Biological aspects of minimal residual disease in acute leukaemia

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Acknowledgements: Our thanks go to the cytometrists in the French Intergroup ALFA-MRD-LAM-LSC for all their efforts in implementing and harmonising this protocol in 30 BIG protocol centres (ALFA and FILO), in particular to Dr Florent Dumezy for the development of ScriptR, to generate integrated FC files, in addition to the initial data and the data of unsupervised analyses viewable on KALUZA analysis software. Thanks also go to the patients and participants in the ALFA and ELAM02 groups, for agreeing to lend their support to this theme over many years.
To the ELN MRD members (especially to Professor Sylvie Freeman [Birmingham, UK] for our close collaboration and all the fruitful discussions around MRD flow).

Conflicts of interest: None of the authors have any conflicts of interest to disclose.

Maladie résiduelle dans les leucémies aiguës myéloïdes : aspects biologiques

minimal residual disease, acute myeloid leukaemia, acute promyelocytic leukaemia
maladie résiduelle, leucémies aiguës myéloïdes, leucémies aiguës promyélocytaires

Abstract

Acute myeloid leukaemia (AML) is a highly heterogeneous disease with a poor prognosis, despite considerable progress in recent years due to a better understanding of the pathophysiology and technological advances in molecular biology and flow cytometry. In theory, an overview of the response to treatment can be achieved based on evaluation of minimal residual disease (MRD), as a single test. However, although MRD is one of the key parameters in acute

Résumé

Les leucémies aiguës myéloïdes (LAM) constituent une entité très hétérogène dont le pronostic reste sombre malgré des progrès considérables réalisés ces dernières années, procédant d'une meilleure compréhension de la physiopathologie ainsi que d'avancées technologiques dans les champs de la biologie moléculaire et de la cytométrie de flux. La maladie résiduelle (MRD) permet, sur le plan théorique, d'avoir en un seul test une vision globale de la réponse au

To cite this article: Preudhomme C, Plesa A, Roumier C. Biological aspects of minimal residual disease inacute leukaemia. *Hématologie* 2020; xx(x): 1-14. doi: 10.1684/hma.2020.1611

doi: 10.1684/hma2020.1611

lymphoblastic leukaemia, its use in developing personalised treatment for AML remains limited to a few subgroups of the disease such as core binding factor leukaemia, *NPM1*-mutated leukaemia and acute promyelocytic leukaemia. This is mainly due to a lack of sensitivity of the techniques used for the other subgroups, a lack of standardisation of the techniques, and the heterogeneity of the biological material used (blood versus bone marrow). It is these latter parameters that will need to be rapidly standardised before MRD can be used as a surrogate marker for AMLs.

traitement. Si elle est l'un des paramètres clés dans les leucémies aiguës lymphoblastiques, son utilisation pour une stratification personnalisée dans les LAM reste limitée à quelques sous-groupes comme les leucémies aiguës promyélocytaires, à *core binding factor* ou à *NPM1* muté. Les causes principales en sont, d'une part, la sensibilité insuffisante des techniques utilisées pour les autres sous-groupes moléculaires, et d'autre part l'absence d'homogénéisation tant des techniques employées que du matériel utilisé (sang *versus* moelle osseuse) et du moment où les analyses sont réalisées dans les études protocolaires rétrospectives. Ce sont ces derniers paramètres qu'il sera nécessaire de standardiser rapidement avant de faire de la MRD un *surrogate marker* dans les LAM.

Acute myeloid leukaemia (AML) is probably one of the most morphologically, phenotypically and genotypically heterogeneous subgroups of haematologic malignancies. The identification of many diagnostic markers – including cytogenetics, age, leukocytosis and molecular markers – has led to better analysis and improved survival rates. However, prognosis remains poor, especially in the elderly [1, 2]. The use of post-therapeutic tests to assess the overall response or persistence of minimal residual disease (MRD) should further improve this analysis and the therapeutic management of patients [3].

What is minimal residual disease?

MRD refers to the persistence of a small number of residual leukaemia cells that are undetectable by morphological techniques (complete blood count or myelogram), resistant to chemotherapy, and are most often responsible for relapse.

MRD is a dynamic process; the kinetics of MRD suppression during treatment reflect the chemosensitivity of the tumour cells. Both pre-therapeutic prognostic factors and the host response (anti-tumour immunity) therefore play a role in MRD. MRD is therefore a powerful prognostic factor which may, however, often be independent of pre-therapeutic factors.When leukaemia is diagnosed, the body is invaded by 10^{11} or 10^{12} tumour cells. The goal of induction therapy is to achieve haematological complete remission (HCR) with, in the case of AML, a normalisation of the blood count and fewer than 5% blasts in the marrow, *i.e.* a reduction by approximately 1-2-log10 (10 to 100-fold) (*figure 1*).

However, even when HCR has been achieved, the tumour load can still be very large; theoretically between 0 and 10^9 tumour cells [4].

The goal of consolidation therapy is therefore, ideally, to completely eradicate the tumour mass and achieve a cure for AML. If this is not achieved, the residual tumour cells may trigger a relapse at any time, depending on the host's depth of response and anti-tumour immunity.

It is important to note that even the most sensitive techniques currently available do not demonstrate sensitivity $<10^{-6}$. This grey area therefore restricts investigation, and complete remission based on molecular techniques (undetectable MRD) does not necessarily indicate an absence of persistence of the disease.

