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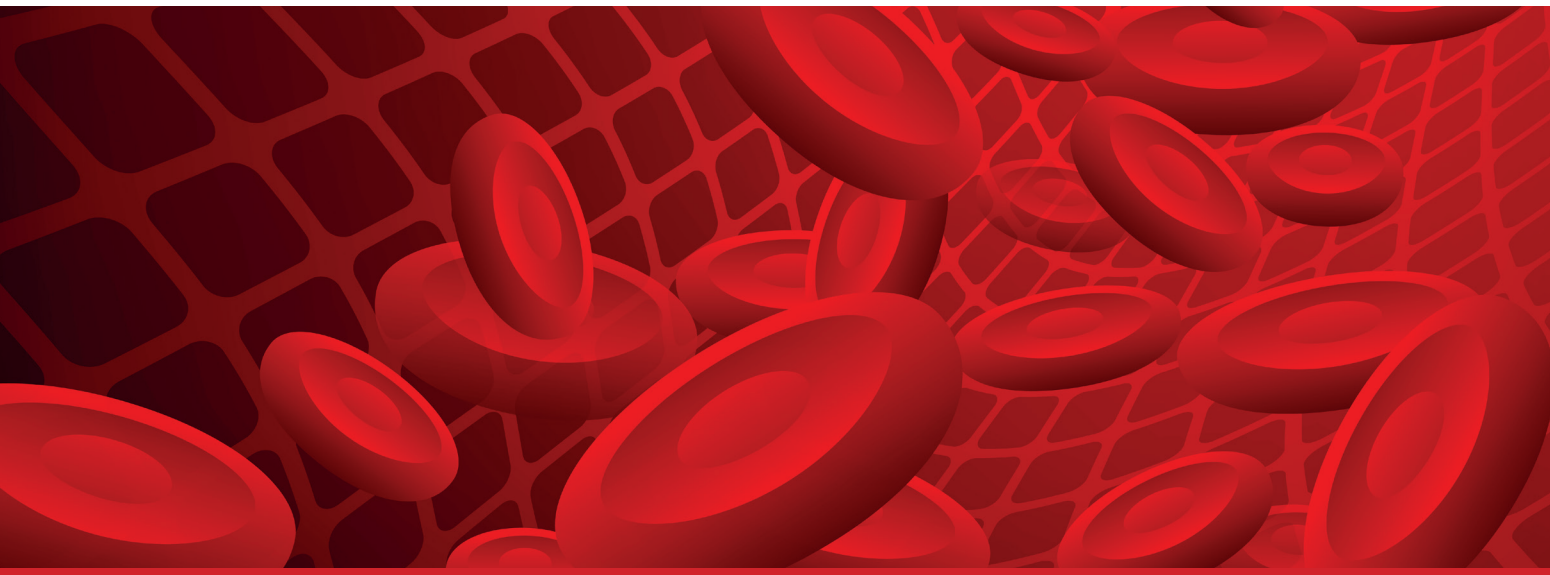

Acta Haematologica Polonica

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- **MRD in multiple myeloma**
  Agnieszka Krzywdzińska et al.
- **FAM therapy in BOS**
  Barbara Tejza
- **Central neurotoxicity in ALL**
  Joanna Krasińska et al.
- **Primary testicular lymphoma**
  Monika Joks et al.
- **Very late disease relapse in B-ALL**
  Anna Płotka et al.

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Table of Contents

SHORT COMMUNICATION

CLINICAL VIGNETTES

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EDITORIAL

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Revolution in management of CMV infection after hematopoietic cell transplantation

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Cytomegalovirus (CMV) infection is a major complication after allogeneic hematopoietic cell transplantation (allo-HCT), and a serious trigger for other sequelae [1–3]. The introduction of letermovir into the prophylaxis of CMV infection has led to a large reduction of the CMV reactivation rate in seropositive adult patients after allo-HCT [4–5]. This effect obviously means fewer direct and indirect complications caused by the virus [6–8]. Successful prophylaxis alleviates the negative impact of recipient CMV-seropositivity and eventually results in lower non-relapse mortality and better overall survival [4, 5, 9].

Progress is still being maintained. Recent trials have shown the safety and efficacy of letermovir use for prolonged (200 days) prophylaxis, and also in other patient populations such as children, as well as in secondary prophylaxis [10].

Yet another new anti-CMV drug, maribavir, is in the pipeline. This antiviral has been shown to have efficacy in preemptive treatment and in resistant CMV infections [1]. Fortunately, both letermovir and maribavir have very beneficial safety profiles, unlike the old-generation anti-CMV antivirals. Interestingly, during the most recent Annual Meeting of the European Society for Blood and Marrow Transplantation (EBMT), half of the highest-scoring abstracts on infectious complications were dedicated to the topic of CMV, confirming that this is one of the most pressing issues in the current management of allo-HCT.

The coronavirus disease 2019 (COVID-19) pandemic is over, as announced by the World Health Organization (WHO) on 5 May. CMV infection after allo-HCT is not over, but, with new antivirals, it can be vastly reduced. The next step must be to find an effective vaccine against CMV. With the new mRNA technologies used for the anti-SARS-CoV-2 vaccine, hopes are high that transplant patients will finally be able to overcome the problem of CMV.

Authors' contributions

JS — sole author.

Conflict of interest

The author declares no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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REVIEW ARTICLE

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Role of flow cytometric measurable residual disease assessment in multiple myeloma

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Abstract

Despite the high rates of complete response achieved with current treatments, patients with multiple myeloma (MM) continue to relapse due to the presence of minute amounts of residual MM cells. These are referred to as "minimal" or "measurable" residual disease (MRD).

As conventional serological and morphological techniques have become suboptimal for evaluating the depth of response, high sensitivity methods, next-generation flow (NGF) cytometry and next-generation sequencing are recommended in MRD assessment in the bone marrow. Under optimal conditions, these methods can detect one MM cell among 1,000,000 normal cells (a sensitivity of 10^{-6}). Furthermore, imaging techniques, particularly positron emission tomography–computed tomography, have an important role to play in MRD assessment outside of the bone marrow, and alternative blood-based methods for MRD assessment are under investigation. There is a growing consensus that MRD is the most relevant prognostic factor in MM, and achieving a negative MRD status significantly prolongs progression-free survival and overall survival.

This review examines the various methods used to detect MRD, including methodological aspects of NGF. It also presents considerations for implementing MRD as a surrogate biomarker to accelerate drug development and guide MM therapy.

Key words: multiple myeloma, measurable/minimal residual disease, next-generation flow, next-generation sequencing

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Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, accounting for c.10% of all hematological cancers. The annual incidence in Europe is 4.5–6 cases per 100,000 [1]. In Poland, c.1,600 new cases of MM are reported each year [2]. The disease is caused by a proliferating clone of neoplastic plasma cells that destructively affect the bone marrow (BM) microenvironment and, in most cases, secrete a non-functional monoclonal protein (paraprotein, M – protein) into the blood [3].

Advances in diagnostics and risk stratification, and more importantly the increasing availability of new therapies, have improved long-term outcomes for patients with MM [4]. Current treatment regimens using immunomodulatory drugs and second- and third-generation proteasome inhibitors in combination with autologous stem cell transplantation (auto-SCT) are achieving complete responses (CR) in

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up to 70–80% of patients [5–7]. New therapeutic options such as monoclonal and bispecific antibodies or chimeric antigen receptor T-cell (CAR-T) therapy, offer the opportunity to advance treatment in refractory and relapsed disease [8, 9]. The repertoire of therapeutic options is constantly expanding, and the quality of responses achieved will increase as new drugs are used in earlier lines of therapy.

Nevertheless, MM remains an incurable disease for most patients, and the clinical course of MM is characterized by relapses, increasingly short periods of remission, and the development of refractory disease [10].

Improvements in the frequency and quality of responses observed with new drugs and treatment regimens have necessitated the development of more sensitive methods to measure MM clone eradication [11]. Since 2016, the response criteria used to assess treatment efficacy have included deep response categories with measurable/minimal residual disease (MRD) in the bone marrow (BM) aspirate assessment and evaluation of extramedullary disease using imaging techniques. MRD should be assessed by high-sensitivity methods: multiparameter flow cytometry (MFC) or next-generation sequencing (NGS), with a recommended sensitivity of at least 10^{-5} [12]. It has been shown that post-treatment MRD negativity is associated with significantly better progression-free survival (PFS) and overall survival (OS) in newly diagnosed and relapsed/refractory MM patients [13]. In the era of intensive development of modern therapies, the introduction of the MRD criteria has opened up a number of possibilities for the application of this parameter.

In this article, we describe the currently used techniques for MRD testing, including methodological aspects of the flow cytometric method, as well as emerging techniques for improved characterization of residual populations that could be adapted for MRD monitoring in the future. We also discuss the relevance and applicability of MRD testing in clinical trials to determine the potential role of MRD assessment in clinical practice.

Evolution of response criteria in MM

Standardized criteria for assessing the efficacy of anti-myeloma therapies date back to the 1990s, when the prognostic role of achieving a complete response in patients following high-dose chemotherapy and auto-SCT was defined [14, 15]. Since then, in response to progressive improvements in the efficacy of new drugs and patient survival, the International Myeloma Working Group (IMWG) expert panel has updated and defined new categories [12, 16, 17]. These category definitions are based on biochemical test parameters assessing serum and urine M-protein and laboratory methods with varying sensitivity for detecting the degree of BM involvement. CR is defined as undetectable M-protein in serum and urine immunofixation and less than 5% of plasma cells in the BM cytomorphological examination, regardless of their clonality [12]. In contrast, the determinants of 'stringent complete response' (sCR) introduced in 2006 are, in addition to the fulfillment of CR conditions, the normalization of the serum-free immunoglobulin light chain (sFLC) ratio, and the absence of clonal PCs by immunohistochemistry (IHC) or cytometric examination of the BM aspirate using 2–4 markers, the sensitivity of which is estimated to be 10^{-2}–10^{-3} [16].

CR is the primary goal of therapy and its achievement is associated with improved treatment outcomes, including PFS and OS [18]. However, due to its limited sensitivity and the long half-life of the M protein, it does not reflect the true degree of eradication of the tumor clone. In turn, sCR is of limited value in differentiating between patients in CR with different risks of progression [19, 20].

In a retrospective analysis, Cedena et al. found that in a group of patients in CR, obtaining sCR did not identify patients with different PFS (68 vs. 69 months, $p = 0.5$). In contrast, the detection of MRD in patients with sCR with a sensitivity of 10^{-4} (by MFC technique) or 10^{-6} (by NGS technique) was associated with a significantly shorter median PFS compared to the MRD-negative group (for MFC, respectively: PFS 58 months vs. not achieved, $p = 0.04$ and for NGS respectively: PFS 32 months vs. not achieved $p = 0.001$) [20].

The term 'MRD' appeared in the International Myeloma Working Group (IMWG) response criteria as early as 2011, when the categories of immunophenotypic and molecular CR were first introduced, allowing for better risk stratification in an increasing number of patients achieving CR [17]. This required the quantitative assessment of MM cells at the detection level of 10^{-4}–10^{-5} using at least a 4-color MFC and a molecular technique: allele-specific oligonucleotide polymerase chain reaction (ASO-PCR). The sensitivity and specificity of MRD assays have since increased due to advances in cytometry and molecular biology. Second-generation MFC, using eight markers and in most cases achieving a sensitivity of 10^{-5}, have proved to be 30% more effective in detecting MRD than the first-generation MFC, which usually used 4–5 antigens and analyzed 200,000 cells [21]. In the PETHEMA/GEM2010 clinical trial, post-treatment MRD status was not only an independent predictor of time to progression (TTP) [hazard ratio (HR), 2.7; $p = 0.007$] and OS (HR, 3.1; $p = 0.04$), but it was also found that a deeper MM clone reduction overcomes the unfavorable prognosis associated with high-risk cytogenetics and patient age [21]. In subsequent studies, a consistent improvement in PFS and OS outcomes was observed as a function of the logarithmic decrease in the degree of BM involvement by the MM clone [22–24]. This relationship justified efforts to improve existing MRD detection techniques and to search for more sensitive ones.

Table I. Criteria for measurable residual disease (MRD) in multiple myeloma (MM) according to the International Myeloma Working Group (source [12])

All the below require a **complete response** defined as: negative immunofixation on serum and urine and disappearance of any soft tissue plasmacytomas and <5% plasma cells in BM aspirate	
Response criteria	**Definition**
Flow MRD-negative	Absence of phenotypically aberrant clonal plasma cells by NGF in BM aspirate using EuroFlow standard operation procedure for MRD detection in multiple myeloma (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Sequencing MRD--negative	Absence of clonal plasma cells by NGS in BM aspirate in which presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing using LymphoSIGHT platform (or validated equivalent method)* with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Imaging-positive MRD--negative	MRD negativity as defined by NGF or NGS plus disappearance of every area of increased tracer uptake found at baseline or a preceding PET-CT or decrease to less mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue
Sustained MRD-negative	MRD negativity in BM (NGF/NGS, or both) and by imaging, confirmed a minimum of one year apart. Subsequent evaluations can be used to further specify the duration of negativity (e.g. MRD-negative at five years)

*ClonoSEQ assay Adaptive Biotech's was approved by Food and Drug Administation in 2019; BM — bone marrow; NGF — next-generation flow; NGS — next-generation sequencing; PET-CT — positron emission tomography–computed tomography; SUV — standardized uptake value

In 2016, another version of the IMWG response criteria was proposed, increasing the recommended sensitivity level of MRD assays (Table I) [12]. MRD-positive status was defined as the persistence or reappearance of clonal PCs in the BM aspirate of patients with CR, assessed with a sensitivity threshold of at least 10^{-5}, which means the need to detect 1 MM cell of among at least 100,000 normal BM cells. Techniques with a sensitivity of 10^{-5}–10^{-6} were considered the reference methods for MRD assessment: next-generation flow (NGF) cytometry with the test protocol developed by the EuroFlow consortium and NGS of immunoglobulin genes performed using the LymphoSIGHT platform (Sequenta/Adaptative) [12, 25, 26]. At the recommended sensitivity threshold, NGF and NGS are considered equivalent techniques; depending on availability, any platform that achieves adequate sensitivity and reproducibility can be used. Due to the heterogeneous nature of BM involvement and the possibility of extramedullary relapse, imaging techniques, particularly positron emission tomography–computed tomography (PET-CT), is a complementary part of assessing a high-quality response in MM [12]. The measure of obtaining a high-quality response with the most favorable prognosis is the category known as sustained MRD-negative, definined as patients with MRD-negative results in BM and imaging tests, confirmed in at least two consecutive assessments within one year [12].

The specificity of the laboratory techniques, and their limitations, significantly affect the sensitivity of the tests, resulting in different limits of MRD detection. In addition, the dependence of the test quality on pre-analytical factors, and the belief that a negative test result does not mean the absence of disease, are the main reasons why the term "measurable residual disease" has been recommended for several years instead of "minimal residual disease".

IMWG — approved methods for MRD assessment

Flow cytometry

MFC, due to its availability, short turnaround time, and relatively low cost, offers the possibility of real-time monitoring of MRD and has the potential to be used in routine clinical practice. MM cells are detected by specific immunophenotypic features that distinguish them from normal/reactive plasma cells. The total population of plasma cells (PCs) in the test sample is determined by the expression of CD38, CD138, and CD45 and parameters determining the size (FSC, forward scatter) and granularity (SSC, side scatter) of the cells. The phenotype of MM cells is determined by abnormal expression patterns of at least two of the most commonly assessed membrane antigens: CD19, CD20, CD27, CD28, CD56, CD81, CD117, or CD200 in conjunction with the intracellular assessment of kappa (cIgκ) and lambda (cIgλ) immunoglobulin light chains [27].

Over the years, several attempts have been made to standardize the method, and recommendations regarding the test procedure have been published [28–31]. A reproducible and validated approach for the highly sensitive assessment of MRD in MM has been proposed by the EuroFlow consortium and defined as 'next-generation flow' (NGF) [25]. The optimized NGF antibody panel contains two 8-color tubes in which ten PCs markers are evaluated: CD38, CD138, CD45, CD19, CD56, CD27, CD81, CD117, cIgκ and cIgλ (Figure 1). This was intended to maximize the likelihood of defining an aberrant PCs population while simultaneously providing important information on sample quality and internal positive and negative control cell populations [25]. Moreover, the multiparametric panels provide valuable information about the tumor microenvironment

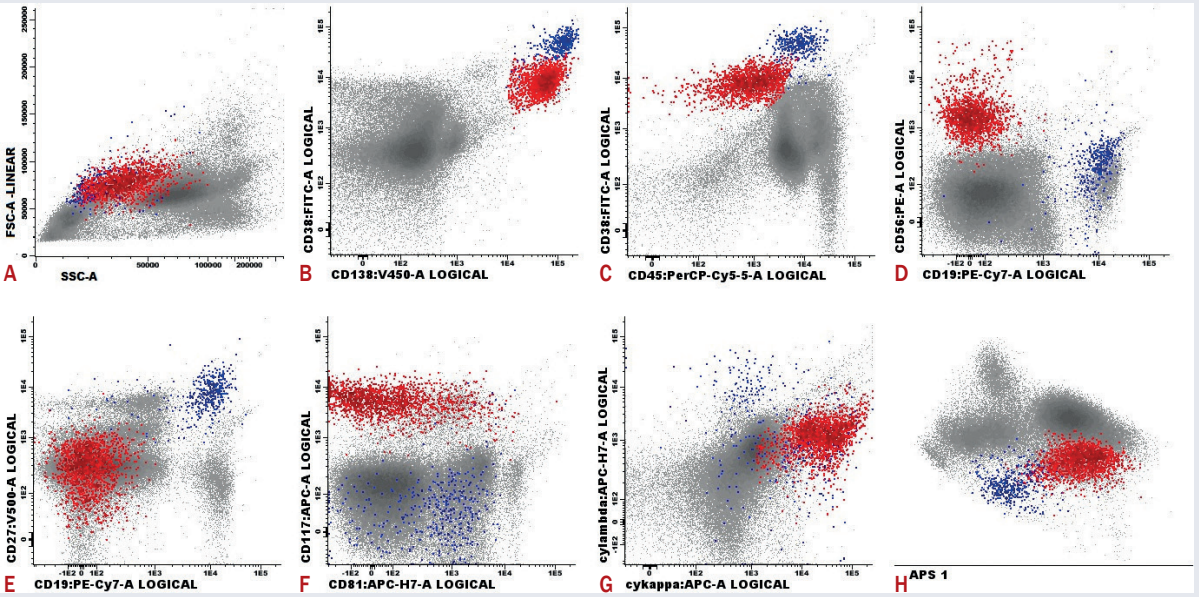

Figure 1A–H. Representative dot plots of analysis of measurable/minimal residual disease assay performed by next-generation flow method using Infinicyt software. Multiple myeloma (MM) cells (red dots) are detected by comparison to normal plasma cells (PCs) (blue dots) and show: high forward scatter (FSC) and medium side scatter (SSC) characteristics, specific high expression of CD138, lower than normal expression of CD38, CD45 and CD27, absence of CD19 and CD81, and aberrant positive expression of CD56 and CD117. Clonal nature of MM cells confirms cytoplasmic kappa light chain restriction. Final plot shows distinct separation of normal PCs and MM cells by automatic population separation (APS) function in Infincyte software. Gray color represents total acquisition events

and the individualized patient immune profile during MRD examination [32, 33].

The simultaneous analysis of at least eight markers is highly specific; thus, detecting PCs immunophenotypic aberrations is possible in all patients. Moreover, a broad antibody panel allows for high sensitivity of MRD assessment without knowledge of the primary antigenic characteristics of MM cells [34]. It is important to note that MM cells can exhibit varying degrees of heterogeneity at the immunophenotypic level. The expression of individual antigens is variable, which may be related to specific molecular alterations and the presence of PC subclones reflecting MM's clonal heterogeneity [35, 36].

In the EuroFlow protocol, the standard **stain-lysis-wash** sample preparation procedure has been replaced by the **lysis-wash-stain-wash** method, in which an appropriate amount of the BM sample is lysed to remove erythrocytes and obtain a suspension of millions of leukocytes in a small volume [25]. NGF requires acquiring and analyzing at least 5 million cells from a test tube (10 mln/test). Considering the losses during preparation, this means that 15–20 million leukocytes must be stained. This guarantees a sensitivity of 4×10^{-6}.

This increases to 2×10^{-6} if we use the special analysis software Infinicyt (Cytognos), which allows us to combine data from two test tubes. This allows for the automatic gating of PCs and identification of the MM cell population, which speeds up the analysis and reduces the risk of subjective evaluation errors. However, at this stage, it will not replace an experienced cytometrist who, when analyzing the MRD examination, must take into account the high immunophenotypic heterogeneity of both MM cells and normal PCs, the presence of MM subclones, and the possibility of modulation of antigen expression after treatment [35–37].

The clinical relevance of the highly sensitive MRD tests was confirmed in a study comparing the NGF approach to the conventional 8-color MFC [25]. 110 BM samples from patients with MM who achieved at least VGPR were evaluated. NGF showed higher sensitivity than 8-color MFC, with 47% versus 34% (p = 0.003) of MRD-positive samples detected. This translated into significantly longer PFS for MRD-negative versus MRD-positive patients (p = 0.01) [25]. The PETHEMA/GEM2014MAIN clinical trial, where the MRD was assessed in 458 patients, confirmed the high sensitivity and efficiency of the MRD NGF method, achieving a sensitivity of $<2 \times 10^{-6}$ in 1% of tested samples, $2 \times \times 10^{-6}$ to $<10^{-5}$ in 88% of samples, $\geq 10^{-5}$ to $<10^{-4}$ in 99.9% of samples, and $\geq 10^{-4}$ in 100% of samples. In only 0.4% of cases was the MRD assessment unreliable due to insufficient quality of the BM sample or technical issues [38].

The sensitivity of MRD assays is significantly affected by sample quality, quantity, and stability. Hemodilution of the BM aspirate sample is the most common pre-analytical

challenge, and is usually due to an incorrect collection procedure. According to current understanding, a patient's MRD status during treatment is the most important prognostic information that can be obtained from the BM aspirate; this is very important to provide high-quality samples for high-sensitivity tests [28]. The first portion of BM from aspiration, not exceeding 2–3 mL, should be collected for MRD studies; further aspiration from the same 'pull' is likely to be hemodilute. Post-acquisition assessment for suboptimal, hemodilute or hypocellular BM samples should be performed, and commented on in the final report. To some extent, hemodilution can be determined cytometricaly by quantifying cell populations that are typically absent in the blood, i.e. precursors of B cells, mast cells and erythroblasts [25]. The MRD report should indicate the potential risk of hemodilution and false negative MRD results in cases with reduced percentages of the cell types mentioned. Reference values for normal BM-associated cell populations have been established [25], although recent studies have shown that their range may vary depending on the time of examination and the type of therapy [39].

It should be noted that the disturbed distribution of the BM cell population may also be the result of impaired hematopoiesis, e.g. due to treatment. Therefore, it is necessary to develop a method with greater specificity to define hemodilution in BM, and the indications for repeated BM aspiration have not yet been clearly defined [39, 40].

According to the recommendations, the laboratory is obliged to determine the sensitivity of the assay obtained in a given test [31]. In particular, the reporting of the limit of detection (LOD) — valid for undetectable MRD, and the lower limit of quantification (LLOQ) — significant for quantitative determinations, are critical parameters for analytical performance. The LOD is defined as the ability of the test to detect MRD at a level that can be reliably distinguished from background noise. The LLOQ is defined as the lowest number or percentage of aberrant PCs that can be reproducibly detected with predetermined bias criteria. These parameters are determined by the identification of at least 20 (for LOD) and 50 (for LLOQ) MM cells and are strictly dependent on the number of BM cells analyzed [31, 41]. As MM MRD assays are highly specialized, the guidance for diagnostic laboratories that wish to perform MM MRD by MFC suggests considering important factors such as the number of MRD tests per year, staff expertise, the fitness of equipment, the availability of a partner laboratory for support and sample exchange, and participation in an external quality control [42].

Caution in the interpretation of single negative MRD results is also warranted by the fact that PCs are underrepresented in BM aspirates, which is particulary evident at diagnosis. This is due to the biology of the disease itself, including remodelling of the extracellular matrix of the BM stroma by the neoplastic process, or the presence of adhesion molecules, e.g. CD56, on the surface of MM cells [43]. False negative MFC results can also be caused by the high sensitivity of PCs in *ex vivo* conditions.

Therefore, it is recommended that the test be performed within 24–48 hours of collection and that appropriate transport conditions be used to maintain a constant temperature. Caution should be exercised in interpreting the CD138 antigen, as it has the greatest expression instability [30].

