# Minimal Residual Disease Assessment in Multiple Myeloma by Multiparametric Flow Cytometry

## Analýza minimální reziduální nemoci u mnohočetného myelomu pomocí multiparametrické průtokové cytometrie

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### Summary

***Background:*** Progress in treatment of multiple myeloma extensively increased patient remission rates, so minimal residual disease (MRD) detection becomes essential to assess the effectivity of treatment and depth of complete response. Nowadays, multiparametric flow cytometry (MFC) is the most used method for monitoring of MRD presence in the bone marrow of multiple myeloma patients; however, detection on molecular level can be used as well. It is evident that choice of protocol used for MFC-MRD assessment can significantly affect required results; nevertheless, standardized and highly sensitive approach of "next generation flow" is already available. Although benefit of MRD assessment as an independent predictor of progression-free survival and overall survival is known, very recent research showed that MRD-negative status surpasses the prognostic value of complete response achievement for progression-free survival and overall survival. ***Aim:*** This review is focused on use MFC in MRD assessment in multiple myeloma. The technical aspects and clinical benefits of this approach are mentioned as well. ***Conclusion:*** The information about MRD level detected by highly sensitive and reproducible MFC can be potentially used as a biomarker to evaluate the efficacy of different treatment strategies, help on treatment decisions and act as a surrogate for overall survival in multiple myeloma patients.

### Key words

multiple myeloma – minimal residual disease – flow cytometry – plasma cells

Supported by Ministry of Health of the Czech Republic, grant No. 17-30089A.

Podpořeno z programového projektu Ministerstva zdravotnictví ČR s reg. č. 17-30089A.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 25. 6. 2017
Accepted/Přijato: 29. 6. 2017

doi: 10.14735/amko20172S21

## Souhrn

***Východiska:*** Díky pokrokům v léčbě mnohočetného myelomu se značně zvýšil počet pacientů dosahujících remise onemocnění, a tedy detekce minimální reziduální choroby (minimal residual disease – MRD) se stala nepostradatelnou ke zhodnocení účinnosti léčby a k posouzení hloubky kompletní léčebné odpovědi. Multiparametrická průtoková cytometrie (multiparametric flow cytometry – MFC) je v současnosti nejpoužívanější metodou pro stanovení a monitorování přítomnosti MRD v kostní dřeni pacientů s mnohočetným myelomem, nicméně mohou být využity také metody na molekulární úrovni. Je zřejmé, že protokol použitý pro stanovení MFC-MRD může významně ovlivnit získané výsledky, přičemž standardizovaný a vysoce senzitivní přístup „next generation flow" je již dostupný. Přínos stanovení MRD pro predikci přežití bez progrese onemocnění a celkového přežití je znám, nicméně nedávné výzkumy ukazují, že MRD negativita dokonce překonává prognostickou hodnotu dosažení kompletní léčebné odpovědi pro přežití bez progrese a celkové přežití. ***Cíl:*** Souhrnný článek je zaměřen na využití MFC v analýze MRD u mnohočetného myelomu. Zmíněny jsou také technické aspekty a zhodnocení klinického přínosu tohoto stanovení. ***Závěr:*** Informace o hladině MRD stanovené pomocí vysoce senzitivní a reprodukovatelné MFC může být využita jako biomarker k hodnocení účinnosti rozdílných léčebných přístupů, k rozhodování o vhodnosti léčby, ale také může sloužit jako parametr nahrazující celkové přežití u pacientů s mnohočetným myelomem.



## Klíčová slova

mnohočetný myelom – minimální reziduální nemoc – průtoková cytometrie – plazmatické buňky


## Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by presence of clonal bone marrow (BM) plasma cells (PCs). Development of new therapies led to the significantly prolonged overall survival (OS) in newly diagnosed patients [1]. Effective treatment comes along with the need for more sensitive approaches to compare the efficacy of different treatment strategies and implementation of individualized therapy monitoring strategies to prevent both under- and overtreatment [2]. New therapies are being developed but the transition to mainstream availability is much slower as randomized phase III clinical trials take years to show a benefit when using progression-free survival (PFS) and OS as study endpoints [3]. Extensive data indicate that minimal residual disease (MRD) information can potentially be used as a biomarker to evaluate the efficacy of different treatment strategies, help make treatment decisions, and act as a surrogate for OS [2–4]. Confirmation of the elimination of myeloma residual cell clones resistant to the therapy should be the way to cure MM.

