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Standardised immunophenotypic analysis of myeloperoxidase in acute leukaemia

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Summary

Given its myeloid-restricted expression, myeloperoxidase (MPO) is typically used for lineage assignment (myeloid vs. lymphoid) during acute leukaemia (AL) diagnostics. In the present study, a robust flow cytometric definition for MPO positivity was established based on the standardised EuroFlow protocols, the standardised Acute Leukaemia Orientation Tube and 1734 multicentre AL cases (with confirmed assay stability). The best diagnostic performance was achieved by defining MPO positivity as ≥20% of the AL cells exceeding a lymphocyte-based threshold. The methodology employed should be applicable to any form of standardised flow cytometry.

Keywords: immunophenotyping, flow cytometry, acute leukaemia, AML, ALL, myeloperoxidase.

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Introduction

Given its myeloid-restricted expression, myeloperoxidase (MPO) is often used for lineage assignment during acute leukaemia (AL) diagnostics. Within acute myeloid leukaemia (AML), subgroups like AML with t(8;21), AML with t(15;17), AML with inv(16) and AML with mutated CCAAT/enhancer-binding protein α (*CEBPA*), are typically MPO positive [World Health Organization (WHO)-POS]. While other subgroups such as acute monoblastic/monocytic leukaemia, acute erythroid leukaemia, acute megakaryocytic leukaemia and AML associated with Down syndrome are typically MPO negative (WHO-NEG).[1] Thus, whereas MPO positivity proves myeloid origin, MPO negativity cannot rule out myeloid origin.

The MPO status (the AL being MPO positive/negative) is classically determined by cytomorphology, which has its advantages (e.g. relatively simple and cheap), disadvantages (e.g. inter/intra-expert variability) and ambiguities (e.g. the WHO considers MPO and Sudan Black B synonymous). Alternatively, the MPO status can be determined by flow cytometry, which has its advantages (e.g. minimise intra/inter-expert and intra/inter-laboratory variability via standardisation and automation) and disadvantages (e.g. relatively complex and expensive).[2–4]

The UK National External Quality Assessment Service (NEQAS) has shown that standardisation of flow cytometric assays (at least in terms of dyes, clones and sample preparation) is crucial for reproducibility.[5] Nevertheless, to our best knowledge, no de facto standard or fully standardised flow cytometric assay exists for the MPO status. However, standardised assays for the initial assessment of samples suspected of AL do already exist, e.g. the EuroFlow Acute Leukaemia Orientation Tube (ALOT), which includes MPO as a marker. Thus, the only missing link for the ALOT to become a fully standardised MPO status assay is a solid definition for MPO positivity.

Methods

The ALOT files from 1180 cases [527 B-cell precursor acute lymphoblastic leukaemia (BCP-ALL), 134 T-cell ALL (T-ALL) and 519 AML], as acquired by the Dutch Childhood Oncology Group (DCOG, 2010–2015) and the Erasmus University Medical Center (EMC, 2010–2018), served as the study cohort. The ALOT files from 554 cases [315 BCP-ALL, 56 T-ALL, 154 AML and 29 mixed-phenotype AL (MPAL)], as acquired at five international EuroFlow centres, served as the validation cohort. Acquisition was performed according to the EuroFlow protocols,[6,7] which rely on the MPO:FITC (fluorescein isothiocyanate) conjugate (clone MPO-7, details in Data S1. The populations of interest (normal and/or leukaemic cells) were gated manually (Data S1). The marker of interest (MPO) was quantified in arbitrary fluorescence intensity units (FIU). Three descriptive statistics were evaluated: the mean fluorescence intensity (MPO.MEAN), the median fluorescence intensity (MPO.MEDIAN) and the percentage of positive cells (MPO.PPC). The area under the curve (AUC) of the receiver operating characteristic (ROC) curve and Youden's *J* statistic (*J*) were used to assess diagnostic performance. The present study was approved by the Ethics Committee of each centre.

Results

The MPO.MEDIAN values of normal lymphocytes, normal neutrophils and ALL cells (BCP/T-ALL together) were confirmed to be homogeneous (unimodal distribution) and stable (over time and across centres; Data S2). The MPO.MEDIAN values for 182 samples, as reported by two cytometrists, showed strong correlations (Data S3). Thus, the ALOT was stable in terms of absolute FIU for MPO, the manual analysis was reproducible, and two suitable negative controls were identified (i.e. normal lymphocytes and ALL cells).