Daratumumab or isatuximab are anti-CD38 IgG kappa monoclonal antibodies that, combined with standard therapy, improve the quality of response and prolong the survival of patients with relapsed and refractory MM, and are increasingly being used in first-line therapy [44, 45]. It must be emphasized that information about a patient's treatment with immunotherapy is crucial for diagnostic laboratories performing serological, biochemical, or cytometric tests [46, 47]. Anti-CD38 therapy significantly reduces the effectiveness of immunophenotypic detection of PCs with CD38 antigen in MRD assays. The solution may be the use of a multi-epitope CD38 antibody that binds to the antigen site not covered by the therapeutic antibody, other markers such as CD229, CD319, CD54, or the VS38c antibody — which binds to the intracellular protein highly expressed in plasma cells and is tested using the protocol for the evaluation of cIgκ/cIgλ [48, 49].

While the variability in data collection and reporting of results in the context of clinical trials still receives attention [50], it appears to be less and less of an issue in how the test is performed in cytometry laboratories. Published recommendations regarding antibody panel design, sample preparation, data analysis, and finally, validation of the EuroFlow method, have all been important steps towards interlaboratory standardization of MRD testing in MM [25, 30, 31]. The diversity of the procedures regarding the number of cells analyzed, the antibody combinations, the analytical strategies, and reporting has significantly impacted the test sensitivity obtained in different centers [51]. The results of a survey analyzing the method of MRD assessment in MM in Poland showed a high variability of procedures and as much as a 100-fold difference in the sensitivity achieved between different laboratories [52]. Subsequently, harmonizing cytometer parameters and assay protocols in four cytometric laboratories allowed a high, 95%, concordance of results obtained in laboratories, even in samples with very few pathological PCs [53]. This confirms the value of the NGF method and legitimizes standardization activities that ensure consistency in the interpretation of MRD assessment results, which is necessary for multicenter projects.

Since the approval of the NGF standard, several groups have demonstrated alternative antibody panel compositions [54–56] and sample preparation methods for cytometric MRD MM assays [57, 58]. According to the IMWG recommendations, the newly introduced MRD method

should be properly validated by comparison with the results obtained using the reference method. Single 10-color tube antibody combinations have been developed, and studies comparing performance and reliability have shown a 95–98% agreement with the results obtained using the NGF method [54, 55]. While the advantages of the single tube method include lower cost due to reduced labor, reagents, and processing time, it should be noted that high agreement was found mostly up to a sensitivity threshold of 10^{-5} (0.001%). The two-tube method has been considered more robust because of the higher number of measurable cells, and the confirmatory value of the second tube for small populations of cells suspicious of MRD found in the first tube [54–56]. It has also been emphasized that including cytoplasmic kappa/lambda light chain markers in the 10-color panel significantly increases the assay's specificity [56].

Next-generation sequencing

Molecular techniques can reliably detect MM MRD since they provide precise disease measurements with high sensitivity. NGS has replaced another molecular method, ASO-quantitative (qPCR), because of its higher sensitivity, lower workload, and ability to be used in a greater percentage of patients with MM (>90% for NGS vs. <70% for ASO-qPCR) [26, 59]. This platform uses specific primers to amplify and sequence immunoglobulin gene segments with locus-specific primers for IgH (VDJ), IgH (DJ), or IgK rearrangements. After amplification, the immunoglobulin gene DNA is sequenced to determine the frequency of different clones. Importantly, MRD monitoring requires the identification of a patient-specific sequence from a pre-treatment sample, and the method has a slightly lower applicability than NGF (c.95% vs. 100%), as in some patients the dominant clonal sequence of MM cells cannot be detected in diagnostic samples [60].

In recent years, several NGS platforms for MRD detection in MM have been tested, achieving high sensitivity in the detection of MRD at the level of 10^{-5}–10^{-6} [61] or even 10^{-7} [62]. The ClonoSeq assay (Adaptive Biotechnologies, Seattle, WA, USA) was the first to be approved by the US Food and Drug Administration (FDA) and is currently the most frequently used for disease assessment in MM patients [63]. Following the promising results of Martinez-Lopez et al. [26], subsequent studies have confirmed the prognostic value of MRD assessment by NGS [61, 62, 64]. Perrot et al. confirmed these important findings in a larger series of MM patients enrolled in the IFM2009 clinical trial. PFS and OS were significantly prolonged in NGS MRD-negative vs. MRD-positive patients at pre- and post-maintenance timepoints [64]. Studies comparing MRD results from NGS and MFC assays at a sensitivity level of 10^{-5} showed an 83–85% concordance between the two techniques and 78% at a 10^{-6} sensitivity level [65, 66]. This suggests that NGS is more likely to reach a sensitivity threshold of 10^{-6} than NGF, but the problem with this type of study is often differences in sample quality. Similar to NGF, the sensitivity of the NGS MRD test is highly dependent on the quality of the BM aspirate. Nonetheless, NGS requires fewer cells for the assay (approx. 3 million vs. 20 million for NGF) [67]. However, unlike cytometry, the NGS method does not allow for sample quality assessment. The feasibility of NGS is limited by its high cost, long turnaround time, and high degree of expertise required. The advantage of this approach is that it can be applied retrospectively to stored material, including cryopreserved cells and archival BM slides. Moreover, the specificity of NGS allows for tracking clonal heterogeneity and the dynamics of molecular changes that occur during the disease. The methods used to assess MRD in BM, and their advantages and disadvantages, are set out in Table II.

Imaging MRD assessment

MM distribution is often heterogeneous, and imaging can be used to complement MRD detection at a single site. According to the IMWG criteria, additional MRD assessment outside the BM is mandatory to define the deepest possible response, and PET-CT is the current optimal technique [12]. The 'imaging plus MRD-negative CR' category further stratifies patients, and normalization of the PET-CT image after treatment correlates with longer PFS [68, 69]. It has been shown that combining MFC MRD and imaging improves outcome prediction, with double-negative and double-positive features defining groups with excellent and dismal PFS, respectively [70]. The presence of focal (metabolically active) areas of fluoro-2-deoxyglucose (FDG) uptake after induction therapy is associated with a higher risk of disease relapse, even in patients who achieved MRD-negative status in BM assessment. Moreau et al. [68] found a concordance of almost 62% between MFC MRD in BM and PET-CT negativity after consolidation, with 6.8% of patients showing PET-CT positivity and a negative MRD result. In the PETHEMA/GEM study, half of the patients with progression confirmed by PET-CT had no serum M protein or BM involvement.

These observations highlight the need to combine NGF or NGS with PET-CT to monitor the effectiveness of treatment, especially in patients with extramedullary and multifocal diseases [37, 70].

Importance and clinical application of MRD assessment in MM

The prognostic significance of MRD in MM was first emphasized in two publications in 2002 by a Spanish and British research groups evaluating the efficacy of auto-SCT in MM therapy [71, 72]. Three months after auto-SCT, with MFC sensitivity of 10^{-4}, MRD was detected in 30% [71] and 60% [72] of patients with negative immunofixation. Both the absence of clonal plasma cells (PCs), and more

Table II. Characteristics of techniques for monitoring multiple myeloma minimal/measurable residual disease in bone marrow (BM)

Variable	NGF	NGS
Method	Clonal cells are identified by their distinct immuno-phenotypic pattern vs. normal plasma cells	Specific immunoglobulin rearrangements are identified and detected by comparison with baseline sample
Reference platform	EuroFlow standardized 2-tube 8-color approach	Lymphosight, CloneSeq
Applicability	~100%	>90%
Baseline sample	Important but not mandatory	Mandatory
Number of cells required	10 million cells/tube	2–3 million cells/20 µg DNA
Sensitivity	10^{-5}–10^{-6}	10^{-5}–10^{-6}
Sample processing	Within 24–48 h Requires fresh sample	Fresh and stored samples can be used
Time required	3–4 h	1–2 weeks
Sample quality control	Concurrent with BM analysis	Not possible
Clonal evolution	Not evaluable	Evaluable
Support required	Automated software; expert flow cytometrist	Bioinformatics support

NGF — next-generation flow; NGS — next-generation sequencing

than 30% of normal PCs in the total plasma cell population, correlated with longer PFS (p = 0.04 and p = 0.02, respectively) [71, 72]. Subsequent analyses using more sensitive MFC, ASO-PCR, and NGS techniques have shown that a deeper response correlates with improved PFS and OS, suggesting that the goal of treating patients should be to achieve the deepest possible eradication of the MM clone [64, 73, 74].

The abundant scientific evidence of the prognostic value of MRD in MM has been summarized in meta-analyses [13, 75–77]. In an analysis of 1,273 patients from 14 studies, undetectable MRD was associated with a significant increase in PFS [hazard ratio (HR) 0.41; 95% CI: 0.36–0.48; p <0.0001]. The effect on OS was assessed in 1,100 patients included in 10 studies, which showed a clear benefit from achieving MRD-negative status (HR 0.57; 95% CI: 0.46–0.71; p <0.0001) [75]. Lahuerta et al. [76], in an analysis of 609 patients from three Spanish clinical trials, demonstrated the superiority of MRD over conventional CR, as MRD-detected CR patients had similar survival to MRD-positive patients who did not achieve CR. This relationship was confirmed in patients eligible and ineligible for auto-SCT, and in subgroups stratified by disease stage (ISS, International Staging System) and cytogenetic risk profile [76]. The recent meta-analysis by Munshi et al. [13] reviewed data from up to 93 publications from 45 studies, including 8,098 patients, and has confirmed the significance and strong prognostic value of MRD in a heterogeneous cohort of patients from different prognostic groups. The benefit of a negative MRD result was evident regardless of treatment, cytogenetic risk, MRD assessment method, or sensitivity level. As expected, the greatest benefit in terms of PFS and OS was observed in patients who had a negative MRD result at a sensitivity level of <10^{-6} [13]. Furthermore, the absence of MRD had a prognostic value in both CR and in very good partial remission (VGPR) patients, which seems to be particularly important in assessing the effectiveness of new immunotherapies that induce rapid and deep responses [13, 76].

While the standard endpoints of PFS and OS provide the most conclusive evidence of treatment efficacy, recent advances in MM treatment have significantly prolonged patient survival, making prospective clinical trials both lengthy and costly. Therefore, the absence of MRD with a sensitivity of 10^{-5} or even more informative at the level of 10^{-6}, is considered as a surrogate endpoint in clinical trials, also due to its speed of reading and its applicability in various clinical scenarios [78]. Data from these meta-analyses, MFC standardization, and FDA approval of the NGS ClonoSEQ platform may contribute to the final acceptance of MRD as a regulatory endpoint in clinical trials aimed at drug approval and those determining the role of MRD testing in routine clinical practice. Ongoing and future clinical trials using MRD as an endpoint would help assess the efficacy of new treatment regimens and, thus, may determine the validity of auto-SCT after four-agent induction [79] or determine the duration of maintenance therapy. Pawlyn et al. observed, using MFC with a sensitivity of 0.004%, that for patients who were MRD-negative after auto-SCT, the PFS advantage of maintenance lenalidomide diminishes beyond three years, compared to beyond 4–5 years in patients who are MRD-positive [80]. To facilitate the design, conduct, and interpretation of clinical trials, an international panel of experts has formulated recommendations regarding the type of MM studies that should include MRD measurement, recommended assessment timepoints, and expected analytical validation for the MRD tests, and recommendations for the reporting of results [41].

In addition to achieving MRD-negativity, an important aspect of therapy is the maintenance and attainment of a sustained MRD-negativity response [81, 82]. Standardized and sensitive MRD testing can provide more information relevant to understanding disease biology and assessing the likelihood of relapse when performed sequentially at multiple timepoints.

Gu et al. [83] monitored 104 patients with MM after induction and 3, 6, 9, 12, 18 and 24 months after auto-SCT with NGF. Patients with undetectable MRD after induction and throughout the post-transplant follow-up had the best prognosis, with PFS and OS similar to those who achieved MRD-negative status only after auto-SCT [83]. In MRD-negative patients after induction, the reappearance of MRD within 24 months after auto-SCT was significantly correlated with a shorter OS compared to the persistently MRD-negative group (35.2 ± 18.6 months vs. not reached), supporting the validity of long-term MRD monitoring. According to the authors, the optimal time for MRD assessment should include the post-induction period and three and 24 months after transplantation. Monitoring MRD-negative patients every six months would allow early detection of disease progression [83]. A recently published long-term follow-up study show that MRD conversion is associated with a high risk of biochemical or clinical relapse and is preceded by a median of 1.0 year (range 0–4.9 years) [84]. Similar results were presented by Schmitz et al. [85], who analyzed the dynamics of MRD quantitative changes in 20 CR/sCR patients. Increasing MRD levels were observed in six cases. They preceded biochemical changes (abnormal FLC ratio and positive electrophoresis) and clinical progression by a mean of 5.5 and 12.6 months, respectively, with the MM doubling mean time of 1.8 months [95% confidence interval (CI): 1.4–2.3 months] [85]. However, about 27% of patients with MRD resurgence can never experience clinical relapse [84]. Rodríguez-Otero et al. [86] found that long-term survival among patients with persistently MRD-positive disease may be explained by an 'MGUS-like' immunophenotypic signature in the BM at diagnosis defined by the relative frequency of BM PCs plus the percentage of clonal and normal PCs within the whole BM PC compartment.

The rate of both eradication and growth of the tumor clone at relapse can be influenced by a number of factors, including those that stratify patients into risk groups [37, 87]. In the Myeloma IX trial, regardless of baseline cytogenetic risk, the absence of MRD at 100 days after auto-SCT was associated with improved PFS (p <0.001) and OS (p = 0.0183), but median PFS was three times longer in the standard cytogenetic risk MRD-negative group compared to the high-risk MRD-negative group (defined as gain(1q), del(1p32), t(4;14), t(14;20), t(14;16) and del(17p) [88]. Also, in patients with persistent MRD, regardless of logarithmic levels, the presence of high-risk cytogenetic abnormalities conferred poorer outcomes [76, 87]. Other studies have reported that high-risk patients who achieve MRD-negative status at the level of 10^{-5} or 10^{-6} after effective therapies have comparable PFS and OS to standard-risk patients [21, 62]. The factors identified by the cytogenetic analysis and the baseline stratification of patients have a significant impact on the prognosis at the time of diagnosis and during disease progression in the MRD-negative group [38]. The ability to identify patients with the deepest responses may optimize the existing risk assessment tools for MM patients.

Risk stratification may need to be reassessed after treatment, as patients with an adverse prognosis can shift into a favorable one after achieving and maintaining deep responses after intensive therapy [38]. Therefore, MRD testing offers the possibility of a better prognosis, dynamic risk assessment, and modification during the course of the disease, but always in the context of risk factors from the moment of diagnosis and earlier treatment [89].

Tracking disease kinetics by numerical or logarithmic changes in the MRD, even at such low tumor weights, may provide greater information resolution. In a prospective study, Diamond et al. evaluated the dynamics of changes in MRD status based on 340 MFC MRD studies performed over five years in 103 patients treated during lenalidomide maintenance therapy [74]. Patients who maintained an MRD-negative response had no disease progression at a median follow-up of 19.8 months. Interestingly, patients who lost the MRD-negative response were more likely to have disease progression than both patients with persistently negative MRD (p <0.0001) and patients with persistently positive MRD (p = 0.015) [74]. Study results by Alonso et al. [90] confirmed the role of lenalidomide maintenance in stabilizing the response and improving its quality. Sequential annual MRD assessments showed that both achieving MRD negativity and gradually decreasing MRD levels alone significantly prolonged PFS [90]. In a recently published study, Paiva et al. [91] assessed the importance of serial monitoring. They examined the dynamics of MRD measured by NGF in 1,362 patients after induction and during the maintenance phase [91]. MRD-negative patients at the end of induction had a median PFS of 38.6 months, compared to 15.6 months for those with MRD-positive result in BM. At the time of evaluation, 9.5% of patients had converted from MRD-negative to MRD-positive, which was associated with worse PFS, similar to patients who were MRD-positive at every timepoint (2-year PFS rate of c.30%). 5.1% of MRD-positive patients achieved MRD negativity and PFS similar to that of the MRD-negative group (2-year PFS rate of 75%) [91]. These observations highlight the importance of sequential MRD monitoring, which may provide a more accurate assessment of prognosis than measurement at a single timepoint. This may indicate the value of the therapy used and distinguish subgroups of patients with different prognoses. The authors also point to the possibility of early relapse warning and the need to implement anti-relapse treatment [90, 91].

The prognostic significance of a deep MRD-negative response is beyond doubt. Even so, the predictive value, and thus the role, of MRD assessment in routine clinical management has not yet been determined. The available preliminary data suggests the benefits of treatment tailored to the response status, and MRD status can be incorporated into the clinical decision-making process at various timepoints, e.g. to determine the duration of induction therapy [92], the validity of the auto-SCT procedure given the availability of effective induction regimens [93], or the intensity and continuation of maintenance therapy [94]. As one of the first, Korde et al. [92] published the results of a study in which the number of cycles of induction therapy with carfilzomib, lenalidomide, and dexamethasone (KRd) was individualized based on MRD status. In the MASTER trial, patients received daratumumab, carfilzomib, lenalidomide, and dexamethasone (Dara-KRd) induction, auto-SCT, and Dara-KRd consolidation, according to MRD status. MRD was assessed by NGS, and patients with two consecutive MRD-negative assessments remained in follow-up without treatment. The 2-year PFS rate in the observation group was 87%, and the risk of relapse within 12 months after treatment discontinuation was significantly higher in patients with a higher cytogenetic risk [94]. Martinez-Lopez et al. [95] published the results of a retrospective analysis of survival in patients monitored with MRD during first-line therapy. Treatment modification based on MRD results (treatment discontinuation, intensification, or new therapy) was performed in 67 patients, resulting in longer PFS than in patients who did not change therapy (mean PFS 104 vs. 62 months, $p = 0.005$). In patients with at least one MRD negative result during maintenance therapy, discontinuation versus continuation did not change PFS ($p = 0.1$). However, in patients who were MRD-positive during maintenance, intensification or therapy change resulted in a better PFS than patients with no therapy adjustments (mean PFS not achieved vs. 39 months, $p = 0.02$) [95].

Several clinical trials are investigating therapeutic strategies based on MRD status (Table III) [96]. The randomized EQUATE study (NCT04566328) will evaluate the effectiveness of intensifying first-line therapy in patients with a positive MRD result after induction. In turn, the DRAMMATIC (NCT04071457) trial may answer whether maintenance therapy can be safely discontinued in patients with persistently negative MRD. The REMNANT study (NCT04513639) will compare the effectiveness of carfilzomib, dexamethasone, and daratumumab in treating MM relapse, defined as the appearance of MRD versus progression of MM defined by IMWG criteria. The Polish Myeloma Consortium's PREDATOR clinical trial (NCT03697655) will evaluate the role of preemptive daratumumab therapy in biochemical relapse or MM progression defined as MRD reaperance measured in BM with a sensitivity of 10^{-5}.

Peripheral blood techniques for MRD assessment

The focal nature of the bone marrow infiltration, the clonal evolution of MM over time, the possibility of recurrent extramedullary lesions, and the invasiveness of the procedure of regular biopsies, all mean that the optimal monitoring scheme and other methods and techniques to obtain complete information about the actual degree of eradication of the MM clone is still being sought.

An alternative approach to BM testing may be **liquid biopsy** — a diagnostic technique that identifies and analyzes circulating tumor plasma cells (CTPC) or cell-free DNA (cfDNA) in peripheral blood. Both CTPC and cfDNA are currently being investigated for quantitative and qualitative characterization of the tumor genome and as a non-invasive monitoring of MM therapy [97]. CTPCs are released from the primary tumor or metastatic sites into the bloodstream and are responsible for dissemination and extramedullary disease. CfDNA consists of degraded DNA fragments released into the circulation from tumor cells and is molecularly distinct in total extracellular DNA [98]. It has been demonstrated that CTPC can be detected in up to 80–90% of newly diagnosed patients and even in 100% of patients at MM relapse [99, 100]. Several studies have confirmed that detectable CTPC at diagnosis, post-treatment, and pre/post-auto-SCT is an unfavorable prognostic factor for therapeutic response and progression, regardless of the ISS/Revised Multiple Myeloma International Staging System (R-ISS) stage and high-risk cytogenetics [101]. Furthermore, it has been suggested that detecting ≥0.01% CTPC may be a new risk factor in novel staging systems for patients with transplant-eligible MM [99]. Moreover, the results of a study by Garcés et al. [99] showed that this adverse effect on PFS can be overcome by effective treatment and achieving an MRD-negative response in BM. Genomic characterization showed a high concordance of mutations detected in CTPCs and paired BM samples; however, some mutations were only detected in blood, indicating that CTPCs represent a more complete picture of disease burden than cells from BM samples obtained from only one region [102]. In the context of MRD testing, a higher degree of sensitivity is needed, and even with next-generation techniques, peripheral blood assessment appears to be significantly less sensitive than BM-based assays. Sanoja-Flores et al. [103] reported that MRD was present in 17% of patients in CR by detection of CTPC and identified a subgroup of patients with significantly shorter PFS. However, in a significant percentage of patients (40–56%) with a positive MRD result in BM, CTPC/ctDNA in the blood may be undetectable. In turn, MRD has been found in the BM in 88–100% of cases with CTPC present in the blood [103, 104].

These observations suggest that persistently positive MRD in the blood may reflect positive BM MRD and avoid

Table III. Selected trials with measurable/minimal residual disease (MRD) adapted treatment strategy in multiple myeloma (MM) (source [96])

Study ID	Title	Phase/ /planned population	Estimated study completion date	MRD methodology/ /sensitivity	Point of MRD-driven decisions	Brief outline	Primary endpoint
PREDATOR-MRD NCT03697655	Pre-emptive Daratumumab Therapy of Minimal Residual Disease Reappearance or Biochemical Relapse in Multiple Myeloma (PREDATOR)	II 274	July 2024	NGF 10^{-5}	At MRD relapse (loss of MRD-negativity)	Patients with loss of previously attained MRD negativity (observation up to 73 weeks) will be given daratumumab immediately vs. standard of care	Event--free survival (EFS)
MRD-STOP NCT04108624	A Multimodality Approach to Minimal Residual Disease Detection to Guide Post-Transplant Maintenance Therapy in Multiple Myeloma (MRD2STOP)	NA 56	December 1, 2024	NGF 10^{-5} NGS $\geq 10^{-6}$ Blood assays	MRD-negative status after at least one year of maintenance	Patients will undergo discontinuation of maintenance therapy if they are MRD negative by multiple modalities (PET-CT, NGF and NGS) after receiving at least one year of maintenance therapy	MRD conversion date
CONPET NCT03314636	Intensified Treatment With Carfilzomib in Myeloma Patients Still PET--positive After First Line Treatment (CONPET)	II 50	March 2025	PET-CT NGF 10^{-5}	PET-positive after a standard first-line treatment	Patients who are PET negative will be excluded from treatment; those who are PET positive will be given KRd	PET conversion rate
AURIGA NCT03901963	A Randomized Study of Daratumumab Plus Lenalidomide Versus Lenalidomide Alone as Maintenance Treatment in Patients With Newly Diagnosed Multiple Myeloma Who Are Minimal Residual Disease Positive After Frontline Autologous Stem Cell Transplant	III 214	May 29, 2026	NGS 10^{-5}	MRD-positive status after auto-SCT	Evaluation of conversion rate of MRD negativity following addition of daratumumab to lenalidomide relative to lenalidomide alone, when administered as maintenance treatment to patients who are MRD positive after auto-SCT	MRD negativity
NCT04140162	Phase 2 Study With Minimal Residual Disease (MRD) Driven Adaptive Strategy in Treatment for Newly Diagnosed Multiple Myeloma (MM) With Upfront Daratumumab--based Therapy	II 50	October 2026	NGS/MFC 10^{-5}	MRD-positive status after induction	Trial will test whether combination of DaraRd as induction therapy, followed by DRVd consolidation therapy if needed, will result in more patients achieving MRD-negative status, relative to standard of care. Consolidation therapy will be administered only to MRD-positive patients after induction	MRD negativity after induction or, if still MRD--positive, after consolidation

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Table III (cont.). Selected trials with measurable/minimal residual disease (MRD) adapted treatment strategy in multiple myeloma (MM) (source [96])

Study ID	Title	Phase/ /planned population	Estimated study completion date	MRD methodology/ /sensitivity	Point of MRD-driven decisions	Brief outline	Primary endpoint
EQUATE NCT04566328	Testing Use of Combination Therapy in Adult Patients With Newly Diagnosed Multiple Myeloma, EQUATE Trial	III 1,450	December 31, 2027	NGS 10^{-6}	Positive MRD result after induction	DaraRD induction followed by addition of bortezomib to DaraRd for consolidation treatment in MRD-positive patients after induction	Consolidation OS
REMNANT NCT04513639	Relapse From MRD Negativity as Indication for Treatment (REMNANT)	III 176	June 1, 2032	NGF 10^{-5}	Upon MRD relapse (loss of MRD negativity)	Randomization to receive second-line treatment (KRd) either at loss of previously attained MRD negativity or at progressive disease, as per IMWG criteria. Study will evaluate whether treating MRD relapse after first line treatment prolongs PFS and OS versus treating relapse at progressive disease	PFS, OS, MRD negativity 30––45 days post consolidation
DRAMMATIC/ /S1803 NCT04071457	Lenalidomide 6 Daratumumab/ /rHuPh20 as Post–ASCT Maintenance for MM w/MRD to Direct Therapy Duration (DRAMMATIC)	III 1,100	July 1, 2040	NGS 10^{-6}	After two years of maintenance (lenalidomide ± ± Dara)	After two years of maintenance, MRD positive patients continue assigned treatment. MRD--negative patients are randomized to continue/discontinue therapy	OS

NGF — next generation flow; NGS — next generation sequencing; PET-CT — positron emission tomography–computed tomography; OS — overall survival; PFS — progression-free survival; auto-SCT — autologous stem cell transplantation; DaraRd — daratumumab, lenalidomide, dexamethasone; DRVd — daratumumab, lenalidomide, bortezomib, dexamethasone; KRd — carfilzomib, lenalidomide, dexamethasone; IMWG — International Myeloma Working Group

invasive BM assessment. Further studies at different treatment timepoints and using a more sensitive methodology (e.g. with immunomagnetic enrichment) would help clarify the role of CTPC assessment in MM prognosis [105].