Why study minimal residual disease?

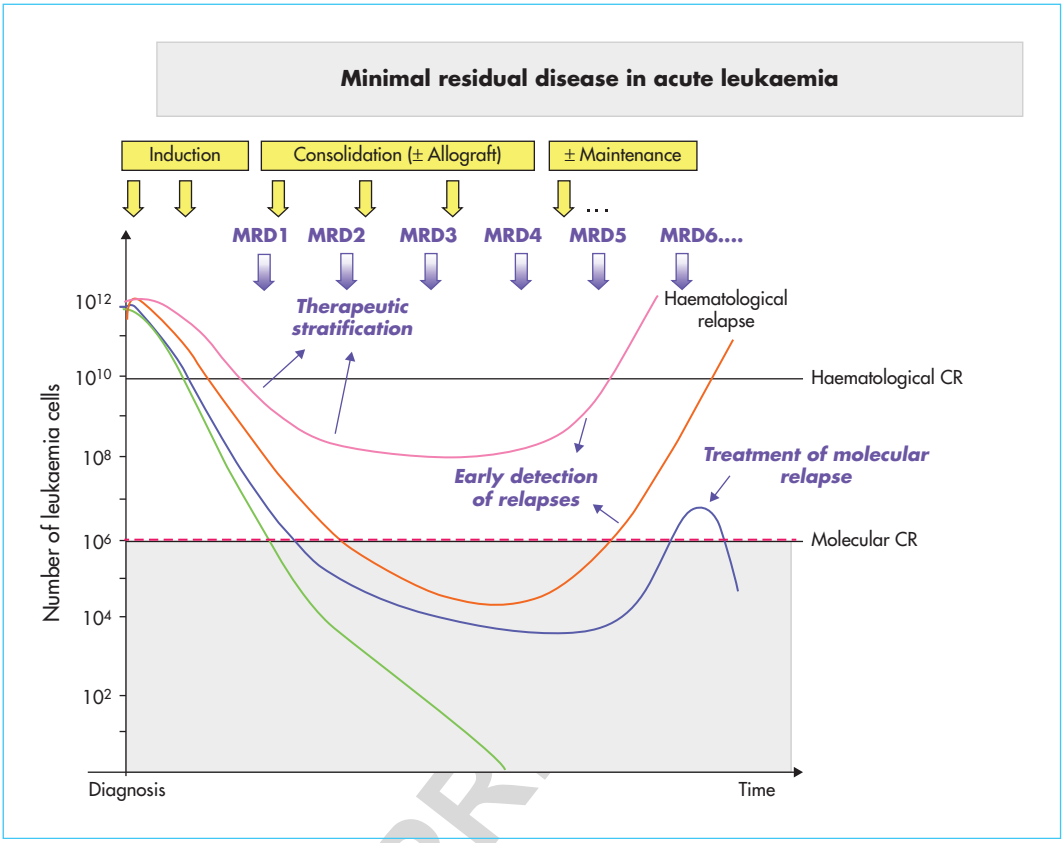
– To establish remission status in a reproducible, objective and sensitive way.

– As a prognostic marker of chemosensitivity, to improve personalised treatment in combination with other prognostic markers.

– To identify any possible relapses as soon as possible during follow-up.

– As a continuous response marker when comparing different therapeutic strategies or new drugs (surrogate marker).

FIGURE 1



Minimal residual disease in acute leukaemia

What are the criteria for choosing the marker(s)? [5]

The major criteria are:

– a marker with the geatest possible sensitivity;
– a specific marker for the disease;
– a marker that is stable between diagnosis and relapse;
– a marker that is applicable to a large number of patients;
– a technique that is easily performed in routine hospital care;
– an acceptably priced technique.

Taking these criteria into account, the best MRD markers for AMLs are:

– fusion transcripts and *NPM1* mutations, which may be used in 40–70% of patients depending on age;
– leukaemia-associated aberrant immunophenotype markers (LAIPs) or leukaemia stem cell (LSC) markers quantified by flow cytometry;
– quantification of *WT1* mutation and other certain mutations.

In acute myeloid leukaemia, what techniques should be used for molecular markers?

The gold standard is currently real-time quantitative PCR (RT-qPCR), which is recommended as a priority for *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA* [6, 7], *WT1* [8] and *NPM1* [9] fusion transcripts. Depending on the transcript, sensitivity is between 10^{-4} and 10^{-6}. It is important to note, however, that unlike chronic myeloid leukaemia (CML), there is no international, or even national, consensus on

how best to express the results. Should this be based on, for example, log reduction relative to diagnosis, an international scale (as is the case for CML) or positivity with respect to a threshold?

For *WT1*, sensitivity is much lower, not because of the technique, but because of the background noise due to the expression of *WT1* in normal haematopoietic stem cells. Since the latter are more scarce in peripheral blood than bone marrow, sensitivity in blood is, paradoxically, better than in marrow due to the lower background noise. Nevertheless, due to the lack of specificity and sensitivity, the European Leukemia Net (ELN) has recently recommended using this marker only in the absence of other molecular markers or in the absence of markers that can be monitored by flow cytometry (FC). This point is discussed in the article in this issue dedicated to clinical applications.

