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CICLO XXXV

BLOOD FLOW: a new applicable and ultra-sensitive test to monitor measurable disease in peripheral blood of Multiple Myeloma

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1. ABSTRACT

BACKGROUND AND AIMS OF THE STUDY.

Assessing Minimal Residual disease (MRD) in bone marrow (BM) using next-generation flow (NGF) or sequencing (NGS) precludes periodic evaluations because of its invasiveness. MRD assessment in PB could overcome this limitation, but its prognostic value is not established and the negative predictive value (NPV) when compared to BM is < 70%. This is because tumor burden in PB is ~3log lower than in BM, and methods capable of detecting MRD below 10^{-6} are thus needed for improved concordance.

We first aimed at investigating the prognostic value of MRD assessment in PB using NGF. Our second aim was to develop a new method with increased sensitivity.

PATIENTS AND METHODS.

MRD was evaluated using NGF in PB of 138 MM patients enrolled in the GEM2014MAIN trial. PB samples were collected after the second year of maintenance, when patients stopped treatment if MRD negative (in BM), or continued on therapy for three additional years if MRD positive at that time. Reaching a minimum sensitivity of 10^{-7} requires analyzing ≥ $2x10^8$ cells (~50mL of PB). To avoid high staining costs and impractically long acquisition periods, a new method integrating immunomagnetic enrichment using MACS® MicroBeads prior NGF was developed and coined as *BloodFlow*. Large PB volumes were magnetically labeled and processed via MACS® columns, and ~100µL aliquots enriched with circulating plasma cells (PCs) were analyzed using EuroFlow NGF. The concordance between MRD assessment using BloodFlow (in PB) *vs* NGF (in PB and/or BM) was analyzed in 389 samples from 351 MM pts.

RESULTS.

Of the 138 patients enrolled in the GEM2014MAIN trial having MRD assessed in PB, 15 (12%) showed positive MRD. Their median progression-free survival (PFS) since MRD testing was 22 months, which was significantly inferior *vs* those with undetectable MRD in PB (median not reached). The respective rates of PFS at two years were 50% and 98% (HR: 11.7; p<.00001). Among the 123 patients with undetectable MRD in PB, 33 (27%) showed persistent MRD in BM and inferior PFS *vs*

those with undetectable MRD in PB and BM. The respective rates of PFS at two years were 62% and 100% (p<0.0001). The results from this part of the study confirmed the prognostic value of MRD assessment using NGF in PB, and emphasized the importance of increased sensitivity to reduce the number of false-negative results in PB *vs* BM.

In the second part of the study, an optimized BloodFlow protocol was developed after comparing various lysing methods and MicroBeads combinations for optimal enrichment of circulating PC. Initial testing in PB samples from healthy individuals showed on average an 82-fold increment in the number of circulating normal PC with BloodFlow *vs* NGF. Dilution experiments with MM cell lines showed detection of up to $1x10^{-7}$ tumor cells.

The performance of BloodFlow *vs* NGF in PB was compared in 353 samples. BloodFlow detected MRD in 33/353 (9%) samples. The lowest MRD level was $6x10^{-8}$. Of the 33 samples with detectable MRD using BloodFlow, 19 (58%) were negative by NGF. All cases with undetectable MRD according to BloodFlow were also negative by NGF.

Subsequently, we compared the performance of BloodFlow *vs* NGF in 199-paired PB and BM samples. Concordance was observed in 137 (69%) double-negative and 19 (9.5%) double-positive samples. MRD was detected in BM and not in 41 (20.5%) PB paired-samples, while 2 (1%) were negative in BM but positive in PB (both showing MRD reappearance in BM soon after). Thus, BloodFlow showed a NPV of 77% when compared to NGF in BM. MRD assessment during induction and intensification was the feature more frequently associated with a false-negative result using BloodFlow (26/41 [63%]), followed by reduced PB cellularity (15/41 [37%]) and MRD levels below 10^{-5} in BM (12/41 [29%]). In the GEM2014MAIN trial, 2 of 4 pts with positive MRD in PB using BloodFlow progressed, whereas none of the 29 patients having undetectable MRD relapsed thus far.

CONCLUSIONS.

MRD assessment in PB using NGF was prognostic in patients under maintenance or observation. Notwithstanding, a new method (BloodFlow) with an unprecedented sensitivity to detect MRD down to 10^{-8} in PB of MM patients, was developed to increase the NPV. BloodFlow detected MRD in PB more frequently than NGF, with a consequent decrease in the number of cases with persistent MRD in BM while undetectable in PB, which were more frequent during early and intensive stages of treatment. These results suggest the possibility of periodic and ultra-sensitive MRD assessment in PB during maintenance or observation.

2. INTRODUCTION

2.1 Role of Minimal Residual Disease in Multiple Myeloma.

Multiple Myeloma (MM) is a blood clonal B-cell malignancy due to an uncontrolled proliferation of plasma cells (PCs) in the bone marrow (BM) or, more rarely, in extramedullary tissues [1].

Treatment for MM has been radically transformed during the past decades by the introduction of several new drugs with different mechanisms of action, which has led to improved survival for patients with MM [2].

With the introduction of more effective multidrug combinations, especially when used with Autologous Stem Cells Transplant (ASCT), nearly all patients achieve a treatment response, with more than 50% of these reaching a complete response (CR). Frustratingly, CR represents one of the greatest paradoxes in MM with devastating consequences for patients and physicians. Indeed, CR does not mean remission and most patients will inevitably relapse, due to the emergence of new subclones. These patients will receive multiple lines of therapy, develop refractoriness to all available drugs, and die from their tumor. This reflects the idea of a persistent disease, called minimal residual disease (MRD) [2], that cannot be detected with the recommended disease evaluation techniques.

A variety of studies have been conducted and virtually all have shown the superiority of undetectable MRD, as the totally absence of pathological PCs in the BM, over CR to evaluate treatment efficacy [2,3]. Indeed, measurement of MRD provides a more sensitive determination of disease burden than CR [3]. For example, in patients with newly diagnosed MM (NDMM) who achieve CR, most of the clinical benefit is in those who are MRD negative, while patients with MRD-positive CR have outcomes similar to those achieving a lesser response [2]. Similar results have also been reported for patients older than 65, high-risk patients, and relapse/refractory MM [4].

For this reason, MRD status was included in the decision-making algorithms for subsequent treatments and has become a primary endpoint for prospective clinical trials [3].

The level of MRD, undetected by conventional methods, is probably one of the most important features contributing to the link between the depth of response and long-term outcomes. Several studies have associated statistically superior survival outcomes with MRD-negative responses relative to MRD-positive responses, with deeper MRD response predicting longer survival [3]. In fact, undetectable MRD emerged as the most relevant prognostic factor in clinical trials, independent of

the method used to define it (cell-based, molecular-based, or imaging-based), for the identification of MM patients at higher risk of relapse [4,5].

In a recent meta-analysis of 44 studies and 8900 patients, *Munshi et al* [6] showed that in all circumstances across disease settings, achieving MRD negativity was associated with improved progression free survival (PFS) (HR, 0.33; 95% CI, 0.29-0.37; *p*:0.001) and overall survival (OS) (HR, 0.45; 95% CI, 0.39-0.51; p:0.001). This improvement in survival was observed regardless of disease setting (newly diagnosed or relapsed/refractory MM), MRD lower limit of detection threshold, cytogenetic risk, method of MRD [7].

Another prognostic factor recently evaluated is the persistence of MRD negativity. Indeed, durable MRD negativity lasting ≥6 or ≥12 months may represent yet a deeper level of response with a higher prognostic value. This suggests that MRD negativity may be a more robust evaluation of disease control if sustained over time. A recent study supports this view by demonstrating improved PFS with sustained MRD negativity [8].

Therefore, we can define MRD as a biomarker to evaluate the efficacy of treatment at different stages (induction, transplantation, consolidation, and/or maintenance) and as a surrogate of survival.

The goal to be achieved is to establish the role of MRD monitoring for tailored therapy in MM, as happened for chronic myeloid leukemia (CML) [9] and acute lymphocytic leukaemia (ALL) [10].

For these reasons, response criteria for MM have evolved and been expanded considerably to redefine and improve CR criteria by implementing MRD assessment (*Table 1*).

2.2 Multiparameter flow cytometry methods for MRD detection.

Multiparameter Flow Cytometry (MFC) is now an integral part of laboratory investigations for the management of PC disorders. MFC is particularly well-suited to study biological samples containing PCs because this technique allows:

- simultaneous identification and characterization of single PC based on multiple parameters;
- evaluation of high cell numbers in a few hours;
- quantitative assessment of different cell populations and their corresponding antigen expression levels,
- combined detection of cell surface and intracellular antigens [4].

Many surface markers have been described for the identification of PCs and for distinguishing MM PCs from normal ones. The most commonly used surface markers for discriminating and categorizing normal and pathological PCs include CD138, CD38, CD45, CD56, CD19, and cytoplasmic κ and λ immunoglobulin light chains. Additional markers, many of which are aberrantly expressed on MM PCs, are also of value and include CD20, CD27, CD28, CD81, CD117, and CD200 [2-11].
The most common aberrancies examined are loss of CD19 expression, decreased expression of CD45, gain of CD56 and aberrant CD117 expression. However, *Perez-Persona et al*, showed that there are also other aberrant phenotypic profiles [12, 13].

MFC can play an important part in monitoring of response to therapy via MRD detection because the aberrant phenotypes of clonal PCs are readily distinguishable from normal PCs [4].
MFC MRD is applicable in virtually every MM patient without requiring patient-specific diagnostic phenotypic profiles [4].
Several studies have demonstrated the use of MFC in the detection of MRD in the BM [14–24]. In particular, *Paiva and colleagues* showed that 4-colour MFC MRD assessment was the most relevant prognostic factor in transplant-eligible MM [14] and also in transplant-ineligible patients too [15]. *Paiva* also showed that combined cytogenetics and MFC MRD were highly-effective to predict unsustained CR after ASCT [16]. Early relapse after achieving a CR was associated with very poor survival. These results highlight the close association between disease biology and depth of response after therapy in determining long-term outcomes, but also highlight the identification of patients with imminent relapse and poor survival (≤ 2 years).
In addition, MFC-based assessment of the post-therapy BM, provides important data regarding the immune-cell profile, which can provide additional prognostic information [2].

2.2.1 First and second generation MFC.

Most of the available data on the prognostic value of MFC MRD assessment were obtained using "first generation" MFC based in 4-colour combinations (CD19, CD38, CD45 and CD56) and the evaluation of 2 x 10^5 cells, with a limit of detection (LOD) of 10^{-4}.

The LOD (and the lower limit of quantification) depend upon the number of cells measured and the relative number of abnormal cells required for a positive vs. negative MRD diagnosis [11].

The "second generation" MFC was progressively introduced and improved the sensitivity and specificity of MRD monitoring. Indeed, the second-generation 8-colour MFC (CD38, CD138, CD19, CD27, CD45, CD56, CD81, and CD117) assay measured 10 times more cells (2 x 10^6 cells) and resulted in a significantly increased specificity and sensitivity [20].

Paiva and colleagues showed that by applying the LOD reached with first-generation MFC (10^{-4}), up to 30% of patients with persistent MRD detectable by second-generation MFC (10^{-5}) would had been wrongly classified as MRD negative. The ability to monitor MRD up to the 10^{-5} sensitivity level is clinically relevant, because this level identifies a subset of patients (those between 10^{-4} and 10^{-5}) with inferior survival than MRD-negative (10^{-5}) cases and similar to that of MRD-positive patients at the 10^{-4} level [20].