Available techniques on cellular (multiparametric flow cytometry – MFC; next generation flow – NGF) and/or molecular (quantitative polymerase chain reaction – qPCR; next generation sequencing – NGS) level including imaging methods showed that persistent MRD means worse survival in MM [2,5–7]. MFC seems to be the most effective of existing approaches. Development of protocols for MFC-MRD followed technical progress of cytometry itself, availability of new antigens and fluorochromes together with standardization requirements [2,8]. A novel validated NGF assay for highly sensitive, fast and standardized quantification of MRD in MM that overcomes previous limitations of conventional MFC-MRD approaches and improves prediction of patient outcome, is ready-to-use and well suited for implementation in clinical trials to establish the diagnostic role of MRD in MM [7].

## Needs for MRD detection

The indicator of treatment effectivity is the number of residual clonal cells. Assessment of MRD is becoming a standard diagnostic care for potentially curable neoplasms, such as acute lymphoblastic leukemia. In MM, the majority of patients will inevitably relapse despite achievement of progressively higher complete response (CR) rates, but new treatment approaches might further increase remission rates and potentially cure rates [2]. Interestingly, few of the patients that reach suboptimal response (near CR and/or very good partial response) are relapse-free at 10 years [9]. In 2008, Paiva et al. already demonstrated the clinical importance of MRD evaluation by MFC and illustrated the need for further refinement of MM response criteria [10]. Analysis of contribution of the serum free light chain ratio (sFLCr) or BM clonality to the prognosis of MM revealed that the sFLCr does not identify patients in CR at distinct risk; by contrast, flow cytometry revealed patients with a significantly inferior outcome. Thus, achieving CR does not mean achieving cure, and new definition of CR is needed even as stringent CR (sCR) is insufficiently informative in terms of expected PFS and/or OS [6]. However, the definition of clinical response criteria and clinical end points largely remained the same over the past 15 years. It was proven that MRD detection is a sensitive and fast approach and an independent predictor of PFS and OS [11,12]. Even more, as was recently demonstrated, MRD-negative status surpasses the prognostic value of CR achievement for PFS and OS across the disease spectrum, regardless of the type of treatment or patient risk group. Thus, MRD negativity should be considered as one of the most relevant end points for transplant-eligible and elderly fit patients with MM [13].

## Analysis of PCs and their phenotype profile by MFC

MFC has been used in MM analyses for a long time. Mostly DNA analysis with cytoplasmic immunoglobulin detection was done [14–15]. Discovery of new monoclonal antibodies (MoAb) against PC antigens helped in the development of immunophenotyping of monoclonal gammopathies (MGs) [16–17]. Combination of surface CD138, CD38 and CD45 allowed identification of whole PC population in BM. The most useful antigens for basic orientation in context of PC normality are CD19 and CD56 which relatively easily discriminate immuno-

**Tab. 1. List of the most useful antigens allowing normal and abnormal PC discrimination [21,22].**

| Antigen | Normal expression | Abnormal expression | Patient's expression | Diagnostics/ /monitoring |
|---|---|---|---|---|
| CD19 | positive (> 70%) | negative | 95% | essential |
| CD56 | negative (< 15%) | strongly positive | 75% | essential |
| CD20 | negative (0%) | positive | 15%* | recommended |
| CD28 | negative* (< 15%) | strongly positive | 15–45% | recommended |
| CD27 | strongly positive (100%) | weak/negative | 30–45% | recommended |
| CD81 | positive (100%) | weak/negative | 20–50%* | recommended |
| CD117 | negative (0%) | positive | 30% | recommended |
| CD200 | negative (0%) | positive | 75%* | useful |