For fusion transcripts involving the *MLL/HRX* gene, as in AML, RT-qPCR is also recommended, however, this should be performed on DNA after identification of the junction between the two partner genes [10]. Finally, for other fusion transcripts, RT-qPCR is not standardised and there are few commercial plasmids available for calibration. Quantification using Droplet Digital PCR (ddPCR) could be an interesting option. The scarcity of these techniques and the technical difficulty of using them for *MLL* would require these cases to be centralised nationally.

Concerning the monitoring of patients with *NPM1* mutations, the MRD group, led by Grimwade, participated in the creation and validation of plasmids corresponding to type A, B and D mutations, marketed by Ipsogen. However, to date, as for fusion transcripts, there are no international recommendations on how the results should be expressed. In order to standardise the results, two new working groups have recently been established; one within the ELN-MRD-AML group (international) and the second within the Groupe des Biologistes Moléculaires des Hémopathies Malignes (GBMHM) (national).

Although A, B and D mutations in *NPM1* account for approximately 90% of the mutations in this gene, monitoring the remaining mutations raises not only technical problems but therapeutic problems (such as the national BIG1 protocol) due to the lack of available results. From a technical point of view, RT-qPCR appears to be unsuitable due to the lack of approved calibration [11, 12]. ddPCR would appear to be a good solution. This does not require calibration, is easy to set up, and is inexpensive. In France, Lesieur *et al.* first validated this approach based on *NPM1A* mutation and then extended its application to rare *NPM1* mutations. Samples from 40 patients with 18 different rare *NPM1* mutations were monitored at Lille University Hospital, with very interesting results [13].

In the absence of fusion or mutation transcripts of *NPM1*, it is possible to monitor mutations either using ddPCR or next-generation sequencing (NGS). For practical reasons, ddPCR is mainly feasible on hot-spots such as mutations in *IDH1/IDH2*, *DNMT3-R882*, *N/KRAS*, *KIT* and *FLT3-TKD* genes. The sensitivity of this technique is between 10^{-3} and 10^{-4} depending on the marker and technology used [14].

The second approach is NGS which, theoretically, should be more sensitive than ddPCR. However, its efficiency depends on the quantity of DNA in the test sample, the quality of the sequences obtained and the background noise (it is recommended to use molecular barcodes). Its application is currently underdeveloped due to technical and bioinformatic difficulties and, above all, its cost, which is very high for prospective analyses.

In general, signalling mutations (*FLT3-ITD* and *-TKD*, *N/K-RAS*, *KIT*) are not recommended as markers because they are inconsistent between diagnosis and relapse. The same is true for age-related mutations, particularly *DNMT3A* mutations, which persist at high levels in the vast majority of patients. Other age-related markers, such as *TET2*, *ASXL1*, and *IDH1/2* mutation, should be quantified alongside other markers if possible [15, 16]. Although the technology is still rarely

used, it should be progressively implemented in haematology laboratories within a few (three to five) years, in two different strategic forms based on:

– a targeted amplicon strategy, which has the advantage of being relatively inexpensive due to the limited size of the libraries, but which requires a quasi-specific design for each patient outside of hot-spots and which only tracks mutations detected at the time of diagnosis;
– or a more generalist approach, making it possible to detect mutations identified at diagnosis but also *de novo* mutations that may appear, for example in the case of resistance to inhibitors of *FLT3* and *IDH1/IDH2*. As before, an amplicon approach is possible, but capture approaches should be preferred due to better homogenisation of coverage and the limited number of PCR cycles to be performed.

The ELN-MRD-AML Group is currently standardising its techniques.
In general, techniques performed on marrow are more sensitive than those performed on blood. However, a better correlation with clinical course (relapse) has recently been published, particularly regarding the follow-up relative to *NPM1* and *RUNX1-RUNX1T1* at the end of treatment as well as *WT1* [8, 17-19].

What are the technical requirements for evaluating minimal residual disease using molecular biology techniques?

For qPCR, the ELN [20] recommends the use of cDNA rather than DNA, especially for fusion transcripts and *NPM1* mutations. Each analysis must be carried out in triplicate. If two of the three replicates have a cycle threshold (CT) value ≤ 40, the sample is considered to be positive according to the recommendations in the European Code Against Cancer (EAC). During each run, four controls should be included: one negative control, two positive controls covering the desired sensitivity range, and one water control.
Conversion from a negative to a positive result must be confirmed four weeks later using a second sample. If the increase in MRD in the second sample is greater than 1-log10, the diagnosis of molecular relapse is confirmed.

For acute myeloid leukaemia, what methodological approach related to flow cytometry should be used?

The study of MRD using FC is based on the characterisation of aberrant protein expression involving proteins that are most often membrane-bound on the surface of blast cells, making it possible to differentiate these cells from normal haematopoiesis cells (LAIP). Three types of aberrant protein expression exist:

– expression of markers from a lineage other than the myeloid lineage;
– modulation of the intensity of expression of markers from the myeloid lineage (absence, decrease or even over-expression);
– asynchronicity of expression.