Mass spectrometry (MS) methods are emerging as a promising new approach for more sensitive detection and monitoring of paraprotein levels in serum [106]. The basis of the MS method is the unique sequence of the antigen binding region, called the 'complementarity determining region' (CDR) of the immunoglobulin. The CDR amino acid sequence is specific for the MM clone. This gives the immunoglobulin a specific isoelectric point (the basis of the electrophoresis method) and mass (the basis of the M-protein detection by MS). Efforts to optimize M protein detection by MS have resulted in two methods varying in analytical sensitivity: matrix-assisted laser desorption/ionization--time-of-flight mass spectrometry (MALDI-TOF-MS), and liquid chromatography-mass spectrometry (LC-MS) [107]. MS techniques can detect and quantify M-protein with a detection limit approximately 100 times lower than immunofixation, translating to concentration ranges of 0.05 to 0.001 g/L [107]. Published data suggests that MS should be considered as part of a multidimensional approach to MRD assessment. Compared to BM NGF, MS in blood displays a fair degree of concordance and is associated with a comparable prognostic value [108]. Eveillard et al. [108] compared the performance of MALDI-TOF-MS to the MRD MFC 10-color single-tube method. Their study demonstrated that the results of MS were concordant with the MFC MRD in BM for 44/71 (62%) patients (p = 0.342). Of the 27 discordant cases, 17 were detectable only by MALDI-TOF MS, and 10 were detectable only by MFC MRD [108].

These results suggest that MALDI-TOF-MS adds value to BM-based MRD testing and is more specific for early detection of relapse than electrophoretic methods. MS could be used as a screening method for MRD testing in patients whose disease is not detectable by immunofixation (IFE) and sFLC testing [107]. A negative MS result could indicate BM aspiration to confirm MRD-negative status. The use of MS in MRD monitoring is currently limited to the research community, nevertheless the IMWG Mass Spectrometry Committee endorses the detection of M-proteins by MS (MALDI-TOF method) as an alternative to IFE and for distinguishing residual M-protein from therapeutic monoclonal antibodies for clinical practice, and for accurate interpretation and determination of complete response in clinical trials [107].

Conclusions

Achieving MRD negativity is one of the strongest prognostic factors in MM, independent of disease status (newly diagnosed or relapsed), cytogenetic risk, MRD assessment, or the sensitivity method achieved. A negative MRD result, and especially sustained MRD negativity, seems to be more important than the treatment used. Flow cytometry and molecular methods guarantee high sensitivity. However, each method in its current form has its limitations, and the most important of these seems to be the limited representativeness of the BM samples. Imaging assessment techniques and new techniques for evaluating peripheral blood complicate the harmonization of MRD evaluation and require further research, but may prove essential for a comprehensive evaluation of a patient's status.

MRD has been incorporated into numerous clinical trials to compare different treatment approaches, adapt therapy intensities according to MRD status, determine maintenance duration, or introduce early intervention strategies. An important issue remains the determination of the frequency of MRD testing, assessing the likelihood and interpretation of false-positive and false-negative results, and combining different evaluation techniques and additional prognostic biomarkers to supplement the MRD results.

This will require a great deal of analysis and application of the MRD parameter in different clinical contexts, but nonetheless offers an unprecedented opportunity to use MRD assessment to optimize and personalize therapeutic strategies in MM.

Authors' contributions

AK — conceptualization, literature analysis, original draft preparation, review and editing. BP — review and editing. KJ — conceptualization, review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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How to manage cytomegalovirus reactivation/infection after hematopoietic stem cell transplantation: practical tips for clinicians

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Abstract

Cytomegalovirus (CMV) reactivation is one of the most common and life-threatening complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT). It is associated with an increased risk of transplantation failure, non-relapse mortality (NRM), and lower overall survival (OS) than in patients without CMV reactivation, even in the era of pre-emptive antiviral treatment. Numerous risk factors for CMV reactivation in the setting of allo-HSCT have been identified. Donor/recipient CMV serological status remains the main risk factor influencing the incidence and mortality of CMV disease after transplantation. Proper selection of donor and recipient, regular and careful monitoring, an early intervention in CMV reactivation, and rapid and effective treatment when the disease develops, remain crucial to decrease the risk of post-transplantation CMV reactivation/disease. The introduction of letermovir as CMV prophylaxis has reduced NRM and improved OS.

Herein we present practical tips as to how to manage CMV reactivation/disease after allo-HSCT through an illustrative case report, with a focus on the risk factors present before and during the procedure.

Key words: allogeneic hematopoietic stem cell transplantation, cytomegalovirus reactivation, letermovir, overall survival, non-relapse mortality, prophylaxis

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Introduction

Treatment with high-dose chemotherapy supported by allogeneic hematopoietic stem cell transplantation (allo-HSCT) has significantly improved the prognosis of patients with malignant and non-malignant hematological disorders [1].

However, despite its proven efficacy, the procedure still carries a significant risk of post-transplant complications. Among these, infections are frequent and remain the major cause of increased morbidity and mortality. Viral infections, especially opportunistic, are the leading cause of death in the post-transplant period with a ~30% mortality rate [1–3]. Cytomegalovirus (CMV) reactivation after allo-HSCT is associated with an increased risk of graft rejection, non-relapse mortality (NRM), and decreased overall survival (OS) [4–7].

CMV is a DNA beta herpes virus carried by up to 90% of the adult population worldwide [8]. Its seroprevalence increases with age. After primary infection, typically asymptomatic in immunocompetent people, CMV remains latent for years and can reactivate at any time in immunocompromised patients [8, 9]. This can be particularly observed in patients after chemotherapy, solid organ transplantation (SOT), and HSCT [9–11]. According to recent studies, delayed reconstitution of the immune system, especially

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Table I. Definitions of cytomegalovirus infection and disease (based on [17–19])

Term	Definition
CMV infection	Isolation of CMV or detection of viral proteins or nucleic acid in any body fluid or tissue sample
Primary CMV infection	First confirmed CMV infection in an individual showing no evidence of CMV exposure before transplantation
Recurrent CMV infection	CMV infection in patient with known previous evidence of CMV infection when virus had not been detected for at least four weeks of active surveillance. This may result from reactivation of latent virus or reinfection (*see below*)
CMV reinfection	Detection of a CMV strain distinct from strain that caused initial CMV infection
CMV reactivation	Detection of two CMV strains (prior and current strain) that are found to be indistinguishable either by sequencing of specific regions of viral genome or by molecular techniques that examine genetic polymorphism
Symptomatic CMV infection	Both presence of general symptoms and/or signs (e.g. fever, bone marrow suppression) and detection of CMV genetic material obtained using sensitive methods. No signs of CMV end-organ disease
CMV disease	Presence of symptoms and/or signs from affected organ and detection of CMV by appropriately sensitive testing of tissue samples obtained by biopsy or other invasive technique (Exception: CMV retinitis — findings observed in ophthalmological examination are sufficient confirmation)

CMV — cytomegalovirus

functional CMV-specific T-cell immunity, may cause CMV reactivation and contribute to the development of CMV disease [10, 12, 13]. The highest rate of CMV reactivation has been reported in recipients of allo-HSCT, with a median of 37% [10, 14].

The development of modern techniques focusing on more sensitive and rapid diagnostic assays, together with the introduction of highly effective drugs against CMV, has helped to reduce the incidence of CMV disease and its serious effects [15, 16]. Nevertheless, CMV reactivation/disease remains one of the most common and life-threatening complications after allo-HSCT [16].

CMV infection is defined as the isolation of viral antigens, genetic material, or the virus itself, in any tissue or body fluid sample. The term "recurrent infection" stands for a new CMV infection in a patient with a confirmed history of a previous CMV infection when the virus had not been detected for at least four weeks of active surveillance, and that can result from reinfection or reactivation of the latent virus. All other definitions concerning CMV infections, reactivation and disease are set out in Table I [17–19].

Below we present how to manage CMV reactivation/disease after allotransplantation through an illustrative case report.

Illustrative case (I)

A 40-year-old CMV-seropositive male diagnosed with a high-risk acute myeloid leukemia (AML) had completed induction treatment with a DAC (daunorubicin, cytarabine, cladribine) regimen with no response. Second line chemotherapy with CLAG-M [cladribine, cytarabine, mitoxantrone and granulocyte colony-stimulating factor (G-CSF)] resulted in complete remission (CR).

A 36-year-old, partially human leukocyte antigen (HLA)-matched, CMV-seronegative female donor was identified for transplantation. Both donor and recipient reported no concomitant diseases and shared the same blood group (B Rh–). Following myeloablative conditioning (MAC) with TBF (thiotepa, busulfan, fludarabine), the patient was transplanted with 1.85×10^6/kg of body weight of CD34-positive cells.

Risk factors for CMV reactivation

Numerous risk factors for CMV reactivation in the setting of allo-HSCT have been identified. These factors can be 1) patient-related (age, sex, CMV serostatus); 2) donor-related (age, sex, CMV serostatus, donor type and HLA-match status; 3) transplant-related (type and intensity of conditioning, stem cell source, use of T-cell depletion); and 4) related to post-transplant immune reconstitution.

Among all those mentioned, three factors seem to be crucial for post-transplant outcome: 1) donor (D)-negative/recipient (R)-positive CMV serological status; 2) occurrence/severity of acute/chronic graft-versus-host disease (GvHD) and its treatment; and 3) unrelated (UD) or mismatched donor (MMD) transplant (Table II).

Donor/recipient CMV serostatus before transplantation

D/R CMV serological status remains the main risk factor influencing the incidence and mortality of CMV disease after allo-HSCT [10, 14]. A positive CMV IgM result confirms ongoing or very recent infection and is a contraindication for transplantation. The presence of CMV IgG antibodies indicates previous contact with the virus and immune competence against CMV. Seropositive individuals carry the latent CMV, and their blood components are potentially infectious to CMV-naïve recipients, leading to transfusion-transmitted CMV. Several recent studies have shown

Table II. Risk factors for cytomegalovirus reactivation

Category	Risk factor
Recipient-related	Age
	Sex
	CMV serostatus
Donor-related	Age
	Sex
	CMV serostatus
	Donor type (family/unrelated)
	HLA-match status
Transplant-related	Conditioning regimen (type and intensity)
	Stem cells source
	T-cell depletion use
	Acute and/or chronic GvHD (prophylaxis, occurrence and treatment)
Related to immune recovery	Recovery of CCTLs
	Immunosuppressive treatment (type and duration)
	Prolonged exposure to anti-CMV drugs
	Speed of immune cells recovery

CMV — cytomegalovirus; HLA — human leukocyte antigen; GvHD — graft-versus-host disease; CCTLs — CMV-specific cytotoxic T lymphocytes

that CMV reactivation is most common in CMV IgG seropositive recipients regardless of donor status [14, 20]. These patients are also nearly nine times more likely to develop CMV disease than seronegative recipients [14, 17, 21]. Moreover, it has been demonstrated that CMV seropositive recipients receiving a graft from a seronegative donor have more frequently CMV reactivation after allo-HSCT when compared to D+/R+ [20, 22–24]. This phenomenon could be explained by the transfer of antiviral cytokines and CMV-specific cytotoxic T lymphocytes (CCTLs) along with the graft from seropositive donor [14, 22].

To sum up, patients can be categorised into those having a high risk (D–/R+ or D+/R+), an intermediate risk (D+/R–), or a low risk (D–/R–) of CMV reactivation [25].

Occurrence of acute and/or chronic GvHD

GvHD is a reaction of donor immunocompetent cells against host tissues and it occurs after HSCT. Two forms of GvHD can be seen: acute and chronic. The two have different pathophysiologies, but involve similar types of cells. GvHD development, together with immunosuppressive treatment especially with corticosteroids, may prolong T-cell reconstitution after allo-HSCT, increasing the patient's susceptibility to opportunistic infections [9, 26, 27]. CMV is reactivated twice as often in patients with acute GvHD than in those without this complication (60.1% vs. 32.1%) [5, 28, 29]. Moreover, patients who experience severe acute GvHD have been shown to be at a much higher risk of CMV infection compared to those with mild severity (92.3% vs. 51.9%) [10, 30, 31]. On the other hand, it has been demonstrated that CMV reactivation increases the risk of death from GvHD [32, 33].

Unrelated (UD) or mismatched donor (MMD) transplant

CMV reactivation risk is higher in UD and MMD transplants compared to a matched sibling donor (MSD) transplant. The risk of CMV disease is ~3 times greater in UD/MMD grafts than in MSD, especially if the recipient is CMV-seropositive [14, 28, 34–36].

Several scoring systems based on the presence of risk factors have been proposed to date, although none has been validated for use in clinical practice [10, 37].

Case continued (II)

GvHD prophylaxis consisted of tacrolimus (TAC), methotrexate (MTX) and anti-thymocyte globulin (ATG). The patient engrafted neutrophils and platelets on days +13 and +17 post-transplant, respectively. On day +18, the patient developed an erythematous, maculopapular rash on <50% of his body surface (grade II aGvHD). Symptoms resolved rapidly after pulses of intravenous methylprednisolone (MP) at 2 mg/kg of bw and topical corticosteroids. Due to the increased serum creatinine level, TAC was switched to mycophenolate mofetil (MMF). Bone marrow assessment performed on day +28 revealed CR with negative minimal residual disease (MRD) and full donor chimerism. CMV was negative on polymerase chain reaction (PCR). Two days later, the patient was discharged on MMF and routine antiviral/antibacterial prophylaxis.

Post-transplantation work-up and CMV prophylaxis

Post-transplant strategy is based on regular monitoring of CMV viremia in peripheral blood. The following diagnostic techniques are used in clinical practice: CMV pp65 antigenemia assay or, preferably, quantitative polymerase chain reaction (qPCR).

It is vital to use the same monitoring technique, PCR assay and sample type for a given patient [18]. CMV tests/viral load monitoring should be performed regularly, at least once a week during the first 100 days for both pre-emptive therapy and during letermovir (LMV) prophylaxis. Until more data on the issue of delayed CMV reactivation/disease is available, some authors postulate that patients with a high risk of CMV reactivation should be provided with prolonged LMV prophylaxis (after day +100) and CMV monitoring at least monthly over the six months after their HSCT. Longer monitoring is also recommended in patients after mismatched, cord blood or haploidentical transplantation, in

those with acute or chronic GvHD, or after prior CMV reactivation or if suffering from an immunodeficiency disorder [18]. PCR assays should be calibrated according to current standards [38].

A real time PCR test determines the amount of CMV genetic material in one milliliter of plasma or serum, and even though this value should be expressed in international units of the viral genome per milliliter [IU/mL], the use of [copies/mL] is still acceptable [39, 40].

It is worth noting that CMV disease can occur with any level of viremia and it is crucial to minimize the risk of CMV replication by the introduction of appropriate prophylaxis [4, 17, 41]. The preventive measures in stem cell recipients include both prophylactic and pre-emptive treatment (PET). Prophylactic treatment is recommended for high-risk patients before any evidence of CMV infection/reactivation occurs. The mainstay of primary prophylaxis is proper donor selection based on the CMV status of donor and recipient. Namely, for a seronegative recipient, a seronegative donor must be sought as the first-line option. For a seropositive recipient, a seropositive donor remains the choice [18]. Matching negative donors to positive recipients should be avoided. Other preventive strategies include a proper transfusion policy of CMV-negative, leukodepleted blood products [11, 42, 43].

The search for the safest and most effective prophylactic agent has been going on for years. Prior therapies have demonstrated numerous adverse effects when used prophylactically [42, 44]. LMV was approved in 2017 by the US Food and Drug Administration (FDA) for the prevention of CMV infection/disease in adult CMV-seropositive allo-HSCT recipients [42, 45]. LMV belongs to a new group of compounds that inhibit the CMV DNA terminase complex disrupting viral genome formation and the maturation of virions. It has been demonstrated in a phase III study that LMV compared to a placebo improved post-transplant survival and decreased CMV-related mortality, and without myelosuppression as a side effect [42].

The positive results from this phase III study have been also confirmed in a real-world setting. Real-world data shows significant improvements in reducing the risk of any CMV viremia and clinically significant CMV infection in studies. CMV primary prophylaxis with LMV has been shown to be effective in reducing the risk of all-cause and non-relapse mortality 200 days after allo-HSCT, and in improving OS during the first 24 and 48 weeks after HSCT [17, 42, 46–48].

LMV not only presents high efficacy and safety in preventing CMV reactivation in seropositive patients, but it also delays the onset of clinically significant CMV infection and at the same time does not delay granulocyte reconstitution. The clearest effect has been seen in high-risk patients.

LMV is a drug that changes the paradigm of PET use in favor of prophylaxis as first-line management strategy against CMV [7, 47, 49]. A Polish experience with LMV prophylaxis, published recently, confirms its low toxicity with no myelosuppressive (or any other) adverse effect and good tolerability [50]. Our own experience with more than 30 patients treated with LMV seems to confirm its efficacy and safety (data not published).

Primary prophylaxis with LMV should be started before day 28 after transplantation and continued for the first 100 days at a single dose of 480 mg per day (or 240 mg during concomitant use of cyclosporine) in the case of seropositive patients. In those with multiple risk factors of CMV reactivation/infection (e.g. seropositive and treated with escalated immunosuppression due to aGvHD), prolonged LMV prophylaxis after day +100 should be considered. Since LMV is active solely against CMV, acyclovir/valacyclovir prophylaxis against other common viruses (herpes simplex and varicella zoster) is required [18]. One should be aware of CMV DNAemia 'blips' that occur frequently after allo-HSCT (with ~32% prevalence), particularly in patients receiving a graft from CMV-seropositive donors and LMV prophylaxis. They are associated with a lower incidence of CMV end-organ disease [51]. A viral 'blip' is defined as an episode of isolated positive PCR test result where both the previous and the subsequent test, performed with seven days, remain negative. Blips could be either an artefact, or a reflection, of transient low-level CMV replication [51, 52]. In the case of first CMV PCR positive samples, blips should always be considered, and ongoing replication must be confirmed before starting anti-CMV treatment.

Case continued (III)

After discharge from hospital, our patient continued immunosuppressive therapy with oral MMF. During check-ups, weekly monitoring of CMV viral load level was continued, and virus remained negative. The symptoms of GvHD were absent and immunosuppressive treatment was gradually reduced. LMV was not reimbursed for Polish patients at that time, and so this medicine was not given despite the presence of unfavorable prognostic factors. On day +64, the patient was urgently readmitted with symptoms of intestinal acute GvHD accompanied by pulmonary infection. Prior CMV assessment had been done a week before and remained negative, making the primary diagnosis of GvHD more likely. On admission, the patient was in a poor condition overall: he presented with nausea, appetite loss, general weakness, and persistent cough. Physical examination was unremarkable except for cachexia. Peripheral blood picture showed pancytopenia. Recurrence of leukemia was ruled out. Laboratory tests revealed hyperbilirubinemia of 31 µmol/L, elevated liver and pancreatic enzymes [alanine aminotransferase (AlAT) = 388 units/L, glutamyl transpeptidase (GGTP) = 179 IU/L, amylase = 83 units/L, alkaline phosphatase = 96 units/L) as well as elevated C-reactive protein (CRP) (115 mg/L). *Clostridioides difficile* infection

was excluded. Blood culture was negative for any bacterial or fungal pathogens. *Mycoplasma pneumoniae* and *Pneumocystis jirovecii* assays were also negative. CMV by qPCR was as high as 14,386 copies/μL in the serum sample and CMV reactivation was confirmed. After CMV confirmation, immunosuppressive treatment was de-escalated. Colon biopsy was not performed due to the patient's worsening overall condition and the high risk of complications.

Clinical manifestations

CMV reactivation is typically reported within the first 100 days after allo-HSCT and is seen mostly in patients not receiving CMV prophylaxis. Late CMV reactivation (up to two years after HSCT) occurs mainly in patients with profound immune suppression, especially after prolonged exposure to anti-CMV drugs, after prophylaxis/PET discontinuation, or during chronic GvHD [11, 18, 23].

In the general population, apart from non-specific fever or mononucleosis-like syndrome, no clinical signs of CMV infection occur [14, 18, 19]. In immunosuppressed patients, a latent infection can reactivate and CMV replication may lead to life-threatening end-organ disease. The incidence of CMV disease in the early post-HSCT period is estimated to be 5–7% in high-risk transplant recipients. Early CMV disease most commonly presents with gastrointestinal (GI) involvement and CMV gastroenteritis/colitis accounts for more than 90% of clinical manifestations [18, 42, 52].

Interestingly, CMV gastroenteritis often develops without detectable CMV DNAemia, making a diagnosis challenging, especially in terms of differentiating from intestinal GvHD. Sometimes these two manifestations will overlap, and a tissue biopsy is necessary [53–56]. Another frequent CMV disease manifestation is CMV pneumonia, defined as the detection of CMV in bronchoalveolar lavage fluid (BAL) or a lung biopsy together with clinical signs/symptoms of pneumonia. Bronchoscopy with BAL is the recommended diagnostic procedure in suspected CMV pneumonia. There is no definitive cut-off value for CMV DNA load in BAL, but quantitative PCR can be used to distinguish CMV-induced pneumonia (viral load of >200–500 IU/mL) from asymptomatic pulmonary shedding (viral load lower than 200 IU/mL) [18]. BAL fluid negative for CMV DNA has a negative predictive value of nearly 100%, and practically rules out CMV pneumonia.

Late CMV disease occurs in up to 15% of high-risk patients, mostly in the form of interstitial pneumonitis [18, 57]. Other frequent CMV disease manifestations are hepatitis, retinitis, encephalitis, and bone marrow suppression [18]. Prophylactic strategies used in the early post-transplant period increase the risk of late CMV disease in up to 25% of patients by delaying the recovery of CMV-specific T-cells [10, 56, 58]. It is well-documented that the greater the viremia the worse the prognosis, but even a relatively low viral load affects the outcome negatively [4].

Case continued (IV)

Starting from day +65, treatment with intravenous ganciclovir (GCV) at 5 mg/kg of bw was implemented, and immunosuppressive treatment and low doses of methylprednisolone and MMF were continued. One week later, the patient presented with a 38°C fever with chills, persistent, non-productive cough, and malaise. Chest X-ray depicted massive pneumonia; high-resolution computed tomography (HRCT) showed bilateral diffuse ground glass infiltrates with interlobular septal thickening. Bronchoscopy with bronchoalveolar lavage was performed and CMV DNA of 502 copies/mL was detected in the BAL, and GCV treatment was continued.

Six days after readmission, the patient's condition rapidly deteriorated with dyspnea and oxygen saturation of 85% despite maintained antiviral treatment. Increasing inflammatory parameters, deepening pancytopenia, and progressive respiratory insufficiency were observed. The patient died on day +72 after transplantation amid symptoms of cardiopulmonary and multiorgan failure.

Treatment and management

Pre-emptive treatment

Despite an attempt to harmonize and standardize CMV-DNA measurements made in 2010 by the World Health Organization (WHO), there is still no universal CMV-DNAemia threshold at which PET should be initiated [19, 40].

The obtained results vary and depend not only on the test sample (plasma or whole blood) but also on the experience of the transplant center. Monitoring of plasma CMV DNA load kinetics with evaluation of viral load doubling time may offer a clue as to when to start therapy. It has been suggested that in those with a doubling time <2 days, therapy should be started [18, 59, 60]. In high-risk patients, it is recommended to start PET at a lower viral load, i.e. >150 IU/mL, and in low-risk patients at >500 IU/mL [58].