The advances in second-generation MFC technology have significantly improved the sensitivity of the assay, particularly when combined with the use of eight colours and the evaluation of greater number of cells than what was previously feasible with 4-colour instruments.

2.2.2 Next-Generation Flow.

In 2017, the EuroFlow group proposed the next-generation flow (NGF) method for MRD detection in MM samples. The greater sensitivity of NGF vs conventional flow-MRD was mostly due to the use of two eight-colour combinations that combine surface antigens for the identification of phenotypically aberrant clonal PCs and cytoplasmic κ and λ light-chain expression to confirm their clonality. The technique has been modified to include an initial bulk lysis step to consistently measure more than 5×10^6 leucocytes per tube. In this way, EuroFlow NGF can increase 10-fold the number of cells evaluated [25] (*Figure 1*). This two-tube NGF approach has been validated for increase specificity at very low MRD levels with a sensitivity of 10^{-6}.

This method is very robust and improves reliability, consistency, and sensitivity because of the acquisition of a greater number of cells [2].

There are mainly three reasons for such a high sensitivity:

1) the evaluation of B-cell precursors, mast cells and nucleated red blood cells by using a standardized approach could detect hemodiluted samples that were considered inadequate for MRD assessment;
2) a high number of nucleated cells was acquired (~10 millions);
3) the use of the automatic population separator eliminated the operator-dependent variability [3, 26].

The innovative EuroFlow-based high-sensitive, standardized and validated NGF-MRD method can be applied to virtually every MM patient for MRD monitoring in BM after therapy. Overall, Montero et colleagues show a similar applicability but a significantly increased sensitivity for the EuroFlow-NGF approach vs conventional MFC-MRD, with around one fourth of all MRD-negative samples by conventional MFC becoming MRD-positive by NGF [25].

The EuroFlow-NGF approach provided similar results in different centers, which further confirms the high standardization level of the method.

2.3 Molecular methods for MRD detection.

2.3.1 ASO-qPCR.

Another method that has been studied is ASO-qPCR that can provide an accurate quantification of MRD. ASO-qPCR can identify clonal MM PCs-specific immunoglobulin heavy chain (*IGH*) gene rearrangements allows the detection of very low levels of MM PCs with a sensitivity that can detect one in 10^5 cells. ASO-qPCR involves making primers complementary to the junctional region of the rearranged *IGH* genes, which are used to interrogate BM samples at different times to determine the response depth. This step requires availability of the baseline diagnostic sample.

Puig and colleagues [18] compared MRD status using ASO-qPCR versus four-colour MFC in a large series of 170 patients from different clinical trials. The authors found a significant correlation in predicting MRD between the two techniques (r=0,881; $p<0{\cdot}001$). However, more than half the patients could not be evaluated by the molecular approach either due to the inability to detect a clone, unsuccessful sequencing, or suboptimal ASO-qPCR performance. These technical limitations are in part due to the presence of multiple somatic mutations in the immunoglobulin genes. In these cases, primers and probes that are adapted to each patient to match the somatic hypermutations are needed[2].

2.3.2 Next-generation sequencing.

Next-generation sequencing (NGS) is of considerable interest for the detection of MM MRD in the BM. Specifically, genomic DNA is amplified using locus-specific primers designed for *IGH-VDJ*$_H$, *IGH-DJ*$_H$, or *IGK*. Once amplified, the immunoglobulin gene DNA is sequenced and the frequencies of the different clonotypes in the sample are determined. To avoid disproportional amplification of the *IGH* and *IGK* rearrangements, the extensive sets of primers need to be attuned and validated to guarantee equal (proportional) amplification of each target rearrangement between the many rearrangements derived from remaining normal B cells [2]. Currently, NGS surpassed ASO-qPCR, because NGS does not need patient-specific primers and probes and showed higher applicability due to a better marker identification rate at diagnosis (90–92% with NGS vs. 50–60% with ASO-qPCR), thus overcoming the failure to detect clonality by ASO-qPCR after IGH somatic hypermutation [27, 28].

The NGS technique allowed the achievement of high sensitivity levels (up to 10^{-6}) in several clinical trials [3, 27].

2.4 Comparison of NGS and NGF assessment.

The ideal MRD evaluation should have several relevant characteristics: high applicability, high sensitivity and specificity, excellent feasibility, easily accessible, rapid turnaround, reproducibility, proven clinical value and cost-effectiveness [2].

NGS and NGF had most of them, therefore they can be uniformly applied. Both methods have the ability to detect one MM cell in 10^5–10^6 cells.

These two approaches have also different features, that are showed in *Table 2* [27]. Since NGF requires an immediate experimental procedure, NGS samples can be frozen and stored for later analyses. On the other hand, NGS relies on the identification of clonotypic rearrangements at diagnosis, making that up to 10% of cases cannot be followed due to somatic hypermutation in primer regions. Another shortcoming of NGS is the turnaround time, which is longer than for NGF (5–7 days compared to 24–48 h). In contrast, interpretation of results is usually more difficult for NGF [29].

Despite these differences, a recent study found a high correlation between NGS and NGF in terms of MRD detection and quantification ability ($R^2 = 0.905$) [29]. For this reason, the International Myeloma Working Group (IMWG) strongly encourage the inclusion of both methods in prospective trials, if possible, to find out the advantages and disadvantages of each approach [2].

2.5 Imaging methods for MRD detection.

MM is characterized by spatial tumor heterogeneity and unfortunately the MFC and molecular MRD evaluations do not allow detection of the disease outside the BM [2, 30].

Extramedullary disease is increasingly seen in the clinic and its estimated incidence is about 15% [31]. In the future, these rates might increase as increasingly sensitive imaging technologies and extended survival of patients with MM.

The patchy type of disease distribution in MM is poorly understood and imaging can be used to complement MRD detection [30].

^{18}F-FDG PET/CT (^{18}F-fluoro-deoxy-glucose positron emission tomography/computed tomography) is a functional imaging procedure with proven good performance in patients affected by MM. It may be considered as a valuable tool to assess tumor metabolic activity and the effect of therapy on tumor-cell metabolism and also to detect extramedullary disease. For this reason, FDG PET/CT is recommended by IMWG as one of the methods for evaluating and monitoring response to therapy and for detecting MRD [32].

Recently, a group of Italian nuclear medicine experts and hematologists defined new visual descriptive criteria (Italian Myeloma criteria for Pet Use: IMPeTUs) to standardize FDG PET/CT evaluation in MM patients [32]. These include the visual interpretation of images to quantify FDG uptake using the five-points scale of Deauville score (DS) proposed for FDG- PET in lymphoma [33], in association with a morphological and anatomical aspect of FDG distribution such as the BM non-focal uptake, focal bone lesions (site, number and uptake), para-medullary, or extra-medullary lesions.

In conclusion, the combination of MRD diagnostics and functional imaging improves prediction of outcome with a benefit for MM patients. In fact, FDG PET/CT evaluation gives an additive value to BM-based assessments of MRD.

2.6 Detection of MRD in peripheral blood.

Evaluation of MRD in peripheral blood (PB) would represent an attractive minimally invasive alternative to circumvent the noted disadvantages of MRD assessment in BM.

In recent years, detection of circulating tumor cells (CTCs), cell-free DNA, or mass spectrometry (MS) have gained interest for MRD assessment in MM [34, 35, 36].

This is mainly because of

1) the minimally invasive nature of PB vs BM analyses,

2) the possibility for more precise quantification of absolute numbers of CTCs than BM MRD resulting from absence of potential hemodilution,

3) the representation of all tumor localizations including extramedullary disease.

Unfortunately, at the moment PB MRD is a less sensitive MRD marker in MM than BM MRD. The problem is that current methods (NGF, NGS, cell-free DNA and MS) are not sensitive enough to measure tumor kinetics in PB [37].

In one study of 274 paired PB/BM samples by NGF [33], NGF in PB was less sensitive than in BM, as 40% of patients with BM residual disease had undetectable PB MRD.

Early data suggest that NGS evaluation of circulating myeloma cells, cell-free DNA and MS are also less sensitive and do not correlate well with BM MRD assessment [38-40].

Several studies have shown that BM MRD reflects persistence of resistant tumor cells [21]. In contrast, CTCs might not only reflect tumor load but, particularly, the ability of persisting tumor cells to disseminate the disease and support tumor growth and progression at distant sites in BM and other tissues, based on their more immature and prominent stem cell-like features [41].

In conclusion, further researches are required to reach similar sensitivity to BM assessment with MFC and NGS and cross-validation is crucial. PB MRD methods have the potential to be used in a complementary role to determine timing for marrow MRD assessment and as indicators of early relapse [37].

2.6.1 Cell-free circulating tumor DNA.

Cell-free circulating tumor DNA (ctDNA) consists of small fragments of nucleic acids extracted from the plasma or serum. The cancer-derived fragments may be identified and quantitative and qualitative information can be obtained. ctDNA sequencing allows the analysis of a tumour profile with high levels of concordance with paired BM samples [36, 42]. However, in some cases, the mutations identified were found only in plasma, which is consistent with the spatial heterogeneity of MM.

In MM, ctDNA may be used to detecting MRD but it may be undetectable in more than half of the patients with positive MRD in the BM [38, 39].

For this reason, ctDNA is currently not yet a robust biomarker for monitoring the disease compared to NGS or EuroFlow NGF in BM.

2.6.2 Mass spectrometry.

PC disorders are identified in the clinical lab by detecting the monoclonal immunoglobulin (M-protein) which they produce. Each M-protein is derived from recombination and somatic hypermutation events of both the heavy and light chain loci of the clonal B cell. Consequently, M-protein has both a unique amino acid sequence and unique molecular mass.

Mass spectrometry (MS) method is ideally suited to making accurate mass measurements or targeted measurements of unique M-protein peptides [43]. Indeed, MS method is based on the search for the unique sequence of the antigen-binding region, also known as the complementarity-determining region (CDR) of immunoglobulin (Ig) [43]. Each PC produces a unique Ig with a specific CDR due to the adaptive immune system's optimization of the CDR via somatic hypermutation. The resulting CDR amino acid sequence is unique and each PC clone has a different peptide sequence and overall mass. Therefore, MS has the advantage of increased accuracy, documented clinical and analytic sensitivity.

Efforts have been made to use MS method for monitoring the circulating M-protein in the serum as a surrogate marker for the presence of neoplastic PCs but its role has to be defined. Therefore, MRD in BM samples provides information that cannot be achieved by MS in PB. In the future, MS will not replace existing MRD tests in BM but will have clinical value as a companion method, especially for helping guide timing of BM test [40, 43].

2.6.3 Circulating Tumor Cells.

CTCs, as defined by the presence of PB clonal plasma cells (PCs), are a powerful prognostic marker in MM. The presence of CTCs has been associated with an increased risk of malignant transformation to symptomatic MM in both MGUS [40] and smoldering MM [44], as well as with an inferior survival among symptomatic newly diagnosed [44] and relapse/refractory MM [45].

CTCs typically represent a unique subpopulation of all clonal PCs, characterized by downregulation (p<0.05) of integrins (CD11a / CD11c / CD29 / CD49d /CD49e), adhesion (CD33 / CD56 / CD117 / CD138) and activation molecules (CD28 / CD38 / CD81). This immune phenotype reflects how hypoxic and pro-inflammatory microenvironment could induce an arrest in proliferation, forcing tumor cells to recirculate in PB [12]. Moreover, CTCs were mostly quiescent (arrested in the G0–G1 phase of the cell cycle) and showed a circadian distribution which fluctuates in a similar pattern to that of CD34+ [4] cells, suggesting that in MM, CTCs could leave the BM, recirculate into PB and home again into the BM at a different localization, in a kind of "metastatic"/dissemination process. In fact, recent observations suggest that tumor cell dissemination is often an early event [46]. Since CTCs are responsible for disease dissemination, they are the most important PCs to monitor for patients' risk-stratification.

