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Flow cytometric minimal residual disease assessment in multiple myeloma

Cytometryczna ocena minimalnej choroby resztkowej w szpiczaku plazmocytowym

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Abstract

Minimal residual disease (MRD) assessment in light of the effectiveness of new multiple myeloma (MM) treatment modalities and related to it increasing ratios of achieved complete remissions (CR), becomes an important tool in recognition of the depth of the response. Multiparametric flow cytometry (MFC) is currently the most popular method for monitoring of MRD presence in bone marrow of MM patients; however, molecular techniques may also be used for MRD assessment. The choice of protocol utilized for MFC-MRD measurement can significantly affect the obtained results, nevertheless standardized and highly sensitive approach of next generation flow (NGF) is already available. The depth of response based on MRD assessment was shown to be an independent predictor of progression-free survival (PFS) and overall survival (OS). Furthermore, the MRD-negative status surpasses the prognostic value of CR achievement for PFS and OS. Thus, MRD status detected by highly sensitive and reproducible MFC is potentially a clinically applicable biomarker for evaluation of different treatment strategies efficacy potentially influencing treatment decisions, and acting as prognostic factor in MM patients.

Key words: multiple myeloma, minimal residual disease, flow cytometry

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Streszczenie

W świetle wysokiej skuteczności nowych metod leczenia szpiczaka plazmocytowego oraz zwiększającego się odsetka osiąganych całkowitych remisji (CR) ocena minimalnej choroby resztkowej (MRD) jest obecnie istotną metodą określającą głębokość odpowiedzi. Wieloparametryczna cytometria przepływowa (MFC) jest obecnie najczęściej wykorzystywaną metodą monitorowania MRD w szpiku kostnym pacjentów ze szpiczakiem plazmocytowym, jednak w tym celu można również stosować metody molekularne. Rodzaj protokołu stosowanego przy badaniu MRD metodą MFC może istotnie wpływać na uzyskiwane wyniki, jednak obecnie jest już dostępny wystandaryzowany i wysoce czuły protokół cytometrii następnej generacji (NGF). Wykazano, że głębokość odpowiedzi oceniona na podstawie pomiaru MRD koreluje z przeżyciem wolnym od progresji (PFS) oraz przeżyciem całkowitym (OS) chorych na szpiczaka plazmocytowego. Ponadto ujemny wynik badania w kierunku MRD jest lepszym czynnikiem prognostycznym w odniesieniu do PFS oraz OS niż osiągnięcie CR. Z tych względów wynik oceny MRD, uzyskany wysoce czułą oraz powtarzalną metodą MFC, jest

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potencjalnie użytecznym klinicznie biomarkerem do oceny skuteczności różnych strategii leczniczych i może być podstawą do podejmowania decyzji terapeutycznej oraz użytecznym czynnikiem prognostycznym w szpiczaku plazmocytowym.

Słowa kluczowe: szpiczak plazmocytowy, minimalna choroba resztkowa, cytometria przepływowa

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Introduction

Multiple myeloma (MM) is a hematologic malignancy characteristic by presence of clonal bone marrow 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]. There are still developed new therapeutics, 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 biomarker to evaluate the efficacy of different treatment strategies, help on treatment decisions, and act as surrogate for overall survival [2–4]. Confirmation of the elimination of myeloma residual cell clones resistant to the therapy should be the way to cure MM.

There are available techniques on cellular (MFC, multiparametric flow cytometry; NGF, next generation flow) and/or molecular (qPCR, quantitative polymerase chain reaction; NGS, next generation sequencing) level including imaging methods which allowed to find that persistent MRD means worse survival in MM (Table 1) [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 high-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 standard diagnostic care for potentially curable neoplasms such as acute lymphoblastic leukaemia. 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 patients that reach suboptimal response (near CR and/or very good partial response) are relapse free at 10 years [9]. Paiva et al. [10] already in 2008 demonstrated the clinical importance of MRD evaluation by MFC and illustrated the need for further refinement of MM response criteria. Analysis of contribution of the serum free light chain ratio (sFLCr) or bone marrow (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. So the achieving of CR does not mean to achieve cure and new definition of CR is needed even as stringent CR (sCR) is not sufficiently informative in term of supposed 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 proved that MRD detection is sensitive and fast approach to acquire 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].