According to the 2017 European Conference on Infections in Leukemia (ECIL-7) recommendations, intravenous GCV at 5 mg/kg of bw twice daily or foscarnet at 60 mg/kg of bw twice daily show comparable efficacy and should be offered as first-line PET [18, 61]. The oral form of GCV — valgancyclovir (VGCV) 900 mg twice a day — is also acceptable except for patients with severe intestinal GvHD. Treatment should last for a minimum of two weeks and be continued until CMV PCR negativity. GCV or foscarnet are also recommended for maintenance treatment. In second-line PET, an alternative drug to that used in the first line should be given. Cidofovir at 3–5 mg/kg of bw weekly can also be recommended, with special caution regarding renal function. Administration of intravenous immunoglobulins (IVIg) is not recommended [18].

For the treatment of symptomatic CMV disease, the therapeutic armamentarium is similar to that available for PET [62]. Therapeutic doses of anti-viral agents should be continued until CMV PCR negativity, but then a 4-week maintenance should be considered. Of note, an increase in viral load observed during the first seven days after treatment initiation does not prove its ineffectiveness or drug resistance [18]. Therefore, discontinuation of therapy in such a situation is not justified. It is also worth noting that starting treatment at a lower CMV viral load results in faster elimination of the virus, which reflects the treatment efficacy and prevents the induction of drug resistance [17, 18, 58].

CMV resistance to antivirals should be considered if therapy has failed after more than three weeks of treatment. A persistent or increasing CMV antigenemia/DNA load or escalating organ manifestations of CMV disease may indicate the development of either clinical or viral resistance. Clinical refractoriness is observed when CMV DNA levels in blood or plasma increase by >1 log10 after at least two weeks of appropriately selected and properly administered antiviral drugs, and clinical resistance occurs when CMV disease symptoms worsen after two weeks of suitable antiviral therapy. Viral resistance is defined by the presence of known mutations that reduce the virus's sensitivity to one or more antivirals. Genetic testing is recommended when the CMV viral load does not decrease by >1 log10 after more than two weeks of properly applied therapy. It is also advised when the plasma viral load exceeds 1,000 IU/mL. It has been demonstrated that mutations in UL97 are mainly responsible for GCV resistance. When resistance is clinically suspected, its type needs to be confirmed, the drug class should be switched, and immunosuppression should be reduced if possible. The choice of drug for a confirmed mutation should be based on the type of mutation, previous exposure to drugs, and acceptable toxicity profile. If high doses of GCV are required, pre-emptive administration of filgrastim (G-CSF) should be considered. Combination therapy is not recommended due to the lack of data confirming its efficacy, with the risk of cumulative nephrotoxicity and myelotoxicity of these drugs [55, 56].

Regarding the issue of managing drug-induced toxicity, experts do not recommend monitoring (V)GCV levels, as peak plasma levels do not correlate with either clinical efficacy or myelotoxicity. In cases of acute kidney failure, the drug dosage should be adjusted, and other potentially nephrotoxic medications should be reduced. In cases of neutropenia, reducing the drug dose when treating active CMV infection is not recommended considering the risk of drug resistance developing. Instead, G-CSF should be used, (V)GCV should be replaced with foscarnet, and myelotoxic drugs such as MMF should be temporarily reduced, replaced, or withdrawn [53].

Conclusions and future directions

The following steps would decrease the risk of post-transplantation CMV reactivation/disease: proper selection of donor and recipient; regular and careful monitoring; an early intervention in CMV reactivation; and rapid and effective treatment when disease develops [1, 58].

The use of PET has resulted in a decline in the incidence of CMV-related end-organ disease and this has translated into better post-transplantation outcomes [18, 61]. To date, LMV prophylaxis (provided at least to day +100) changes the well-established pattern of CMV management policy in seropositive patients from monitoring and pre-emptive therapy to a preventive approach [17, 46].

Although the available anti-CMV drugs have demonstrated efficacy in preventing and managing post-transplant CMV infection, the risk of toxicity and resistance limits their long-term use [63]. Hence, there is a constant need for newer, safer therapies to be developed. Adoptive cell therapy (ACT), by transferring virus-specific donor T-cells to an immunocompetent recipient, has come into use as a rational approach to induce rapid and sufficient viral immunity in patients until they achieve optimal immune reconstitution. However, obstacles such as regulations, logistics and time-consuming virus-specific T-cell selection techniques, limit the widespread implementation of this therapy. CMV vaccines remain under development [18, 53, 64].

Several novel drugs are currently in development. Maribavir is one of them, and is active against CMV including strains resistant to GCV or foscarnet. It was approved by the FDA in 2021 and in November 2022 by the European Medicines Agency (EMA) for the treatment of recurrent CMV infection and/or disease after the failure of at least one prior therapy in adult transplant patients. In a randomized phase III study (NCT02931539), oral maribavir at a dose of 400 mg twice daily showed high efficacy with significantly lower renal toxicity and neutropenia rates. A phase III randomized trial determining the utility of extended (i.e. beyond day 100 from transplant) LMV prophylaxis (#NCT03930615) and a single-center phase II study of LMV use in relapsed/refractory CMV infections (#NCT03728426) are underway. Promising results from a phase II study of posoleucel (#NCT04693637) were unveiled at the 64th American Society of Hematology (ASH) Annual Meeting in 2022. This study evaluated the efficacy and safety of posoleucel in preventing clinically significant viral infections caused by six target viruses, including CMV. It investigated both prophylaxis in patients at high risk of viral reactivation, and PET in those experiencing viral reactivation. As a result, a significant reduction in clinically significant viral infections and also a long-term effect of the drug on the expansion of functional CMV-specific T-cells accompanied by a decrease in viral load were observed. This study has progressed to phase III (#NCT05305040) for further evaluation [65].

Authors' contributions

MW — collected data and wrote manuscript. GH — co-wrote manuscript, critical revision.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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ORIGINAL RESEARCH ARTICLE

VIA MEDICA

FAM therapy in children with bronchiolitis obliterans syndrome after allogeneic hematopoietic stem cell transplantation

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Abstract

Introduction: Bronchiolitis obliterans syndrome (BOS) is one of the most frequent late-onset non-infectious pulmonary complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT). It can lead to progressive respiratory insufficiency, and even death. BOS is a clinical term and does not necessarily require histological confirmation. A retrospective analysis of the clinical course in eight pediatric patients with BOS after allo-HSCT was performed.

Material and methods: The diagnosis of BOS was defined by the NIH-2014 criteria including pulmonary function tests and high resolution computer tomography scanning. Lung function score was simplified to forced expiratory volume in one second (FEV_1) values alone and symptoms score.

Results: FAM therapy (inhaled fluticasone, azithromycin and montelukast) was added to systemic immunosuppressive treatment typical for chronic graft-versus-host disease (cyclosporine, steroids) and continued after cessation of immunosuppressive therapy or was begun from the start as a separate treatment option. An improvement of lung function was observed in seven patients, while one patient deteriorated in FEV_1 test without clinical exacerbation.

Conclusion: Systemic corticosteroids remain the recommended first-line therapy for patients diagnosed with BOS in severe cases. Combination therapy with FAM may spare patients from systemic steroids and attenuate the need for prolonged systemic corticosteroid therapy.

Key words: allogeneic hematopoietic stem cell transplantation, non-infectious pulmonary complications, children

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Introduction

Bronchiolitis obliterans syndrome (BOS) is one of the most common, non-infectious, late-onset respiratory complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. The disease process leads to obturation and/or obliteration of the bronchioles, i.e. the final conductive airways in the respiratory system, up to 1–2 mm in diameter, through the inflammatory and fibrous tissue [2].

A pulmonary complication can present at any time, but in most cases does so within the first two years after transplantation, with various concomitant manifestations of chronic graft-versus-host disease (GvHD) in other organs. The prevalence of BOS in children is estimated at 3–6% [3]. The occurrence of pulmonary complications increases by several times the risk of transplantation-related death [4, 5]. Histopathological confirmation is difficult due to the low sensitivity of transbronchial biopsy and the potential

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complications of open lung biopsy. In 1993, the International Society for Heart and Lung Transplantation provided a clinical definition of BOS based on lung function, rather than histopathology criteria. Studies conducted over the next 20 years confirmed this thesis, and showed that there is no superiority of a histopathological examination over a diagnosis based on clinical symptoms, lung function tests and radiological examinations. Finally, in 2018, it was recognized that BOS is a clinical diagnosis based on functional tests without the need for histopathological confirmation [6, 7].

The multitude of potential causative factors indicates a multifactorial etiology of the disease, including drug-induced toxicity, radiation, opportunistic infection, and immunological reactions as well as individual susceptibility to the development of pulmonary complications, e.g. associated with respiratory efficiency before conditioning. Risk factors for the development of late pulmonary complications include methotrexate use for GvHD prophylaxis, hypogammaglobulinemia, history of acute GvHD, viral respiratory infections within the first 100 days after transplantation, conditioning with busulfan, transplantation of peripheral blood hematopoietic cells, and a history of interstitial pneumonia. Bronchiolitis obliterans affects mainly patients after allogeneic bone marrow transplantation; reports of the development of this disease after autologous transplantation are very rare [8].

Diagnosis of the disease is based on clinical symptoms, respiratory function tests and radiological examinations of the lungs using high resolution tomography. Clinical symptoms include cough, decreased exercise tolerance, and shortness of breath. The onset of the disease is often insidious, and clinical symptoms may only become apparent many years after a bone marrow transplant. High-resolution tomography shows airway damage in the form of thickening of the walls of bronchioles or widening of their lumen, or the presence of an air trap caused by segmental obstruction of the bronchioles [8].

In functional tests, BOS manifests as a new obstructive lung ventilation disorder. FEV_1 is recognized as the most reliable indicator of airway flow restriction and is considered to be a key parameter in the early detection of BOS. Patients with BOS may have an 'occult obstruction' on spirometry due to the early collapse of the bronchioles during forced expiration. This causes an underestimation of forced vital capacity (FVC), and consequently a false overestimation of the FEV_1/FVC obstruction index [9].

Treatment depends on the severity of the clinical course and the presence or absence of GvHD symptoms in other organs. Immunosuppressive treatment (cyclosporin, steroids), FAM regimen, and extracorporeal photopheresis (ECP) are the most commonly used.

The aim of this study was to assess the effectiveness of diagnostic and therapeutic procedures in pediatric patients undergoing allo-HSCT with late pulmonary complications in the form of bronchiolitis obliterans.

Material and methods

Study design

A retrospective analysis of the disease course and treatment results in pediatric patients after allo-HSCT with symptoms of lower respiratory tract disorders suggestive of BOS was performed. The patients underwent functional and imaging tests of the respiratory system, followed by appropriate treatment, and the effects of the adopted diagnostic and therapeutic procedures were assessed.

Patients

Study participants comprised pediatric patients after allo-HSCT performed between 2007 and 2022 at the Department of Bone Marrow Transplantation at the Antoni Jurasz University Hospital No. 1, *Collegium Medicum* in Bydgoszcz, Poland. Lung function tests, including spirometry and body plethysmography, were performed with MES LUNGTEST 1000 apparatus (MES, Kraków, Poland).

BOS diagnosis criteria

The adopted criteria for BOS diagnosis were based on pulmonary functional tests (PFTs) and high-resolution computed tomography (HRCT) according to the National Institutes of Health (NIH) (Table I). A BOS diagnosis requires meeting the listed spirometric criteria or showing functional progression (a decrease in FEV_1), ruling out an infection, and demonstrating the presence of an air trap in a radiological examination or body plethysmography [10].

The NIH criteria do not identify clinical conditions where there is a parallel decrease in FEV_1 and FVC with a normal FEV_1/FVC ratio. Such a spirometry pattern is common, and results from lung distension in the course of bronchiole disease due to BOS [11].

Disease severity criterion

FEV_1 has been established as a disease severity criterion [12], wherein the disease is classified as mild (FEV_1 >60%), or moderate (40–59%) or severe type (<39%).

Clinical evaluation of lung function

The updated National Institutes of Health criteria for clinical evaluation of chronic graft-versus-host disease (cGvHD) were used in this study. Lung Symptoms Score (LSS) included clinical symptoms (dyspnea) and spirometry (FEV_1 measurement) (Table II). When there was a discrepancy between stages, the parameter with the higher score was decisive [13].

Radiological examination

HRCT was performed in all patients at baseline and after treatment. Air trapping, wall thickening, and bronchiectasis were assessed.

Table I. Bronchiolitis obliterans syndrome (BOS) diagnostic criteria

I. Functional criteria — obstructive ventilatory disorders
1. FEV_1/FVC <5 percentile*
2. FEV_1 <75% predicted value with >10% decline in less than two years

II. Clinical criterion — ruling out respiratory infections

III. Confirmation of an air trap — presence of at least one of two BOS features
A. Presence of air trap in expiratory phase on HRCT or thickening of walls of small bronchi or presence of bronchiectasis
B. Presence of lung distension ('air trap') in functional tests: RV >120% of predicted value or RV/TLC ratio >95 percentile

*Normal range 5–95 percentile; FEV_1/FVC — forced expiratory volume in one second to forced vital capacity ratio; FEV_1 — forced expiratory volume in one second; HRCT — high-resolution computed tomography; RV — residual capacity; TLC — total lung capacity

Table II. Lung Symptoms Score

Score	Clinical symptoms	FEV_1 [%]
0	No shortness of breath	≥80
1	Mild degree: shortness of breath when climbing stairs	60–79
2	Moderate degree: shortness of breath when walking on flat ground	40–59
3	Severe degree: shortness of breath at rest, requires oxygen therapy	≤39

FEV_1 — forced expiratory volume in one second

BOS treatment

Recommendations [14] regarding monitoring and treatment of sudden lung function deterioration of obstructive type were adopted. In patients with FEV_1 >70% treatment with inhaled steroids was initiated, and in cases of a lower spirometric index or disease progression, systemic steroids were added. A FAM regimen was introduced additionally to systemic immunosuppressive treatment and continued during tapering and after discontinuation of immunosuppression, or was used as the only therapeutic option. The treatment scheme included oral administration of azithromycin at a dose of 5 mg/kg bw (max 250 mg) once a day, three days a week, for children 0–5 years; the anti-leukotriene drug (montelukast) at a dose of 5 mg for children 6–14 years and 10 mg for children >15 years once a day; and inhaled fluticasone propionate 250 µg twice a day for children 6–11 years, and 500 µg twice a day for children over 12 [15, 16].

Response to treatment

Response was assessed according to the PFTs criteria and clinical symptoms (NIH LSS) [13]:

- complete response (CR): normal FEV_1 after previously decreasing, score 0;
- partial response (PR): 10% increase in FEV_1, score decrease by 1 or more;
- progression: FEV_1 decrease by 10%, score increase by 1 or more except from 0 to 1.

Functional testing plan

Functional tests were performed at diagnosis, and then every 3–4 months until treatment discontinuation.

Results

Patient characteristics

Eight patients were included in analysis of symptoms, diagnosis and treatment of BOS after allo-HSCT. Clinical characteristics of patients according to risk factors for pulmonary complications after HSCT, including BOS, are set out in Table III.

Analysis of clinical course

The symptoms and clinical course of BOS in the eight analyzed patients are set out in Table IV. In two cases, lung function abnormalities preceded radiological changes. In patients 3 and 4, the diagnosis of BOS was associated with a sharp decrease in FEV_1, respectively 26% within three months and 25% within two months.

Obstructive ventilatory disorders were confirmed in 6/8 patients. The obturation reversibility test was negative and the obstruction was irreversible. Spirometry with concomitant decrease in FEV_1 and VC with normal FEV_1/VC ratio occurred in two patients. In these two, the reduced FEV_1 and VC parameters also did not meet the improvement criteria after the use of bronchodilators. Examinations in the body plethysmography cabin in seven patients showed features of an air trap. Mild BOS was found in four patients and moderate BOS in the other four. Patient 1 had an upgraded Lung Symptoms Score due to the severity of clinical symptoms (score 3 with FEV_1 = 42%) (Table V):

- vital capacity (VC) includes FVC or maximum vital capacity (VC_{max}), whichever is higher;
- in Patient 1, body plethysmography was not performed due to severe airway obstruction. After clinical improvement, he met an air trap functional criterion (RV >120%). A normal TLC value excludes restrictive changes in lung parenchyma.

Therapy results

In the therapy of patients with BOS, the FAM regimen was used, either in combination with systemic steroid therapy (in five patients) or alone (in the other three) (Table VI). Four patients had a complete response, three patients had a partial response, and the other patient progressed

Table III. Patient characteristics and risk factors for bronchiolitis obliterans syndrome (BOS)

BOS risk factor	Patient							
	1	2	3	4	5	6	7	8
Age at time of transplantation [years]	17	6	6	6	8	8	3	14
Gender	M	F	M	M	M	F	F	F
Underlying disease	T-ALL-HR	ALL-HR, relapse after allo-HSCT (2015)	ALL-HR	AML	Transformation of MDS into AML	SAA	ALL — bone marrow relapse	ALL — second late bone marrow relapse
Transplantation type: 10/10 HLA-matched unrelated donor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Source of hematopoietic cells: peripheral blood	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Conditioning with busulfan	No	Yes	Yes	Yes	Yes	No	No	No
Total body irradiation (TBI)	Yes	No	No	No	No	No	No	Yes
Infections within 100 days after transplantation	No	Yes (UTI, sepsis, CMV)	Yes (cystitis, BKV)	Yes (UTI, CMV, BOOP)	Yes (invasive pulmonary fungal disease)	Yes (pneumonia)	Yes (CMV, adenovirus, cystitis, BKV)	Yes (CMV, invasive pulmonary fungal disease, UTI)
Interstitial pneumonia	No	No	No	Yes	Yes	Yes	No	Yes
Hypogammaglobulinemia (substitution in months)	Yes (12)	No	Yes (6)	No	Yes (5)	Yes (19)	Yes (9)	Yes (10)
Acute GvHD	No	No	Yes (skin and intestine, stage II)	No	Yes (skin, stage I; intestine stage II)	Yes (skin, stage III; intestine, stage I)	Yes (skin and intestine, stage I)	Yes (skin, stage II)
Chronic GvHD in other organs	No	No	No	No	No	Yes (skin, oral cavity, genitourinary organs, eye, GI)	No	Yes (skin, oral cavity, eye, GI, liver)
Methotrexate in prevention of GvHD	No	Yes	No	Yes	No	Yes	Yes	No

ALL-HR — acute lymphoblastic leukemia, high-risk group; allo-HSCT — allogeneic hematopoietic stem cell transplantation; AML — acute myeloblastic leukemia; BOOP — bronchiolitis obliterans organizing pneumonia; CMV — cytomegalovirus; GI — gastrointestinal; GvHD — graft-versus-host disease; HLA — human leukocyte antigen; MDS — myelodysplastic syndrome; SAA — severe aplastic anemia; T-ALL-HR — T-cell acute lymphoblastic leukemia, high-risk group; UTI — urinary tract infection

with deterioration of functional tests and simultaneous improvement of clinical ventilatory efficiency. Three patients were qualified for extracorporeal photopheresis (ECP) due to complications after systemic steroid therapy and the presence of chronic GvHD in other organs, while continuing the FAM regimen [17, 18]. Patient 2 was taking inhaled ciclesonide due to fluticasone intolerance. Due to the progression shown in the spirometric examination, she was qualified for further extracorporeal photopheresis procedures. The remaining patients are under clinical observation and undergoing control respiratory function tests.

Table IV. Signs and symptoms of bronchiolitis obliterans syndrome (BOS) in study group

Patient	1	2	3	4	5	6	7	8
Signs	Dyspnea, decreased exercise tolerance	Decreased exercise tolerance			Cough	Cough	Decreased exercise tolerance	Decreased exercise tolerance
Symptoms	Tachypnea	Crackling, wheezing	None	None	Wheezing, bronchi	None	Crackling	None
Time since transplantation	54 days	13 months	38 months	54 months	17 months	9 months	8 years	11 months
Changes in HRCT typical for BOS	None	Yes	Yes	None	Yes	Yes	Yes	Yes

HRCT — high-resolution computed tomography

Table V. Bronchiolitis obliterans syndrome diagnosis in analyzed patients

Patient	1	2	3	4	5	6	7	8
FEV_1/VC	<1 percentile	Normal but FEV_1 and VC <1 percentile	<1 percentile	3 percentile	<1 percentile	<1 percentile	<1 percentile	Normal but FEV_1 and VC <5 percentile
Obturation reversibility test	Negative	No improvement	Negative	Negative	Negative	Negative	Negative	No improvement
FEV_1	42% <1 percentile	71% <1 percentile	72% <1 percentile	65% <1 percentile	53% <1 percentile	74% <1 percentile	57% <1 percentile	65% <5 percentile
RV >120%	Not tested	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Air trap in HRCT	No	Yes	Yes	No	Yes	Yes	Yes	Yes
Thickening of bronchial walls	No	No	No	No	Yes	No	Yes	No
Bronchiectasis	No	No	No	No	No	No	Yes	No
Infection ruled out	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Histopathological confirmation	No	Yes	No	No	No	No	No	No
Ruling out of inflammatory infiltrates in X-ray	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
TLC	Not tested	Normal	>90 percentile	>90 percentile	Normal	>90 percentile	>90 percentile	>90 percentile
Lung Function Score	3	1	1	1	2	1	2	1

FEV_1 — forced expiratory volume in one second; FEV_1/VC — forced expiratory volume in one second to vital capacity ratio; RV — residual volume; HRCT — high-resolution computed tomography; TLC — total lung capacity

Discussion

The development of BOS is closely related to the presence of chronic GvHD in other organs, and according to some authors it is a manifestation of chronic GvHD in the lungs. Isolated BOS without symptoms of chronic GvHD in other organs is often observed in pediatric patients [19–21]. In the study group, chronic GvHD outside the respiratory system occurred in two patients. Early clinical signs include non-productive exercise-induced cough, decreased exercise tolerance, and wheezing. There is also a group of patients without clinical symptoms in the initial period in whom a decrease in lung function is detected in subsequent functional tests. In our patients, we performed functional tests after the occurrence of respiratory symptoms or chronic GvHD in other organs. Physical examination findings are nonspecific (diffuse crackles, wheezing) and may be absent despite NIH Lung

Table VI. Therapeutic response in study group

Patient	Duration of FAM treatment	Baseline FEV_1, LSS	Treatment discontinuation FEV_1, LSS	Response to treatment
1	26 months	FEV_1 = 42%, score 3	FEV_1 = 63%, score 0	CR
2	33 months	FEV_1 = 64%, score 1	FEV_1 = 42%, score 0	Progression
3	14 months	FEV_1 = 72%, score 1	FEV_1 = 97%, score 0	CR
4	16 months	FEV_1 = 65%, score1	FEV_1 = 76%, score 0	CR
5	23 months	FEV_1 = 53%, score 2	FEV_1 = 48%, score 1	PR
6	14 months	FEV_1 = 74%, score 1	FEV_1 = 99%, score 0	CR
7	14 months	FEV_1 = 57%, score 2	FEV_1 = 55%, score1	PR
8	14 months	FEV_1 = 65%, score 1	FEV_1 = 74%, score 0	PR

FAM — inhaled fluticasone, azithromycin and montelukast; FEV_1 — forced expiratory volume in one second; LSS — Lung Symptoms Score; CR— complete response; PR — partial response

Symptoms Score >0. In the study group, four patients had no signs with a score of 1.

The disease has differing clinical courses. It may manifest as a sudden deterioration of lung function with shortness of breath and a decrease in saturation. This situation occurred in 1/8 patients in the study group in the early post-transplant period (day 54). After completion of combination therapy, including continued FAM treatment for 26 months, this patient achieved a complete clinical response.

In some patients, there is a gradual decline in respiratory efficiency, although periodic exacerbations with long periods of stable lung function are also observed. Clinical symptoms and specific functional tests results may precede typical BOS-related radiological changes in HRCT, and therefore meeting the imaging tests criterion is not necessary in order to make a diagnosis. In the study group, two patients had no symptoms of BOS in lung tomography at the time of diagnosis. All patients met the spirometric criteria and presented clinical respiratory symptoms. Evaluation of lung function based on NIH LSS including only the signs and FEV_1 value in % correlates with survival rates [22]. Obturation, which increases over time, is a symptom of progressive bronchiole fibrosis and, ultimately, a decrease in vital capacity (VC). Revealing the deterioration of lung function in functional screening tests may contribute to the earlier detection of patients at risk of developing BOS after bone marrow transplantation.

The introduction of treatment according to the FAM framework in oligosymptomatic patients can limit the development of the disease and reduce the use of systemic steroids. In other cases, detection of progression via a spirometric test will allow for the swifter introduction of more intensive immunosuppressive treatment in order to stop the irreversible process of bronchiole fibrosis. According to published reports, the results of functional tests (VC, FEV_1) correlate with survival rates in patients after allogeneic bone marrow transplantation [22, 23]. After the treatment, in seven of the eight patients receiving FAM therapy, improvement of lung function parameters was observed, including improvement of exercise tolerance and an increase in or a stabilization of the FEV_1 parameter in spirometry. The treatment was safe. No side effects were observed, but in one patient the inhaled drug was switched due to intolerance.