A recent study showed that the transcriptional state of CTCs resembles that of patient-matched BM clonal PCs, except for a few genes that are involved in interferon and inflammatory response, hypoxia, cell cycle and migration [47]. The study also demonstrated that patients with BM clonal PCs overexpressing CTC-related genes have more aggressive disease behavior. Thus, deep characterization of CTCs might unveil novel therapeutic targets to overcome disease dissemination and prolong survival of patients with MM [47].

Indeed, CTCs could become a promising noninvasive tool also for monitoring response to treatment and MRD. In fact, the EuroFlow has recently shown a ~2-fold increased frequency of cases with detectable CTCs by NGF as compared to low-sensitive flow cytometry [48], which resulted in unprecedented rates of MRD detection in PB (15% of MM patients in CR) [34]. Unfortunately, there were also 40% of cases with persistent MRD in the BM but undetectable CTCs in the volume of PB that can be currently tested by these methods [34].

Therefore, EuroFlow NGF is very sensitive to detect CTCs at diagnosis, but needs something else to become sensitive enough to measure CTC kinetics during treatment.

3. AIMS OF THE STUDY

A variety of studies conducted in the last decades have shown the superiority of assessment of MRD to evaluate treatment efficacy; indeed, undetectable MRD emerged has the most relevant prognostic factor in MM. Because of its surrogacy for long-term survival, there are ongoing efforts to adopt MRD as primary endpoint in clinical trials to accelerate drug development.

There is consensus that MRD will be one (if not) the most useful biomarker for precision medicine in MM but the problem is that MRD is evaluated in BM and not in PB.

BM aspirates are an invasive and painful procedure and thereby not performed periodically. In MM, this is aggravated by the likelihood that BM aspirates are non-representative due to patchy infiltration or even extramedullary disease. This reduces confidence on response assessment due to the uncertainty behind a negative MRD result.

The solution to have clinically useful response assessment in MM is to monitor tumor kinetics periodically in PB. In fact, the requirement for PB rather than BM as the tissue of choice for molecular monitoring will improve the quality of life of patients.

The first aim of this thesis was to investigate the prognostic value of MRD assessment in PB using EuroFlow NGF, one of the methods approved by the IMWG and validated in the clinical setting.

The problem is that EuroFlow NGF is not sensitive enough to measure tumor kinetics in PB. Indeed, unfortunately, EuroFlow NGF in PB was less sensitive than in BM, as 40% of patients with BM residual disease had undetectable PB MRD.

The second aim was to develop a new method with increased sensitivity to detect CTCs with NGF after optimized immunomagnetic enrichment from large volumes of PB (BLOOD-FLOW). This approach was empowered with a LOD reaching 10^{-7}, which is one-log more sensitive than the current state-of-the-art.

Table 3 demonstrates the potential innovation of BLOOD-FLOW.

4. PATIENTS, MATERIALS AND METHODS

All patients provided written informed consent and the study was conducted in accordance with the Declaration of Helsinki after approved by the University of Navarra ethics committees.

4.1 Previous work.

Firstly, we had tested the performance of EuroFlow NGF vs the concept of BLOOD-FLOW. Briefly, PB from healthy adults were collected and the total number of circulating normal PCs as well as the number of PCs per mL were compared between both methods; NGF performed according to the EuroFlow standard operation procedures (SOP) vs immunomagnetic isolation of $CD138^+$ circulating PCs followed by NGF. The main difference between the two approaches is that, whereas the volume of PB used for NGF is adjusted to measure approximately 10 million cells and reach a LOD of 2×10^{-6} (typically ranging from 3 to 5mL), with BLOOD-FLOW we can readily start with 50mL of PB since after immunomagnetic enrichment of circulating PCs, a small aliquot would be ultimately stained following the EuroFlow SOP for NGF.

By increasing the PB volume more than 10-fold, we measured a higher number of circulating PCs with BLOOD-FLOW than with NGF; however, we had to show that by adding beads for immunomagnetic enrichment before NGF, the whole methodology remained equally effective (i.e., a similar number of PCs/mL). By achieving this, the cost of NGF after immunomagnetic isolation would remain unchanged, as it would be performed in a PC-enriched fraction, and the acquisition time in cytometers would be minimal, as the total number of cells in an enriched aliquot should be low.

Initially, we conducted these experiments with PB samples from 5 healthy adults. We confirmed a significant enrichment of circulating PCs from NGF to BLOOD-FLOW (27-fold average) and also we found that the number of circulating PCs per mL was the same by adding immunomagnetic isolation before NGF (*Figure 2 and 3*).

4.2 Development phase.

Our aim was to develop a protocol that resulted in maximum recovery of circulating PCs from 50mL of PB, based on our estimation that this volume was needed to reach a LOD that covered the logarithmic range of 10^{-7} and reached 10^{-8} in a considerable number of PB samples. This estimation was based on the fact that:

- The standard NGF protocol was designed to analyse 10-15 million nucleated cells and thereby, reached a LOD of $2x10^{-6}$ (i.e., a cluster of 20 tumour cells within 10^6 normal cells).

- Based on a fixed concentration of 5.000 cells/µL of PB (expected mean value in these patients), the NGF protocol would start from 4mL of PB taking into account a maximum of 50% cell loss, particularly during centrifugation steps.
- In 50mL of PB one could expect 250 million or more leukocytes and the goal was to select circulating PCs with maximum efficacy into an aliquot of 200µL.
- NGF would be performed directly in that aliquot without need of red blood cell (RBC) lysis and no centrifugation steps (no cell loss).
- The identification of a cluster of 20 CTCs by NGF from a starting number of 250 million or more leukocytes would set the LOD of BLOOD-FLOW in $8x10^{-8}$.
- Anticipating a variation of ±20% in the starting number of cells, this approach could still cover the entire logarithmic range of 10^{-7}, and would be 1-log more sensitive than NGS or NGF MRD assessment in BM and PB samples. Noteworthy, a LOD around $5x10^{-8}$ could be reached in cases with increased cellularity (>5.000 cells/µL).
- We estimated that a 1-log increment in sensitivity could increase the detection rate of CTCs in PB of MM patients in CR, from 15% with NGF/NGS up to 30% with BLOOD-FLOW. This estimation was based on the 2-fold increment in the detection of MRD in BM and CTCs in PB, observed with the transition from conventional into NGF cytometry [25, 48].

A detection rate of 30% of CTCs in PB from MM patients in CR, would match the detection rate of MRD in BM aspirates (25-35% depending on the quality of CR) [22]. BLOOD-FLOW would be ready to be compared with gold-standard MRD methods for non-inferiority performance.

During the development phase of the project we focused our efforts in testing different protocols for optimal enrichment of circulating PCs. Amongst others, we tested in PB samples from healthy adults, different methods for RBC lysis or sedimentation (*Figure 4*) as well as different concentrations of immunomagnetic microbeads targeting CD138 (an antigen that is specific of PCs within the hematopoietic system).

Of note, we prioritized recovery and enrichment of PCs over the purity of the enriched aliquot, since subsequent immunophenotyping with NGF allowed the unequivocal detection of CTCs regardless the extent of "contamination" with other (CD138-negative) leukocytes (*Figure 5*). In fact, there could be as many as 10 million cells in the 200µL aliquot (consisting in circulating PCs plus contaminating leukocytes) and NGF could still be performed following the SOP developed by EuroFlow.

It should also be noted that based on our stability tests performed during the implementation of standard NGF for CTC detection in PB, we concluded that samples were valid up to 72h after collection, in duplicate measurements made every 24h in 5 samples (*Figure 6*).

All variations in the protocol were tested and compared against the performance of NGF in the same sample (gold-standard). The protocol with the highest recovery of circulating PCs was then tested for sensitivity and specificity based on serial dilution experiments with MM cell lines available in our laboratory. A schematic representation is shown in *Figure 7*.

A go/no-go decision was made based on the ability of the selected protocol to cover the entire logarithmic range of 10^{-7} though we expected that this approach would reach a LOD around $5x10^{-8}$ in PB samples with increased cellularity (>5.000 cells/μL). Afterwards, a SOP was developed for implementation of BLOOD-FLOW in a series of MM patients in CR after therapy.

4.3 Validation phase.

Briefly, the validation phase was performed as follows:

a) Patients were informed and signed their consent to draw 50mL of PB in parallel to the BM aspirate that was part of the diagnostic procedure to confirm CR.
b) PB and BM samples were directly collected from patients of the Hematology Unit of Clinica Universidad de Navarra (Pamplona), or sent at ambient temperature via Courier (<24h for national shipment) from patients in other Spanish Hospitals.
c) Samples were received, registered and the BLOOD-FLOW SOP was implemented:
    i. Determine the cellular concentration of PB.
    ii. Collect the volume corresponding to 20 million cells for the NGF SOP.
    iii. Use all sample leftover for BLOOD-FLOW.
    iv. Positive selection of circulating PCs with MACS® Technology (following the final version of the protocol established during the development phase).
    v. Stain the aliquot enriched in circulating PCs with the NGF panel of monoclonal antibodies.
    vi. Incubate for 30min in the dark and at ambient temperature.
    vii. Measure the aliquot in a flow cytometer with standard NGF settings.
    viii. Determine the absolute number of circulating PCs in the sample processed with the NGF SOP based on automated analysis using the Infinicyt software.
    ix. Quantify the percentage of CTCs within total circulating PCs in the sample processed with the BLOOD-FLOW SOP.
    x. Determine the absolute number of CTCs by extrapolation from the absolute number of circulating PCs.
d) The key performance indicator (KPI) was the categorical comparison of tumour detection in BM vs PB (negative or positive). We also compared tumour levels in BM and PB.

e) Raw data and reports are stored in the secure servers of the University of Navarra for a period of time not inferior to 5-years.

4.4 Clinical application of the data.

Upon the demonstration of non-inferiority between NGF MRD assessment in BM vs BLOOD-FLOW monitoring of CTCs in PB, we continued testing both methods in three clinical trials of the PETHEMA/GEM cooperative group: GEM2014MAIN (NCT 2014-024061-44), GEMCESAR (NCT 2014-002948-40), GEM2017FIT (NCT 2017-037422-97). We specifically selected these trials based on their design (*Figure 8, 9, 10*) since after induction/consolidation, patients were stratified according to their MRD status (in BM by EuroFlow NGF). Paired MRD assessment in BM by EuroFlow NGF and in PB by BLOOD-FLOW during the maintenance phase of the clinical trials could create a unique opportunity. Indeed, these results could establish the clinical utility of BLOOD-FLOW in the most important setting where response assessment is expected to play a fundamental role in guiding individualized treatment decisions: continuous therapy until (eventual) disease progression or fixed-duration therapy. Our questions are:

- If BLOOD-FLOW can be equally effective when performed during treatment as compared to off-treatment.
- If BLOOD-FLOW can measure the efficacy of maintenance therapy based on progressively decreasing/increasing levels of CTCs in PB.
- If BLOOD-FLOW can predict biological relapse before current criteria (based on MRD and M-protein) in patients on- and off-therapy.

We also confirmed the robustness of the results obtained in the validation phase of the project with multiple patient-paired analytical comparison between BM-MRD and BLOOD-FLOW.

4.4.1 GEM 2014MAIN.

The GEM 2014MAIN is a randomized, open-label, national, multicenter trial studying maintenance treatment with lenalidomide and dexamethasone versus lenalidomide, dexamethasone and MLN9708 (Ixazomib) after ASCT in patients with newly-diagnosed symptomatic MM.