Flow cytometric assessment of MRD

Although flow cytometry is not a diagnostic tool in MM analyses (where morphology is widely available and still plays an irreplaceable role; on

Table 1. Characteristics of available techniques to monitor minimal residual disease (MRD) in multiple myeloma (source [2])

Tabela 1. Charakterystyka dostępnych technik monitorowania minimalnej choroby resztkowej (MRD) w szpiczaku plazmocytowym (źródło [2])

Applicability [%]	**~100**	**60–70**	**~90**	**~100**
Reproducibility	High	High	Not reported	Moderated
Availability	High	Intermediate	Limited	Intermediate
Diagnostic sample	Not mandatory	Mandatory	Mandatory	Not mandatory
Fresh sample	Needed (< 36 h)	Not needed	Not needed	NA
Time	2–3 h	≥ 5 days	≥ 7days	2 h
Cost per sample [EUR]	~300	~450	~600	~1800
Sensitivity	10^{-5}–10^{-6}	10^{-5}–10^{-6}	10^{-6}	High
Global cell characterisation	Yes	No	No	No
Standardization	Euroflow	EuroMRD	Not reported	No

NA — not available data

Table 2. List of the most useful antigens allowing normal and abnormal plasma cells (PC) discrimination (sources [14, 15])

Tabela 2. Lista najbardziej użytecznych antygenów do różnicowania prawidłowych i nieprawidłowych komórek plazmatycznych (PC) (źródła [14, 15])

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	30	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

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 PC. MFC is generally applicable to majority of myeloma patients with a sensitivity ranging from 10^{-4} to 10^{-6}. The progress in MFC technology and wide availability of used antibodies allows MFC to be an integral part of laboratory investigations and the management of plasma-cell disorders (PCD) and can play an important part in the diagnosis, prognostic stratification, and monitoring of response to therapy via minimal residual disease detection, the understanding of the biology of disease progression, the study of the role of the tumour microenvironment in PCD and the identification of potential therapeutic targets on the malignant PC (Table 2) [2, 16–18]. 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 was demonstrated by several studies from 2002. The sensitivity of the flow cytometry assay was highlighted by the presence of detectable PC in nearly a third of the patients with negative immunofixation (IFx–) results and patients who were MRD-positive (MRD+) had a worse outcome [19, 20]. Flow cytometry was mentioned for the first time in stringent CR (sCR) definition, where absence of BM clonal cells by immunohistochemistry or

immunofluorescence was required [21]. Presence/absence of clonal cells was based upon the κ/λ ratio which required a minimum of 100 PC for analysis, an abnormal ratio reflecting presence of an abnormal clone was κ/λ of > 4:1 or < 1:2 [21]. Then the term MFC remission was used and patients were considered to be in MFC remission when MM-PC were undetectable in the BM sample at the MFC sensitivity limit of 10^{-4} (ie, 1 MM-PC in 10^4 N-PC). Only 4-colour MFC was used and a minimum of $3 \times \times 10^5$ 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, 22]. Recently published IMWG MRD criteria defines flow MRD-negative (MRD–) sample as an absence of phenotypically aberrant clonal plasma cells 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 [18].

Highly sensitive MFC and standardisation

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 patient regardless of the knowledge of the phenotype in time of diagnosis. The initial lower sensitivity of the method (when used 4-colour flow cytometry) was increased by simultaneous detection of 8 markers/fluorochromes and acquisition of sufficient number of leukocytes in 1 run up to 10^{-7}, thus MFC is comparable with techniques based on molecular level [7]. The technique has been modified to include an initial bulk lysis step to consistently measure more than 5×10^6 leucocytes per tube. Transition to the quantitative assessment of residual clonal PC will improve predictive potential of analyses as higher logarithms of clonal PC depletion significantly improved the length of OS [12]. But harmonisation and/or standardisation of MFC in MM are still relatively open as majority of clinical labs use their own protocols which are not inter-laboratory comparable (Figure 1) and often not sufficient enough in a sensitivity manner [4].