In conclusion, the FEV_1 parameter is the best recognized and most reliable indicator of airflow in the airways: its decrease indicates the severity of airflow obstruction. Performing follow-up spirometry tests in patients after bone marrow transplantation enables earlier identification of patients at risk of developing BOS. Earlier implementation of treatment increases the chance of stopping the fibrosis process and the decline in lung function [24].

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Authors' contributions

BT — sole author.

Conflict of interest

The author declares no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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Central neurotoxicity as complication in course of treatment of acute lymphoblastic leukemia in children: a single center experience

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Abstract

Introduction: Neurotoxicity is a common and severe complication of the treatment of acute lymphoblastic leukemia in children, and affects 10–15% of patients. The aim of this study was to show the characteristics of this group over the course of time, the outcomes of patients, and to evaluate possible clinical risk factors for central nervous system toxicity.

Material and methods: Clinical data from patients hospitalized between 2003 and 2018 was obtained from hospital records and assessed retrospectively. Additional data was obtained to characterize the group of neurotoxic events. Statistical analysis was used to describe study group and intragroup associations, as well as event-free survival (EFS), relapse-free survival (RFS), and overall survival (OS).

The cohort comprised 224 patients (median age 5.64 years), consisting of 130 boys (58%) and 94 girls. 129 of them were treated with Protocol ALLIC BFM 2002 (57.6%), and 95 with Protocol ALLIC BFM 2009.

Results: Twenty-one patients (9.37%) developed subacute central neurotoxicity, which comprised posterior reversible encephalopathy syndrome, stroke-like syndrome and seizures, defined according to the Ponte di Legno working croup criteria. The 5-year OS and EFS of the analyzed group were 85.11% [95% confidence interval (CI): 8.32–89.82%] and 80.03% (95% CI: 74.69–85.38%) respectively. There was a statistically significant difference in EFS and RFS between neurotoxic and non-neurotoxic patients ($p = 0.00082$ and $p < 10^{-5}$ respectively), but this did not affect overall survival ($p = 0.10$). In multivariate analysis, the risks of death, adverse events and relapses were increased in patients belonging to the neurotoxicity group [hazard ratio (HR) 3.18, 95% CI: 1.26–8.06, HR 4.96, 95% CI: 2.4–10.22, HR 7.22 95% CI: 3.21–16.24, respectively).

Conclusion: The occurrence of neurotoxicity might be associated with poorer prognosis among pediatric patients with ALL.

Key words: acute lymphoblastic leukemia, children, neurotoxicity, survival

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common neoplastic disease in children, with a frequency of 35.4/1,000,000 patients [1]. Due to intensive multi-agent chemotherapy, treatment stratification directed by somatic mutations and early responses to chemotherapy, and good supportive care therapy of complications, 5-year overall survival (OS) is 90% [2]. Mortality among patients is caused more frequently by the therapy rather than by the leukemia itself. Understanding of non-infectious chemotherapy-associated acute toxicities remains limited, including the best ways to prevent and treat them. Neurotoxicity affects 10–15% of children with ALL over the course of treatment [3]. Frequently, these symptoms can endanger the lives of the patients.

Among many cytostatic drugs used in the treatment of ALL, methotrexate (MTX), cytarabine, vincristine, cyclophosphamide, iphosphamide and etoposide are the most commonly found to cause neurological complications [4]. Their interactions with many other medical substances or clinical states promote the appearance of neurotoxic symptoms.

In this paper, we present a single center experience of central subacute neurotoxic events in children with ALL treated between 2003 and 2018, show the characteristics of this group over the course of time and the outcomes of patients, and try to indicate clinical risk factors and discuss problems and controversies in the management of this clinical state, as well as to contemplate possible ways of preventing such incidents.

Material and methods

Patients and therapy

This retrospective analysis included children suffering from ALL who were treated in the Department of Pediatrics, Oncology and Hematology in the Medical University of Lodz, Poland between 2003 and 2018. All patients diagnosed with ALL and treated according to the ALLIC BFM 2002 and ALLIC BFM 2009 protocols were included in the study. Clinical data was obtained from hospital records and assessed retrospectively. We identified treatment protocol, age at onset, sex, leukemia variant, prognostic risk group [standard risk (SR), intermediate risk (IR)and high risk (HR)], central nervous system (CNS) status, steroid response, white blood cell count at diagnosis, main cytogenetics information (MLL rearrangement, *BCR/ABL* mutation), date of diagnosis, date of relapse or death, and date of last follow-up. Among all patients, a group of children who suffered from neurotoxic events was created, and additional data concerning these complications was collected — i.e. laboratory test abnormalities, the presence of other infectious or non-infectious complications of the chemotherapy in the last three weeks, any other drugs administered in the last seven days (especially MTX), symptoms of the incident, prophylaxis with theophylline, and information as to whether the neurotoxicity influenced further treatment schedule. Descriptions of the imaging diagnostic procedures were performed by an experienced radiologist and neurological events were qualified by an interdisciplinary team. This study was approved by the Bioethics Committee of the Medical University of Lodz (RNN/96/19/KE).

The analyzed central subacute neurotoxic incidents were divided into three specific groups according to the descriptions of toxic organ impairments specific for childhood acute lymphoblastic leukemia created in 2016 by the Ponte di Legno working group: i.e. seizures, methotrexate-related stroke-like syndrome (SLS), and posterior reversible encephalopathy syndrome (PRES). Although one patient did not meet the time criterion of diagnosing SLS, due to a very characteristic clinical and radiological image the child was qualified as SLS. Another case, of a girl who had typical stroke-like syndrome after liposomal cytarabine given intrathecally, was also qualified to the study as an SLS case.

Methotrexate-related stroke-like syndrome

According to the Ponte di Legno working group criteria, SLS can be diagnosed in cases of neurotoxic symptoms occurring within 21 days of intravenously (i.v.) or intrathecal (i.t.) administration of MTX with three characteristics, provided that all three are fulfilled:

1) new onset of one or more of the following: paresis or paralysis; movement disorder or bilateral weakness; aphasia or dysarthria; altered mental status including consciousness (e.g. somnolence, confusion, disorientation, and emotional lability); and/or seizures with at least one of the other symptoms;
2) characteristic, though often transient, white matter changes indicating leukoencephalopathy on magnetic resonance imaging (MRI) or a characteristic clinical course with waxing and waning symptoms, usually leading to complete (or sometimes partial) resolution within seven days;
3) no other identifiable cause [5].

Although seizures are a common feature of SLS, they can also occur without the other symptoms of SLS syndrome and may have a completely different pathophysiology. Therefore, although seizures are listed among the neurological symptoms, as an isolated symptom they do not fulfill the diagnostic criteria for SLS. The great majority of patients make a full recovery, although there have been reports of persistent neurological deficits in some cases.

To make a diagnosis of SLS, there is no need to perform imaging diagnostic procedures, although the great majority of patients have this done in order to differentiate from other pathologies e.g. stroke, hemorrhagic stroke or infiltrations of the CNS. In MRI T2-weighted scans in some patients there are visible hyperintensive white matter changes

around lateral ventricles. A few days later, an increase in intensity of those changes is usually observed, despite clinical recovery. Magnetic resonance imaging allows clinicians to distinguish between stroke-like syndrome and posterior reversible encephalopathy syndrome by opposing effects on measured apparent diffusion co-efficient (ADC) values, with SLS having reduced ADC values (due to cytotoxic edema), and PRES having increased values (due to vasogenic edema) [6].

The management of stroke-like syndrome is mainly symptomatic treatment: anti-edematous, anticonvulsant, administration of folinic acid (leucovorin) 15 mg/m^2 i.v. or higher doses depending on MTX serum concentration, as well as aminophylline — adenosine receptor antagonist [doses 2–5 mg/kg of body weight (bw)/dose i.v. or orally] [7]. Other therapeutic options include the administration of dextromethorphan 1–2 mg/kg of bw orally [8].

Posterior reversible encephalopathy syndrome

PRES is a clinical diagnosis including transient headache, confusion, seizures, and visual disturbances combined with characteristic, but transient, contrast-enhanced and diffusion-weighted imaging MRI findings. In making this diagnosis, electroencephalography (EEG) changes, as well as the presence of hypertension, may be helpful [5]. The incidence of PRES in the pediatric ALL population varies from 1.6% to 4.5% [9]. Most often it occurs during the three first months of therapy [10]. Immunosuppressive or cytotoxic drugs, autoimmune disorders, renal failure, and sepsis are all known triggers of PRES [11]. Endothelial and blood–brain barrier dysfunction are the main underlying pathophysiological mechanisms causing PRES [12]. Typical MRI findings include: patchy (and confluent) cortical (and subcortical) territory lesions in the cortex, and subcortical white matter mainly in the parietal and occipital lobes and cerebellum. Despite the word 'reversible' in its title, PRES generally has a good outcome but is not always reversible. Many sequelae, such as cerebral hemorrhage, cerebral infarction, focal gliosis, brain atrophy, and cerebral necrosis have been observed when vasogenic brain edema develops into cytotoxic brain edema [13]. Mortality in patients admitted to the intensive care unit has been assessed at 3–6%. The recurrence of PRES takes place in 4–8% of cases [14].

Older age, T-cell ALL (T-ALL), the involvement of the CNS, the presence of hypertension or hypomagnesemia, and treatment with calcineurin inhibitors or steroids are known risk factors for the occurrence of PRES [14]. Banerjee et al. [15] noted that the general outcomes of PRES patients are worse than other children with ALL (5-year OS 79.5% vs. 88.4%) and also that this group more often suffers from relapse of leukemia (45% vs. 20%). Treatment is mainly symptomatic and there is no consensus on the preferable drugs, nor on how long to maintain antiepileptic therapy [14].

Seizures

Seizures are defined by the Ponte di Legno working group as sudden, involuntary skeletal muscle contractions of cerebral or brainstem origin. They can be graded according to CTCAE: Grade 1 — brief partial seizure; Grade 2 — brief generalized seizure; Grade 3 — multiple seizures despite medical intervention; Grade 4 — life-threatening prolonged repetitive seizures; and Grade 5 — death [5]. Children with ALL treated with various protocols have had an incidence of seizures of between 1.5% and 13% [10]. Female sex, older age T-cell leukemia, CNS involvement at diagnosis and induction with dexamethasone are known to cause a higher risk of seizures [16, 17]. Epilepsy diagnosis after seizures has been reported in more than 10% of ALL survivors [17]. Long-term anticonvulsant therapy increases the systemic clearance of several antileukemic agents (e.g. teniposide, MTX), and is associated with lower efficacy of chemotherapy [18].

Statistical analysis

Study group characteristics and intragroup associations were established using Chi^2 and Mann-Whitney U-tests as well as Spearman's rank correlation coefficient and Pearson contingency coefficient. Event-free survival (EFS), relapse-free survival (RFS) and OS of the diagnosed population were evaluated using Kaplan-Meier curves and univariate Cox proportional hazards regression modelling. A log-rank test and an F Cox test was used to compare the survival of subgroups. EFS, RFS and OS were calculated from date of diagnosis to date of first event. EFS as an event was defined as time to relapse or death, and OS was defined as time to death resulting from any cause. The observation time was ceased at last follow-up if no event had occurred. p values ≤ 0.05 were considered statistically significant. All analyses have been performed using STATISTICA software version 13.1.

Results

224 children treated at the Department of Pediatrics, Oncology and Hematology at the Medical University of Lodz, Poland between 2003 and 2018 were included in this analysis. The study group comprised 130 boys (58%) and 94 girls; median age at diagnosis was 5.64 years (interquartile range: 3.29–11.65 years) and was equal in girls and boys ($p = 0.55$). More detailed characteristics of the study group are set out in Table I.

In total, 21 children experienced neurotoxicity incidents (9.37%), 13 girls (62%) and eight boys (38%). The 21 consisted of four in the ALLIC BFM 2002 protocol (19%) and 17 in the ALLIC BFM 2009 protocol (81%).

In all 21 patients, we observed 28 incidents of three different types: 16 SLS (57%), seven PRES (25%), and five seizures (18%). Seven children experienced recurrence of incident (33.3%). Most incidents took place during the

Table I. Characteristics of study group

Clinical characteristic	Median (interquartile range) or N [%]
Age at diagnosis [years]	5.64 (3.29–11.65)
Number of patients	224
Patients in Protocol 2002 Patients in Protocol 2009	129 (57.58) 95 (42.41)
Sex (female/male)	94/130
BCP-ALL T-ALL	194 (86.60%) 30 (13.40%)
WBC at diagnosis [per µL]	14,275 (4,860–48,900)
Risk group SR Risk group IR Risk group HR	44 (19.64%) 124 (55.36%) 56 (25%)
CNS1 CNS2 CNS3	180 (80.36%) 31 (13.84%) 12 (5.36%)
Poor steroid response Good steroid response	26 (11.60%) 197 (87.94%)
Death Survival	37 (16.52%) 187 (83.48%)
Event Event-free	47 (21%) 177 (79%)

BCP-ALL — B-cell progenitor acute lymphoblastic leukemia; T-ALL — T-cell acute lymphoblastic leukemia; WBC — white blood cells; SR — standard risk; IR — intermediate risk; HR — high risk; CNS — central nervous system

Table II. Detailed characteristics of study group according to neurotoxicity status

Clinical characteristic	Neurotoxicity	No neurotoxicity	*p* level
Age at diagnosis [years]*	8.48 (4.5–12.05)	5.29 (3.09–11.43)	0.05523
Number of patients	21 (9.37%)	203 (90.63%)	
Patients in Protocol 2002 Patients in Protocol 2009	4 (3%) 17 (18%)	125 (97%) 78 (82%)	0.00014
Sex (female/male)	13 (62%)/8 (38%)	81(40%)/122 (60%)	0.08675
B-ALL T-ALL	16 (17%) 5 (8%)	178 (83%) 25 (92%)	0.25605
Risk group SR Risk group IR Risk group HR	1 (2%) 14 (11%) 6 (11%)	43 (98%) 110 (89%) 50 (89%)	0.19535
CNS1 CNS2 CNS3	16 (9%) 3 (10%) 2 (17%)	164 (91%) 28 (90%) 10 (83%)	0.67009
Poor steroid response Good steroid response	2 (8%) 18 (9%)	24 (92%) 179 (91%)	0.90226
Death Survival	6 (16.22%) 15 (8.02%)	31 (83.78%) 172 (91.98%)	0.20989
Event Event free	11 (23.4%) 10 (5.65%)	36 (76.6%) 167 (94.35%)	0.00060
WBC at diagnosis [per µL]*	11,700 (5,300–23,200)	14,400 (4,860–50,000)	0.62599

*Age and white blood cells (WBC) at diagnosis are presented as median with interquartile range; B-ALL — B-cell acute lymphoblastic leukemia; T-ALL — T-cell acute lymphoblastic leukemia; SR — standard risk; IR — intermediate risk; HR — high risk; CNS — central nervous system

induction phase (42.86%), and during the treatment of relapse (25%), and more rarely during Protocol M or HR blocks (17.86%), II Protocol (10.71%) and in maintenance treatment (3.57%). Median time from beginning treatment to the occurrence of neurotoxicity was 0.52 years [interquartile range (IR): 0.14–0.97 years]. There were no significant differences between times to the occurrence of neurotoxicity and its type (p = 0.8094). 75% of incidents were associated with the presence of seizures. There was no statistically significant association between type of incident and occurrence of contractions (p = 0.18). In more than half of incidents with seizures, patients were treated with any epileptic drugs for six months. In 23% of cases, no chronic treatment was introduced and 14% finished the therapy within two months after the neurotoxicity incident.

Specific MRI presentations were revealed in 82% of incidents, whereas CT was relevant only in 18% of cases. 75% of incidents were accompanied by leucopenia and neutropenia, and in the majority of cases (53.57%) the inflammatory markers were elevated. Almost all children had been given any antibiotic in the last seven days before the incident: 25% of them had obtained meropenem, 42% piperacillin and tazobactam, and 60% other cytostatic drugs. There was no statistically significant difference between type of incident and mean time from administration of MTX (p = 0.2949). Median time was eight days (IR: 5–14 days). In 86% of patients it was intrathecal administration, while 11% got this drug via a combination of intravenous and intrathecal ways. After 50% of neurotoxic incidents, theophylline prophylaxis was introduced. Of the seven patients who suffered from a recurrence of neurotoxicity, four of them did not have prophylaxis with theophylline, two of them were given theophylline before planned lumbar puncture with administration of MTX, and there is a lack of information about one patient. Almost half of neurotoxic incidents (42.8%) caused modification of chemotherapy.

The occurrence of neurotoxicity was associated with the study protocol (p = 0.00014), although it was not a strong association (Pearson contingency correlation coefficient C = 0.2432954).

There was no significant association between occurrence of neurotoxicity and other clinical characteristics such as group of risk (p = 0.19535), sex (p = 0.08675), status of involvement of CNS (p = 0.67009), type of leukemia [B-cell ALL (B-ALL) or T-ALL] (p = 0.25605), steroid response (p = 0.90226), death (p = 0.20989), age at diagnosis (p = 0.05523), or white blood cells (WBC) at diagnosis (p = 0.62599). Detailed characteristics are set out in Table II.

The 5-year OS and EFS of the analyzed group were 85.11% [95% confidence interval (CI): 80.32–89.82%] and 80.03% (95% CI: 74.69–85.38%) respectively.

Regarding neurotoxicity occurrence, there was a statistically significant difference in EFS (p = 0.00082) (Figure 1A), but not in OS (p = 0.10135), (Figure 1B). The difference in relapse-free survival between groups of neurotoxicity and no-neurotoxicity was statistically significant (p <10^{-5}) (Figure 1C).

5-year OS in the group of children affected by this complication was 73.14% (95% CI: 54.34–91.65%) while 5-year OS in the group without neurotoxicity was 86.64% (95% CI: 81.79–91.35%). 5-year EFS, in turn, was found to be 50.41% (95% CI: 29.41–71.19%) in the neurotoxicity group, and 83.26% (95% CI: 77.91–88.38%) in the group without neurotoxicity. 5-year RFS in the neurotoxicity group was 50.63% (95% CI: 28.54–72.92%), but in the group without neurotoxicity it was 90.39% (95% CI: 86.27–94.57%).

In a model where risk factors of an adverse event were neurotoxicity and protocol, 5-year OS in the group of children affected by neurotoxicity was 51.96% (95% CI: 22.6–84.26%) in the 2002 protocol and 79.67% (95% CI: 63.51–95.43%) in the 2009 protocol, whereas in the no-neurotoxicity group it was 82.13% (95% CI: 75.51–88.67%) in 2002 and 93.06% (95% CI: 88.09–98.07%) in 2009.

5-year EFS in the group of children with neurotoxicity was 31.58% (95% CI: 5.1–57.29%) in the 2002 protocol and 57.75% (95% CI: 37.48–78.41%) in the 2009 protocol, and in the no-neurotoxicity group it was 79.04% (95% CI: 72.24–85.92%) in the 2002 protocol and 89.62% (95% CI: 83.64–95.54%) in the 2009 version. 5-year RFS for the no-neurotoxicity group was 89.66% (95% CI: 84.41–94.85%) in the ALLIC BFM 2002 protocol and 91.51% (95% CI: 86.07–97%) in the ALLIC BFM 2009 protocol, and for neurotoxicity was 45.91% (95% CI: 15.17–75.57%) in ALLIC BFM 2002 and 52.4% (95% CI: 29.66–75.35%) in ALLIC BFM 2009.

The risk of death in patients belonging to the neurotoxicity group more than trebled (HR 3.18, 95% CI: 1.26–8.06, p <0.05), the risk of an adverse event was increased by almost five times (HR 4.96, 95% CI: 2.4–10.22, p <0.05), and the risk of relapse was increased by more than seven times (HR 7.22 95% CI: 3.21–16.24, p <0.05). The ALLIC BFM 2002 protocol in this model was also a relevant risk factor of a worse outcome compared to ALLIC BFM 2009, as set out in Table III.

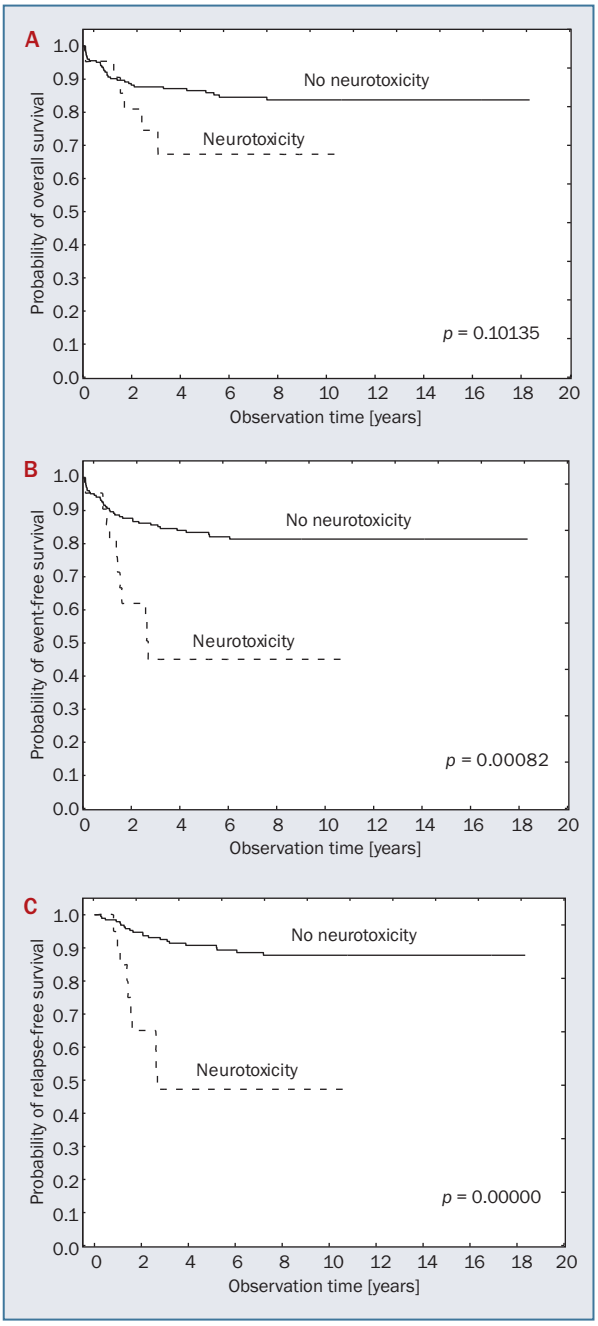

Figure 1. Survival analysis according to neurotoxicity status: **A.** Probability of overall survival; **B.** Probability of event-free survival; **C.** Probability of relapse-free survival

Because we noted that the great majority of neurotoxicity incidents and adverse events occurred to the patients in the intermediate and high risk group, and because it is widely known that they have a poorer prognosis, we checked whether there was an association between the occurrence of neurotoxicity and a higher risk group than standard. There was no statistical significance (p = 0.12989) due to the limited number of patients in the standard risk group. Differences between the neurotoxic and the no-neurotoxic

Table III. Multivariate analysis of probability survival using Cox regression modelling

Survival	Risk factor	HR	95% CI	*p* level
OS	Risk group	3.57	2.04–6.24	0.000008
	Neurotoxicity	3.18	1.26–8.06	0.14309
	ALLIC BFM 2002 protocol	0.87	0.77–0.97	0.012962
EFS	Risk group	3.45	2.1–5.69	0.000001
	Neurotoxicity	4.96	2.4–10.22	0.000014
	ALLIC BFM 2002 protocol	0.9	0.82–0.99	0.23256
RFS	Risk group	2.72	1.5–4.95	0.001032
	Neurotoxicity	7.22	3.21–16.24	0.000002
	ALLIC BFM 2002 protocol	0.97	0.87–1.08	0.607885

HR — hazard ratio; CI — confidence interval; OS — overall survival; EFS — event-free survival; RFS — relapse-free survival

group in each risk group were assessed regarding the probability of OS, EFS and RFS as shown in Figure 2, which depicts that regardless of the risk group, neurotoxicity diminished the probability of survival.

The model where risk factors of an adverse event were neurotoxicity and risk group showed that 5-year OS, 5-year EFS, and 5-year RFS were shorter in the neurotoxicity group within the risk groups of the protocols. All the results are summarized in Table IV. Hazard ratio of risk factors depicts that neurotoxicity, as well as higher protocol risk group, increased the risk of death and the occurrence of adverse events and relapses, whereas the risk was lower in the ALLIC BFM 2009 protocol. All the results are set out in Table III.

Discussion

There is a limited number of papers about neurotoxicity in the literature, and most of them are descriptions of individual cases. Few studies have been carried out on small and medium populations, and there are no clear consensus algorithms for management in this state. According to the literature, the incidence of neurotoxicity as a complication during treatment of ALL is 10–15% [3].

This is consistent with our observations (9.37%). The most common type of incident in our material was SLS (57%), although the experimental population was small (21 children, 28 incidents). We classified cases to a specific type of neurotoxicity based on the criteria of the Ponte di Legno working group 2016. However, not all the incidents met the criteria and eventually two cases were classified as SLS due to the characteristic clinical course of the incident.