During haematopoietic maturation from progenitors to lineage endpoints, a succession of membrane markers accompany each step of differentiation, in a highly rigorous sequence. However, this order is often disrupted during the leukaemia process, and blasts often express a mosaic of markers on their surface that should not be expressed at the same stage of differentiation. These anomalies are highlighted using FC based on two strategies:

– a strategy to monitor LAIPs identified in the diagnosis;
– or the so-called “different from normal” (DfN) strategy which is based on identifying cells with characteristics that differentiate them from those associated with normal haematopoiesis. This approach does not require knowledge of the immunophenotype at diagnosis.

This technique can also detect the appearance of new markers on blasts during the course of the disease and its treatment (immunophenotypic shift). The difference between the two approaches is minimal since, in both cases, clear knowledge of the immunological characteristics of each cell type observed in the bone marrow, at each stage of haematopoietic differentiation, is a prerequisite for any FC MRD study. With the advancement in flow cytometers, it is now possible to monitor between eight and 14 markers with different fluorescence in a standard laboratory. This approach enables the use of large panels of antibodies that are increasingly being used to differentiate leukaemia cells from normal haematopoietic cells at all stages of maturation (see ELN recommendations) [20]. Finally, with the advent of immunotherapy –using monoclonal antibodies and/or chimeric antigen receptor T-cells (CAR-T) – it is crucial to consider possible phenotypic shifts in response to immunotherapy before considering the relevance of a combination of LAIPs. Similarly, regarding the fact that therapeutics, such as IDH1/2 inhibitors, may induce differentiation, it is important to integrate the concept of differentiation molecules into the analysis strategy. These inhibitors also induce a phenotypic shift, but this occurs within the same myeloid lineage, towards a more differentiated stage than the diagnostic blasts. Technology based on at least eight colours is therefore necessary. The ELN recommends using a combination of the two approaches known as the LAIP-based approach (the fastest and most sensitive) and the DfN approach (which is not constrained by possible antigenic variation during treatment, but often shows lower sensitivity).

For FC, it is recommended to take a bone marrow sample as this is much more likely to reveal MRD since it allows a larger number of cells to be studied and investigation of the tissue where the leukaemia originates from; a bone marrow sample is therefore the gold standard. Analysis of bone marrow regeneration is possible in parallel with investigation of blasts as well as the stem cell compartment. When a myelogram reveals, for example, a small excess of blasts associated with aplasia, it is possible to confirm whether these correspond to the disease or regeneration. The reasonably practical threshold for each patient is 10^{-3} (0.1%) with the possibility of dropping to 10^{-4} (0.01%) in the case of very robust LAIPs. A DfN analysis is limited to a threshold of 10^{-3}.

The disadvantages of marrow analysis are, firstly, the invasive aspect of the collection and, secondly possible dilution of the blood sample.

FC enables the early stages of haematopoiesis in bone marrow to be described in detail. The future of FC for MRD probably, therefore, like the myelogram, lies in the immunophenotypic description and interpretation of the immunoblastogram; from the stem cell stage to the first morphologically identifiable cells.

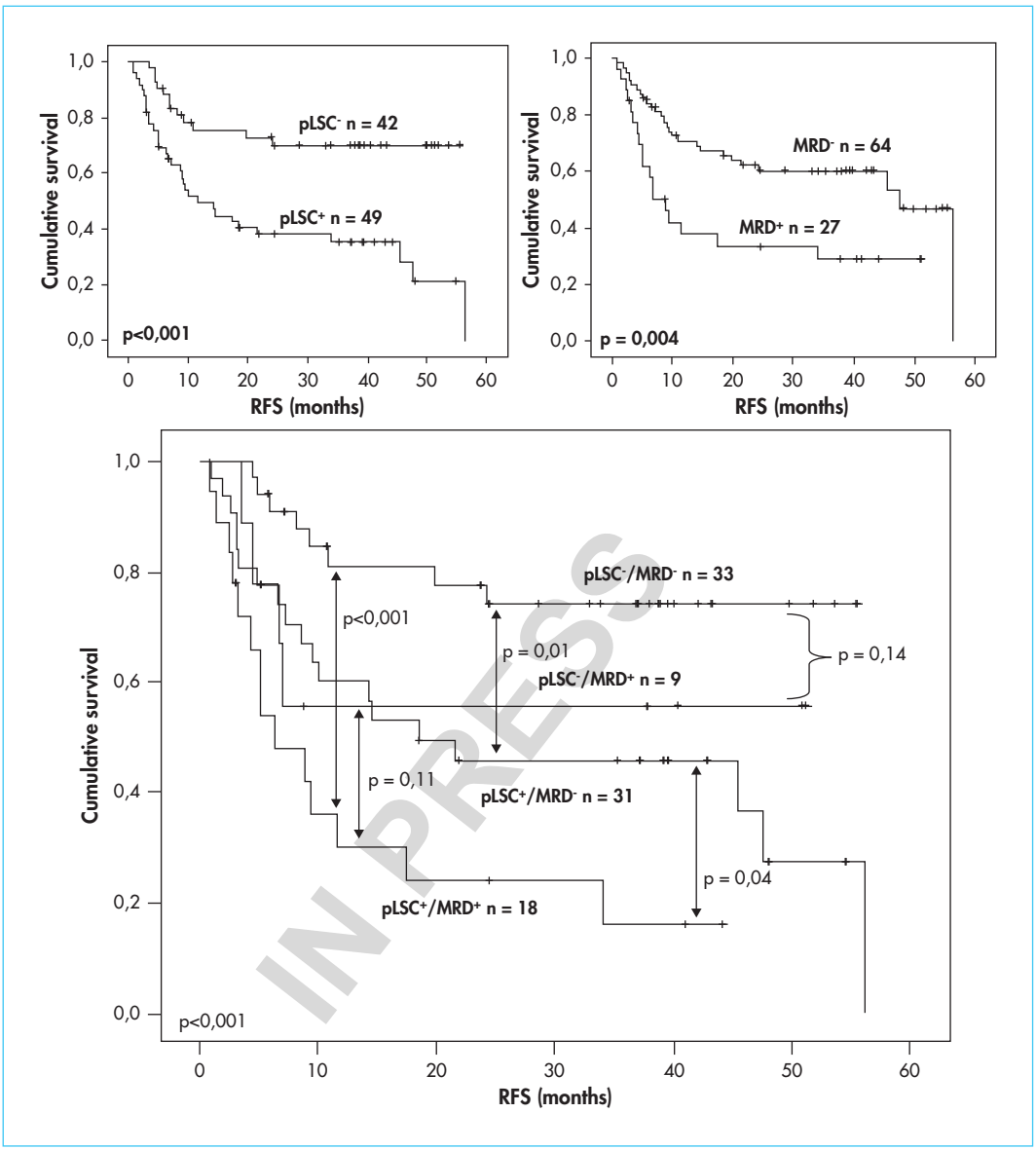
In order to meet these requirements, the French AML Intergroup (Acute Leukaemia French Association [ALFA] + French Innovative Leukaemia Organisation [FILO] + Myechild) is carrying out major structuring and standardisation, based on the recent study by the French group, France Flow [25]. Currently, the 0.1% threshold appears robustly achievable for all combinations and platforms used (*figure 2A*). It is also the threshold that seems most relevant in the published retrospective studies, even though significant technical efforts are required to reach 10^{-3}.