Project EuroFlow offers standardized process of sample preparation and data acquisition when defined validated panels of selected and verified markers are used for every type of haematological malignancy. Also innovative software Infinicyt for data analysis is used [23]. Original EuroFlow panel designed for PCD was not sufficient for MRD detection so 2nd generation of panel was developed (Table 3). This can identify clonal PC on a background of normal regenerating BM (Figure 1) [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 PC 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 $\geq 10^7$ cells/sample. Prospective validation of the whole procedure at two distinct centers confirmed its robustness and significantly greater sensitivity vs. conventional 8-color MRD approaches, comparable to current NGS methods, with an improved prediction of patient outcome [7]. On the other hand, semi-standardised approach is available from Beckman Coulter Company as premixed dry tubes combining 8 surface markers (CD38/CD45/CD81/CD27/CD19/CD200/CD138/CD56) for effective detection of clonal PC without clonality assessment (Figure 2).

Preanalytical rules

It is very important to obtain BM sample not diluted by peripheral blood, where marrow elements must be present, for obtaining a high quality results. There is a preference of EDTA anticoagulants as Heparin decrease CD138 intensity and PC should be less recognisable in context of whole leukocytes. When transportation of sample is needed, only the room temperature must be used. Analysis must be done until 36 hours from sample acquisition [4, 24].

Trouble shooting

Because 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 [25]. Using of targeted therapies as anti-CD38 (daratumumab) may complicate analysis by home-made protocols, but replacement of CD38 by CD229 and/or using multiepitope CD38 resolve that problem [26]. The therapy-induced clonal selection could be already present at the MRD stage, where chemoresistant PC show a singular phenotypic signature that may result from the persistence of clones with different genetic and gene expression profiles [27]. Although characteristic phenotype profile of clonal PC is