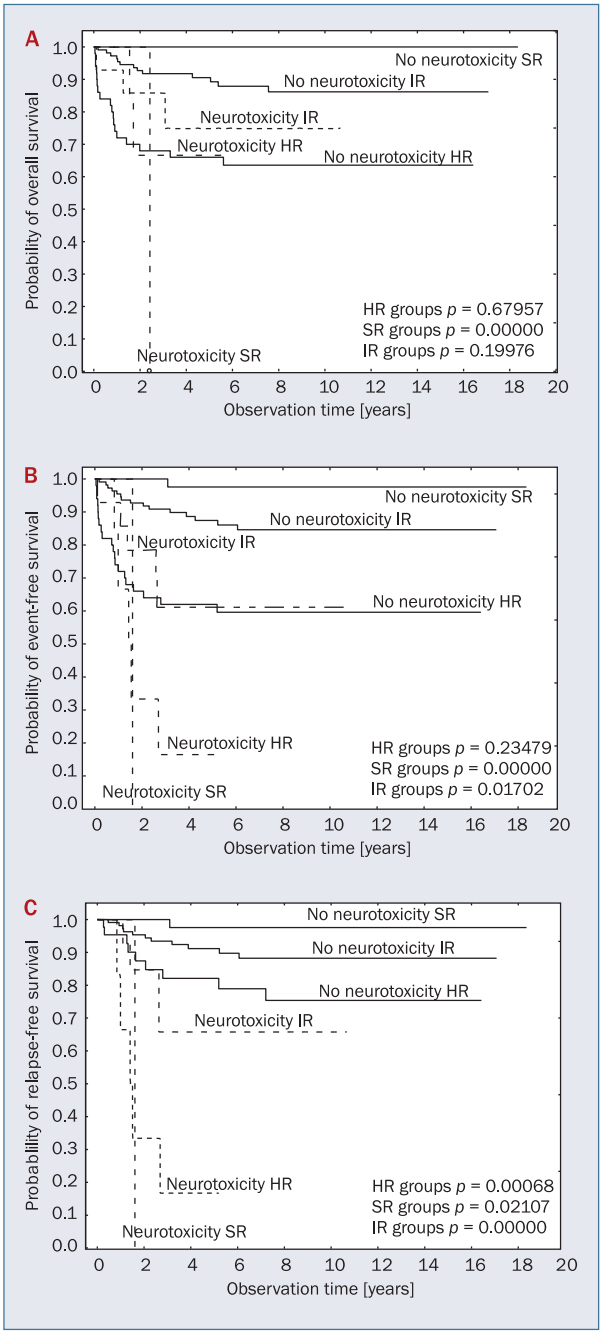

Figure 2. Survival analysis according to neurotoxicity status within risk groups of protocols: **A.** Probability of overall survival; **B.** Probability of event-free survival; **C.** Probability of relapse-free survival

The weak point of this paper is a lack of radiological verification of images — they were classified as characteristic for each type of neurotoxic event because of the description performed as the moment of diagnosis by many radiologists over the course of time. This shows the need to verify the criteria of diagnosing and differentiating between types of neurotoxicity, along with clarifying radiological features for each type of incident.

Table IV. Probability of survival according to the risk group in patients of neurotoxic and the no-neurotoxic group

Probability of survival	Risk group	Neurotoxicity	No neurotoxicity
5-year OS	SR	96.59% (95% CI: 89.87–100%)	98% (95% CI: 94.38–100%)
	IR	82.15% (95% CI: 67.36–96.68%)	89.08% (95% CI: 83.7–94.41%)
	HR	52.78% (95% CI: 23.66–81.63%)	68.88% (95% CI: 56.48–81.12%)
5-year EFS	SR	87.54% (95% CI: 70.09–100%)	96.04% (95% CI: 90.51–100%)
	IR	64.27% (95% CI: 45.07–83.62%)	87.18% (95% CI: 81.45–92.89%)
	HR	21.11% (95% CI: 0–43.72%)	61.74% (95% CI: 49.01–75.02%)
5-year RFS	SR	80.21% (95% CI: 52.42–100%)	96.44% (95% CI: 91.61–100%)
	IR	60.55% (95% CI: 38.97–82.15%)	92.13% (95% CI: 87.6–96.71%)
	HR	23.62% (95% CI: 0–50.32%)	79.06% (95% CI: 68.03–90.36%)

OS — overall survival; SR — standard risk; CI — confidence interval; IR — intermediate risk; HR — high risk; EFS — event-free survival; RFS — relapse-free survival

The majority of incidents took place in the induction phase of chemotherapy (57% in our material), irrespective of the type of incident, and that is consistent with the literature [19, 20]. Recurrence of incident for SLS is reported to be 10–56% [21], for PRES 4–8% [14], and for seizures 25% after antiseizure drug withdrawal [16].

The question arises as to how to prevent the occurrence of another incident. In our study, after nearly half of incidents (42.8%) changes in chemotherapy were introduced. In five cases, these changes depended on a reduction of the MTX dose in Protocol M, in four cases MTX i.t. was changed to cytarabine i.t. in prophylaxis of CNS, and three children had major changes in chemotherapy protocol — in the first case the parents refused the continuation of intensive treatment, the second patient had maintenance treatment introduced due to complete remission and having received long-term intensive treatment up to that moment, and the third patient after a second incident received further treatment but Protocol M was omitted.

Owing to the fact that most incidents were connected to the administration of methotrexate, there is some advice as to how to manage future administration of these drugs. According to Inaba et al. [21] in a study of six cases of neurotoxicity from a sample of 754 patients (0.8%), it seems that in most cases MTX i.t. may be re-administered without the recurrence of symptoms, although there have been no randomized trials in larger patient populations.

Most protocols state that re-exposure to MTX can be attempted (or possibly discussed with trial coordinators) once the neurotoxicity resolves [5] According to Atra et al. [22], a delay of high-dose MTH (HD-MTX) or MTX i.t. for a short time may be necessary to avoid a further neurotoxicity episode, but major changes in the chemotherapy regimen are rarely required. In a situation of recurrent episodes, in some cases in the literature MTX has been discontinued from therapy, with prophylaxis with hydrocortisone and cytarabine i.th. being maintained. This was also done in one of our patients (although we observed another neurotoxic incident after a few lumbar punctures with the administration of these drugs). The efficacy of both drugs without MTX is unknown [23].

In the literature there is grounds for the use of aminophylline as an adenosine receptor antagonist — the detection of increased adenosine in the cerebrospinal fluid of ALL children with toxic symptoms prompted Bernini et al. [7] to use an infusion of aminophylline at a dose of 2.5 mg/kg of bw, i.e. displacing adenosine from its receptors with good effect. However, there are no papers that definitively confirm the action of aminophylline, and there are no studies on the use of this drug in the prevention of methotrexate-induced neurotoxicity [24]. In our clinic, prophylactic theophylline i.v. on the day of the lumbar puncture with MTX was used, and then oral ingestion was continued for five days with the desired effect — only two of the patients had a re-incident of neurotoxicity out of the 13 who had this prophylaxis introduced.

It is an important issue in the prevention and treatment of neurotoxicity to avoid drug interactions that may also affect the overall prognosis in the disease.

MTX, the crucial drug in treatment and prophylaxis of the sanctuary sites, interacts with a range of different substances, including ciprofloxacin, non-steroidal anti-inflammatory drugs (NSAIDs), leflunomide, probenecid, penicillin, tetracyclines, chloramphenicol, cytarabine, cyclophosphamide, proton pump inhibitors, nitric oxide, theophylline, mercaptopurine, phenytoin, sulfonamides, salicylates, furosemide, folic acid, and valproic acid [25, 26]. This is a significant problem when it comes to the treatment of convulsions. They are the most common symptom of neurotoxicity; in the course of treatment this complication affects about 10% of patients with ALL, although their pathomechanisms may be varied [3]. Antiepileptic drugs have been shown to reduce the effectiveness of chemotherapy with MTX through acting with hepatic cytochromes [26] and some of them (phenobarbital, carbamazepine) also affect the active folate transporter in another mechanism [27].

The prognosis among ALL patients treated with antiepileptic drugs is worse than in a group of peers who did not receive such treatment [18]. Therefore, a safer drug was found — levetiracetam, which did not induce hepatic enzymes, and for many neurologists became the first-choice drug for convulsions in children undergoing chemotherapy [16]. A study of 81 adults who received a total of 280 MTX cycles, and 12% (33 cycles) together with levetiracetam, did not confirm the previously described interactions between levetiracetam and MTX. Indeed, they were found not to be likely without additional risk factors for prolonged MTX elimination [28]. The question as to how long to continue and when to stop anti-epileptic treatment is difficult to answer. Bond et al. have suggested that prolonged treatment is not often required after chemotherapy [4].

In this study, we observed a statistically significant association between event-free survival defined as relapse or death, and the occurrence of neurotoxicity. Due to the fact that there was no statistically significant difference in overall survival between groups with and without neurotoxicity, we can assume that these are relapses that contribute to a statistically significant poorer prognosis in these patients. Relapse referred to 47.6% of patients with neurotoxicity and death occurred in 28.6% in our study. This is consistent with the literature [29]. It is unclear whether this is due to an intrinsic tendency for some ALL cases to become complicated based on genetic predisposition, or if the increased relapse rate comes about because of suboptimal therapy. Nearly half of the patients had some modifications of the chemotherapy scheme performed, although only in three cases were these very significant. Antiepileptic drugs have been reported to be associated with faster antileukemic drug clearance and a higher risk of relapse in ALL [18], and may have contributed as a risk factor in seven of our patients. However, none of the patients died due to the occurrence of a neurotoxicity incident, which has been reported as a rare outcome previously [30].

Although our study also describes incidents of neurotoxicity in the pediatric population, it mainly focuses on survival and event-free survival in children with ALL after a neurotoxic event, in a way that is unique in the literature.

However, it has some limitations. Firstly, since this is a single center observational study, our findings should be repeated in another larger independent cohort. And the retrospective character of our study obviously limited the obtaining of some data.

Conclusions

The occurrence of neurotoxicity is associated with a poorer prognosis due to relapse and, possibly, treatment modifications. Further investigations aimed at better understanding the mechanism and predictors of subacute central neurotoxicity, as well as establishing clear classifications and guidelines of treatment, are required in order to improve treatment outcomes in pediatric ALL.

Authors' contributions

JK — conceptualization, formal analysis, writing draft manuscript; JT — providing clinical data, reviewing draft manuscript; JW — providing clinical data, reviewing draft manuscript; IDK — providing clinical data, reviewing draft manuscript; WM — conceptualization, formal analysis, reviewing and supervision of study.

Conflict of interest

The authors declare no conflict of interest.

Financial support

None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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ORIGINAL RESEARCH ARTICLE

Invasive fungal disease presenting as septic shock in immunocompromised pediatric and adult patients: summary of reported cases

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Abstract

Introduction: Septic shock is a very rare presentation of invasive fungal disease (IFD) in immunocompromised patients. The objective of this paper was to summarize reported cases of pediatric and adult patients with IFD presenting as septic shock in non-*Candida* infections. Literature data describing etiology, age, and outcome of septic shock as a presentation of IFD, is summarized.

Material and methods: The available pediatric data included 23 patients, most of them with underlying non-hematological disease.

Results: Only 6/23 (26.1%) were reported to survive this infection. Respective data in adults with invasive fungal disease presenting as septic shock were reported in 28 patients. Most of these patients were treated for acute leukemias (including three patients after hematopoietic cell transplantation); only 5/28 (17.9%) survived the infection.

Conclusion: Invasive fungal disease presenting as septic shock in immunocompromised patients is a highly unusual presentation.

Key words: invasive fungal disease, septic shock, hematopoietic cell transplantation, leukemia

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Introduction

Patients after allogeneic hematopoietic cell transplantation (allo-HCT) belong to a high-risk group of invasive fungal disease (IFD) [1]. The distribution of pathogens in an allo-HCT setting include aspergillosis in 55–60%, candidiasis in 25–30%, mycormycosis in 7–8%, and rare species e.g. fusariosis, scedosporiosis, geotrichosis in 2–3% [2]. With the widespread introduction of antifungal prophylaxis with azoles, this epidemiology is tending to change, with the rise of rare and sporadic species. No major differences in etiology between children and adults have been reported [3–6], although in one study the incidence of IFD after allo-HCT was significantly higher in children than in adults [6]. Regardless of age, patients in the following groups are considered high-risk for IFD: acute myeloblastic leukemia (AML); recurrent acute leukemia; allogeneic hematopoietic stem cell transplantation; and high risk acute lymphoblastic

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leukemia (ALL) [4, 5, 7–9]. Coexisting cytomegalovirus replication increases the risk of fungal complications [9].

Clinical symptoms of IFD in immunocompromised patients are dependent on the localization of the infection, which in most cases involves the lungs, abdomen, paranasal sinuses, skin or brain. In most cases, general symptoms occur including fever, followed by other systemic symptoms and laboratory markers of severe infection (e.g. C-reactive protein, procalcitonin). Fungemia or fungal sepsis might occur in a case of bloodstream infection with *Candida*; nonetheless septic shock is an infrequent presentation [10]. The objective of this paper was to analyze a series of cases of pediatric and adult patients with non-*Candida* IFD presenting as septic shock.

Material and methods

Studies and case reports regarding non-*Candida* invasive fungal disease presenting as septic shock in pediatric and adult patients were searched for in 'PubMed'. Search queries included 'invasive fungal disease' OR 'invasive fungal infection' AND 'septic shock'. The following data was retrieved from these reports: number of patients, age, gender, underlying disease, identification of fungal etiology, antifungal therapy, and treatment outcome.

Papers were included into analysis according to the diagnosis made by the respective authors. No additional judgment of sepsis and/or septic shock was made. According to the Third International Consensus Definitions for Sepsis and Septic Shock, sepsis was defined as 'life-threatening organ dysfunction caused by a dysregulated host response to infection' [11]. Septic shock was defined as 'a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone'. Patients with septic shock can be clinically identified by the presence of two factors: the need to use a vasopressor and increased serum lactate concentration despite adequate volume resuscitation [11].

Results

Literature data describing etiology, age and outcome is very limited. The available pediatric data includes 22 patients, most of them with an underlying non-hematological disease (Table I) [12–22]. Only 6/22 (27.3%) are reported to have survived this infection, including 2/4 with acute leukemia. Etiology of the infection was highly variable, however infection with *Saprochaete spp.* (formerly *Geotrichum spp.*, now proposed as *Magnusiomyces spp.*) presenting as septic shock was reported in four children, all with acute leukemias. Only two of them survived the infection. IFD-related non-*Candida* septic shock was reported also in 28 adults (Table II) [21, 23–40]. Among them, high mortality has been observed, with only 5/28 (17.9%) patients survival.

Discussion

Invasive fungal infections represent a serious medical problem worldwide and are a major cause of morbidity and mortality in patients with hematological malignancies. Other immunocompromised patients are also at high risk of IFD mainly because of the increased use of immunosuppressive and cytotoxic therapies, as well as improved diagnostic techniques. *Candida* and *Aspergillus spp.* are major causative agents.

However, with the new prevention strategies, new species are increasingly being reported as agents of bloodstream infections (BSI) or disseminated fungal disease. While *Candida spp.* is the most common etiology of fungal BSI and septic shock, such presentation is rare in case of other fungal pathogens.

Septic shock is the most dangerous presentation of infection, bringing the risk of poor prognosis. In this paper, we have searched for patients with non-*Candida* invasive fungal infection presenting as septic shock. We have found a limited number of reports, both in children and adults.

Septic shock is always a medical emergency. It requires the immediate administration of antimicrobials. Usually, bacterial etiology is suspected and adequate empirical treatment broadly covering the most probable pathogens is promptly started. In immunocompromised patients, in the presence of severe infection with symptoms of septic shock, atypical agents including fungal etiology should also be considered. One should also be aware of mixed etiology of septic shock in patients.

The most striking finding of our analysis is the low survival of patients, as only 26.1% of children and 17.9% of adults survived fungal infection with septic shock. Even with the long time period of inclusion, this survival rate was low in comparison with the outcome of IFD seen in leukemic patients both in the first [3, 41] and second [42, 43] decades of this century.

Immunocompromised patients, especially those with hematological malignancy who develop septic shock caused by fungal infection, are at very high risk of mortality. There are two options to improve therapeutic effect. Firstly, starting the empirical antifungal therapy as soon as possible is essential to increase the chance of survival in these patients. Secondly, it is recommended that early source control, including catheter removal, is a key factor influencing the outcome of leukemic or transplant patients with septic shock or sepsis of fungal etiology [4, 8].

The limitation of this study was the heterogenous population in terms of primary diagnosis, primary treatment, and etiology of fungal infection. We also limited the

Table I. Literature data on septic shock in children with invasive fungal disease

Source	Age	Primary disease	Etiology	Treatment	Outcome
Zeng et al., 2021 [12]	Median age 22 months; age range: 3–44 months in 12 children	Various	*Talaromyces marneffei*	Amphotericin B 7/11 Itraconazole 2/11 Fluconazole 2/11 Voriconazole 3/11 Caspofungin 3/11	Died 9/12 Cured 3/12
Romanio et al., 2017 [13]	1-year-old boy	Down's syndrome in postoperative period of congenital cardiac disease correction	*Saccharomyces cerevisiae*	Amphotericin B	Alive
Watson et al., 2016 [14]	12-year-old girl	Juvenile idiopathic arthritis	*Blastomyces*	Amphotericin B, adjunctive inhaled amphotericin; liposomal amphotericin B was changed to amphotericin B lipid complex	Died
El Dib et al., 2014 [15]	5-year-old boy		*Coccidioidomycosis*	Patient died before receiving required antifungal therapy	Died
Cavalcante et al., 2014 [16]	10-year-old girl	JSLE	*Cryptococcus neoformans*	Amphotericin B (liposomal) (3 mg/kg)	Died
França et al., 2012 [17]	14-year-old girl	JSLE	*Histoplasma capsulatum*	Amphotericin B (liposomal) (1 mg/kg)	Died
Pereira et al., 2004 [18]	2-year-old girl	Lymphoproliferative syndrome	*Paracoccidioides brasiliensis*	Intravenous sulfamethoxazole-trimethoprim (10 mg/kg trimethoprim)	Died
Hsu et al., 1998 [19]	23-month-old boy	AML	*Trichosporon beigelii*	Amphotericin B	Died
Wee et al., 2019 [20]	13-year-old boy	ALL	*Saprochaete clavata*	Amphotericin B and voriconazole (10 weeks)	Alive
	17-year-old boy	AML	*Saprochaete clavata*	Amphotericin B (60 mg/d)	Died
Parahym et al., 2015 [21]	15-year-old boy	AML	*Saprochaete capitata*	Caspofungin, amphotericin B (lipid) (5 mg/kg) (24 days), and voriconazole (400 mg/d)	Alive
Trabelsi et al., 2015 [22]	17-year-old boy	AML	*Saprochaete capitata*	Amphotericin B (60 mg/d)	Died

ALL — acute lymphoblastic leukemia; AML — acute myeloid leukemia; JSLE — juvenile systemic lupus erythematosus

analysis to non-*Candida* etiology, because the symptoms of sepsis are relatively more frequent in *Candida* bloodstream infection.

Conclusion

Septic shock is a very rare presentation of fungal infection in immunocompromised patients, but the mortality is very high both in children and adults.

Authors' contributions

KC, JS — design of study. RD, MRP, KC — provision of clinical data. All authors — analysis of clinical data. TS, JS — literature search and analysis of data. TS, JS — writing manuscript. All authors — critical revision and final approval.

Conflict of interest

The authors declare no conflict of interest.

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None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments

Table II. Literature data on septic shock in adults with invasive fungal disease

Source	Age	Primary disease	Etiology	Treatment	Outcome
Caldas et al., 2022 [23]	43-year-old woman	Crohn's disease on treatment with infliximab and azathioprine	*Saprochaete clavata, Legionella pneumophila* serogroup 1	Amphotericin B (liposomal)	Died
Duarte et al., 2021 [24]	66-year-old woman	AML	*Saprochaete capitata*	Amphotericin B (liposomal) and flucytosine	Died
Lo Cascio et al., 2020 [25]	20-year-old woman	ALL	*Saprochaete clavata*	Caspofungin	Died
Buchta et al., 2019 [26]	Patient 1 — 45-year-old man Patient 2 — 61-year-old woman Patient 3 — 58-year-old woman Patient 4 — 50-year-old woman Patient 5 — 66-year-old woman	1 — AML after allo-HCT 2 — AML 3 — AML after allo-HCT 4 — AML after auto-HCT 5 — DLBCL	1 — *Saprochaete clavata* 2 — *Saprochaete clavata* 3 — *Saprochaete clavata* 4 — *Saprochaete clavate* (+ *Candida albicans* were cultivated from nasopharyngeal swab) 5 — *Saprochaete clavata* + *Candida glabrata*	1 — amphotericin B (1 mg/kg) 2 — amphotericin B (1 mg/kg), amphotericin B (lipid) (5 mg/kg) 3 — amphotericin B (0.7–1 mg/kg), amphotericin B (lipid) (5 mg/kg), voriconazole (2 × 200 mg) 4 — amphotericin B (0.7–1 mg/kg) 5 — micafungin (100 mg), voriconazole (2 × 200 mg)	1 — died 2 — survived (but died from early relapse of AML later) 3 — died 4 — died 5 — died
Ben Neji et al., 2019 [27]	39-year-old man	AML	*Saprochaete capitata*	Amphotericin B (deoxycholate)	Died
Alobaid et al., 2019 [28]	67-year-old woman	Diabetes, hypertension, ischemic heart disease, left ventricular failure, peripheral vascular disease, bronchial asthma and obstructive sleep apnea	*Saprochaete capitata*	No antifungal therapy	Died
Bansal et al., 2018 [29]	29-year-old woman	AML	*Saprochaete capitata*	Amphotericin B (liposomal)	Died
Hajar et al., 2018 [30]	82-year-old man	Kidney transplant recipient	*Saprochaete capitata*	Amphotericin B (liposomal)	Died
Pamidimukkala et al., 2017 [31]	48-year-old woman	Biphenotypic acute leukemia	*Saprochaete capitata concomitant Enterococcus gallinarum*		Died
Fernández-Ruiz et al., 2017 [32]	55-year-old man	Refractory acute leukemia	*Saprochaete capitata*	Amphotericin B and voriconazole	Died
Del Principe et al., 2016 [33]	50-year-old woman	Mantle cell lymphoma	*Saprochaete clavate*	Amphotericin B (liposomal) (3 mg/kg), 47 days	Died on day 60 from chemotherapy initiation because of lymphoma progression

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Table II (cont.). Literature data on septic shock in adults with invasive fungal disease

Source	Age	Primary disease	Etiology	Treatment	Outcome
Subramanya Supram et al., 2016 [34]	Patient 1 — 77-year-old woman Patient 2 — 80-year-old man	Patient 1 — hypertension, Alzheimer's disease Patient 2 — COPD, hypertension	Patient 1 — *Saprochaete capitata* + + *Flavobacter spp.* (endotracheal aspirate) Patient 2 — *Saprochaete capitata*	Patient 1 — no antifungal therapy Patient 2 — fluconazole (400 mg/day for 4 days)	Patient 1 — died Patient 2 — died
Trabelsi et al., 2015 [22]	Patient 1 — 25-year-old woman Patient 2 — 57-year-old man	AML	*Saprochaete capitata*	Patient 1 — amphotericin B (60 mg/d) Patient 2 — amphotericin B (80 mg/d) + voriconazole (2 × 200 mg)	Patient 1 — died Patient 2 — died
Picard et al., 2014 [35]	Patient 1 — 46-year-old woman Patient 2 — 70-year-old man Patient 3 — 63-year-old woman	AML	*Saprochaete clavata*	Patient 1 — amphotericin B (liposomal) and voriconazole Patient 2 — caspofungin Patient 3 — amphotericin B (liposomal) and voriconazole	Patient 1 — survived septic shock, died because of hemorrhage Patient 2 — died Patient 3 — died
García-Ruiz et al., 2013 [36]	55-year-old man	ALL	*Saprochaete capitata*	Amphotericin B (liposomal) (4 mg/kg/d) + voriconazole	Died
Saghrouni et al., 2012 [37]	47-year-old man	AML	*Saprochaete capitata*	Amphotericin B (1 mg/kg) for 14 days followed by voriconazole 2 × 200 mg	Died
Avelar Rodriguez et al., 2017 [38]	28-year-old man	Cocaine abuse and Child-Pugh class C alcoholic liver cirrhosis	*Rhinocerebral mucormycosis*, *Candida glabrata*	Amphotericin B (liposomal) 3 mg/kg	Died
Fernández-Ruiz et al., 2017 [32]	56-year-old man	Solid cancer, abdominal surgery, prolonged ICU stay	*Wickerhamomyces anomalus*	Amphotericin B for 9 days followed by fluconazole for 5 days)	Alive
Taniguchi et al., 2009 [39]	18-year-old man	Mitochondrial encephalomyopathy accompanied by refractory anemia and chronic renal failure	*Lecythophora mutabilis*	Micafungin 5 mg/kg, amphotericin B (liposomal) (3 mg/kg)	Died
Hennequin et al., 2000 [40]	47-year-oldman	Adenocarcinoma of lower esophagus	*Saccharomyces boulardii*	Fluconazole (100 mg/d) initiated on day 35 for six weeks	Alive

ALL — acute lymphoblastic leukemia; allo-HCT — allogeneic hematopoietic cell transplantatin; AML — aute myeloid leukemia; auto-HCT — autologous hematopoietic cell transplantatin; COPD — chronic obstructive pulmonary disease; DLBCL — diffuse large B-cell lymphoma; ICU — intensive care unit

involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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Rare primary testicular lymphoma: a single-center analysis

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Abstract

Introduction: Primary testicular lymphoma (PTL) is a rare disease, accounting for <5% of all testicular malignancies and 1–2% of non-Hodgkin lymphoma cases. Diffuse large B-cell lymphoma (DLBCL) is the most common histological diagnosis. The literature data concerning PTL is scarce and based mainly on small series or retrospective studies.