The MPO.MEAN and MPO.MEDIAN values were comparable within each normal cell population and within each ALL cell population (Fig 1A). As expected, neutrophils had the highest values followed by monocytes, whereas lymphocytes had the lowest values (lower than ALL cells). The MPO.MEAN and MPO.MEDIAN values were highly variable between AML cases (Fig 1A), ranging from MPO negative (like lymphocytes) to strongly MPO positive (like neutrophils). The MPO.MEAN and MPO.MEDIAN values were different within most AML cases (Fig 1A), which is indicative of heterogeneity, e.g. due to clear skewness (~76% of cases, Data S4) or subclones (~5% of cases, Data S4).

The expression of MPO between and within AML subgroups was highly heterogeneous (Fig 1B). As expected, the MPO.MEAN and MPO.MEDIAN values were highest for the WHO-POS subgroups, and lowest for the WHO-NEG subgroups (Fig 1B). For the WHO-NEG and WHO-POS cases, the MPO.MEAN and MPO.MEDIAN values were similar (i.e. robust MPO negativity/positivity), while for the other AML

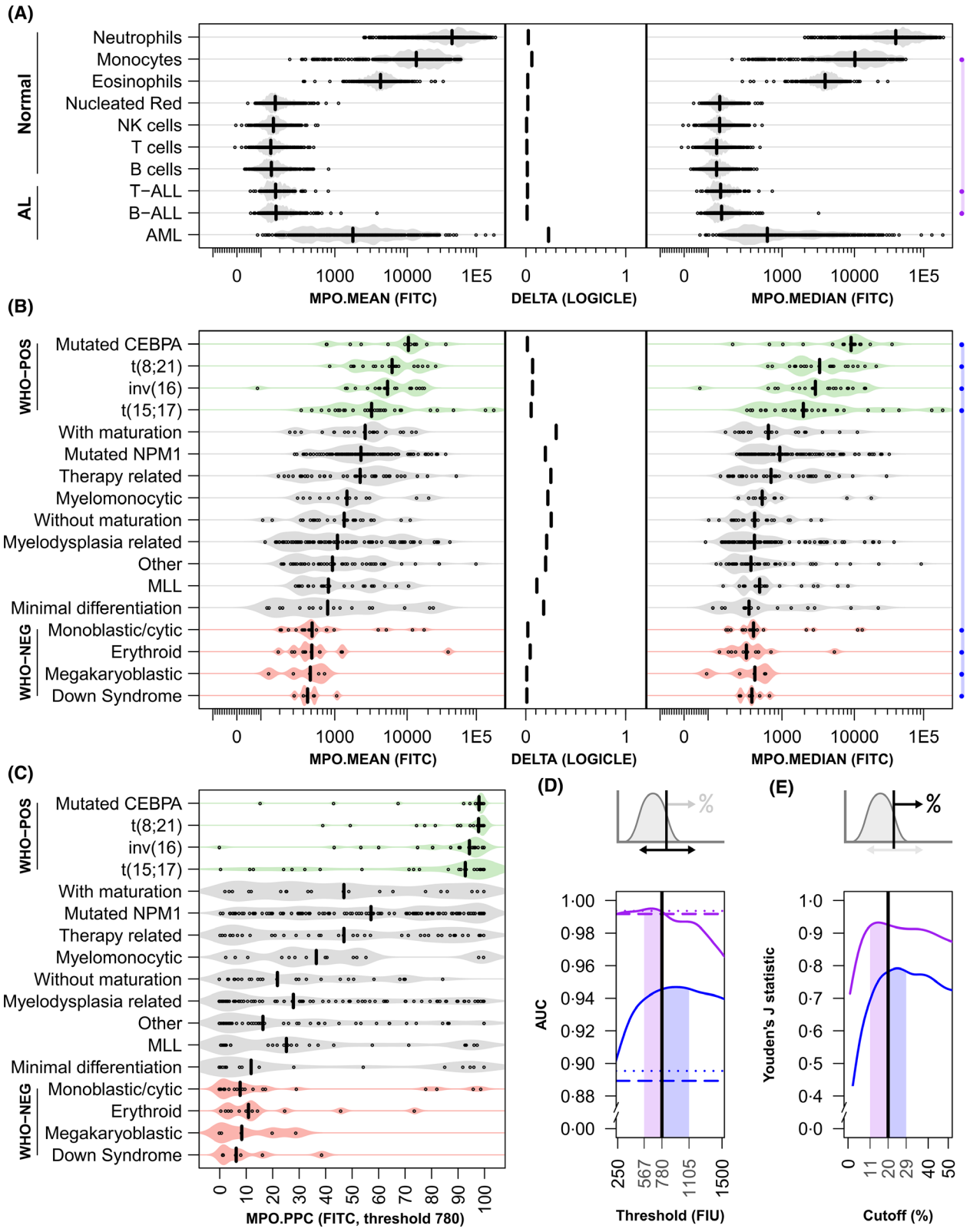

Fig 1. (A, B) MPO expression, in terms of MPO.MEAN and MPO.MEDIAN, on default "logicle" scales, for normal cells, AL cells and AML subgroups. Circles visualise individual populations. Vertical bars represent; in left panel MPO.MEAN, in the right panel MPO.MEDIAN and in the middle panel absolute differences between average MPO.MEAN and average MPO.MEDIAN (in terms of "logicle" units). The WHO groups are ordered by average MPO.MEAN. Self-explanatory abbreviations were used for various WHO classes, and rare cases were combined into 'other' [e.g. Runt-related transcription factor 1 (RUNX1) and nucleophosmin 1 (NPM1) + CCAAT/enhancer-binding protein α (CEBPA)]. WHO-NEG are shown in red, and WHO-POS in green. (C) MPO expression, in terms of MPO.PPC, based on the lymphocyte based positivity threshold (=780 FIU), for the AML subgroups. (D) The performance of MPO.MEAN (in dotted lines), MPO.MEDIAN (in dashed