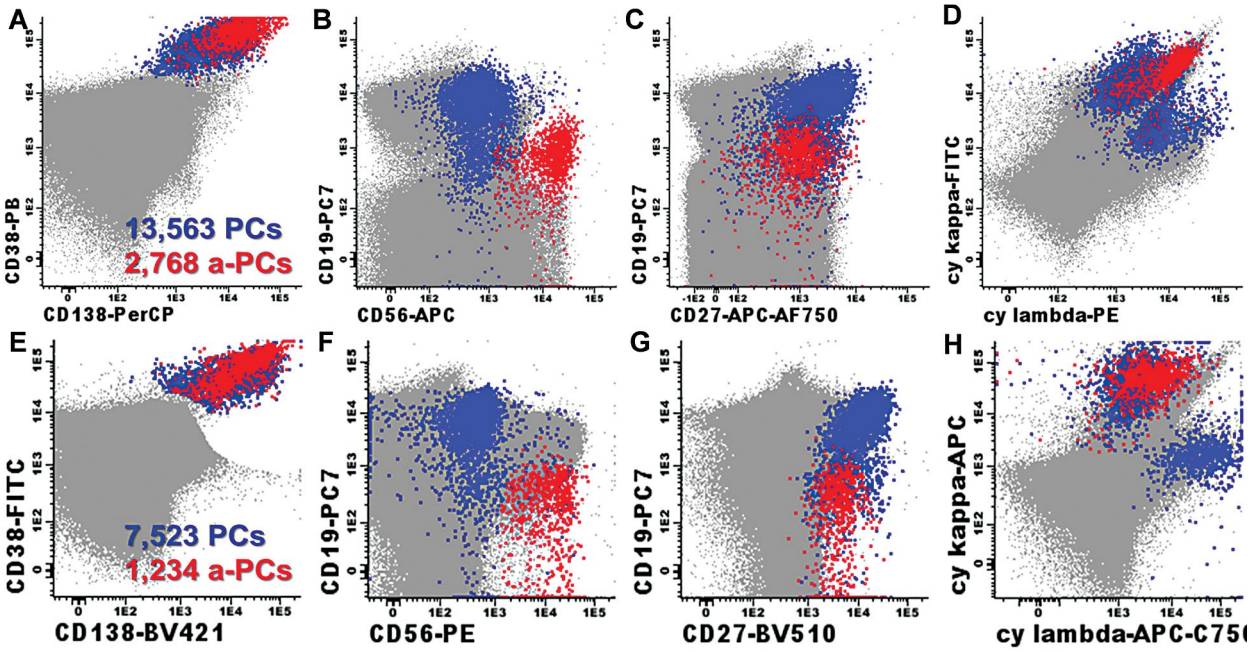

Figure 1A–H. Different approaches of multiparametric flow cytometry of minimal residual disease (MFC-MRD) analysis. Comparison of "home-made" (**A–D**) and 2nd generation of Euroflow PCD (**E–H**) protocol was done. Almost similar position of polyclonal (n-PCs, blue dots) and clonal (a-PCs, red dots) plasma cells (PCs) are visualised. Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos)

Rycina 1A–H. Odmienne podejścia do analizy minimalnej choroby resztkowej (MRD) metodą wieloparametrycznej cytometrii przepływowej (MFC). Przeprowadzono porównanie analizy za pomocą własnego protokołu typu *home-made* (**A–D**) i protokołu *Euroflow PCD* drugiej generacji (**E–H**). Zwraca uwagę podobne położenie poliklonalnych (n-PCs, niebieskie punkty) i klonalnych (a-PC, czerwone punkty) plazmocytów (PC). Akwizycję danych przeprowadzono na cytometrze przepływowym *BD FACSCanto II* z oprogramowaniem *Diva SW* (*BD Bioscience*); ponownej analizy danych dokonano przy użyciu oprogramowania *Infinicyt SW* (*Cytognos*)

already known (Table 2), their detection should be impossible in small part of patients with atypical and/or changed profile (diagnostic vs. MRD antigenic profile), where different spectrum of antibodies should be used. Analysis of listmodes without SW assistance requires well educated operator and it is relatively time consuming.

Results report

The reporting of results to the clinicians should be descriptive and clear including % of plasma cells from leukocytes, % of clonal PC 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 demonstrated the clinical importance of MFC in MRD detection, whichwere published in 2002, when Rawstron at al. shown that analysis of normal and neoplastic PC levels is more sensitive than IFx [19]. Patients with detected neoplastic PC had a significantly shorter PFS than those with no detectable disease (median 20 months vs. > 35 months, $p = 0.003$). Neoplastic PC 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 PC ($p = 0.04$) [19]. Similarly San Miguel et al. [20] found out that treatment-induced changes in the PC compartment correlated with disease outcome and patients in whom at least 30% of gated PC had a normal phenotype after treatment with a significantly longer PFS (60 ± 6 vs. 34 ± 12 months, $p = 0.02$). Detailed analysis of newly diagnosed MM