A total of 316 patients were enrolled in the study. During the treatment period, patients received maintenance treatment with lenalidomide/dexamethasone (Arm A) versus lenalidomide/dexamethasone/MLN9708 (Arm B).

At two years, patients with negative MRD finished maintenance treatment. Patients with positive MRD continued treatment with lenalidomide/dexamethasone until they had completed five years of maintenance treatment.

Once this phase of active treatment was complete, patients began the long-term follow-up phase, during which they were visited every three months to evaluate progression and survival.

MRD was evaluated by EuroFlow NGF after induction, ASCT, consolidation, maintenance and annually thereafter [49].

4.4.2 GEM CESAR.

The GEM-CESAR is a phase II single-arm trial studying a potentially curative strategy for high-risk smoldering MM (HR-SMM) patients in which the primary endpoint is the achievement of BM MRD negativity [50]. In this clinical trial, 90 HR-SMM patients at high-risk of progression (>50% at 2 year), younger than 70 year and transplant candidates were included.
Induction therapy consisted on six 4-weeks cycles of KRd (K: Carfilzomib, R: Revlimid/Lenalidomide, d: dexamethasone). Melphalan at dose of 200 mg/m^2 followed by ASCT was given as intensification therapy and three months later, patients received two KRd consolidation cycles followed by maintenance with Lenalidomide at dose of 10 mg on days 1-21 plus dex 20 mg weekly for up to 2 years.
MRD was evaluated by EuroFlow NGF after induction, ASCT, consolidation and annually thereafter during follow-up [51].

4.4.3 GEM 2017FIT

GEM 2017FIT is a phase III trial by PETHEMA group (NCT03742297) enrolling elderly fit patients aged between 65 and 80 years who randomizes patients to nine cycles VMP (Velcade/Bortezomib-Melphalan-Prednisone) followed by nine Rd *vs* 18 cycles KRd *vs* 18 cycles Dara (Daratumumab)-KRd [50]. The study is designed as a randomized, controlled, open-label, assessor blind, multicenter superiority trial with three parallel groups, and primary endpoint was immunophenotypic complete response and MRD rates at 18 months after randomization. Block randomization was performed with a 1:1:1 allocation ratio. Patients were randomized up front to 3 arms. Patients could receive:

- arm 1 (control arm), VMP x 9 + Rd x 9
- arm 2a, KRd regimen: 18 cycles
- arm2b, KRd regimen combined with Dara: 18 cycles.

MRD was evaluated by EuroFlow NGF after induction, consolidation, maintenance and every six months thereafter [53].

4.5 Statistical methods.

Crosstab tables were used to compare the distribution of cases presenting pathological PC by EuroFlow-NGF and BLOOD FLOW in paired blood (BLOOD FLOW) vs. BM samples (NGF).

The Kaplan–Meier method and either the (two-sided) log-rank were used to plot and compare progression-free survival (PFS) curves between two or more than two patient groups, respectively.

PFS was defined as the time from MRD assessment to either disease progression or the last follow-up visit.

Statistical significance was set at p-values <0.05.

For all statistical analyses, the Statistical Package for Social Sciences (SPSS version 23; IBM, Armonk, NY), was used.

5. RESULTS

5.1 The prognostic value of MRD assessment using NGF in PB.

MRD was evaluated using EuroFlow NGF in PB of 138 MM patients enrolled in the GEM2014MAIN trial. PB samples were collected after the second year of maintenance, when patients stopped treatment if MRD negative (in BM), or continued on therapy for three additional years if MRD positive at that time.

Of the 138 patients enrolled in the GEM2014MAIN trial having MRD assessed in PB, 15 (12%) showed positive MRD. Their median PFS since MRD testing was 22 months, which was inferior *vs* those with undetectable MRD in PB (median not reached, *Figure 11*). The respective rates of PFS at two years were 50% and 98% (HR: 11.7; $p<0.0001$).

Among the 123 patients with undetectable MRD in PB, 33 (27%) showed persistent MRD in BM and inferior PFS *vs* those with undetectable MRD in PB and BM (*Figure 12*). The respective rates of PFS at two years were 62% and 100% ($p<0.0001$).

The results from this part of the study confirmed the prognostic value of MRD assessment using EuroFlow NGF in PB, and emphasized the importance of increased sensitivity to reduce the number of false-negative results in PB.

5.2 Comparison between BloodFlow and NGF in PB.

In the second part of the study, an optimized BloodFlow protocol was developed after comparing various lysing methods and bead combinations for optimal enrichment of circulating PCs. Initial testing in PB samples from healthy individuals showed on average an 82-fold increment in the number of circulating normal PCs with BloodFlow *vs* EuroFlow NGF.

Dilution experiments with MM cell lines showed detection of $1x10^{-7}$ tumor cells.

Reaching a minimum sensitivity of 10^{-7} requires analyzing $\geq 2x10^{8}$ cells.

Large PB (~50mL of PB) volumes were initially processed in MACS® columns and ~100µL aliquots enriched with circulating PCs were analyzed using EuroFlow NGF.

We used BLOOD-FLOW in 389 PB samples from 351 MM patients.

The concordance between MRD assessment using BloodFlow (in PB) *vs* EuroFlow NGF (in PB) was analyzed in 353 samples.

BloodFlow detected MRD in 33/353 (9%) samples. The lowest MRD level was $6x10^{-8}$. These data confirm the higher sensitivity of BLOOD-FLOW compared to the conventional EuroFlow NGF.

Of the 33 samples with detectable MRD using BloodFlow, 19 (58%) were negative by conventional EuroFlow NGF (*Figure 13*).

All cases with undetectable MRD according to BloodFlow (320/353, 91%) were also negative by EuroFlow NGF in PB.

5.3 Comparison between BloodFlow and NGF in BM.

Subsequently, we compared the performance of BloodFlow *vs* EuroFlow NGF in BM in 199-paired PB and BM samples.

199-paired PB and BM samples came from the following three clinical trials of the PETHEMA/GEM cooperative group: GEM2014MAIN (NCT 2014-000554-10), GEMCESAR (NCT 2014-002948-40), GEM2017FIT (NCT 2017-037422-97).

Concordance was observed in 137 (69%) double-negative and 19 (9.5%) double-positive samples. MRD was detected in BM and not in 41 (20.5%) PB paired-samples, and 2 (1%) were negative in BM but positive in PB (both showing MRD conversion from negative to positive in BM soon after). Thus, BloodFlow showed a negative predictive value (NPV) of 77% when compared to EuroFlow NGF in BM.

MRD assessment early during treatment (ie, induction or intensification) was the feature more frequently associated with a false-negative result using BloodFlow (26/41 [63%]). Reduced PB cellularity (15/41 [37%]) and MRD levels below 10^{-5} in BM (12/41 [29%]) contributed to a lesser extent (*Figure 14* and *Table 4*).

As regards the GEM2014MAIN trial, we analized 70 samples of GEM2014MAIN trial with BLOOD FLOW and EuroFlow NGF BM from 33 patients. 29 patients were in follow-up without any treatment while 4 patients were in maintenance treatment with lenalidomide. 2 of 4 patients with positive MRD in PB using BloodFlow progressed, whereas none of the 29 patients having undetectable MRD relapsed thus far.

6. DISCUSSION AND CONCLUSION

MM is a blood clonal B-cell malignancy due to an uncontrolled proliferation of PCs in the BM or, more rarely, in extramedullary tissues [1]. Due to recent advances in immune chemotherapy, the OS has increased in the last decade [54], but most patients still relapse [55]. At the moment, follow-up monitoring is still largely based on the evaluation of neoplastic PCs in the BM, that could be quantified with different approaches based on MFC, morphology assessment and molecular biology [2, 54].

After treatment, the most relevant prognostic factor is the achievement of negative MRD, defined by the absence of PCs in BM by NGS or EuroFlow NGF. These two methods have a minimum sensitivity of 1 in 10^6 nucleated cells in patients with CR. This result has to be confirmed at least 1 year apart to be considered "sustained", regardless of treatment, cytogenetic risk and ISS stage [2, 56].

Unfortunately, both NGS and EuroFlow NGF using BM samples are invasive procedures, with the possibility of potential hemodilution of the samples and not represent all tumor localizations (due to the spatial clonal heterogeneity, typical of MM). To overcome these limitations, new approaches have been explored, including quantifying CTCs. To understand the whole tumor heterogeneity, CTCs could be used as an indicator in detecting MRD without invasive procedures [57, 58].

Differently from neoplastic PCs in the BM, CTCs in MM are mostly apoptopic (arrested in the G0–G1 phase of the cell cycle) [59], with a significantly lower proliferation index than BM PCs. Thus, CTCs follow a circadian rhythm similar to $CD34^+$ cells [41] and are highly informative about both intra and extramedullary disease [60].

CTCs have successfully been quantified in PB of MM patients. However, it must be noted that CTCs burden in PB is 100-fold lower than in BM [61, 62].

To overcome the main limitations in CTC detection, novel technological platforms are required to improve their enrichment by increasing sensitivity and specificity [63].

Noteworthy, recent studies using the methods approved by the IMWG for MRD assessment in BM, were able to detect CTCs or clonal immunoglobulin sequences in PB of approximately 15% of patients in CR [4,5]. Unfortunately, there were also 40% of cases with persistent MRD in the BM but undetectable tumor cells or DNA in the volume of PB that can be currently tested by these methods.

Taken together, these are promising results but not good enough to replace BM for PB as the gold-standard sample to evaluate treatment efficacy in MM.

Firstly, we confirmed the prognostic value of MRD assessment in PB using Euroflow NGF in patients under maintenance or observation in the clinical trial GEM2014 MAIN. We specifically selected this trial for the design since after induction/consolidation, patients were stratified according to their MRD status (in BM by EuroFlow NGF).

Then, we used another method consisting of the use of NGF to detect CTCs after immunomagnetic enrichment of circulating PCs from large volumes of PB (Blood-Flow).

Briefly, PB samples would be labelled with nano-sized immunomagnetic microbeads coupled with CD138 antibodies and applied onto columns, to enrich circulating PCs into a small aliquot for cost-effective NGF cytometry immunophenotyping.
This innovative method, developed to reduce false-negative results, is at least 1-log more sensitive than current NGF or NGS techniques (10^{-6}) [3,25,26] by covering the logarithmic range of 10^{-7} in all patients and, in high-cellularity samples, reaches a limit of detection (LOD) of $5x10^{-8}$.
We used BLOOD-FLOW in 389 PB samples from 351 MM patients.
The concordance between MRD assessment using BloodFlow (in PB) *vs* EuroFlow NGF (in PB) was analyzed in 353 samples.
BloodFlow detected MRD in 33/353 (9%) samples. The lowest MRD level was $6x10^{-8}$. These data confirm the higher sensitivity of BLOOD-FLOW compared to the conventional EuroFlow NGF.
Of the 33 samples with detectable MRD using BloodFlow, 19 (58%) were negative by conventional EuroFlow NGF. All cases with undetectable MRD according to BloodFlow (320/353, 91%) were also negative by EuroFlow NGF in PB.
Subsequently, we compared the performance of BloodFlow *vs* EuroFlow NGF in BM in 199-paired PB and BM samples.
Concordance was observed in 137 (69%) double-negative and 19 (9.5%) double-positive samples. MRD was detected in BM and not in 41 (20.5%) PB paired-samples, and 2 (1%) were negative in BM but positive in PB (both showing MRD conversion from negative to positive in BM soon after). Thus, BloodFlow showed a negative predictive value (NPV) of 77% when compared to EuroFlow NGF in BM.
MRD assessment especially during early treatment (ie, induction or intensification) was the feature more frequently associated with a false-negative result using BloodFlow (26/41 [63%]). Reduced PB

cellularity (15/41 [37%]) and MRD levels below 10^{-5} in BM (12/41 [29%]) contributed to a lesser extent to false-negatives.