Finally, the Schuurhuis team demonstrated the complementarity of the leukaemia stem cell (LSC) approach in the analysis of MRD and the added value of the three approaches: FIPA, DfN and LSC (*figure 2*) [21]. Again, the French Intergroup has developed a strategy for prospectively validating the three approaches mentioned above [22].

A FC report on MRD should include (*figure 3*):

– an assessment of bone marrow in the sample (dilution);
– a description of the combinations used to identify the MRD (FIPA/DfN/LSC);

FIGURE 2



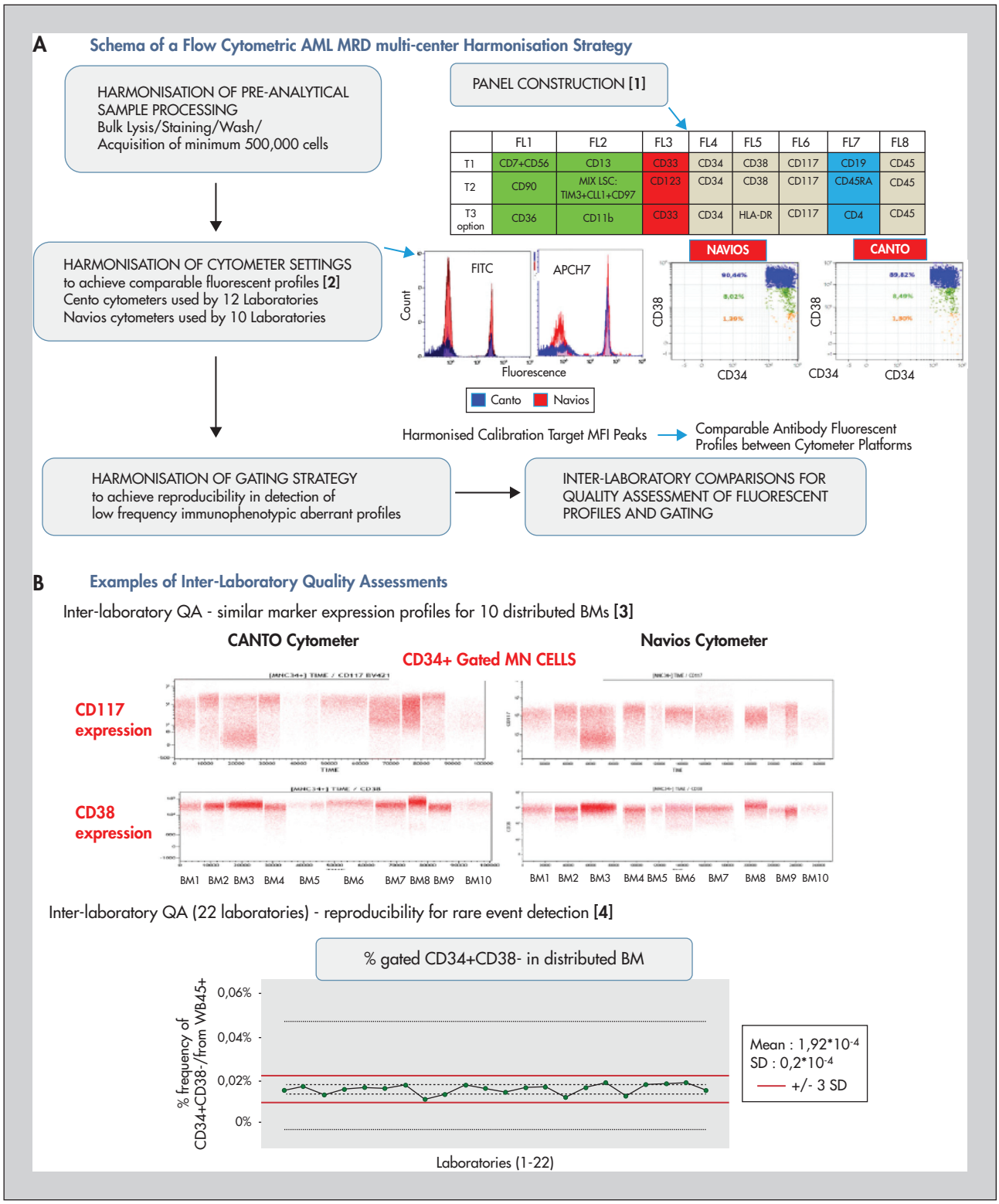
Results of the study relative to MRD based on LAIP/DfN FC and LSCs impact on relapse-free survival **(A)**; LSC approach **(B)**; MRD LAIP/DfN approach **(C)**; LSC + MRD approach [21] (Terwijn 2014).