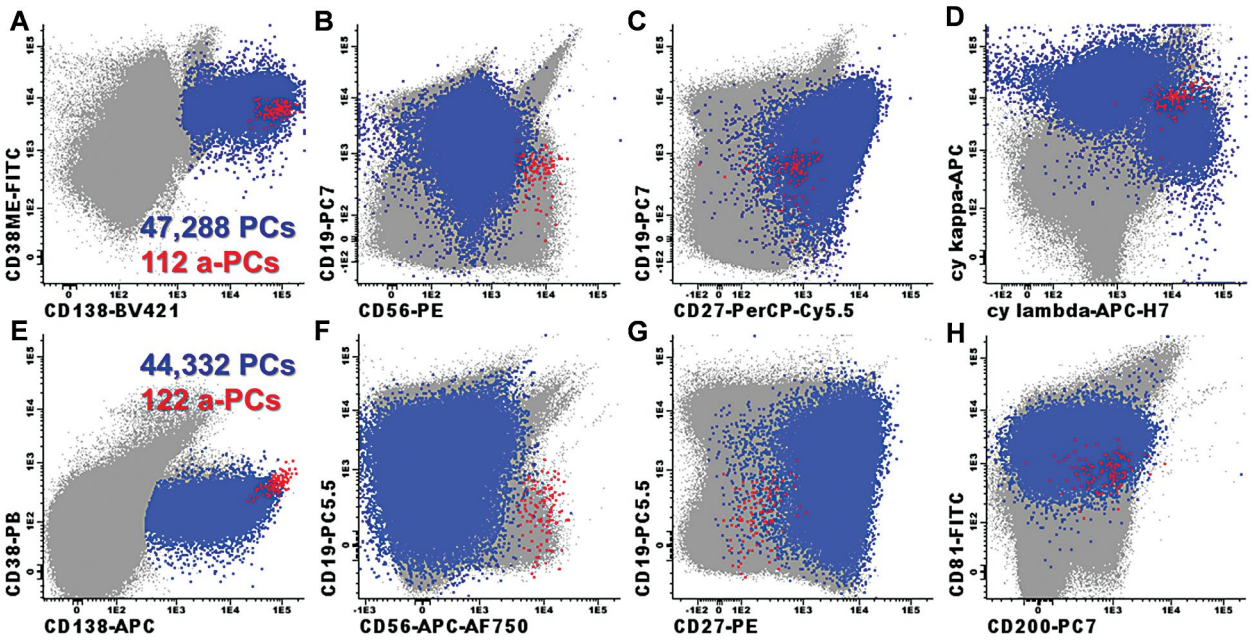

Figure 2A–H. Polychromatic analysis of plasma cells (PCs) in daratumumab treated multiple myeloma. Comparison of "home-made" (**A–D**) and Duraclone RE PC (**E–H**) protocol was done. Phenotype of clonal PCs (red dots) is similar CD19–CD56+CD27– (**B, C, F, G**) with high CD138 expression (**A, E**) in both approaches. Clonality assessment confirmed cytoplasmic expression of lambda light Ig chain (**D**), low CD81 and higher CD200 expression is relevant to pathological PCs as well (**H**). Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos)

Rycina 2A–H. Wielokolorowa analiza komórek plazmatycznych (PC) chorego na szpiczaka plazmocytowego leczonego daratumumabem. Przeprowadzono porównanie analizy za pomocą własnego protokołu typu *home-made* (**A–D**) i protokołu *Duraclone RE PC* (**E–H**). W obu podejściach fenotyp PC jest podobny: CD19–CD56+CD27– (**B, C, F, G**) z wysoką ekspresją CD138 (**A, E**). Ocena klonalności potwierdziła cytoplazmatyczną ekspresję lekkiego łańcucha Ig lambda (**D**); niska ekspresja CD81 i wyższa Cd200 jest również typowa dla patologicznych plazmocytów (**H**). Akwizycję danych przeprowadzono na cytometrze przepływowym *BD FACSCanto II* z oprogramowaniem *Diva SW* (*BD Bioscience*); ponownej analizy danych dokonano przy użyciu oprogramowania *Infinicyt SW* (*Cytognos*)

Table 3. Development of Euroflow PCD panel (sources [7, 23])

Tabela 3. Opracowanie panelu *Euroflow PCD* (źródła [7, 23])

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	b2m	CD19	cy Igk	cy Igl
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 Igk	cy Igl

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

patients treated in Spanish the 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(–) *versus* 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 has

shown 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 $p < 0.001$, respectively) 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, $p = 0.1$) [28]. Also was demonstrated that the prognostic impact of MRD following ASCT is independent of the induction therapy received [29]. Surprisingly, MFC-MRD monitoring has the 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, 30]. Using NGF in multicentre 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, respectively; median OS, 59, 64, and 65 months, respectively). MRD(–) status was strongly associated with prolonged PFS (median 63 months, $p < 0.001$) and OS (median not reached, $p < 0.001$) overall and in subgroups defined by prior transplantation, disease stage, and cytogenetics, with prognostic superiority of MRD negativity *versus* CR particularly evident in patients with high-risk cytogenetics [13].

Conclusion

As treatment strategies for MM become more effective and PFS becomes longer, assessing the 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 standardisation of sample processing, sample and data analysis and verification of this approach in clinical studies.

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Piśmiennictwo

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