lines) and MPO.PPC (in solid lines), in terms of AUC values, for two pairs of controls, namely BCP/T-ALL cells *versus* monocytes (in purple) and WHO-NEG AML *versus* WHO-POS AML (in blue). Within both pairs, the MPO.MEAN (dashed lines) and MPO.MEDIAN (dotted lines) yielded similar AUC, and the MPO.PPC yielded superior AUC. For the first pair (purple), thresholds between 567 and 780 FIU yielded near-optimal AUC (>0·993). For the second pair (blue), thresholds between 780 and 1105 FIU yielded near-optimal AUC (>0·946). Thus, the lymphocyte-based positivity threshold (=780 FIU) yielded near-optimal AUC for both pairs, and was therefore chosen. (E) Finally, the same pairs of controls were used to find the optimal positivity cut-off (i.e. the positivity cut-off that results in the highest *J* statistic, i.e. the lowest proportion of misclassified results). This analysis was based on the previously established positivity threshold (=780 FIU). For the first pair (purple), cut-offs between 11% and 20% yielded near-optimal *J* statistics, and for the second pair (blue), cut-offs between 20% and 29% yielded near-optimal *J* statistics. Thus, a positivity cut-off of 20% yielded a near-optimal *J* statistic for both pairs, and was therefore chosen. [Colour figure can be viewed at wileyonlinelibrary.com]

cases, the MPO.MEAN and MPO.MEDIAN values were different (i.e. heterogeneous MPO expression).

The MPO.PPC is an ambiguous statistic, as any positivity threshold may be used, either arbitrarily chosen or based on negative controls. In the present study, two solid negative controls were evaluated: lymphocytes and ALL cells. 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 the threshold for the MPO.PPC calculation (Fig 1C and Data S5). The same procedure was repeated for ALL cells, resulting in a threshold of 1503 FIU (Data S5). Obviously, the control of choice influenced the threshold (780 vs. 1503 FIU), and thereby the resulting MPO.PPC values (Data S5).