As regards to the GEM2014MAIN trial, we analized 70 samples of GEM2014MAIN trial with BLOOD FLOW and EuroFlow NGF BM from 33 patients. 29 patients were in follow-up without any treatment while 4 patients were in maintenance treatment with lenalidomide. 2 of 4 patients with positive MRD in PB using BloodFlow progressed, whereas none of the 29 patients having undetectable MRD have relapsed to this date.

In conclusion, BloodFlow detected MRD in PB more frequently than EuroFlow-NGF, with a consequent decrease in the number of false-negative cases with persistent MRD in BM while having undetectable MRD in PB. These results suggest the possibility of periodic and ultra-sensitive MRD assessment in PB during maintenance treatment or follow-up. If our results are confirmed, BLOOD FLOW could be potentially used in a complementary role to determine timing for BM MRD assessment and as indicators of early relapse.

7. TABLES AND FIGURES

Response Category	Definition
CR	Negative serum and urine immunofixation, disappearance of soft tissue plasmacytomas and < 5% PC in the BM
MRD-negative by NGF	CR + the absence of aberrant clonal PC in BM by Euroflow or a validated equivalent method with a minimum sensitivity of 10^{-5}
MRD-negative by NGS	CR + the absence of clonal PC in BM by an NGS-based clonality assay with a minimum sensitivity of 10^{-5}
Imaging + MRD-negative	Marrow MRD negativity (NGF/NGS) plus disappearance of all areas of increased tracer uptake from baseline/preceding PET/CT, or decrease to < mediastinal blood pool SUV or to < SUV of surrounding normal tissue
Sustained MRD	Marrow MRD negativity (NGF/NGS) and negative imaging, confirmed at a minimum of 1 year apart
BM, bone marrow; CR, complete response; MRD minimal residual disease; NGF, next-generation flow cytometry; NGS, next-generation sequencing; PC, plasma cells; SUV, standardised uptake value.	

Table 1: IMWG MRD Criteria

	Next-Generation Sequencing (NGS)	Next-Generation Flow (NGF)
Availability	Adaptive Biotechnologies (Seattle, US-WA)	Worldwide
Applicability	90-92%	100%
Baseline assessment	Required for identification of dominant clonotype	Not required
Processing requirements	Both fresh and stored samples	Fresh samples are required
Standardization	Yes; Adaptive Biotechnologies (Seattle, US-WA)	Yes; EuroFlow Consortium
Quantitative	Yes	Yes
Sensitivity	1 in 10^{-5} - 10^{-6}	1 in 10^{-5} - 10^{-6}
Turnaround and complexity	1-2 weeks; bioinformatic support required	3-4 hours; flow cytometry skills required
Clonal evolution	Evaluable by tracking minor clonotypes	Not evaluable
Patchy disease evaluation	No	No
Costs	Roughly 1500 USD/sample	Roughly 300 USD/sample

Table 2: MRD techniques for MM recommended by IMWG

State-of-the-art	Innovation
There are no effective methods to monitor tumor kinetics in the PB of MM patients.	We will develop a new method based on widely available, cost-effective CE-IVD products.
Current MRD methods reach a limit of detection in the logarithmic range of 10^{-6}.	Our innovative approach will reach a limit of detection that covers the logarithmic range of 10^{-7}.
Except NGF, other MRD methods are not applicable to all MM patients.	The solution proposed builds up on NGF and will have 100% applicability at reasonable cost.
MRD assessment is confined to BM aspirates that carry risk of false-negative results	BLOOD-FLOW will monitor CTCs from distant BM and extramedullary sites of disease.
Other hematological malignancies lack effective methods to monitor MRD in PB.	Our solution for MM can be readily escalated to other tumors by adapting beads and antibody panels.

Table 3: Demonstration of Innovation Potential of BLOOD FLOW

199 patients (+/- 3 months)		BM	
		NGF-	NGF+
PB	BF-	137 (69%)	41 (21%)
	BF+	2 (1%)	19 (9%)

Table 4: Comparison in MRD results with BloodFlow vs NGF in BM.

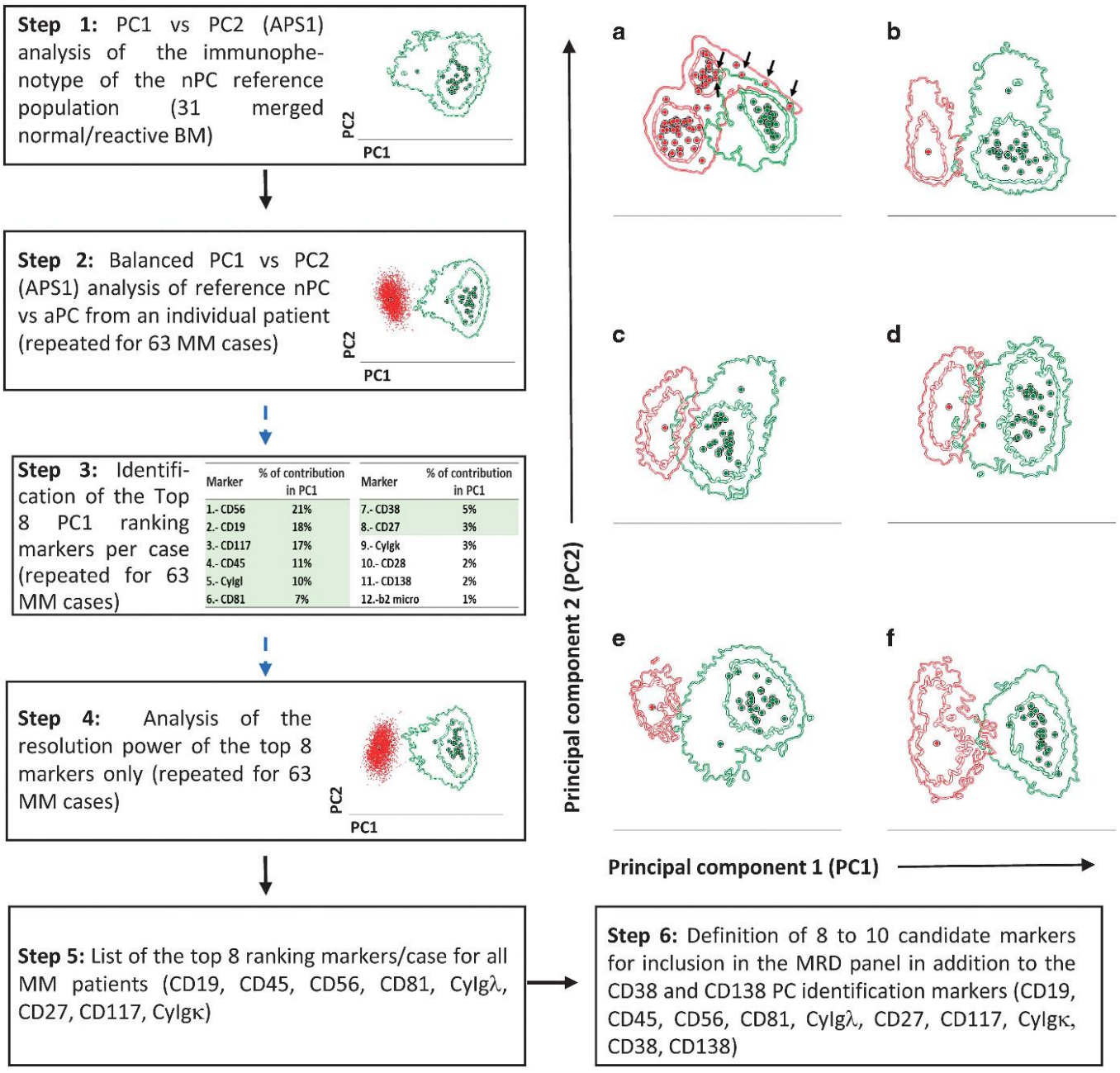


Figure 1: Diagram illustrating the process used for the selection and evaluation of markers for the NGF MRD panel (from J Flores-Montero et al, *Leukemia* 2017).

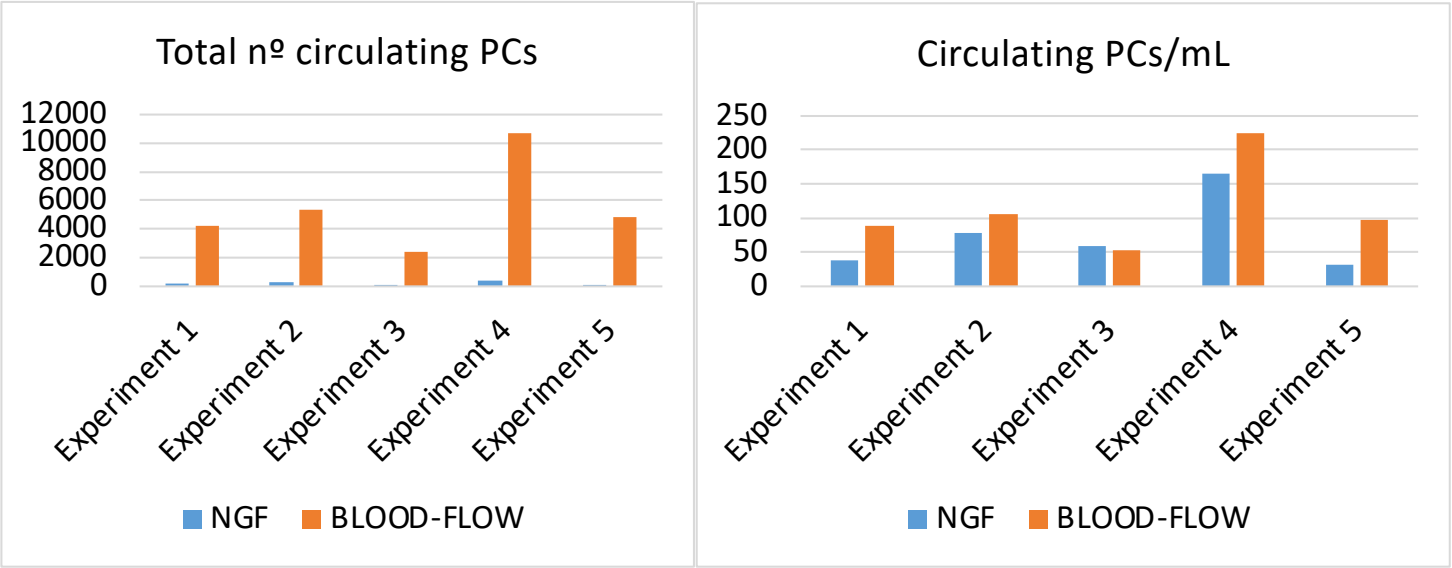


Figure 2: Comparison of the total number of circulating PCs and circulating PCs/ml with NGF and Blood Flow with PB samples from 5 healthy adults