Methods and results: In this paper, we present six patients with DLBCL-PTL who were treated in the Department of Hematology and Bone Marrow Transplantation at Poznan University of Medical Science, Poland between 2006 and 2022. All the patients obtained complete remission (CR) after six cycles of R-CHOP-21 (cyclophosphamide, doxorubicin, vincristine and rituximab on day 1, and prednisolone on days 1–5, administered every 21 days for a total of eight cycles) as immunochemotherapy. Five of them additionally received prophylaxis of central nervous system involvement with intrathecal methotrexate/arabinoside cytosine. One patient received scrotal radiotherapy, and in another one radiotherapy is planned. Relapse was confirmed in one patient after 72 months in the contralateral testis, and the patient was successfully retreated. After a median follow-up of 146 (range 5–196) months, all patients remain alive and in CR.

Conclusion: Despite all interpretative limitations, the current standard DLBCL-PTL therapy seems to be six courses of CHOP-R-21 combined with intrathecal metothrexate and scrotal irradiation.

Key words: primary testicular lymphoma, extranodal lymphoma

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Introduction

Primary testicular lymphoma (PTL) is an aggressive form of non-Hodgkin lymphoma (NHL) representing c.1–2% of all NHLs and c.1–7% of testicular malignancies [1]. It is worth noting that PTLs comprise the most common testicular tumors in men with a median age of 66–68 years. It is estimated that the annual incidence amounts to 0.09–0.26 per 100,000 population [1, 2]. Inguinal orchiectomy is recommended when PTL is suspected. A proper panel of immunohistochemical staining and an evaluation by an experienced pathologist is crucial for appropriate diagnosis, because PTL is rare and can be difficult to distinguish from seminoma in some cases [3].

The vast majority of PTLs exhibit the histology of diffuse large B-cell lymphoma (DLBCL; 80–90%) [4]. The 5th edition of the 'World Health Organization Classification of Hematolymphoid Tumors: Lymphoid Neoplasms' included PTL-DLCBLs in a category of primary large B-cell lymphomas of immune-privileged sites in addition to the primary

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central nervous system DLBCL and primary large B-cell lymphoma of the vitreoretina [5]. Nevertheless, isolated cases of other histological subtypes such as follicular lymphoma, marginal zone lymphoma, Burkitt's lymphoma, anaplastic large cell lymphoma, lymphoblastic lymphoma, mantle cell lymphoma, natural killer (NK)/T lymphoma, and peripheral T-cell lymphoma have also been found [6–12]. DLBCL-PTL differs from nodal DLBCL in some aspects including the cell of origin, genetics and pathophysiology. PTLs, similarly to the other primary extranodal DLBCLs, are mainly of activated B-cell-like origin (ABC). PTL usually demonstrates with a non-tender, firm mass of a medium size of 6 cm, difficult to separate from the involved testis [13]. The right testis is as frequently involved as the left, while bilateral testicular involvement is rare, accounting for only c.6% of cases [1]. Recommended staging and response assessment procedures are no different from other forms of NHL, and include computed tomography (CT) or positron emission tomography–computed tomography (PET-CT) for routine staging of ^{18}F-fluoro-deoxy-glucose (FDG)-avid lymphomas which include almost all histologies [14].

Moreover, PTL may be associated with central nervous system (CNS) involvement, and therefore lumbar puncture and magnetic resonance imaging (MRI) are mandatory at diagnosis. According to the Lugano classification, extranodal lymphomas can be diagnosed only in stage I, when a single extranodal lesion is diagnosed or in stage II when nodal extent with limited contiguous extranodal involvement is observed. Stages III and IV are not applicable for extranodal lymphomas, because they are the equivalent of advanced disease [14]. Patients with isolated bilateral involvement of the testes have a prognosis similar to that of patients with stage I/II disease, and therefore they should be considered as stage I [15]. In PTLs, most patients experience a relapse in numerous regions of their body as long as 10–15 years after the initial diagnosis. Relapses are often observed in sanctuary sites such as the contralateral testis and CNS [2, 16–19]. The other sites of relapse can include skin, Waldeyer's ring, lung, pleura, adrenal glands, kidney, liver, and bone marrow, and even eye and heart [2, 16–20]. PTL cases are mainly reported as small series or small retrospective studies. To the best of our knowledge, there have only been three PTL prospective studies [21–23].

Therefore, to date there is a lack of standardized treatment regimen guidelines. In this paper, we summarize our center's experience of PTL and discuss that in the context of the literature data.

Methods and results

Among the lymphoma patients treated in the Department of Hematology and Bone Marrow Transplantation at Poznan University of Medical Science Poland between 2006 and 2022, we identified six patients with truly PTL lymphoma. Five testicular lymphoma patients with concomitant other sites involvement were excluded from the analysis. The median age of PTL patients was 62 years (range: 54–74), and all patients were in a good performance status according to the Eastern Cooperative Oncology Group (ECOG) 0–1. In all of them, DLBCL was diagnosed. Based on the Hans algorithm, it was possible to confirm the cell of origin subtype in three of the patients. All patients were initially treated with inguinal orchidectomy. PTL was diagnosed after exclusion of other sites involvement, based on CT scans or PET-CT, lumbar puncture, and bone marrow examination. None of the patients presented with B symptoms. All patients were treated with six courses of chemotherapy: in four of them, CHOP-R-21 (cyclophosphamide, doxorubicin, vincristine and rituximab on day 1, and prednisolone on days 1–5, administered every 21 days for a total of eight cycles) was given in all courses of treatment; in two of them, 2-3 initial courses of CHOP-21 was not combined with rituximab. Additionally, five of them received primary CNS involvement prophylaxis including repeated doses of intrathecal (i.t.) methotrexate (MTX) 15 mg and/or arabinoside cytosine 40 mg.

After the treatment, all patientsachieved complete remission (CR) based on CT or PET-CT. In one patient, scrotal radiotherapy 30 Gy was performed, and in another one radiotherapy (RT) is planned. Unfortunately, in one patient who did not receive RT, relapse in the contralateral testis was confirmed after 72 months. This patient underwent orchidectomy combined with CHOP-R modo Travade/COP-R immunotherapy and liposomal arabinoside cytosine i.t. prophylaxis all with subsequent the second CR. After a median follow-up of 146 (5–196) months, all patients remain under observation without signs of active disease. Detailed patient characteristics are set out in Table I.

Discussion

Clinical characteristics of, and clinical course in, our patients are consistent with literature data. Most literature analyses are difficult to interpret because treatment protocols differ. Moreover, the studies often include patients presenting with testicular involvement being a part of disseminated disease, which nowadays cannot be regarded as truly PTL. In the literature, the proportion of ABC subtypes in PTLs ranges between 60% and 96% and is dependent on the diagnostic method, with a higher incidence when using gene expression profiling than when using immunohistochemistry [24–26]. Additionally, in PTL *TP53* mutations are unlikely, but a high proportion of patients show active STAT signaling, and expression of nuclear p50 suggesting the activity nuclear factor κB (NF-κB) signaling pathway is observed [27]. It must be underscored that there is a similarity of PTLs to primary central nervous system lymphomas (PCNLs), in that both present with genomic

Table I. Patient characteristics, treatments and outcomes

Patient number	Age	Laterality	IPI	Stage	Chemotherapy	Intrathecal prophylaxis	Response	Radiotherapy	Relapse	Alive	Time of follow-up (months)
1	64	Left	1	IE	6 × CHOP-R	3 × MTX + Ara-C	CR	No	Yes (contralateral testis)	Yes	143
2	58	Left	1	IE	3 × CHOP 3 × CHOP-R	No	CR	No	No	Yes	156
3	54	Left	1	IIE	6 × CHOP-R	3 × MTX	CR	No	No	Yes	149
4	60	Right	ND	IE	2 × CHOP 4 × CHOP-R	3 × MT	CR	No	No	Yes	196
5	66	Left	1	IE	6 × CHOP-R	2 × MTX	CR	Planned	No	Yes	5
6	74	Right	1	IE	6 × CHOP-R	3 × MTX	CR	Yes (30 Gy)	No	Yes	20

CHOP-R — cyclophosphamide 750 mg/m² intravenous (i.v.) on day 1, rituximab 375 mg/m² i.v. on day 1, doxorubicin 50 mg/m² i.v. on day 1, vincristine 2 mg (pts <70-years) or vincristine 1 mg (pts >70-years) i.v. on day 1, prednisone 100 mg per os daily, day 1–5; MTX — metothrexate; Ara-C — arabinoside cytosine; CR — complete remission; ND — no data

instability, and near-uniform, often biallelic, CDKN2A loss with rare *TP53* mutations [28]. PCNSLs and PTLs also utilize multiple genetic mechanisms to target key genes and pathways and exhibit near-uniform oncogenic Toll-like receptor signaling due to *MYD88* mutation and/or NFKBIZ amplification, frequent concurrent B-cell receptor pathway activation, and BCL6 deregulation. Interestingly, PCNSLs and PTLs have frequent 9p24.1/PD-L1/PD-L2 CNAs and additional translocations of these loci, structural bases of immune evasion that are shared with primary mediastinal large B-cell lymphoma.

In PTL, high *MYD88* expression is typically observed and *MYD88L265P* mutation is found in c.70% of patients [29, 30]. The morphology of testicular lymphoma is not pathognomonic in ultrasound, presenting with unifocal, multifocal or diffuse hypoechogenic areas and can be difficult to distinguish from an inflammatory process [31]. Magnetic resonance imaging (MRI) is a more sensitive technique which allows simultaneous evaluation of both testes, paratesticular spaces, and spermatic cord. In MRI, typical PTL findings include T2-hypointensity and strong heterogeneous gadolinium enhancement [32]. Similarly to the other extranodal lymphomas, in PTL the prognostic utility of International Prognostic Index (IPI) and its components seems to be limited, because they are surrogate markers of a high tumor burden and disseminated disease. Indeed, all our patients had IPI 1. In the available literature, the following biological, clinical, and laboratory factors were considered as adverse prognostic factors: age >70 years, left testis involvement, a lack of surgery or RT [1], an infiltration of adjacent tissues including either spermatic cord or epididymis or scrotum, ECOG ≥2, and bulky disease (tumor mass >9 cm) [33], high lactate dehydrogenase (LDH) or high beta$_2$-microglubulin, B symptoms, tumor size >10 cm [2].

Orchidectomy is necessary for both diagnostic and therapeutic purposes, but the outcomes of patients treated with orchidectomy alone are poor. Additionally, treatment with surgery combined with RT but omitting immunochemotherapy is considered as an independent predictor of worse survival [1] and should be reserved only for PTL patients who are ineligible for systemic chemotherapy. Due to the rarity of PTL, no prospective randomized trial has been performed so far.

The results of selected studies are set out in Table II. With regard to the PTL treatment strategies, the time of treatment, the choice of the first line treatment, the role and type of CNS prophylaxis, and finally the impact of RT on patient outcome, have all been discussed in the literature. First, independent of the limited character of PTLs, the literature data suggests that PTL patients have a significantly better long-term outcome when treated with at least six cycles of chemotherapy compared to an abbreviated treatment schedule [2, 17]. MD Anderson Cancer Center analysis showed significant improvements on both progression-free survival (PFS) and overall survival (OS) over time, reflecting the refinement of treatment strategy. The authors reported 5-year OS of 15.4% and 5-year PFS of 15.4% in patients treated before 1977 predominantly with chemotherapy without doxorubicin, while patients treated in 1977–1999 with doxorubicin-based chemotherapy without rituximab had a 5-year OS and PFS of 56.3% and 51.7% respectively. Patients treated since 2000, mainly with R-CHOP, had a 5-year OS and PFS of 86.6% and 59.3%, respectively (p = 0.019 for OS and p = 0.138 for PFS). Additionally, the patients treated after 2,000 were more likely to receive i.t. prophylaxis [34]. Finally, a CHOP-21 regimen was the most widely used regimen for PTL prior to the introduction of rituximab, with 5-year OS ranging from 30% to 52% [34, 35]. Similarly to the other extranodal lymphomas, the impact of rituximab addition to the chemotherapy in PTL seems to be less important than in the nodal forms. However, some retrospective analyses have confirmed its benefit in terms of time to progression, OS, PFS or the risk of relapse [34,

36, 37]. In the literature data, the rates of CNS relapses are divergent ranged from 0 to 15% [2, 20]. CNS relapses are often detected up to 10 years after an initial presentation of PTL [2, 23, 24]. CNS relapse i.t. prophylaxis with MTX has been reported in many studies with differing MTX application doses [23, 34, 37]. Of the prospective clinical trials previously reported, two used intrathecal chemotherapy alone and reported CNS relapse rates of 6% [21, 22], while one used both intrathecal and systemic MTX. Given the fact that more relapses have a parenchymal pattern, and that the penetration of MTX into the brain may be limited, it seems to be rational to use high-dose systemic MTX for CNS prophylaxis. Aviles et al.'s prospective study with the use of 4 × MTX 6 g/m^2 intravenous (i.v.) every 28 days found mild hematological and nonhematological toxicity. After a median follow-up of 64.8 months,

Table II. Results of prospective and selected retrospective studies containing more than 50 patients presenting with testicular lymphoma

Authors, type of study	No of pts	Age	Stage I/IIE [%]	Chemotherapy	Radiotherapy	Rate of response	Relapses	Outcome
Vitolo et al., 2011 (IELSG-10 trial) [20]	53	54 (22–79)	100	6–8 CHOP-R-21 i.v. + 4 × MTX i.t.	30 Gy contralateral testis + 30–36 Gy regional LN	52 pts (98%) CR	9 pts (2 LN, 5 EN*, 3 CNS)	FU 65 mo 5-y PFS 74% 5-y OS 85%
Linassier et al., 2002 GOELAMS Study Group [21]	16	62 (29–73)	100	Age 18–60 y: VCAP i.v. Age 61–75 y: VECP--bleo i.v. MTX i.t. all	RT: inguinal, iliac and para--aortic	100% CR	LN 1 pt EN 3 pts (1 testis) CNS 1 pt	FU 73 mo ≤60-y DFS 66% OS 83% >60-y DFS 74% OS 56%
Aviles et al., 2009 [22]	38	52 (53–70)	100	COEP-R-14 CNS prophylaxis 4 × MTX 6 g/m^2 i.v. every 28 days	30 Gy to scrotum and contralateral testis (stage IE) or scrotum, contralateral testis, paraaortic iliac and pelvic lymph nodes (stage IIE)	86%	10 EN	FU 64.8 mo 5-y EFS 70% 5-y OS 66%
Gundrum et al., 2009 [1]	769	68 (21–98)	75	CHT ND	Surgery and RT 35.9% Surgery 59.3% RT 1.6%	ND	ND	Median OS 4.6-y DSS in: • 3-y 71.5% • 5-y 62.4% • 15-y 43%
Zucca et al., 2003 [2]	373	66 (19–91)	79	Systemic CHT 279 (75%) Aggressive regimens 255 (68%) CHOP or 2nd-line CHT 191 (51%) MACOP-B, proMACE/ /proMACE/3rd generation regimens 45 pts (12%) High-dose CHT and/or auto-SCT 19 pts (5%) Non-anthracycline-based regimen (CVP) or single alkylating agent 24 (6%) MTX i.t. 73 (20%) High-dose MTX i.v. 29 (8%)	RT 196 (53%) + CHT 145 (39%) RT — contralateral testis 133 (36%) Anthracycline--based CHT + + i.t. prophylaxis and scrotal irradiation 34 (9%)	ND	195 (52%) EN ± ND 140 (72%) including contralateral testis in 31 pts (16%)	Median OS: • 4.8-y/whole group • 5.8-y/pts in stage I/II 5-y PFS 48% 10-y OS 27% 5-y PFS 48% 10-y PFS 33% Median PFS of 4 years

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Table II (cont.). Results of prospective and selected retrospective studies containing more than 50 patients presenting with testicular lymphoma

Authors, type of study	No of pts	Age	Stage I/IIE [%]	Chemotherapy	Radiotherapy	Rate of response	Relapses	Outcome
Mazloom et al., 2010 [33]	75	64 (22–82)	62	Non-doxorubicin based CHT + RT 4 pts (6%) Doxorubicin based CHT alone (without rituximab) 15 pts (23%) Doxorubicin based CHT (without rituximab) + RT 8 pts (12%) Doxorubicin based CHT (without rituximab) + i.t. prophylaxis 3 pts (5%) Doxorubicin based CHT (without rituximab) + RT + i.t. prophylaxis 11 pts (17%) R-CHOP + RT 1 R-CHOP + i.t. prophylaxis 5 pts (8%)	RT alone 6 pts (9%)	CR 67% PR 2%	40 pts (57%) CNS 9 (23%) LN 8 pts (20%) Contralateral testis 5 (13%)	Pts after 2000 treated predominantly with R-CHOP + i.t. prophylaxis, and scrotal RT 5-y OS 86.6% 5-y PFS 59.3% Pts treated between 1977 and 1999 with doxorubicin based CHT without rituximab, not uniformly treated with i.t. prophylaxis 5-y OS 56.3% 5-y PFS 51.7% Pts treated prior to 1977 without doxorubicin based chemotherapy, or i.t. prophylaxis 5-y OS 15.4% 5-y PFS 15.4%
Deng et al., 2016 [36]	280	65 (10–96)	77	Anthracycline-containing regimen CHT (CHOP/CHOP-like regimen) 223 pts (91%) Rituximab treatment 161 pts (64%) Prophylactic i.t. 83 pts (34%) CHT + RT + i.t. prophylaxis 56 pts (24%)	Prophylactic RT to contralateral testis 96 pts (39%)	95% CR	212 pts (30%) 5-y/10-y cumulative risk of relapse: CNS 15%/21%, contralateral testis 6%/21% 10-y cumulative risk: EN 46% vs. ND 15% CNS and contralateral testis most common sites of relapse	5-y/10-y OS 58%/24% 5-y/10-y PFS 46%/38% 5-y/10-y DSS 66%/58% 5-y OS Age ≤60 vs. age >60 65% vs. 52%
Fonesca et al., 2000 [15]	62	68	79	CHT 22 pts (37%) CHT + RT 10 pts (16%) MTX i.t. × 4	RT 10 pts (16%)	ND	CNS 13 pts 80%	Median DFS/OS 2.7-y

*No relapses in contralateral testis; auto-SCT — autologous stem cell transplantation; CHOP-R-21 cyclophosphamide, doxorubicin, vincristine and rituximab on day 1, and prednisolone on days 1–5, administered every 21 days for a total of eight cycles; CHT — chemotherapy; CNS — central nervous system; COEP-R-14 — cyclophosphamide, vincristine, etoposide and rituximab on day 1, and prednisolone on days 1–5, administered every 14 days; CR — complete remission; CVP — cyclophosphamide, vincristine, etoposide and rituximab on day 1, and prednisolone on days 1–5; DFS — disease-free survival; DSS — disease-specific survival; EN — extranodal; FU — follow-up; GOELAMS — *Groupe Ouest Est d'Etude des Leucemies Aigues et Maladies du Sang*; i.t. — intrathecal; IELSG — International Extranodal Lymphoma Study Group; LN — lymph nodes; MACOP-B — methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin; mo — month; MTX — methotrexate; ND — no data; OS — overall survival; PFS — progression-free survival; proMACE/cytaBOM — prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine; pts — patients; RT — radiotherapy; VCAP — vindesine, doxorubicin, cyclophosphamide, prednisolone; VECP-bleo — vindesine, epirubicin, cyclophosphamide, prednisolone, bleomycin

neither evidence of late neurological or cardiac toxicity nor of treatment-related mortality were observed [23]. Contradicting the abovementioned studies, a large retrospective analysis including 280 patients revealed no impact of i.t. prophylaxis on CNS relapse [37]. Our patients were treated with six courses of CHOP-R-21 immunochemotherapy, in 83% combined with i.t. prophylaxis with MTX and arabinoside cytosine, all resulting in durable complete remission. To date, scrotal RT has been performed in only one patient. Unfortunately, one patient who did not receive RT experienced relapse in the contralateral testis 72 months after treatment completion. After retreatment, he received the second CR and remains without signs of active disease after 63 months of further follow-up. This is very unusual, because the prognosis is poor in such cases and median survival in general does not exceed two months [38]. The National Comprehensive Cancer Network (NCCN) guidelines recommend 25–30 Gy RT to the contralateral testis as part of the treatment for PTL of any stage [38, 39]. Despite its benefit being supported by the results of retrospective and prospective trials, only 20–84% of PTL patients in 'real-world' analysis receive RT, although today this percentage is higher than it used to be [1, 2, 21, 37, 40, 41].

Adjuvant scrotal/contralateral testis RT has been found to decrease the risk of contralateral testis relapse and to prolong both OS and PFS [1, 2, 21, 37]. In a large retrospective analysis among PTL patients treated between 2004 and 2015, 49.8% of them received RT with a median dose of RT of 30 Gy, including 77% who received 30–39.9 Gy delivered over a median 16 treatments. The authors underscored that although RT requires multiple daily visits (delaying return to work), and may incur high out-of-pocket costs, it should never be omitted, especially in older patients with comorbidities, as these groups may be the least likely to tolerate aggressive salvage therapies at relapse [42].

All our patients remain in CR, although under strict observation, because very late relapses may occur. To conclude, even given all the limitations mentioned above, according to the available literature data it seems that the current standard treatment of PTL patients should include six courses of R-CHOP-21 with intrathecal MTX and scrotal adjuvant RT. Randomized, or even new, prospective studies are expected.

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Authors' contributions
MJ — concept, data collection and analysis, drafting article. LG — critical revision and approval of article. PC, ED — data collection and analysis. JRM — data analysis and critical revision of article. LG, KL — critical revision and approval of article.

Conflict of interest
The authors declare no conflict of interest.

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Ethics
The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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Use of Mindray MC-80 digital morphology analyzer's estimated platelet counts as adjunct to automated hematology analyzer

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Abstract

Introduction: Automated hematology analyzer platelet counts can be performed using either the impedance (PLT-I) method or the fluorescent nucleic acid staining (PLT-O) method. Estimated platelet counts (PLT-E) can be calculated using a digital morphology analyzer by evaluating the peripheral blood smear. Our objective was to compare the platelet values detected on a Mindray BC-6200 device to the PLT-E count on a Mindray MC-80 digital morphology analyzer.

Material and methods: Complete blood cell count findings between 1 September and 11 October, 2022 were obtained from the data storage units of the devices. We selected two groups of blood: a first group with thrombocytopenia (n = 49) and a second group that gave an aggregation and/or platelet clumping flag (n = 32). The results of 190 consecutive patients with normal platelet counts, and no aggregation flag, were evaluated as a control group. Pearson's correlation coefficients, Bland-Altman plots, and paired t-tests were calculated.

Results: The plot of the difference between PLT-I and PLT-O counts showed that the mean difference was –43.6 (95%: –17.2 to –69.9); when we compared PLT-O to PLT-E, bias was improved to –6.1 (95%: –18.26 to 6.1) in samples with aggregation and/or clumping flags (Bland-Altman plots).

In samples with thrombocytopenia without aggregation and/or clumping, on the Bland-Altman plot, the differences in means were all close to zero, and there were no definite biases.

Conclusions: Examining blood samples using the Mindray MC-80 digital morphology analyzer system on samples that show platelet clumps has the potential to improve PLT-I results in day-to-day laboratory routine.

Key words: method comparison, platelet estimation, digital morphology analyzer, thrombocytopenia, auto analyzer

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Introduction

The main function of platelets is to maintain vascular integrity after injury to the vascular system. In addition to hemostasis and thrombosis, platelets also play an important role in the biology of inflammation, immunity, and cancer [1].