*own results

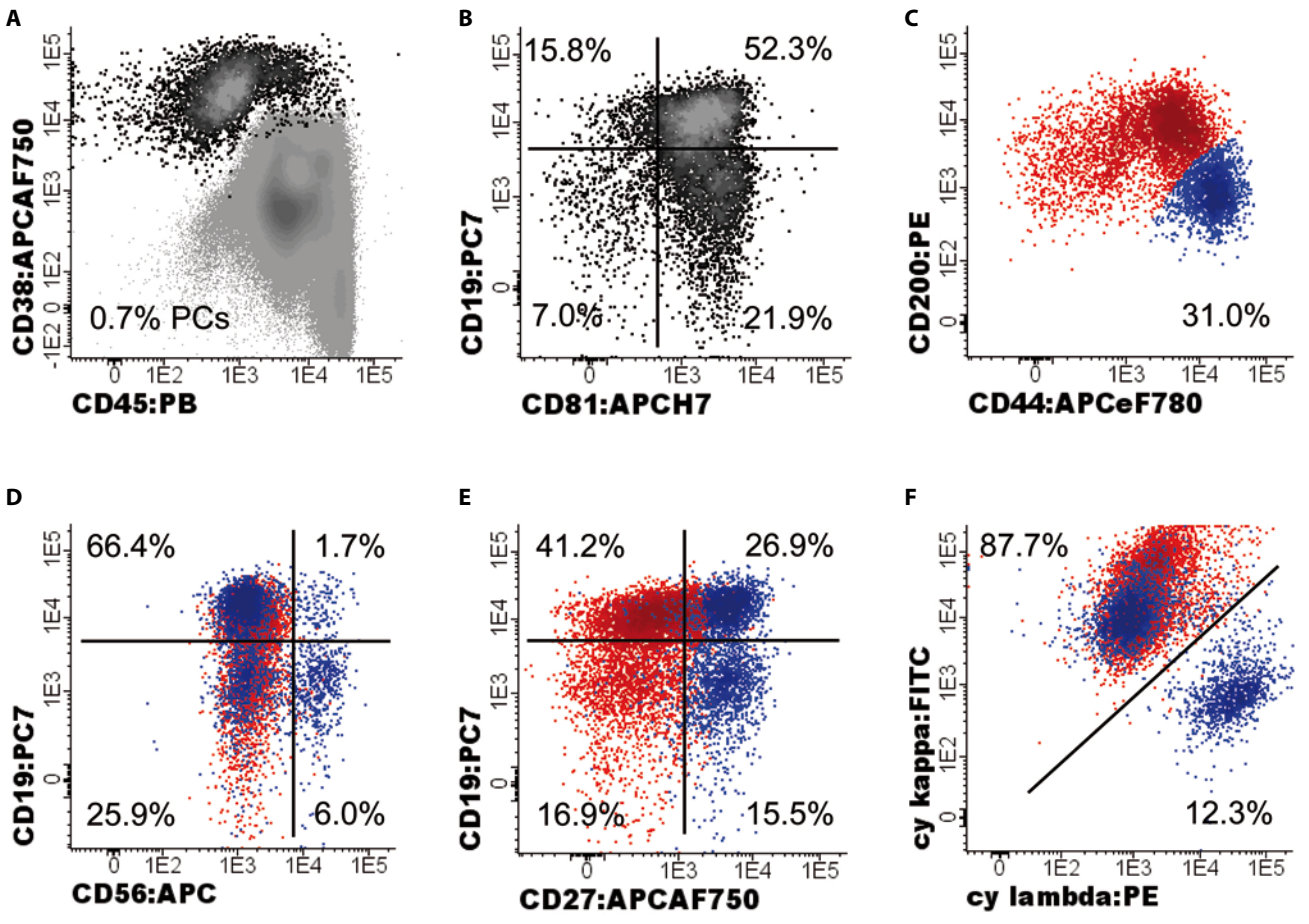


**Fig. 1. Polychromatic analysis of PCs in newly diagnosed MM.**

Clonal CD45$^-$ PCs (A) with atypical phenotype profile CD19$^+$CD81$^{dim+}$ (B) are visualised. Using other surface markers and clonality assessment revealed CD56$^-$CD27$^-$CD200$^+$cykappa$^+$ clonal PCs (red dots; C–F) with slightly lower expression of CD44 (C). Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos).

phenotypically normal (N-PC; CD19$^+$ CD56$^-$) from aberrant/pathological (A-PC; CD19$^-$CD56$^{+/-}$) PCs [18–19]. Combination with other surface Ag further identifies phenotypic profile of pathological PCs (Tab. 1) [20–21]. Detection of immunoglobulin light chains kappa and lambda cytoplasmic expression is used for confirmation of PC clonality.

