differentiation.⁷ CXCR5 shows low or negative expression on pre-B cells, plasma cells and their malignant counterparts^{7,8} and high expression on mature B-cell neoplasms including chronic lymphocytic leukaemia, mantle cell lymphoma and follicular lymphoma.^{7–10} Positive PAX-5 expression and negative to low CXCR5 expression in this case pointed against plasmacytic differentiation and mature B-cell neoplasms, respectively, and suggested a precursor B-cell stage of maturation.

At the same time, distinct CD34 positive subsets detected by flow cytometry, though forming only a minority of the blasts, were reproducible on both samples and on different flow cytometry tubes tested in the bone marrow. However, CD34 was negative on IHC. Discrepancies between IHC and flow cytometry have been reported in the literature and the reason underlying this is not clear but may reflect differences in sensitivity and antibody recognition. Moreover, normal cytogenetic karyotype occurs in 20–40% of lymphoblastic leukaemias but is highly uncommon for aggressive forms of B-cell lymphoma, wherein often complex abnormal karyotypes are found in the majority of cases.¹¹

In conclusion, this case illustrates complementarity of histology and flow cytometry in the diagnosis of TdT and CD34 negative LBL. CD138 expression in B-LBL is highly unusual, but there have been sporadic studies which had previously demonstrated that some lymphoblastic leukaemias and myeloid leukaemias express CD138 at the electron microscopic level.¹² We believe that CD138 is an under-appreciated marker of immature B-cells and may be used to support the diagnosis in challenging cases of TdT and CD34 negative precursor B-cell lymphoblastic leukaemia.

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Re: Myeloblasts in normal bone marrows expressing leukaemia-associated immunophenotypes

Sir,

We noted with interest the recent publication of Camburn *et al.*,¹ who evaluated the expression of a set of five leukaemia-associated immunophenotypes (LAIPs) in 14 normal bone marrow (NBM) samples. The authors underlined the value of this effort for their own laboratory in improving MRD assessment, but also raised general concerns about the liberal criteria of LAIP definition on myeloid blasts and whether LAIP expression percentages on normal myeloid blasts might exceed the cut-off used in clinical studies. We fully support the notion that standardisation of multicolour flow cytometry (MFC) determined measurable residual disease (MRD) is of great importance for accurate and reliable results.^{2,3} However, in our opinion, the concerns raised are specific to the presented data and simply extrapolating these to the latest, more evolved MFC MRD protocols would be suggestive and an invalided diminishment of the clinical relevance of MFC MRD. Therefore, we share our considerations on the described LAIP background expression in MFC MRD.

First, we stress that the specificity of LAIPs using pre-defined thresholds depends on the applied MFC antibody panels and analyses. The authors acknowledge the limitations of their antibody panel and the consequences for their gating strategy and LAIP assessment, but do not address its direct implications for the estimation of LAIP positive cells in NBM samples. They use a four-colour antibody panel with limited cell surface markers, containing only CD45 and CD34 in combination with six markers for the detection of aberrant immunophenotypes. In contrast, current antibody panels as recommended by the European LeukaemiaNet use eight or more fluorochromes and besides CD45 and CD34 at least nine markers for LAIP assessment, which enables background levels (far) below 0.1%.^{4–6} Prior panels were specifically improved by the addition of myeloid markers CD13 and/or CD33, aiding the specific gating of relevant LAIPs [e.g., CD7+ (Fig. 1A), CD22+, CD2+ and CD11b+ on CD117+ blasts]. Beyond the nine markers for LAIP detection, additional markers are recommended to exclude non-blast populations (e.g., CD14 for identification of monocytes) and reduce background

Fig. 1 Background leukaemia associated immunophenotypes on normal and regenerating bone marrows. (A) Addition of CD13 shows that CD7 background expression observed is higher on CD13^{low} cells in NBM CD34+ blasts (grey). Correct gating of LAIP CD7 in patients' CD34+ blasts (red) thus need addition of myeloid markers. (B) Background of LAIP CD15+, CD7+, CD11b+ and CD56+ evaluated in NBM and regenerating BM (RBM) in our institute. RBM were selected according to the following criteria: patients had no LAIP at time of diagnosis, and no new emerging LAIPs were identified during and after therapy. Measurements were performed after two cycles of standard induction chemotherapy. (C) Expression of background in NBM and RBM show that background does not interfere with the 0.1% threshold. Expression differences between NBM and RBM were analysed with unpaired t test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

expression introduced by healthy cell populations. Hence, the concerns raised on LAIP specificity do not apply to the modern MFC MRD protocols that are implemented in novel clinical studies.