– the level of MRD for which the most relevant denominator has yet to be defined (total leukocytes, CD34/CD117 progenitors, *etc.*);
– and, in the case of non-detectable MRD, the detection limit of the method used relative to normal haematopoietic regeneration.

The use of quantifying and characterising leukaemia stem cells in acute myeloid leukaemia

In recent years, several studies have shown the value of evaluating the frequency of the most immature blasts (LSCs or leukaemia initiating cells, LICs) identified in the

FIGURE 3



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CD34+ CD38- population of bulk leukaemia cells at diagnosis and during follow-up [21]. When CD45+/SSC blasts are visualised in the CD34/CD38 biparametric space, heterogeneity of the bulk leukaemia cells is observed, and several patient groups associated with distinct genomic and molecular profiles can be identified [22] (*figure 4*). Recently, the study by Jentzsch *et al.* [23], on 169 allograft patients, showed that the frequency of LSC CD34+ CD38- in total blasts at diagnosis was an independent prognostic factor for overall and relapse-free survival, likely reflecting the immune escape of the graft-versus-leukaemia effect (GVL) in this population.

Alongside the LAIP/DfN approach in the MRD FC protocols, ALFA thus plans to include in its future clinical trials, an evaluation at diagnosis and, at the various MRD monitoring points, quantification of LSCs in the CD34+ CD38- compartment, based on ELN recommendations. The panel also includes markers related to blocking differentiation or aberrant expression related to leukaemogenesis: CD90/CD45RA, CLL1/TIM3/CD97, CD123 and CD7, CD56, CD19, CD13, CD33, CD117, *etc*.

Finally, of major interest is the study of the expression profile of the different markers in the CD34+ CD38- LSC compartment and evaluation of the potential therapeutic targets in order to propose personalised treatment to each patient. Recently, the team from the Lyon CHU [24] has shown the effectiveness of bispecific immunotherapy against CD19/CD3 using blinatumomab in a CBF-AML patient with post-transplant relapse, in whom CD19 expression was found in bulk and CD34+ CD38- LSCs. Identifying, quantifying and characterising the CD34+ CD38- LSC compartment via FC involves evaluating new markers and studying the functions and dormancy of LSCs (GPR56, CD81, CD9, *etc.*). The ongoing study within the group is opening up promising avenues for the development of new targeted therapies and the evaluation of their effectiveness.

Unsupervised analysis of flow cytometry in the diagnosis and monitoring of minimal residual disease in acute myeloid leukaemia

As the number of accessible parameters using FC increases, the need has arisen to implement new analysis strategies, no longer based on sequential window acquisition, but designed to simultaneously exploit all the characteristics that define a cell as abnormal. This can be achieved using bioinformatic approaches and unsupervised analysis systems in addition to the classic model. Two main approaches are possible:

– dimension reduction algorithms for data visualisation in principal component analysis or t-distributed stochastic neighbour embedding (t-SNE),
– unsupervised clustering algorithms associated with the use of a self-organising map (SOM) Flowsom [27-29].

These methods imply perfect standardisation of acquisition sensitivity for each of the fluorescence channels, enabling platforms involved in patient monitoring to be harmonised or better yet, standardised. Indeed, it is very difficult to normalise files *a posteriori,* as is the case for gene expression profiles on microarrays. The use of these methods should lead cytometrists to reconsider some of the old dogmas associated with FC, such as placing unlabelled cells in the first decade, as the signal distortions generated by compensation methods are particularly deleterious for unsupervised algorithms. Extensive data clean-up to remove debris, duplicates, *etc*., is crucial before injecting data into the analysis scripts.