Thus, polychromatic MFC (minimum of six markers, but usually eight markers) is required for sufficient PC analysis and combination of surface and intracellular antigens is necessary for identification and clonality assessment of pathological myelomatous PCs (Fig. 1) [22–24]. Peripheral blood circulating clonal PCs are not detectable by routine analysis in all MM in time of diagnosis or are present in very low number, so their further monitoring during treatment needs a highly sensitive approach [25].

## Flow cytometric assessment of MRD

Although flow cytometry is not a diagnostic tool in MG analyses (morphology is widely available and plays an irreplaceable role; on the other hand, the underestimation of PC number by flow cytometry is known), it provides important information about the presence and number of especially clonal PCs. MFC is generally applicable to majority of MM patients with sensitivity ranging from $10^{-4}$ to $10^{-6}$. Progress in MFC technology and wide availability of used antibodies allows MFC to be an integral part of laboratory analyses and management of plasma-cell disorders (PCD). It can play an important part in diagnosis, prognostic stratification and monitoring of response to therapy via MRD detection, understanding of biology of disease progression, study of the role of the tumor microenvironment in PCD and identification of potential therapeutic targets on malignant PCs [2,26–28]. Valid and/or even better standardized MRD detection will ensure superior uniform assessment of response and clinical prognostication.

### MFC in MRD definition

The use of MFC in the detection of MRD in BM has been demonstrated by several studies since 2002. The sensitivity of the flow cytometry assay was highlighted by the presence of detectable PCs in nearly a third of the patients with negative immunofixation ($IFx^-$) results and patients who were MRD-positive ($MRD^+$) had a worse outcome [29–30]. Flow cytometry was mentioned in sCR definition for the first time, where absence of BM clonal cells by immunohistochemistry or immunofluorescence was required [31]. Presence/absence of clonal cells was based on the κ/λ ratio which required a minimum of 100 PCs for analysis, an abnormal ratio reflecting presence of an abnormal clone was κ/λ of > 4 : 1 or < 1 : 2 [31]. Then, the term MFC remission was used and patients were considered to be in MFC remission when MM-PCs were undetectable in the BM sample at the MFC sensitivity limit of $10^{-4}$ (i.e., 1 MM-PC in $10^4$ N-PCs). Only 4-colour MFC was used and a minimum of $3 \times 10^5$ BM cells was acquired [10]. Later, the updated International Myeloma Working Group (IMWG) response criteria in 2011 incorporated some new designations to traditional CR definitions and immunophenotypic CR (iCR, $sCR^+$) was defined as mentioned in previous sentence [10,32]. Recently published IMWG MRD criteria defines flow MRD-negative ($MRD^-$) sample as an absence of phenotypically aberrant clonal PCs by NGF on BM aspirates using the EuroFlow standard operation procedure for MRD detection in MM (or validated equivalent method) with a minimum sensitivity of 1 in $10^5$ nucleated cells or higher [28].

### Highly sensitive MFC and standardization

MFC seems to be the most perspective approach for detection of MRD in MM in terms of speed, price and availability of method. Applicability of MFC is over 90% of MM patients regardless of the knowledge of the phenotype at the time of diagnosis. The initial lower sensitivity of the method (when 4-colour flow cytometry was used) was increased by simultaneous detection of eight markers/fluorochromes and acquisition of a sufficient number of leukocytes in one run up to $10^{-7}$, thus MFC is comparable with molecular techniques based on molecular level [7]. The technique has been modified to include an initial bulk lysis step to measure consistently more than $5 \times 10^6$ leukocytes per tube. Transition to quantitative assessment of residual clonal PCs will improve predictive potential of analyses as higher logarithms of clonal PC depletion significantly improved length of OS [12]. However, harmonization and/or standardization of MFC in MM are still relatively open as majority of clinical labs use their own protocols, which are comparable and sufficiently sensitive [4].