Obviously, thresholds to determine MRD status determine specificity and sensitivity of the assay. Many combinations of markers can be assessed and each of these LAIPs will have different background levels on healthy cells. Therefore, threshold levels to assess MRD status should be chosen such that maximal specificity is obtained. Camburn *et al.* show that based on a threshold of 0.1% or 0.01% for MFC MRD, interference of background expression is observed in 57–100% of NBM samples tested. These results are not in line with the recently published consensus document on MRD assessment,² in which the threshold of 0.1% was recommended as universally applicable, indicating that all LAIPs have a background in NBM below 0.1%. In our experience, background expressions on NBM using strict gating strategies are far below the 0.1% cut-off, ranging between 0.00% and 0.04% (not published, Fig. 1B,C). Expression of LAIPs may vary in regenerating BM samples after intensive chemotherapy. In preliminary analyses of regenerating BM (selected based on following criteria: patients' blasts had no LAIP at time of diagnosis, and no new emerging LAIPs were identified during and after therapy), background LAIP levels do not interfere with the 0.1% cut-off levels for all frequently used LAIP markers (Fig. 1B,C).

Furthermore, the difference in chosen analysis method of MFC MRD [LAIP approach versus the different-from-normal (DfN) approach] is likely to influence the specificity and sensitivity of the assay. Specificity is likely more warranted in the LAIP approach compared to DfN, possibly at the expense of sensitivity, as clones with different LAIPs emerging after therapy are not being considered.

Overall, we would like to highlight that current clinical studies that implement MRD assessment according to the European LeukaemiaNet consensus guidelines are, in our experience, not impeded by excess of false-positive MRD

cases. In fact, false-negative cases could be explained by the use of the one cut-off for all patients and all LAIPs, thereby overestimating background for some of the LAIPs and depriving the true sensitivity of the assay.

Finally, we would like to emphasise the importance of directing the discussion to the use of regenerating BM instead of NBM as a reference for background expression, especially in the light of future automated data analysis programs currently explored for MFC MRD.

In conclusion, we have full trust in the robustness of LAIP specificity under the currently applied 0.1% threshold of the European LeukaemiaNet protocols and MFC MRD assessment stands as a strong prognostic factor for acute myeloid leukaemia patients in complete remission.

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Re: Myeloblasts in normal bone marrows expressing leukaemia-associated immunophenotypes: author reply

Sir,

We thank Hanekamp and colleagues for their comments on our paper and sharing their experience with the multiparametric flow cytometry for acute myeloid leukaemia measurable residual disease (MFC AML MRD) using the European LeukaemiaNet recommendations.¹

The findings in our paper are valid and relevant only for a setting where MRD is determined by MFC in the strict leukaemia-associated immunophenotype (LAIP) approach with four-colour tube assays. We and others have discussed the limitations of this approach,^{2,3} and we agree that the ELN recommendations⁴ on which Hanekamp *et al.* base their analysis are significant steps in advancing, harmonising and standardising the AML MFC MRD. Until more laboratories change to eight or higher colour MFC and the LAIP-based different-from-normal (DfN) approach is universally adopted with the required technical expertise and reference database, the four-colour based MFC assays will still be used in clinical practice and findings reported.⁵

The inherent difficulties with AML MFC MRD and the areas for development have been described in previous publications. We want to highlight again the threshold of 0.1% for determining the positive/negative status, and the implication on the specificity of the MFC MRD results. As commented by Paietta⁶ and others, and summarised in the ELN Working Party Consensus Document,⁴ the 0.1% cut-off was a consensus derived from retrospective studies, and there are studies which show that MRD of <0.1% has important prognostic and outcome significance. This may be more important with novel treatment schemes, new therapeutic agents and supportive interventions, which may reduce the MRD to an undetectable level and can be considered 'cure' for the disease. While the present status of AML treatment is not quite at this stage, it has been shown that MRD of 0.00% is associated with prolonged and near-normal survival in children,^{7,8} and in a recent German-Austrian study in a subtype of AML in adults.⁹ The German-Austrian study used molecular

MRD, but with the developments of next-generation flow cytometry, MFC should achieve the sensitivity of <0.01%. In this regard, the ability to distinguish the minute population of normal or regenerative myeloblasts from residual leukaemic blasts becomes relevant, and a level of up to 0.04% in the experience of Hanekamp and colleagues will still be a limiting factor in the specificity of the MFC MRD assay.

Finally, we agree with the comment from Hanekamp *et al.* that using regenerating bone marrow after chemotherapy should be the future reference for assessing AML MFC MRD, as regenerating myeloblasts may have immunophenotypic deviations from normal myeloblasts. Adopting the LAIP-based-DfN approach may overcome this problem, but the normal maturation pattern is derived from normal myeloblasts, and there is still not enough knowledge about immunophenotypic deviations in regenerating blasts. We believe collaborative effort using standardised analysis to build up the database of regenerating myeloblast profile is worthwhile.

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