. The original diagnostic reports for these six cases revealed that the myeloid involvement was not underpinned by MPO-positivity (i.e. they were never considered to be MPO-positive), but by expression of other myeloid markers (CD13, CD33, and/or CD117) and partial lack of lymphoid-defining markers. Thus, these six cases should formally (according to WHO criteria) not be classified as MPAL by flow cytometry. For these cases, the expression of various markers is shown; in terms of being negative (“neg”), positive (“pos”), heterogeneous (“het”), partially expressed (“part”) or dimly expressed (“dim”). The percentage of MPO-positive MPAL cells is given, based on flow cytometry (MPO<sup>flow</sup>, using the positivity threshold of 780 FIU), and based on cytomorphology (MPO<sup>cyto</sup>, using the Sudan Black B staining). Some cases were not tested (“NT”). Notably, these six cases (five M/T and one M/B) were treated with ALL regimens; and five achieved long-term complete remission.