Once these modalities are taken into account, these approaches look promising. The algorithms can integrate data from the diagnosis, from several monitoring points and finally from reference data (physiological haematopoiesis). This makes

FIGURE 4

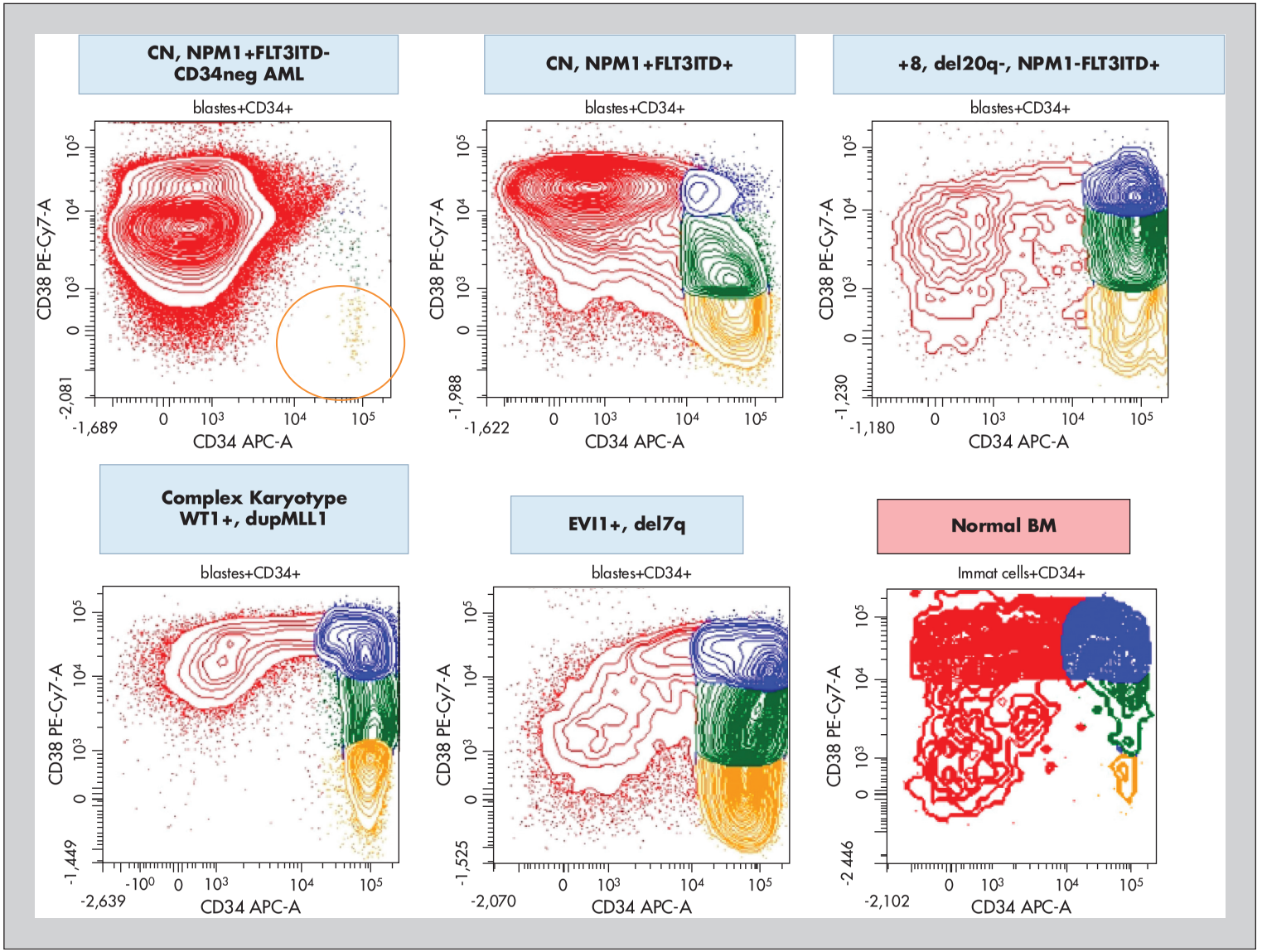

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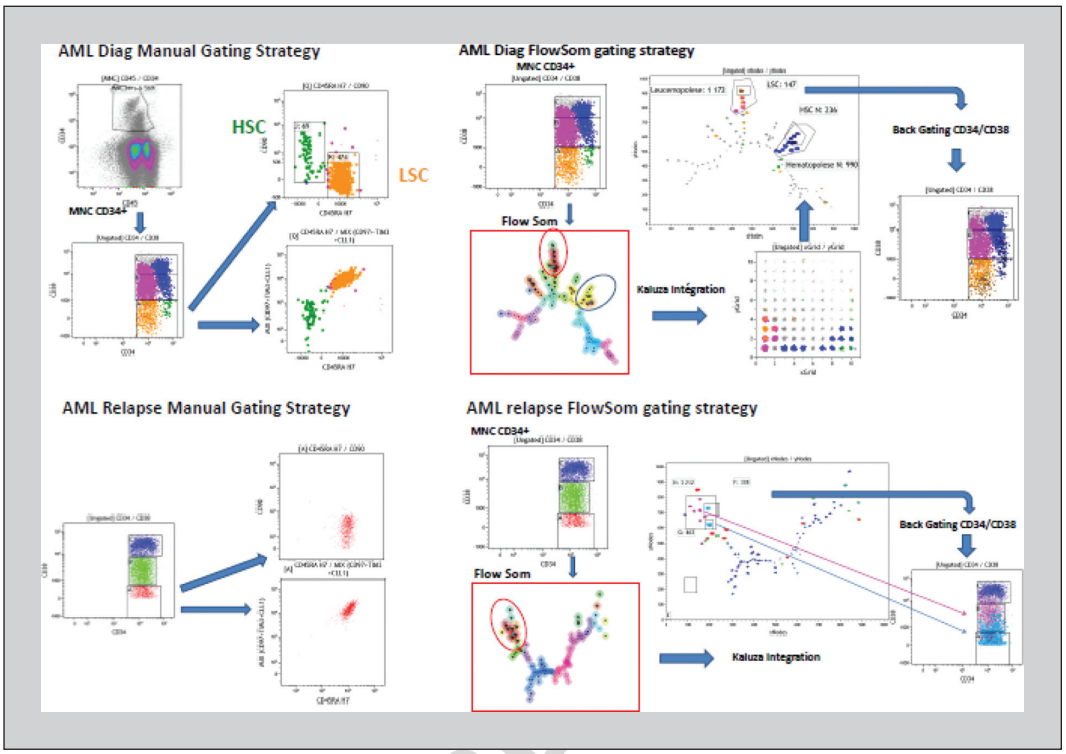
Figure 5