Project EuroFlow offers a standardized process of sample preparation and data acquisition when defined validated panels of selected and verified markers are used for every type of hematological malignancy. In addition, innovative software Infinicyt for data analysis is used [33]. Original EuroFlow panel designed for PCD was not sufficient for MRD detection, so 2nd generation of panel was developed (Tab. 2). This panel can identify clonal PCs on a background of normal regenerating BM (Fig. 2) [8]. The novel NGF-MRD approach takes advantage of innovative tools and procedures recently developed by the EuroFlow Consortium for sample preparation, antibody panel construction (including choice of type of antibody and fluorochrome), and automatic identification of PCs against reference databases of normal and patient BM. An optimized 2-tube 8-color antibody panel was constructed in five cycles of design-evaluation-redesign. In addition, a bulk-lysis procedure was established for acquisition of ≥ $10^7$ cells/sample. Prospective validation of the whole procedure at two distinct centers confirmed its robustness and significantly greater sensitivity vs. conventional 8-color MFC-MRD approaches, comparable to current NGS methods, with an improved prediction of patient outcome [7]. On the other hand, semi-standardized approach is available from Beckman Coulter Company as premixed dry tubes combining eight surface markers (CD38/CD45/CD81/CD27/CD19/CD200/CD138/CD56) for effective detection of clonal PCs without clonality assessment.

### Preanalytical rules

It is very important to obtain BM sample not diluted by peripheral blood, where marrow elements must be present, to receive high quality results. There is a preference of EDTA anticoagulants as heparin decreases CD138 intensity and PCs should be less recognizable in context of

**Tab. 2. Development of EuroFlow PCD panel [7,34].**

| 1st PCD generation | PB | PO | FITC | PE | PerCP-Cy5.5 | PC7 | APC | APC-H7 |
|---|---|---|---|---|---|---|---|---|
| PCD1 | CD45 | CD138 | CD38 | CD28 | CD27 | CD19 | CD117 | CD81 |
| PCD2 | CD45 | CD138 | CD38 | CD56 | $\beta^2$m | CD19 | cy Igκ | cy Igλ |
| **2nd PCD generation** | **BV421** | **BV510** | **FITC** | **PE** | **PerCP-Cy5.5** | **PC7** | **APC** | **APC-C750** |
| PCD1 | CD138 | CD27 | CD38 | CD56 | CD45 | CD19 | CD117 | CD81 |
| PCD2 | CD138 | CD27 | CD38 | CD56 | CD45 | CD19 | cy Igκ | cy Igλ |

PB – Pacific Blue, PO – Pacific Orange, FITC – Fluoresceinisothiocyanate, PE – Phycoerythrin, PerCP-Cy5.5 – Perridin Chlorophyll--Cyanine 5.5, PC7 – Phycoerythrin-Cyanine 7, APC – Allophycocyanin, APC-H7 – Allophycocyanin-H7, BV421 – Brilliant Violet 421, BV510 – Brilliant Violet 510, APC-C750 – Allophycocyanin-C750