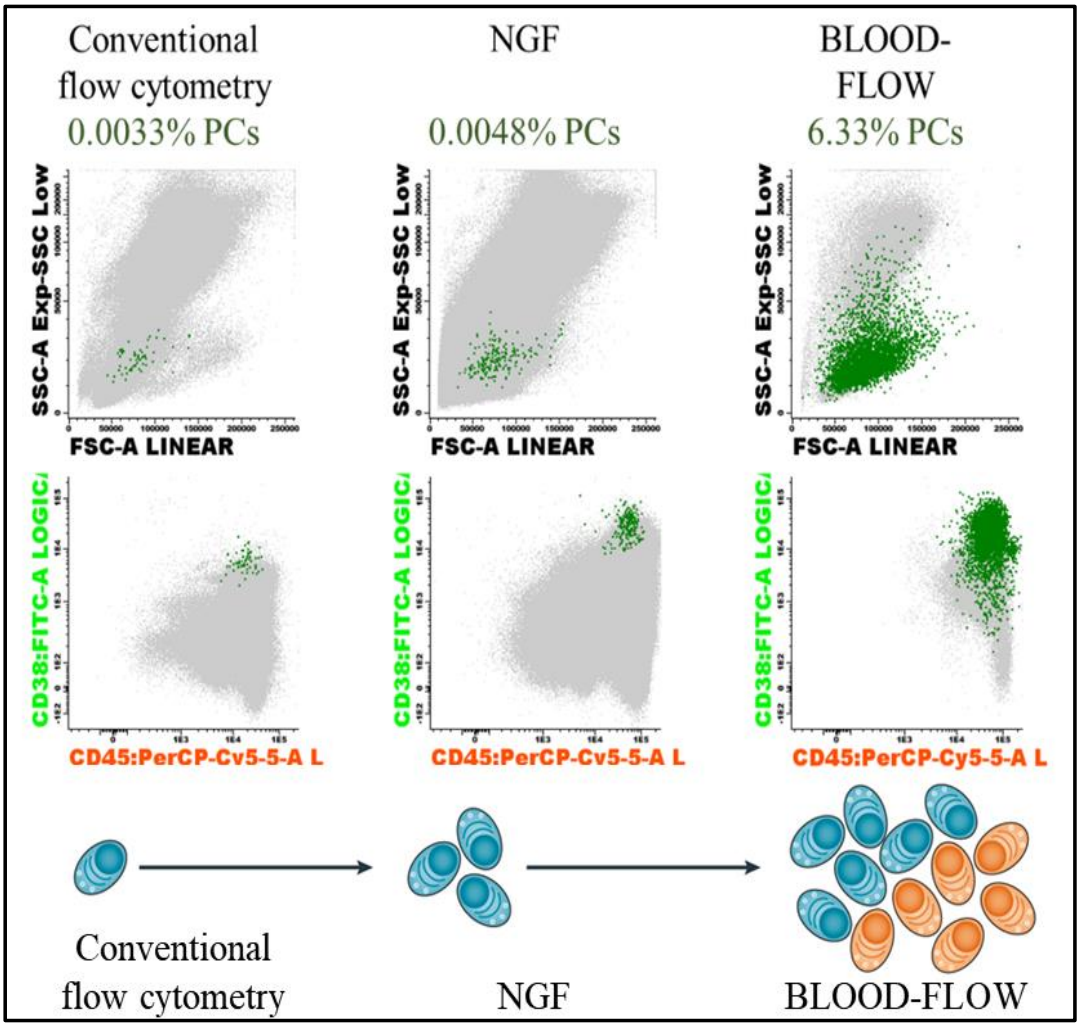


Figure 3: Enrichment of circulating normal and clonal PCs using BLOOD-FLOW.

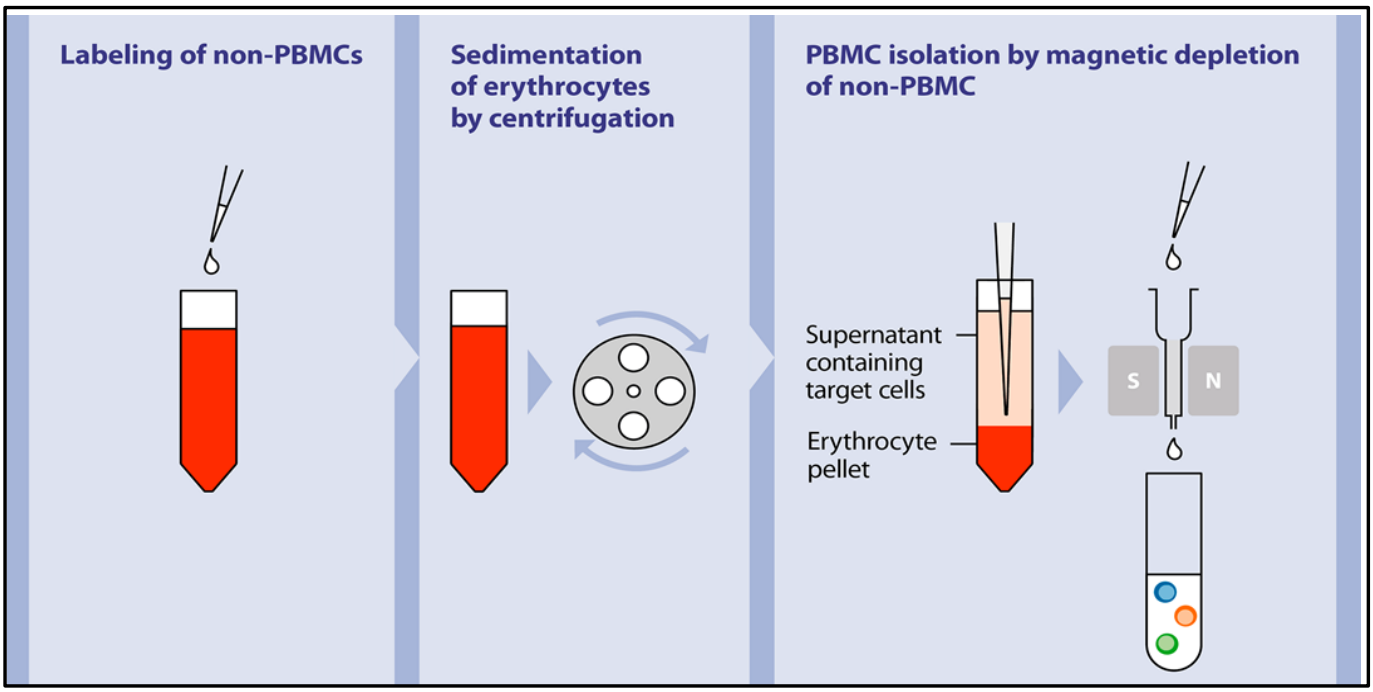


Figure 4: Example of one of the methods that was tested for optimal enrichment of PCs.

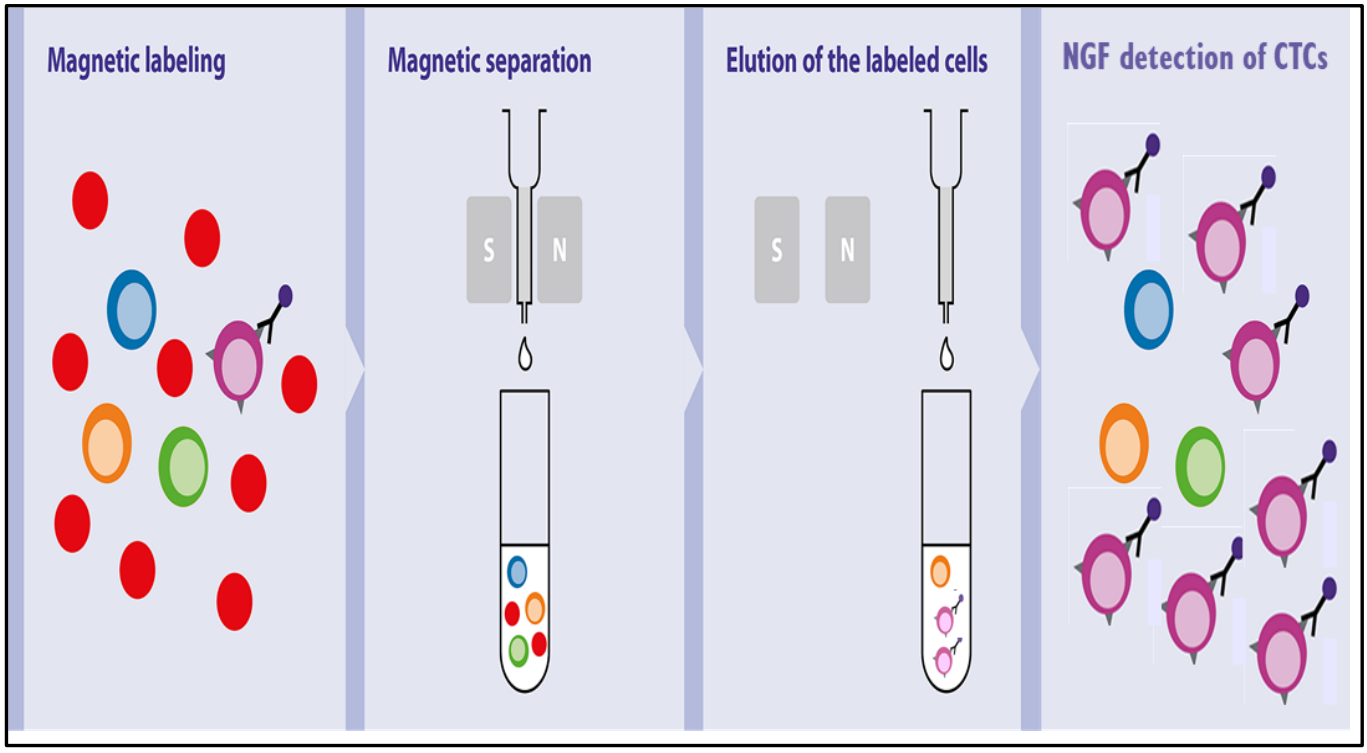


Figure 5: Schematic representation of BLOOD FLOW.

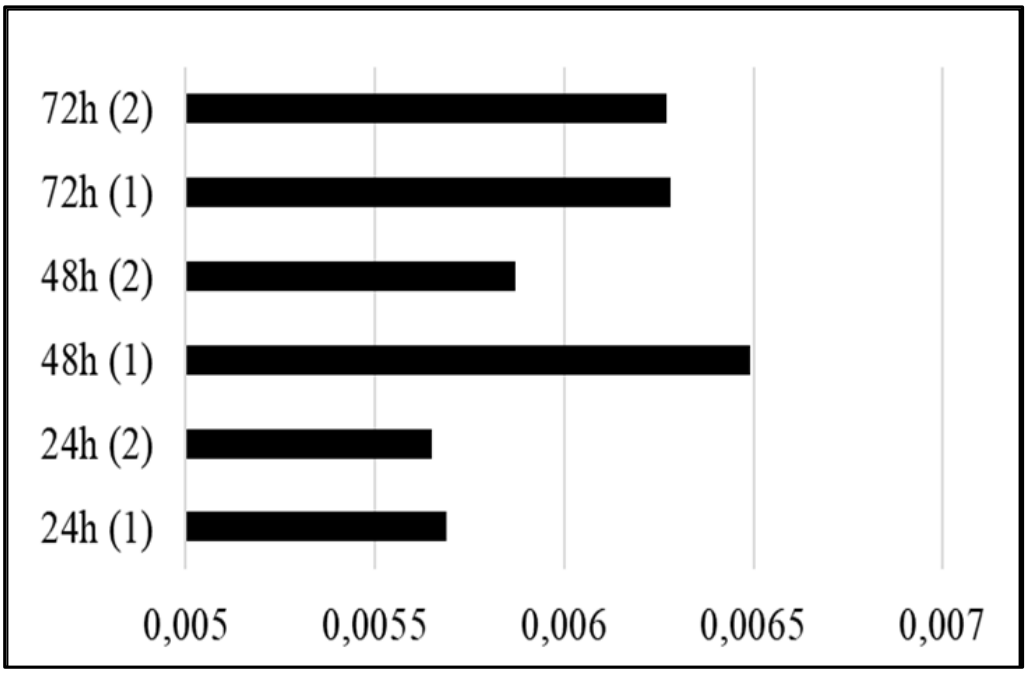


Figure 6: Percentage of CTCs every 24h. Mean results of duplicate measurements in 5 samples.