Alternatively, the positivity threshold may be optimised for a specific purpose by taking a pair of controls, and finding the threshold that results in optimal discrimination. Two pairs of controls were chosen for this purpose: ALL cells *versus* monocytes (for negative vs. weak positive) and WHO-NEG *versus* WHO-POS (for AML). For both pairs, thresholds from 250 to 1500 FIU were evaluated (Fig 1D, details in Data S6) and 674 and 943 FIU were found to be optimal (AUC = 0·995 and AUC = 0·947 respectively). Interestingly, the MPO.PPC based on the lymphocyte-based threshold (780 FIU) resulted in a near optimal AUC, for ALL cells *versus* monocytes (AUC = 0·993), and for WHO-NEG *versus* WHO-POS (AUC = 0·946). Furthermore, the MPO.PPC based on this threshold (780 FIU) outperformed the MPO.PPC based on the ALL-based threshold, and outperformed the MPO.MEAN and MPO.MEDIAN (Fig 1D).

To derive the MPO status (a binary MPO classifier) from the MPO.PPC (a continuous MPO measurement) a positivity

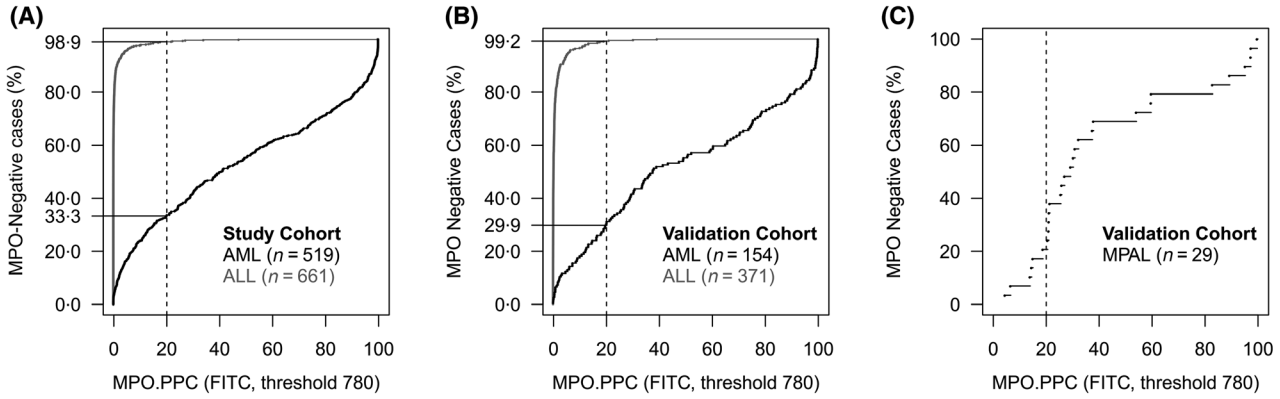

Fig 2. The MPO.PPC values, based on the established positivity threshold (=780 FIU), for the study and validation cohort, shown in terms of empirical cumulative distribution functions (ECDF). The percentage of MPO-positive cases can be easily obtained for any cut-off. The established positivity cut-off (20%) is shown by the vertical dashed line. (A) Based on the established definition for MPO positivity (i.e. the aforementioned threshold and cut-off together), within the study cohort 98·9% of the ALL cases and 33·3% of the AML cases were MPO negative. (B) Similar percentages were found in the validation cohort, namely 99·2% of the ALL cases and 29·9% of the AML cases. (C) In addition, 29 MPAL cases with myeloid involvement (originally classified as such based on the same ALOT files) were re-evaluated based on the definition established here 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.

cut-off needed to be established (i.e. how many AL cells must exceed the positivity threshold for the AL to be considered MPO positive). For both pairs of controls, a cut-off of 20% resulted in near optimal diagnostic performance (Fig 1E), as measured by the Youden's *J*statistic (i.e. the proportion of misclassified results).

Altogether, for optimal diagnostic performance, MPO positivity had to be defined as ≥20% of the AL cells exceeding a lymphocyte-based threshold (=780 FIU for the ALOT with EuroFlow protocols). Within the study cohort (661 ALL and 519 AML), this definition resulted in 98·9% (654/661) of ALL cases being MPO negative and 33·3% (173/519) of AML cases being MPO negative (Fig 2A). Within the validation cohort (371 ALL and 154 AML) similar percentages were found: 99·2% (368/371) and 29·9% (46/154) respectively (Fig 2B). The phenomenon of MPO-positive ALL cases (n = 10, details in Data S7) was reported by others as well, and attributed to either false or true positivity.[8,9]

Finally, 29 MPAL cases with myeloid involvement (originally classified as such based on the same ALOT files) were re-evaluated based on the definition established here for MPO positivity, resulting in 23 MPO-positive and six MPO-negative cases (Fig 2C). 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 (Data S8), as practiced by others as well.[10] Thus, these six cases should formally (according to WHO criteria) not be classified as MPAL by flow cytometry.