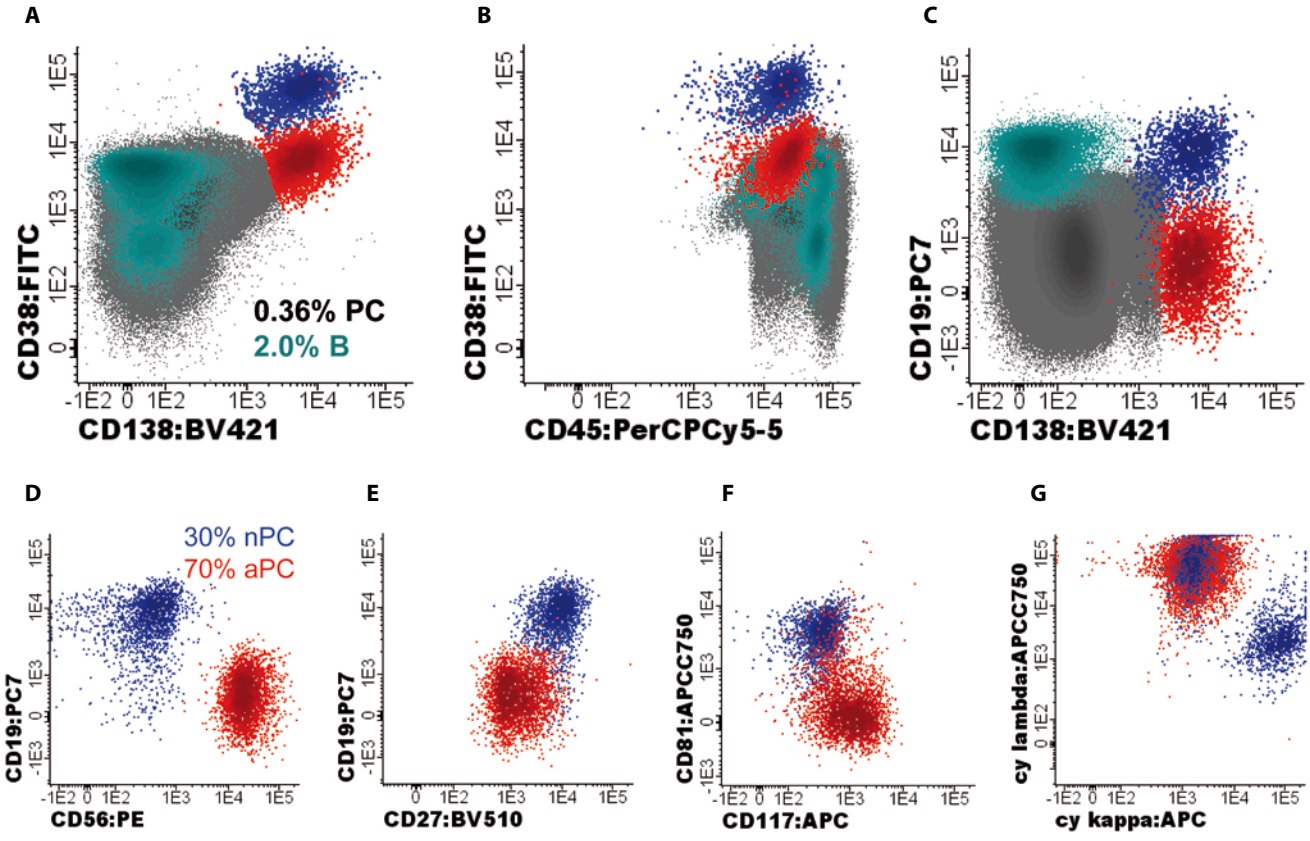


**Fig. 2. Standardized analysis by 2nd generation of EuroFlow PCD protocol.**

Polyclonal (nPCs, blue dots) and clonal (aPCs, red dots) PCs together with total B-cells (turquoise dots) are visualised in context of whole BM leukocytes (A–C). Typical phenotype of aPCs $CD19^{-}CD56^{+}CD27^{-}CD81^{-}CD117^{het+}$ with cytoplasmic lambda expression was detected (D–G). Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos).

whole leukocytes. When transportation of sample is needed, only room temperature must be used. Analysis must be done within 36 hours from sample acquisition [4,34].

## Trouble shooting

As a result of reduced number of PC after treatment, the procedure for cell concentration must be used (bulk lysis), to allow MRD assessment without loss of cell subpopulation, and to acquire at least limit of detection (LOD) and better limit of quantification (LOQ) of method [35]. Using targeted therapies as anti-CD38 (daratumumab) may complicate analy-

sis by homemade protocols, but replacement of CD38 by CD229 and/or using multiepitope CD38 resolve that problem [36]. Therapy-induced clonal selection could be already present at the MRD stage, where chemoresistant PCs show a singular phenotypic signature that may result from the persistence of clones with different genetic and gene expression profiles [37]. Although characteristic phenotypic profile of clonal PCs is already known, their detection should be impossible in a small subgroup of patients with atypical and/or changed profile (diagnostic vs. MRD antigenic profile), where different spectrum of antibodies should be used. Analysis of list-modes without SW assistance requires a well-educated operator and it is relatively time consuming.