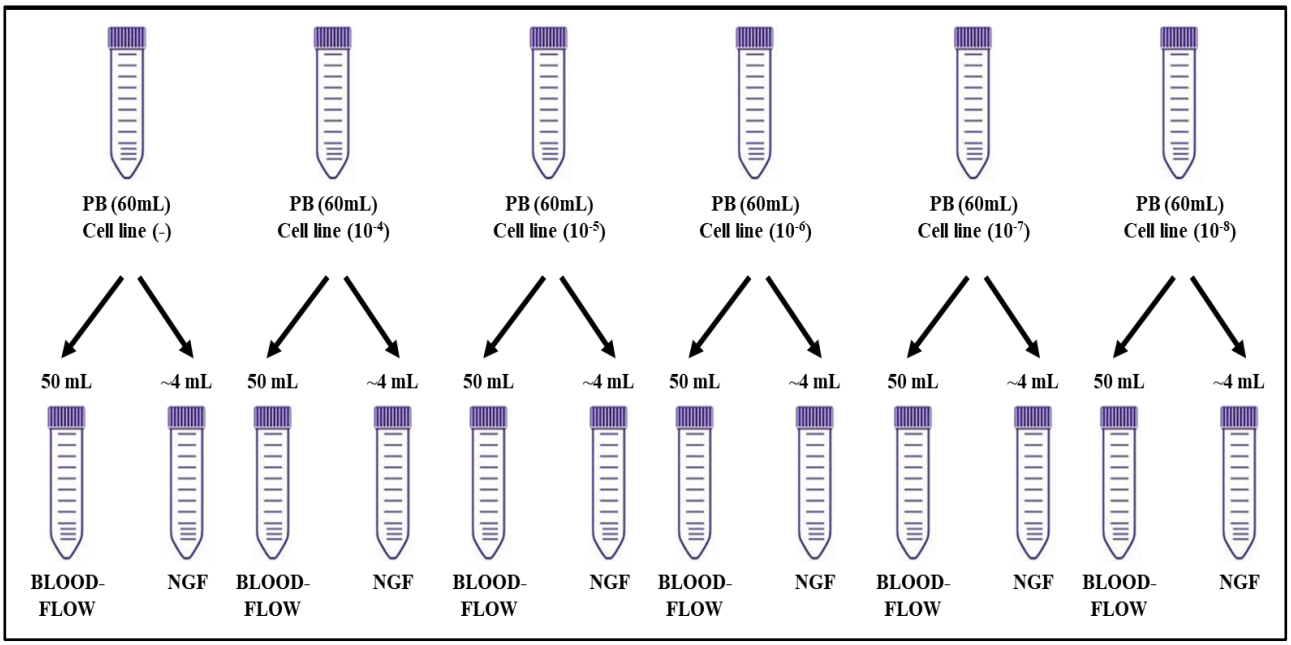
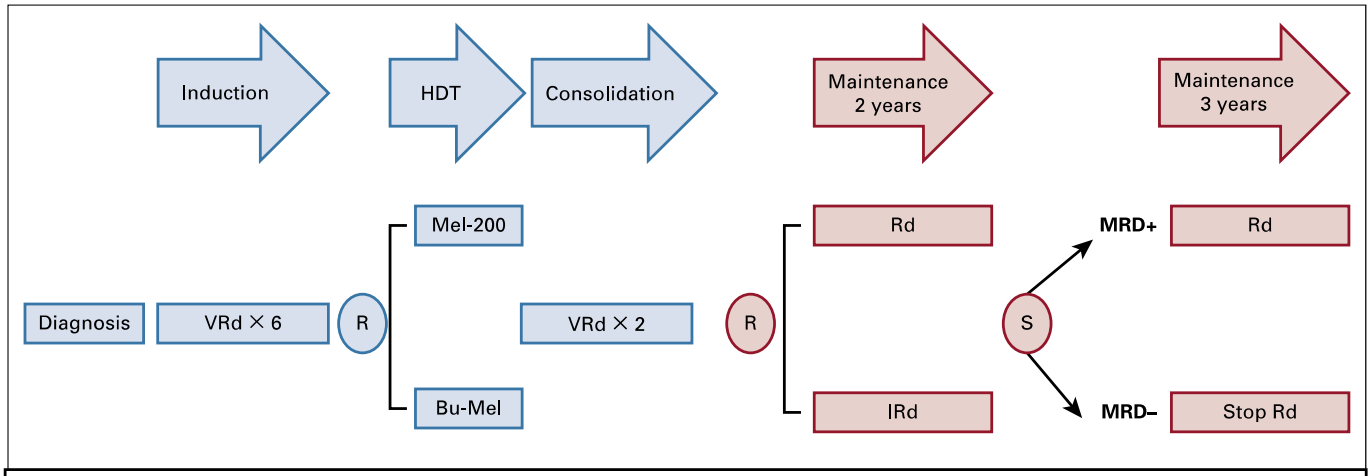


Figure 7: Schematic representation of serial diluition experiments to evaluate sensitivity and specificity of BLOOD-FLOW.



Bu-Mel, busulfan + melphalan; d, low dose dexamethasone; HDT, high-dose therapy; I, ixazomib; Mel, melphalan; R, lenalidomide; V, bortezomib

Figure 8: Design of the PETHEMA/GEM2014MAIN trial.

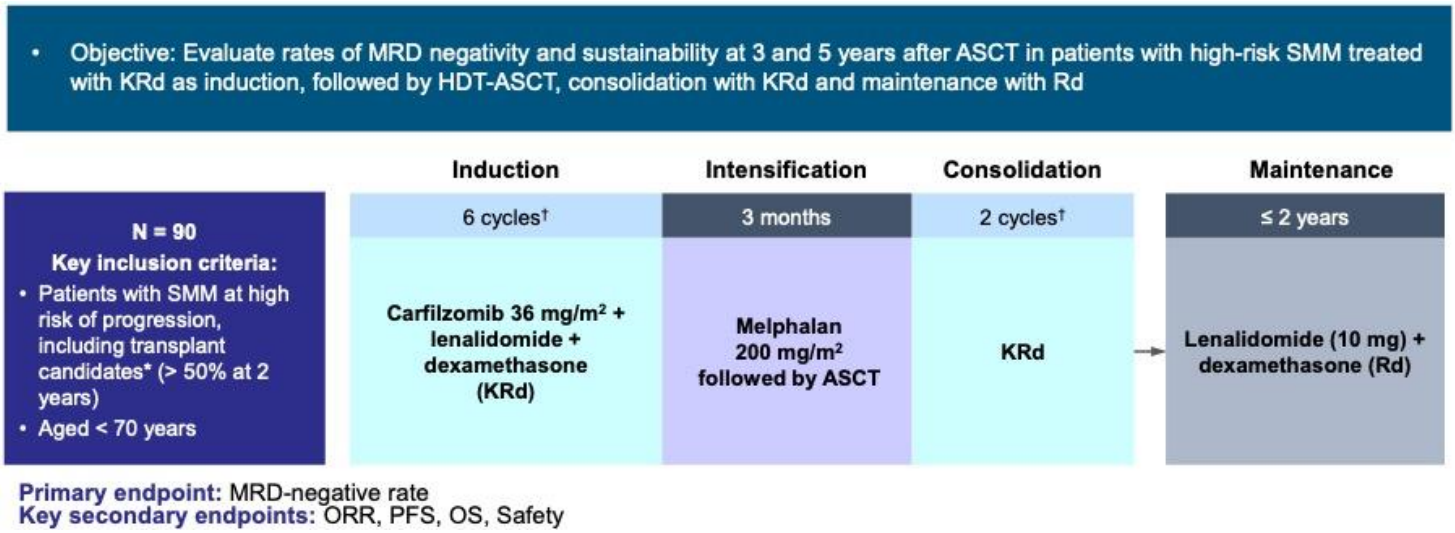


Figure 9: Design of the PETHEMA/GEMCESAR clinical trial.

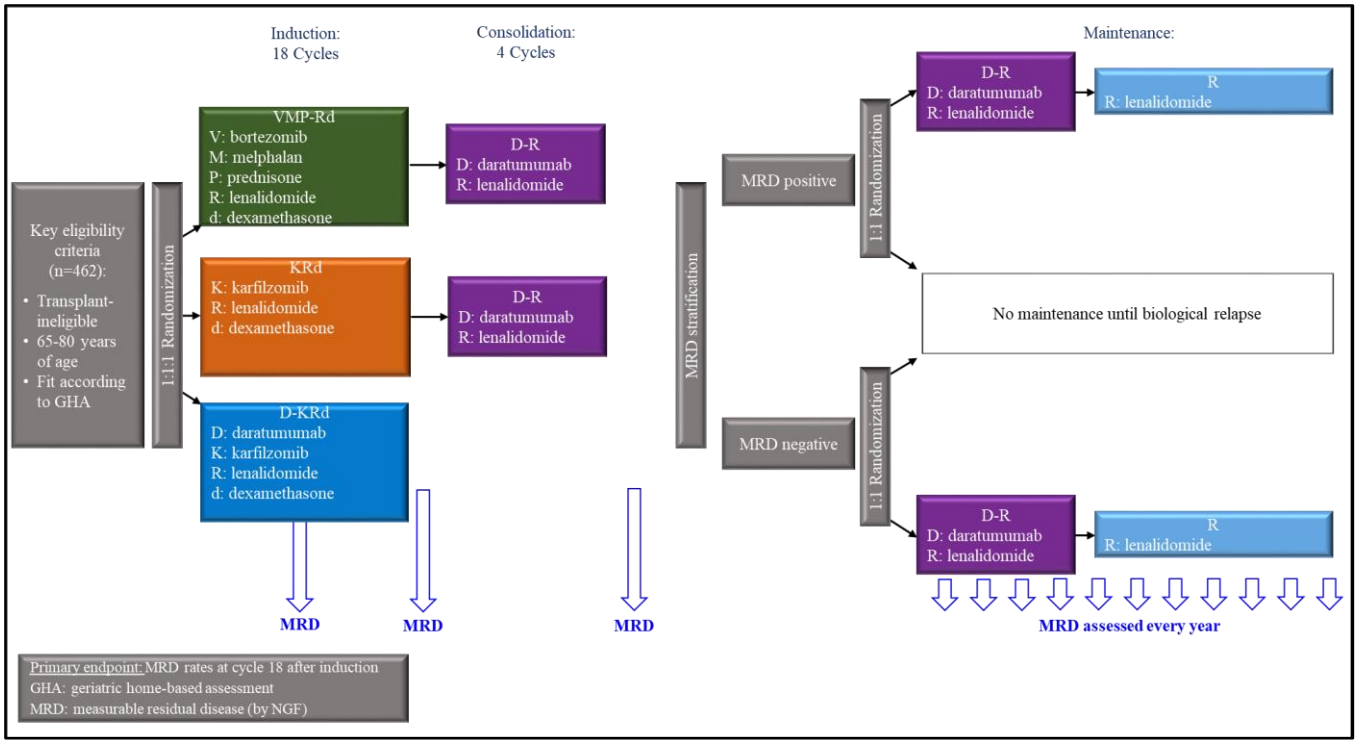


Figure 10: Design of the PETHEMA/GEM2017FIT clinical trial, including the identification of time points where BM and PB will be collected for MRD assessment.

