Author's response to reviews

Title: If it is in the marrow, is it also in the blood? An analysis of 1,000 paired samples from patients with B-cell non-Hodgkin lymphoma

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Author's response to reviews: see over
We are grateful to the Editor and the Reviewer for useful comments and constructive criticism. Following in most detail their comments, the manuscript has been amended as following:

- The definition of concordance in the abstract and in the Methods section (page 6) (“...the presence of a positive or negative result in BOTH BM-TP and BM-FC OR BM-FC and PB-FC.”) is confusing. In fact, the authors analyzed 3 different parameters: BM trephine biopsy (that is currently the standard), BM flow and PB flow. The comparison between BM flow and PB flow is perfectly performed (in page 8 and in table 2), with a nice concordance of 95.2%.

The comparison between BM biopsy and BM flow is more confusing probably because in table2 the data on BM trephine biopsy is not informative on the concordance with BM flow. Perhaps a separate table would be of help in this sense. So, there are two different “concordances” and this should be clarified in the methods, results and abstract (i.e., in the current version of the abstract the sentence “discordant cases (ie presence of pathological B-lymphocytes in BM under the sensibility of the technique in the PB)” is confusing, since it is not clear if the authors are comparing PB flow with BM flow or with BM trephine biopsy or with both).

The abstract and the text have been amended according to the suggestions of the Reviewer:

**Background:** Staging of B-cell non Hodgkin’s lymphoma (NHL) routinely involves bone marrow (BM) examination by trephine biopsy (BM-TB). The evidence of disease in the BM-TB results in a clinical stage IV classification affecting therapeutic strategies for NHL patients. BM immunophenotyping by flow cytometry (FC) is also used, although its clinical value is still under debate.

**Methods:** Using FC we analyzed 1,000 paired BM aspirates and peripheral blood (PB) samples from 591 NHL patients to investigate the concordance between BM and PB. B–lymphocytes were defined monoclonal when a ratio of $0.3 < \kappa/\lambda > 3$ was observed. Aberrant immunophenotypes present in the B-cell subpopulation were also investigated. BM-TB was also performed in 84.1% of samples (841/1000), and concordance between BM-TB and BM-FC was evaluated. Concordance was defined as the presence of a positive (in terms of disease detection) or negative result in both BM-FC and PB-FC or BM-TB and BM-FC.

**Results:** Using FC, the overall concordance between BM and PB was 95%. Among the discordant cases (ie presence of neoplastic B-lymphocyte in the BM but under the sensibility of the technique in the PB) the most frequent diagnosis was Waldenstrom’s macroglobulinemia (WM, accounting for 20.8% of all discordant cases). The expression of CXCR4, a receptor involved in B-cell trafficking and homing, was found to be down regulated in WM compared to other NHL types, thus suggesting a possible role of CXCR4 in WM cell homing in the BM. WM excluded, FC investigation of BM and PB in NHL patients gives overlapping information.

BM involvement was observed by FC in 38% of samples, and concordance between BM-FC and BM-TB was 85%.

**Conclusions:** The finding that FC data from BM and PB samples overlap in NHL might have major implications for the design of future clinical studies and for patients’ follow-up.
Data in former Table 2 are now presented in two tables. Table 2 shows the concordance between BM-FC an PB-FC. Table 3 shows BM-TB results.

The analysis of discordance between BM-TB and BM-FC is reported in Fig2

An additional analysis that may have some interest would be to compare the data on BM trephine biopsy with the combination of BM plus PB flow cytometry. At the end, the aim of the study from the clinical standpoint would be to have a less aggressive technique to detect lymphoma infiltration in the bone marrow.

As now better reported in the results, the overall FC concordance between BM and PB was 95.2% (Tab 2) In all NHL subtypes, the sensitivity of BM-FC was higher than that of PB-FC. In all discordant cases (4.8%), monoclonal B lymphocytes were present in the BM, whereas in PB the monoclonal cell population was absent or under the sensitivity of the procedure.

The concordance between BM-FC and BM-TB was 84% (83% at diagnosis and 85% after treatment).

Following the suggestion of the Reviewer, when comparing data about BM-TB with the combination of BM-FC and PB-FC, the overall concordance between FC and BM-TB was 87% (vs 84%). However, as we don’t know which is the clinical relevance of a BM-TB-/BM-FC+ results, we are not sure on whether that analysis should be not included and would like to leave the final decision to the Editor.

The histological distribution is not the standard for a series of unselected non-Hodgkin’s lymphoma (35% FL, 24% DLBCL, etc), but rather suggest a selection, probably based on the availability of the samples. This is a potential bias of the study and should be at least commented in the Discussion section

As now better reported in the Methods section, Hairy Cell Leukaemia, T-cell NHL, Hodgkin disease and multiple myeloma patients were not included in this study. As now better reported in the Discussion, for all the other LNH subtypes we retrospectively analyzed BM aspirates and PB samples from NHL patients (i.e 1000 BM samples along with 1000 PB samples from the same day) consecutively collected in our Institute from 2000 to 2007. AS discussed, we did not select samples.