### Results report

Reporting of results to the clinicians should be descriptive and clear including percentage of PCs from leukocytes, percentage of clonal PCs from whole PC population, phenotype of clonal cells and sensitivity of analysis. Unsuitable and/or not representative samples should be reported.

## Clinical relevancy of MFC-MRD assessment

Previous approaches to measurement of MRD levels were based on morphological assessment of BM, analysis of the paraprotein levels, or polymerase chain reaction (PCR) analysis of the immunoglobulin heavy chain variable-diversity-joining (VDJ) region. Historically, first results demonstrating the clinical importance of MFC in MRD detection were published in 2002, when Rawstron at al. showed that analysis of normal and neoplastic PC levels is more sensitive than IFx [29]. Patients with detected neoplastic PCs had a significantly shorter PFS than those with no detectable disease (median 20 vs. > 35 months; $p = 0.003$). Neoplastic PCs were detectable in 27% (9 of 33) of IFx$^-$ CR patients. These patients had a significantly shorter PFS than IFx$^-$ patients with no detectable neoplastic PCs ($p = 0.04$) [29]. Similarly, San Miguel at el. found that treatment-induced changes in the PC compartment correlated with disease outcome and patients in whom at least 30% of gated PCs had a normal phenotype after treatment had a significantly longer PFS (60 ± 6 vs. 34 ± 12 months; $p = 0.02$) [30]. Detailed analysis of newly diagnosed MM patients treated in Spanish GEM2000 protocol showed that PFS (median 71 vs. 37 months; $p < 0.001$) and OS (median not reached vs. 89 months; $p = 0.002$) were longer in patients who were MRD$^-$ vs. MRD$^+$ at day 100 after autologous stem cell transplantation (ASCT). Similar prognostic differentiation was seen in patients who achieved IFx$^-$ complete response after ASCT. Moreover, MRD$^-$IFx$^-$ patients and MRD$^-$IFx$^+$ patients had significantly longer PFS than MRD$^+$IFx$^-$ patients. Multivariate analysis identified MRD status by MFC at day 100 after ASCT as the most important independent prognostic factor for PFS (hazard ratio (HR) 3.64; $p = 0.002$) and OS (HR 2.02; $p = 0.02$) [10]. Results from Medical Research Council (MRC) Myeloma IX trial showed that absence of MRD at day 100 after ASCT was highly predictive of a favorable outcome (PFS, $p < 0.001$; OS, $p = 0.0183$). This outcome advantage was demonstrable in patients with favorable and adverse cytogenetics (PFS, $p = 0.014$ and $p < 0.001$, resp.) and in patients achieving IFx$^-$ CR (PFS, $p < 0.0068$). The effect of maintenance thalidomide was assessed, with the shortest PFS demonstrable in those MRD$^+$ patients who did not receive maintenance and longest in those who were MRD$^-$ and did receive thalidomide ($p < 0.001$). Further analysis demonstrated that 28% of MRD$^+$ patients who received maintenance thalidomide became MRD$^-$. MRD assessment after induction therapy in the non-intensive-pathway patients did not seem to be predictive of outcome (PFS, $p = 0.1$) [38]. In addition, it was demonstrated that prognostic impact of MRD following ASCT is independent of the induction therapy received [39,40]. Surprisingly, MFC-MRD monitoring has a prognostic value also in relapsed MM and is one of the most relevant prognostic factors in elderly MM patients, irrespectively of age or cytogenetic risk [11,40]. Using NGF in multicenter evaluation of 110 follow-up BM from MM patients in very good partial response (VGPR) or CR showed a higher sensitivity for NGF--MRD vs. conventional 8-color MFC-MRD with MRD$^+$ rate of 47 vs. 34% ($p = 0.003$). Thus, 25% of patients classified as MRD$^-$ by conventional 8-color MFC were MRD$^+$ by NGF, translating into a significantly longer PFS for MRD$^-$ vs. MRD$^+$ CR patients by NGF (75% PFS not reached vs. 7 months; $p = 0.02$) [7]. Very recent publication showed that achievement of CR in the absence of MRD negativity was not associated with prolonged PFS and OS compared with near-CR or partial response (median PFS 27, 27, and 29 months, resp.; median OS, 59, 64, and 65 months, resp.). MRD$^-$ status was strongly associated with prolonged PFS (median 63 months; $p < 0.001$) and OS (median not reached; $p < 0.001$) and in subgroups defined by prior transplantation, disease stage, and cytogenetics, with prognostic superiority of MRD negativity vs. CR particularly evident in patients with high-risk cytogenetics [13].