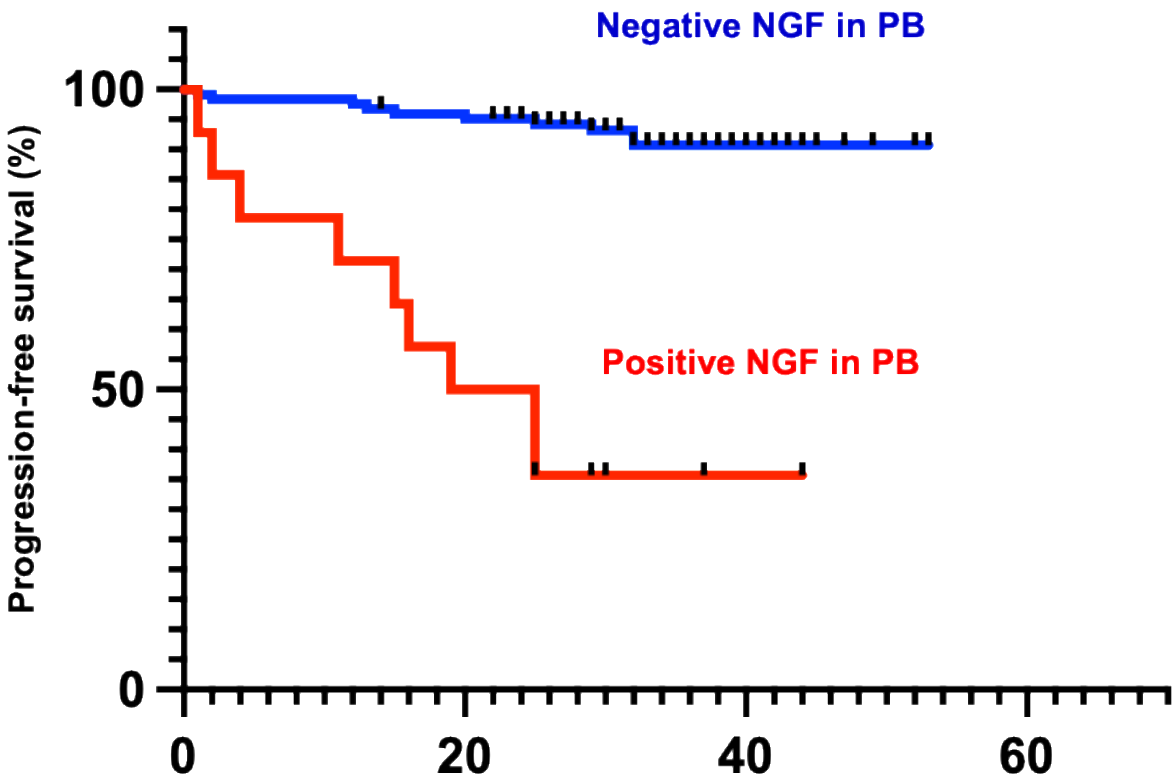


Figure 11: Kaplan–Meier curves comparing PFS of MRD-positive and MRD-negative subsets of NGF-based results in PB (p<0.0001).

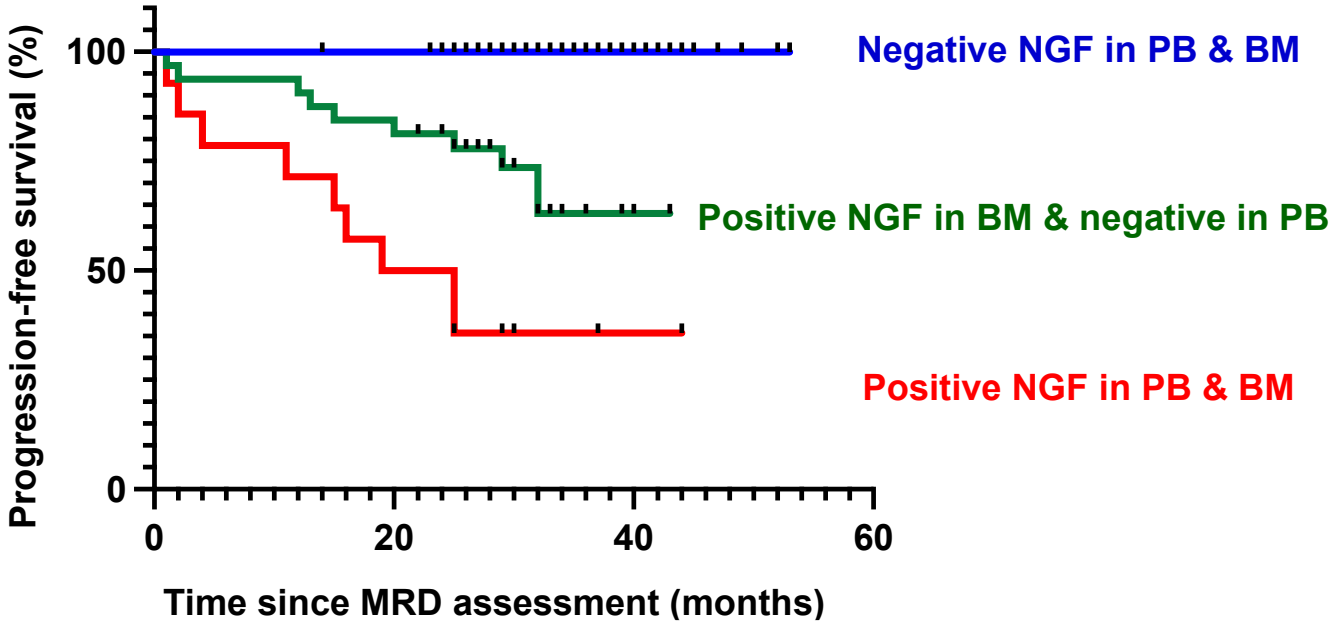


Figure 12: Kaplan–Meier curves comparing PFS of MRD-positive and MRD-negative subsets of NGF-based results in PB and BM (p<0.0001).

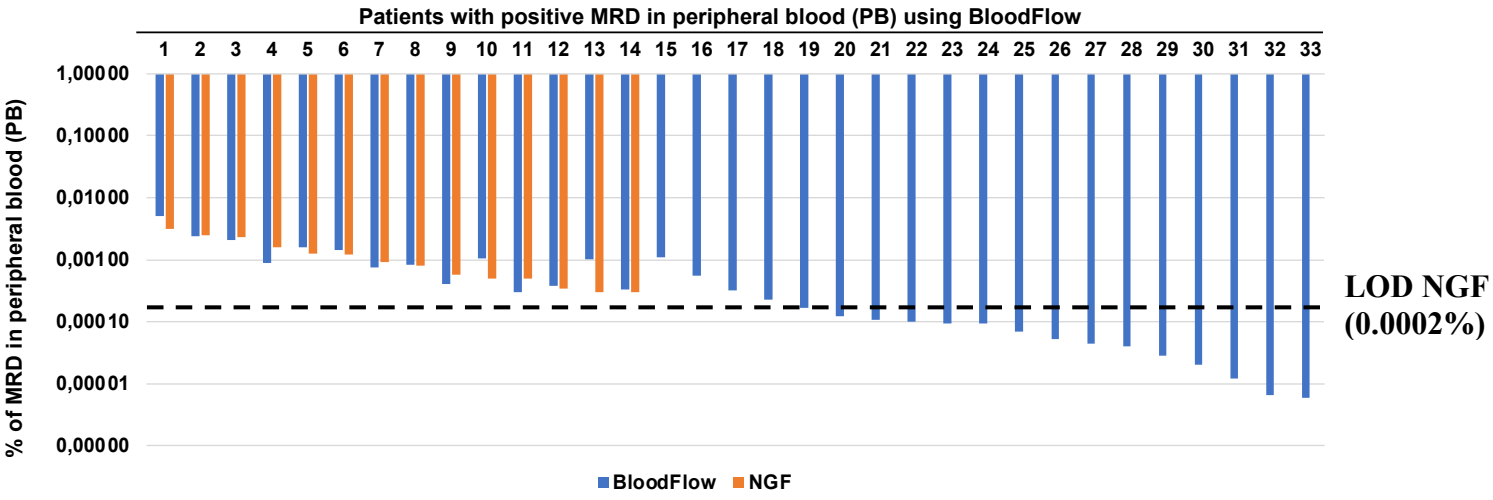


Figure 13: Comparison in positive MRD results with BloodFlow vs NGF in PB.

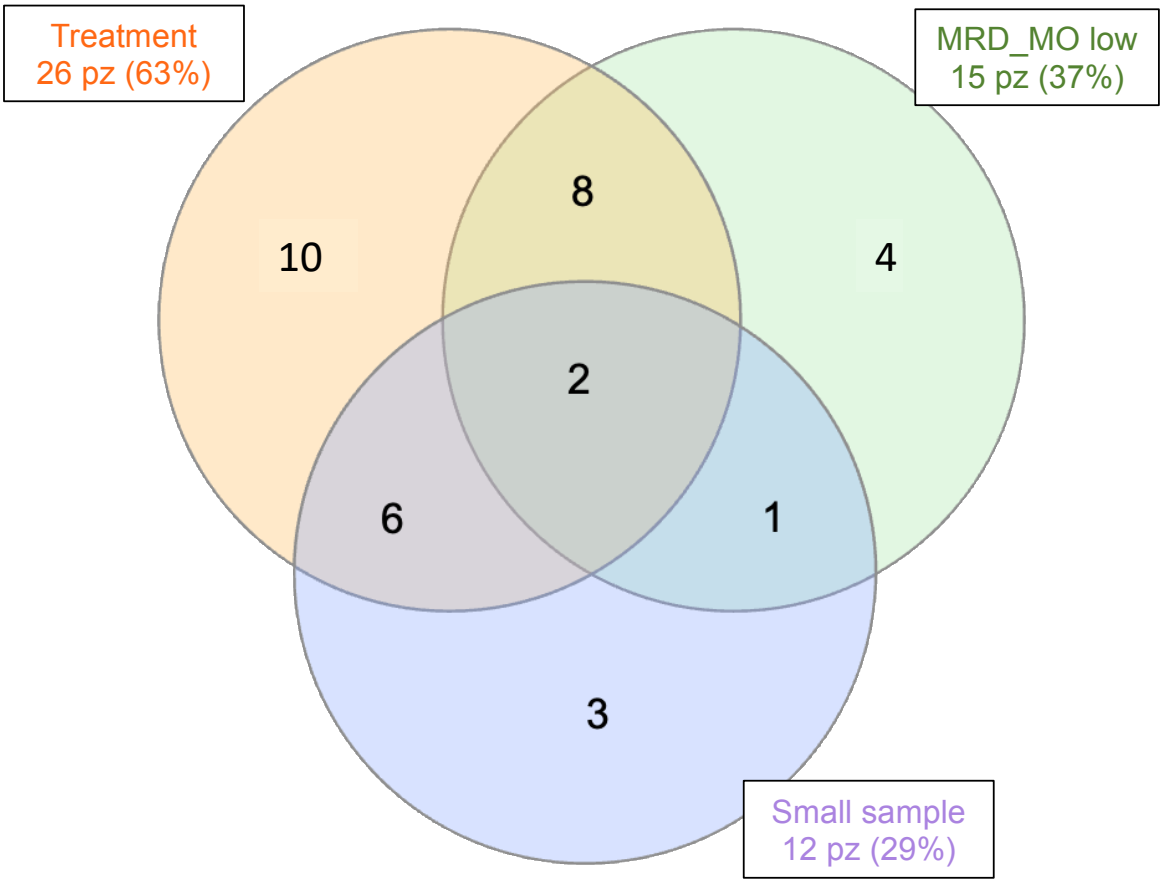


Figure 14: Comparison in MRD results with BloodFlow vs NGF in BM. Possible causes of false-negative results using BloodFlow.

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