In addition, another potential bias is the fact that only 31% of paired samples corresponded to patients at diagnosis. The selection of the samples probably is much higher in relapsed patients. On the other hand, the therapy used, including Rituximab, may significantly influence the results. These aspects should also be commented

As now better reported in the results, the concordance between BM-FC and BM-TB (84%) was not statistically different at diagnosis or after treatment (83% at diagnosis and 85% after treatment). This result is in line with previous studies dealing with smaller series of patients that have shown a 79-90% concordance between the two methods. For this reason, we do not believe that overall concordance between these two techniques is dependent on patient selection.

No clinical data are provided, in terms of initial features, response or survival. This information would be very interesting, particularly in discordant cases.
We agree with the Reviewer that the outcome of patient in discordant case would be of great interest. However, the analysis of the clinical follow-up of the discordant cases did not offer any meaningful and significant information, because of the very large variety of different treatment procedures in the patients. As we describe in the text, the investigation of NHL patients’ clinical outcome was beyond the scope of the present study, which encompassed a large variety of NHL types and treatment.

- The reasons to explain the discordant cases are probably diverse and the data on CXCR4 here in presented are only a small part. Moreover, the information provided on CXCR4 expression is really scarce (there is no information on the number of patients analyzed for each histological subtype, whether or not the CXCR4 expression was different in BM or PB from the same patient, etc). Detailed information on CXCR4 expression would be the subject of a different paper.

We performed the suggested analysis and the text has been amended as following:

Methods: We also evaluated CXCR4 expression on B-cells in 54 paired BM-aspirates and PB (10 Waldenstrom, 14 DLBC, 18 FL and 12 LLC).

Results: CXR4 expression on B lymphocyte in the BM vs PB was not statistically different.

- The discussion is too concise; some of the above mentioned limitations of the study should be at least commented in this section.

As suggested by the Reviewer, and following her/his requests, all the issues described above were better organized and described in the discussion section.

Here are the amended and improved discussion and conclusion sections:

Discussion:

BM examination by BM-TB is an integral part of the clinical staging and follow up of NHL patients. Along with BM-TB, BM-FC is used as an ancillary investigation, even though its use is still debated. The correlation between these two techniques was found to be very high, and our results are in line with previous studies dealing with smaller series of patients (85% vs 80%-90%, see ref. 9).

However, as BM aspiration might be a painful procedure for patients, we retrospectively analyzed 1,000 paired BM aspirates and PB samples from NHL patients to assess if PB analysis could be an alternative reliable procedure for staging and follow up of these patients. The overall FC concordance between BM and PB was 95.2%. Among the discordant cases, 62% of samples were collected after chemotherapy and the most frequent treatment was Rituximab alone or in association with chemotherapy. This result suggests that during treatment with Rituximab alone or in association with chemotherapy, neoplastic B cells may be depleted in the PB but still present in the BM. Therefore, a negative PB sample obtained during treatment with Rituximab should be considered with caution (8).

Among discordant cases, the most frequent diagnosis was WM (20.8%). Among the different biological reasons that might explain this discrepancy, we focused on CXCR4. The crucial role of CXCR4 in WM cell homing in the BM has been recently described (10) and our results, even though obtained in a small number of patients and samples, confirm this hypothesis. In fact, CXCR4 expression on B lymphocytes was found to be down-regulated when compared to other NHL types (p<0.001).
Albeit the investigation of NHL patients’ clinical outcome was beyond the scope of the present study, encompassing a large variety of NHL types and treatment plans, we tried to investigate the clinical outcome of the discordant cases. However, the clinical follow-up of the discordant cases did not offer any meaningful and significant information, because of the very large variety of different treatment procedures in the patients.

To the best of our knowledge, only two previous papers have reported about the role of FC in clinical NHL outcome. Perea et al (9) reported discordance between BM-FC and BM-TB (BM-FCpos/BM-TBneg) in 9% (36) of low-grade NHL patients, in most cases (66%) affected by FL. In this study, discordance had no apparent influence on the clinical outcome when compared with BM-negative patients (median follow-up 14 months). In the second study, Gronich et al (11) investigated 70 low-grade NHL patients and reported that the outcome in patients who had BM involvement defined by FC alone or by morphology was similar.

Conclusions:

Our data indicate that - WM excluded - FC investigation of BM and PB in NHL patients gives overlapping information. This finding might have major implications for the design of future NHL clinical studies and suggest that the FC investigation of PB has potential as a possible alternative to BM-TB for the follow-up of NHL patients.

- Minor point: currently, Waldenström disease is not considered a specific entity in the WHO classification.

The text has been amended: Waldenström’s disease has been changed in Waldenström’s macroglobulinemia (WM) according to the most recent WHO classification.

In conclusion, we would like to thank once again the Editor and the Reviewer for their useful comments, and hope that the manuscript amended following their request might now be considered of enough scientific merit and interest to be accepted for publication.

Yours sincerely

Patrizia Mancuso and Francesco Bertolini on behalf of all co-authors