## Implementation of MFC-MRD assessment in the Czech Republic – a single center experience and cooperation

Our laboratory provides flow cytometry analyses of MGs for almost 15 years. MRD monitoring began at our department in 2006 when 4-colour flow cytometry (CD38/CD56/CD45/CD19) was used for detection of pathological PCs in MM patients after ASCT. Switch to 5-colours in 2007 (CD38/CD138/CD45/CD56/CD19) helped to better discriminate subpopulations of PCs. Although surface expression of CD19 and CD56 on CD38$^+$CD138$^+$ PC is able to discriminate N-PCs from A-PCs, in some cases more detailed analysis using other markers was necessary. Therefore, other markers, which are aberrantly expressed on PCs (CD28, CD117 and CD20), were analyzed, but these were present in only a limited cohort of MM patients. CD27 was used as well, as its higher expression is specific for N-PCs, while lower intensity and/or lack of its expression are typical for A-PCs. Unfortunately, there was still no clear evidence which subpopulation is really polyclonal and/or clonal in selected cases. Thanks to new labora-

tory equipment, we replaced "minimalistic" 4-color flow cytometry analysis with 8-color MFC including also cytoplasmic kappa and lambda immunoglobulin light chains expression (CD38/CD45/ /cyκ/cyλ/CD138/CD19/CD56/CD27) in March 2011. Together with a progressive increase in number of acquired events, we are now able to reach sensitivity up to $10^{-6}$.

As Czech central laboratory for EMN02 HOVON study, we have repeatedly analyzed 29 patients achieving CR by EuroFlow 1st generation protocol and MRD negativity was confirmed in 44.8% (13/29) of them. Unfortunately, only about $2 \times 10^6$ leukocytes are acquired for MRD assessment in that study, so sensitivity of analyses is only $10^{-5}$. Interestingly, when we compared our MFC-MRD results to other European laboratories in this study (the same BM sample was sent, prepared and analyzed by others), there was an absolute concordance [41].

Nowadays, we are testing EuroFlow 2nd generation protocol for research purposes and clinical studies as well [42].

We meet other university laboratories in the Czech Republic involved in clinical studies during annual meetings of the Czech myeloma Group (CMG) and Myeloma Workshops and discuss innovations and problems in MFC-MRD analyses. They usually use homemade methods, EuroFlow-like and/or standardized EuroFlow approach (1st generation) and commonly acquire about $2 \times 10^6$ leukocytes. When tested similarity of acquired data from one patient BM in two different laboratories using the same EuroFlow protocol of 1st generation but different flow cytometers (BD FACS Canto vs. Navios, both set up according to EuroFlow rules), these looked similar. So rather harmonized than standardized MFC-MRD is going to be provided by different centers in Czech Republic.

## Conclusion

As treatment strategies for MM become more effective and PFS becomes longer, assessing treatment efficacy according to MRD levels becomes increasingly important. Different approaches for MFC--MRD monitoring are available. Implementation of highly sensitive automated MFC-MRD assessment by NGF should confirm a new biomarker for treatment effectivity assessment and replace obsolete indicators defining clinical response and prediction of OS in MM. The prerequisite for that is standardization of sample processing, sample and data analysis and verification of this approach in clinical studies.

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