Discussion

In the present study, we established a robust flow cytometric definition for MPO positivity based on the standardised EuroFlow protocols, the standardised ALOT and 1734 multicentre AL cases. For optimal diagnostic performance, MPO positivity had to be defined as ≥20% of the AL cells exceeding a lymphocyte-based threshold. Others have reported similar findings, e.g. lymphocytes being an advantageous control and cut-offs between 13% and 28% being optimal.[11] However, the present study is uniquely characterised by its large cohort (others at most a few hundred cases), standardised protocols (publicly available), assay stability checks (over time and across centres/experts), continuous evaluations (for thresholds and cut-offs), comprehensiveness (multiple descriptive statistics and controls) and detailed insight in MPO expression.

It should be emphasised that the conversion from MPO.PPC (i.e. the underlying continuous MPO measurement) to the MPO status (i.e. the binary MPO classifier, as requested by clinicians, and used by the WHO classification) causes significant loss of information. Thus, reporting the MPO.PPC along with the MPO status seems desirable.

Despite being the 'gold standard', the cytomorphological MPO status was not used as reference, primarily due to limited availability, but also due to lack of standardisation (e.g. different protocols across participating centres) and limited correlations being reported by others.[4,11–14]. Instead, two pairs of controls were selected, which were unfortunately not fully MPO negative or MPO positive. For example, acute megakaryocytic leukaemia was part of the WHO.NEG group, being MPO negative according to the WHO classification.[1] However, two cases were clearly MPO positive, and therefore one might argue that these cases should be excluded. On the other hand, MPO positivity in acute megakaryocytic leukaemia has also been reported by others.[15]

Anyhow, temporarily excluding such cases barely influenced the final threshold and/or cut-off, proving their robustness. In the end, one solid definition for MPO positivity could be established, which was robust (e.g. barely influenced the control of choice and/or outliers), and yielded good diagnostic performance.

Thus, by using the ALOT with EuroFlow protocols, together with the definition established here for MPO positivity, the MPO status can be defined in a reproducible manner, with good diagnostic performance. The methodology employed should be applicable to any form of standardised flow cytometry.

Authorship contribution

V.H.J. van der Velden and A.E. Bras designed the study; V. de Haas, M. Jongen-Lavrencic, C.M Zwaan, E. Mejstrikova, P. Fernandez, T. Szczepanski and A. Orfao provided patient material and clinical data; J.G. te Marvelde performed laboratory research; A.E. Bras, Z. Osmani and V.H.J. van der Velden analysed data; A.E. Bras, Z. Osmani and V.H.J. van der Velden interpreted results; A.E. Bras and V.H.J. van der Velden wrote the manuscript; finally, all authors critically reviewed the manuscript and gave their approval.

Conflicts of interest

E. Mejstrikova, T. Szczepanski, J.J.M. van Dongen, A. Orfao and V.H.J. van der Velden each report being one of the inventors on the EuroFlow-owned patent PCT/NL2010/050332 (Methods, reagents and kits for flow cytometric immunophenotyping of leukaemia and lymphoma). The related patents are licensed to Cytognos (Salamanca, Spain) and BD Biosciences (San José, CA, USA), which companies pay royalties to the EuroFlow Consortium. J.J.M. van Dongen and A. Orfao report an Educational Services Agreement from BD Biosciences and a Scientific Advisory Agreement from Cytognos. V.H.J. van der Velden reports a Laboratory Services Agreement with BD Biosciences. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Manual analysis.
Data S2. Stability.
Data S3. Reproducibility of manual analysis.
Data S4. Heterogeneity.
Data S5. Positivity threshold.
Data S6. ROC and AUC.
Data S7. MPO-positive BCP/T-ALL cases.
Data S8. MPO-negative MPAL cases.

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