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it possible to study the evolution of the FC pattern between diagnosis, MRD and relapse in the same patient. This approach also makes it possible to investigate and distinguish the pattern of normal cells (NCsh) compared to leukaemia (LSC) cells within the CD34+ CD38- compartment. Finally, these approaches make it possible to conduct a comparative study of the LSC flow pattern versus the LSC signature in genomics using the LSC17 nanostring score (*figure 5*) [26].

When and for what purpose should minimal residual disease in acute myeloid leukaemia be measured? *(figure 1)*

Six situations may be considered:

– measurement of early response for the purpose of personalising treatment, with a focus on allograft marrow transplantation;
– assessment at the end of consolidation, in order to consider maintenance treatment;
– early detection of relapse through close follow-up and initiating the search for a donor for allograft or other therapy;
– adaptation of allograft conditioning?
– after CSH allograft: modulation of immunosuppression, donor lymphocyte injections (DLI), implementation of pre-emptive treatment or follow-up of maintenance treatment (hypomethylating agent, tyrosine kinase inhibitor, IDH1/2 inhibitor, *etc.*);
– comparison of the effectiveness of one branch of treatment with another.

FIGURE 6

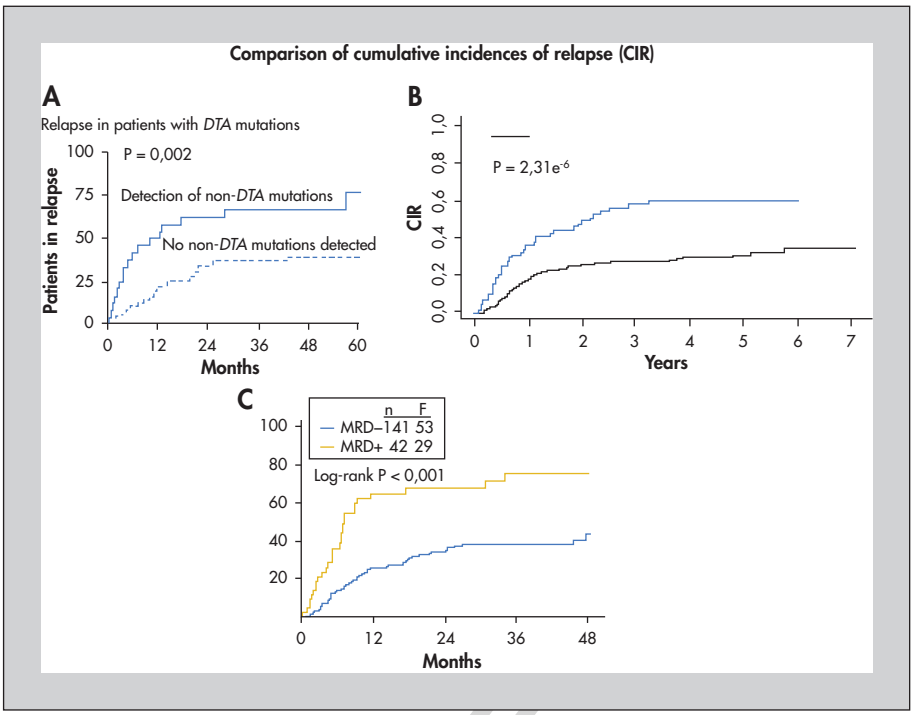

Illustration comparing the cumulative incidence of relapse (CIR) according to the technique used: NGS **(A)**, WT1 and RQ PCR **(B)**, and flow cytometry **(C)**.

In conclusion (*figure 6*)

In AML, MRD is a strong prognostic marker regardless of the technique and biological material used or when it is performed (*figure 6*). The use of different techniques and MRD markers is highly complementary, and this should, in the future, allow for better personalisation of treatment and introduction of new drugs, including immunotherapy. The role of MRD as a surrogate marker of risk of relapse or survival is still unclear.]

Uncited referencesQ2

[11, 12].

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Questions à l'auteur

Q1 Figures 3 and 6 have not been cited in the text in the original manuscript. They have been cited in the text, please check the citations. If not appropriate, please cite them at appropriate places.

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