Accurately determining the platelet number is of the utmost importance because a deficiency can cause bleeding [2]. However, conditions such as cold platelet agglutinins, paraproteinemias, platelets coming into contact with foreign surfaces (such as a dialysis membrane), giant platelets, hyperlipemia, platelet aggregation due to ethylenediaminetetraacetic acid (EDTA), or overfilled blood samples can also cause spuriously low platelet counts incompatible with patient's clinics (pseudothrombocytopenia) [2, 3]. Pseudothrombocytopenia has been reported in patients suffering from various conditions and, more recently, in patients with coronavirus disease 2019 (COVID-19) [4, 5]. Pseudothrombocytopenia can lead to misdiagnosis and, ultimately, the selection of inappropriate treatments for the patient [4, 6].

In automated hematology analyzers, platelets are counted as part of the complete blood cell count (CBC) by impedance, optical methods (light diffraction or fluorescence techniques), and immunofluorescence techniques using monoclonal antibodies directed against glycoproteins of the surface membrane of platelets [2]. Automated hematology analyzers can produce accurate CBC results for virtually any sample. However, from time to time, inaccurate results may occur [7]. Flags or messages from the devices regarding these 'spurious' changes differ depending on the analyzer and the method [8].

With impedance-type instruments, platelet and red blood cell count (RBC), which are both analyzed in the same channel(s), are discriminated according to their volume, and volume histograms are then generated. With the Mindray BC-6200, platelet counts as a part of CBC can be done in two ways: by platelet counts based on the DC sheath-flow impedance (PLT-I) method (based on DC sheath-flow impedance), or by platelet counts based on fluorescent nucleic acid staining and done in the reticulocyte channel (PLT-O) method. If there is a 'platelet aggregation' or a 'low platelet count' alarm from the hematology analyzer, a reflex test can be performed using the PLT-O method. It has been claimed that PLT-O detection technology can effectively correct platelet counts [9].

In routine laboratory practice, microscopic examination of peripheral blood smear slides by a trained specialist is required for thrombocytopenic samples. However, manually examining the platelet count is time-consuming and labor-intensive. Recent advances have allowed platelet count estimation using digital morphology analyzer imagery and software algorithms [10]. These increase efficiency and reduce inconsistency between observers, especially in laboratories with a large number of patient samples.

Our objective was to compare the PLT-I and PLT-O values detected by the Mindray BC-6200 device to the MC-80 digital morphology analyzers' estimated platelet counts (PLT-E) test results, and to determine its efficacy for the confirmation of pseudothrombocytopenia.

Material and methods

This was a retrospective data analysis study. The study protocol was approved by the Ethics Committee of the Institution (2011-KAEK-25 2022/11-10).

CBC findings between 1 September and 11 October, 2022 were obtained from the data storage units of the devices. In our laboratory, blood is taken into K3-EDTA-containing vacutainer tubes (Ayset Medical Products, Adana, Turkey) for CBC analysis. According to laboratory protocol, CBC analysis is completed within two hours after blood collection. During this period, a total of 59,856 hemograms were studied in our laboratory, including from outpatients, inpatients, and asymptomatic individuals who came to the hospital only for the purpose of health screening.

Platelet counts (as a part of the CBC) were performed using an automated hematology analyzer, the Mindray BC-6200 (Mindray, Shenzhen, China). Internal quality control tests were performed in accordance with the manufacturer's instructions. External quality control was performed in accordance with KBUDEK (External Quality Control Program, Istanbul, Turkey).

In our laboratory routine, PLT-I results were reported after the first measurement. Once the samples with a thrombocytopenia and/or platelet aggregation flag were detected, they went under the verification protocol for PLTs, which includes opening the reticulocyte channel. The result that was analyzed in the PLT-O mode by staining with a specific fluorescent dye with high specificity and sensitivity was reported at the second measurement. At the same time, peripheral blood smear (PBS) staining and examination were also performed [9, 11].

SC120 automated slidemakers (Mindray, Shenzhen, China) were used to prepare PBSs stained with Wright-Geimsa dye [12]. PBSs were evaluated using an MC-80 digital morphology analyzer. The analyzer classifies white blood cells (WBCs) and red blood cells (RBCs) and include functionality for estimation of platelet count. For our analysis, only the platelet count data was used.

Statistical analysis

Statistical analysis was performed using MedCalc® Statistical Software version 20.121 (MedCalc Software Ltd, Ostend, Belgium) and SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

Table I. Mindray BC-6200 automated hematology analyzer complete blood count platelet indices analyzed usingimpedance method

Parameter	Group 1*	Group 2**	Control
N	49	32	190
PLT-I count, mean ± SD [× 10^9/L]	49.5 ± 34.2	75.0 ± 33.3	289.8 ± 77.4
PLT-O count, mean ± SD [× 10^9/L]	45.9 ± 33.5	112.5 ± 71.0	
PLT-E count, mean ± SD [× 10^9/L]	44.7 ± 32.0	115.1 ± 68.3	270.8 ± 77.6
PDW [%]	16.7 ± 1.12	16.5 ± 1.01	16.1 ± 0.30
MPV [fL]	11.7 ± 1.8	12.7 ± 1.9	11.4 ± 1.0

*Thrombocytopenia; **aggregation/clumping flag; N — number of patients' samples; PLT — platelet; PLT-I — impedance method; SD — standard deviation; PLT-O — fluorescent nucleic acid staining method; PLT-E — calculated using a digital morphology analyzer; PDW — platelet distribution width; MPV — mean platelet volume; fL — femtoliters

The mean ± standard deviation (SD) and median values of the variables were calculated. One-way repeated measures analysis of variance (ANOVA) was used to determine significant differences between the related means. We performed a paired sample t-test or aWilcoxon matched paired test where appropriate (depending on the distribution of the measurements) to determine the significance of the differences between groups. Agreement between assays were evaluated by Passing-Bablok regression analysis, Spearman's rank correlation coefficient (rho), and Bland-Altman plots. A p value ≤0.05 was considered statistically significant.

Results

We selected two groups of blood specimens with a PLT-I level ranging from 2 × 10^9/L to 146 × 10^9/L: the first group with thrombocytopenia, and the second group that gave an aggregation and/or platelet clumping flag using a Mindray BC-6200 automated hematology analyzer and/or an MC-80 digital morphology analyzer. The results of 190 consecutive patients with normal platelet counts (158 × 10^9/L to 448 × 10^9/L) and no aggregation flag were evaluated as a control group.

During the study period, the results of the blood specimens, a total of 81 specimens from CBC with thrombocytopenia and/or platelet aggregation/clumping flag, were evaluated. The age range of patients was 1–94 years. Twenty-two patients were followed up for malignancy: five for idiopathic thrombocytopenic purpura, five for chronic renal failure, six for chronic viral hepatitis, four for connective tissue disease, and the remaining two patients with various diagnoses.

Of the patient samples, one with thrombocytopenia and another one with an aggregation flag were analyzed 10 times with the Mindray BC-6200 device in PLT-I and PLT-O modes, and calculated CVs were found to be acceptable, in the range 2.58–4.61% [13].

The thrombocytopenic samples without aggregation and/or clumping flag from the Mindray BC-6200 analyzer and/or Mindray MC-80 (n = 49) had a mean platelet level of 49.5 ± 34.2 × 10^9/L and a median platelet level of 46.0 × 10^9/L [interquartile range (IQR): 65] in PLT-I mode. The CBC was reanalyzed in the reticulocyte channel PLT-O, and the mean PLT count was 45.9 ± 33.5 with a median count of 43.0 × 10^9/L (IQR: 54). The PLT-E was 44.7 ± 32.0 × 10^9/L with a median of 36 × 10^9/L (IQR: 59) using the MC-80 system (Table I). One-way repeated measures analysis of variance (ANOVA) showed no significant group difference between the methods ($F = 2.83$, $p = 0.064$).

After reanalyzing thrombocytopenic samples without aggregation/clumping flags, PLT-E values with PLT-O and with PLT-I were strongly correlated, Spearman's rho values were 0.914 and 0.902, respectively (p <0.001, p <0.001).

In samples with thrombocytopenia without aggregation and/or clumping, the plot of the difference between the automated analyzer Mindray BC-6200's PLT-O and PLT-I count values against their means according to the Bland-Altman design showed that the difference in means was 3.6 (95% CI: 0.04–7.18; Figure 1). On the Bland-Altman plot, the differences in means were all close to zero, and there were no definite biases, although outliers were identified.

In samples from the aggregation and/or clumping group, 11 gave the aggregation flag using the Mindray BC-6200 analyzer, 24 gave the clumping flag using the MC-80 analyzer, and six gave flags from both analyzers.

The mean platelet level of 32 patients with aggregation and/or clumping flags using the Mindray BC-6200 analyzer and/or MC-80 was 75.0 ± 33.3 × 10^9/L, with a median of 72.5 × 10^9/L (IQR: 51.2) in PLT-I mode. The reanalyzed PLT-O mean in the reticulocyte channel of the patients was 112.5 ± 71.0 × 10^9/L, and the median was 97 × 10^9/L (IQR: 104.7). With the MC-80 system, the PLT-E mean was 115.1 ± 68.3 × 10^9/L, with a median of 92.5 × 10^9/L (IQR: 123.5). However, the platelet counts were still lower than 100 × 10^9/L with 18 specimens, although these counts had increased upon reevaluation.

One-way repeated measure analysis of variance (ANOVA) showed a significant difference between the methods ($F = 11.7$, p <0.001). The concentrations obtained by the PLT-I method were lower than those obtained by the PLT-E and PLT-O methods (p <0.001 and $p = 0.002$, respectively).

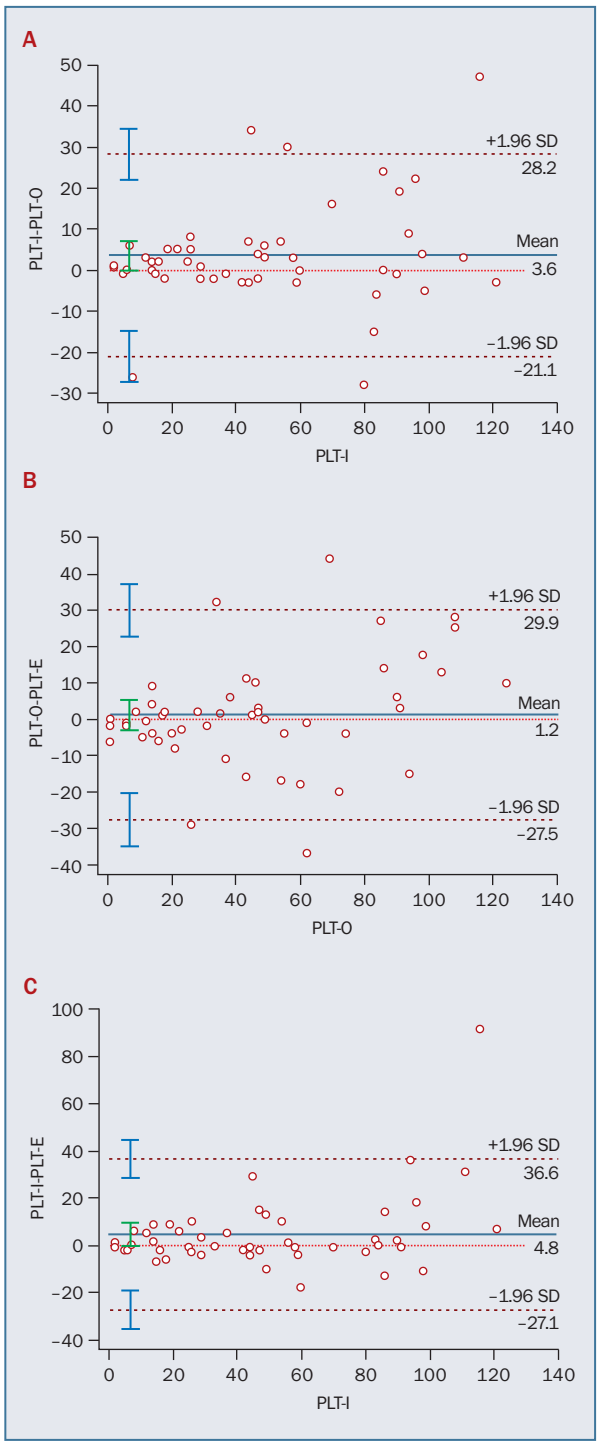

Figure 1. In samples with thrombocytopenia without aggregation and/or clumping, plot of difference between: **A.** Platelet counts based on DC sheath-flow impedance method (PLT-I) and platelet counts based on fluorescent nucleic acid staining and done in reticulocyte channel (PLT-O) values against their means according to Bland-Altman design shows that difference in means was 3.6 [95% confidence interval (CI): –0.04–7.18]; **B.** PLT-O and estimated platelet counts (PLT-E) values against their means was 4.8 (95% CI: –0.08–9.42); **C.** PLT-I and PLT-E count values against their means was 1.18 (95% CI: –3.02–5.39). Outer solid lines are upper and lower limits of agreement; SD — standard deviation

The concentrations obtained by the PLT-E and PLT-O methods were not statistically different (p = 0.318).

Regression analysis of samples with aggregation and/or clumping flags using the Mindray BC-6200 analyzer and/or MC-80 between PLT-O and PLT-I was rho = 0.367, p = 0.039 (Figure 2), and for PLT-I with PLT-E was rho = = 0.157, p = 0.391. A comparison of the digital morphology analyzer system PLT-E count to the PLT-O count showed a rho value of 0.807 (p <0.001). For samples with aggregation/clumping flags, the plot of the difference between the PLT-I and PLT-O counts against their means according to the Bland-Altman design showed that the mean difference was –43.6 (95% CI: –17.2 to –69.9) (Figure 3), and when we compared PLT-O to PLT-E, bias was improved to –6.1 (95% CI: –18.26 to 6.1) (Figure 3).

The platelet count values were obtained by the PLT-I and PLT-E values on the same blood samples of the control samples respectively (Table I).

Discussion

We found that platelet counts with PLT-I and repeat-test PLT-O modes using the Mindray BC-6200 analyzer's and morphology analyzer's PLT-E values of samples with thrombocytopenia without aggregation were close to each other.

However, in samples with aggregation and/or clumping flags from the Mindray BC-6200 and/or MC-80, the results of PLT-I and PLT-O counts were quite different from each other.

Clinically, a platelet count below 100 × 10^9/L indicates the risk of bleeding [14]. Using the repeat tests, the aggregation- and/or clumping-related outcomes of eight patients' platelet values changed significantly from high-risk bleeding.

Most clinical laboratories use the impedance technique for platelet counting, which involves changing the density of an electrical current as the blood particle passes through two electrodes. However, this method has some limitations [15]. It does not distinguish platelets from other blood elements with similar size ranges, which is highly imprecise in various clinical situations, despite the application of computerized algorithms [16]. Interferences such as platelet aggregation and giant platelets can cause a false decrease, and fragments of red and white blood cells can cause a false increase, in platelet counts using the PLT-I method [16].

While the impedance method (PLT-I) is generally used in routine settings, PLT-O is established as a 'reflex test' in laboratories when thrombocytopenia or an aggregation flag is found. More importantly, it has recently been described as an effective method for correcting falsely low platelet counts [11, 13].

While PLT-I and PLT-O results were different in the samples with aggregation, the PLT-E values obtained by

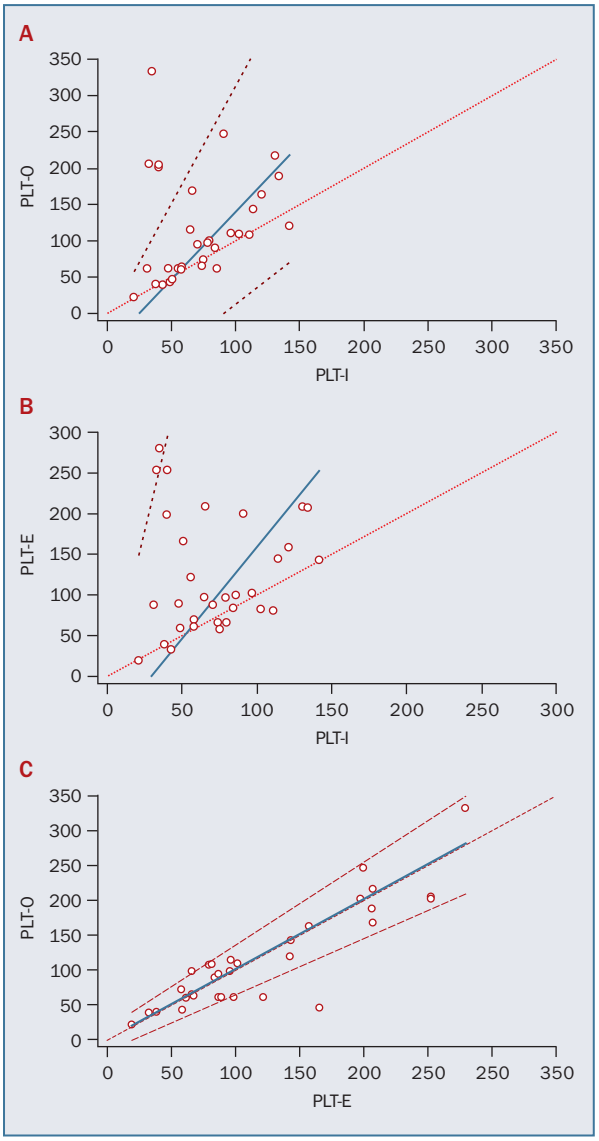

Figure 2. Regression analysis of samples with aggregation and/or clumping flags using Mindray BC-6200 analyzer and/or MC-80 between: **A.** Platelet counts based on fluorescent nucleic acid staining and done in reticulocyte channel (PLT-O) and platelet counts based on impedance method (PLT-I) (rho = 0.367); **B.** MC-80 digital morphology analyzer's estimated platelet counts (PLT-E) and PLT-I counts (rho = 0.157); **C.** PLT-E and PLT-O counts (rho = 0.807). Confidence intervals for regression lines are dashed

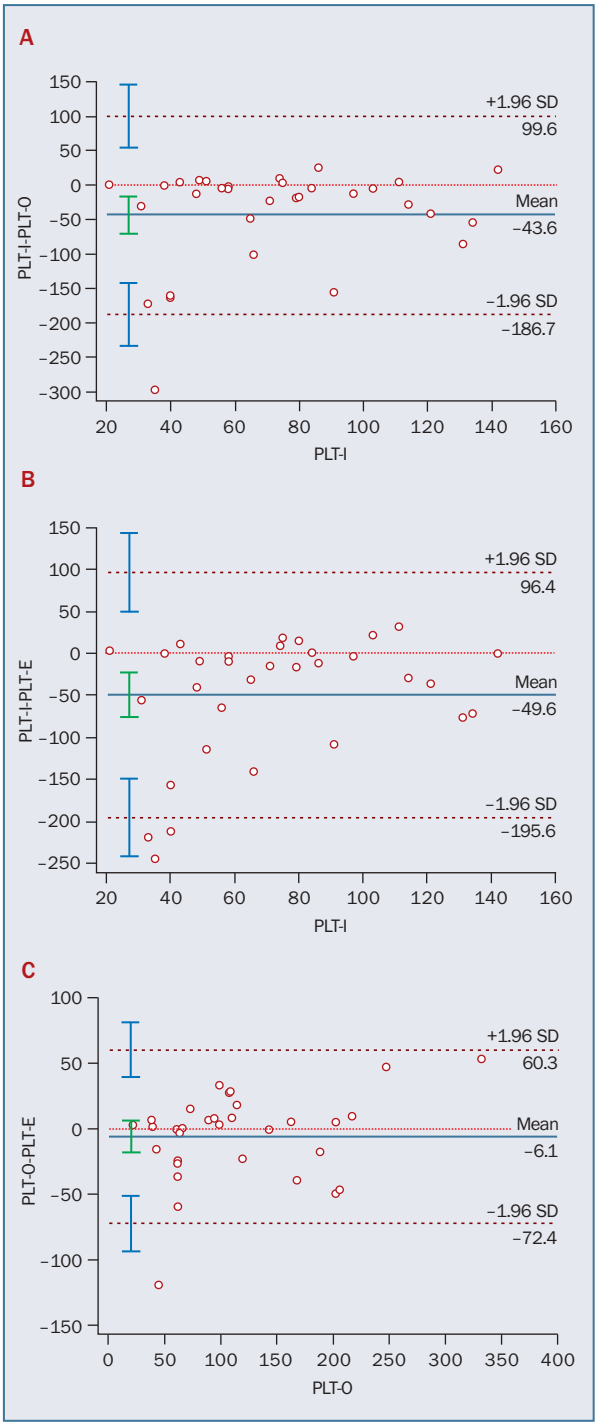

Figure 3. In samples with aggregation and/or clumping flags, plot of difference between: **A.** Platelet counts based on impedance method (PLT-I) and platelet counts based on fluorescent nucleic acid staining and done in reticulocyte channel (PLT-O) values against their means according to Bland-Altman design shows that difference in means was –43.6 (95%: –69.9 to –17.2); **B.** PLT-I and PLT-E count values against their means was –49.6 (95%: –76.5 to –22.8); **C.** PLT-O and MC-80 digital morphology analyzer's estimated platelet counts (PLT-E) values against their means was –6.1 (95%: –18.3 to 6.1). Outer solid lines are upper and lower limits of agreement; SD — standard deviation

evaluating and calculating stained preparations with a digital morphology analyzer and PLT-O results were correlated with each other, and the bias was quite low. Manual microscopy has been reported to be a more specific option for rechecking platelet counts. However, the manual microscopy method also has many limitations: the test is difficult, time consuming, and has low reproducibility [9]. The newly introduced digital morphology analyzer method, on the other hand, is automated, has good reproducibility,

and allows discussion between clinicians regardless of their physical location.

In recent years, several automated digital morphology analyzer systems, such as the Cobas m511, CellaVision systems and Sysmex systems, have been developed for use in medical laboratories [17]. Gao et al. [18] compared platelet counts obtained using the CellaVision DM96 system (CCS; CellaVision, Lund, Sweden) to automated hematology analyzers (Beckman Counter LH 780 or Unicel DXH 800 analyzers). They found that the two analyzers showed good compatibility with manual platelet estimates. Kim et al. [19] observed that the DI-60 system (Sysmex, Kobe, Japan) and the Sysmex XN hematology analyzer had results comparable to each other for PLT estimation, but that there was a tendency to underestimate platelet counts in samples with marked thrombocytosis. However, to the best of our knowledge, this has never been evaluated in the presence of platelet clumps. With the continuing development of artificial intelligence methods, digital microscopes in our labs in the near future will replace manual processes, just as automatic urine analyzers are replacing manual urine microscopy [20]. Reliable platelet counts from blood smear images using image processing techniques will increase standardization and efficiency by reducing laboratory staff involvement [21, 22].

Limitations

This study has several limitations. Firstly, we did not confirm the platelet count using the immunological platelet counting method [23, 24]. Secondly, since the specificity and sensitivity of platelet-related alarms/flags vary widely, and any abnormal distribution in the platelet curve from the CBC result from the hematology analyzer should be examined, we did not evaluate that. There were no peer-reviewed manuscripts whether platelet count validation with the MC-80 digital morphology analyzer was performed by the manufacturer.

Further well-designed studies with a large number of participants are needed to demonstrate whether the combined use of a digital morphology analyzer and the Mindray PLT-O method achieves the most accurate reporting of spuriously low platelet counts. Further investigations should be performed, including the necessary verification of the results obtained by the MC-80 digital morphology analyzer in combination with the reference method.

Conclusions

Laboratory professionals should recognize unreliable results and identify possible causes. Evaluation of a decreased platelet count to rule out the presence of pseudothrombocytopenia, thus avoiding unnecessary treatment, is required.

In a laboratory that routinely analyzes large numbers of patient samples and requires rapid turnaround of results, evaluating platelets with aggregation could be missed. The simultaneous measurement of platelet counts using the PLT-O and PLT-E methods gives a more specific recognition of platelets. The PLT-O and PLT-E methods should also be rerun in patients with thrombocytopenia, even if there is no aggregation flag from a hematology analyzer.

Examining blood samples using the Mindray MC-80 digital morphology analyzer system on samples that show platelet clumps has the potential to improve PLT-I results in day-to-day laboratory routine.

Authors' contributions

YU contributed to concept and design. KH, EGK drafted work or revised it critically for important intellectual content. All authors have acquired, analyzed or interpreted data. All authors accept responsibility for entire content of this manuscript and approve submission.

Conflict of interest

The authors declare no conflict of interest.

Financial support

None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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VIA MEDICA

Characterization and prognostic factors of secondary to MDS/MPN and therapy-related AML: a single-center study

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Abstract

Introduction: Secondary acute myeloid leukemia (sAML) accounts for 15–30% of overall AML cases and is associated with shorter survival compared to de novo AML. The pathogenetic spectrum of sAML is heterogeneous, i.e. therapy-related AML (tAML) arises from prior cytotoxic, radiation, or immunosuppressive therapy, while myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN)-AML develops from a previous clonal disorder of hematopoiesis.

Material and methods: We performed a single-center retrospective analysis of MDS/MPN-AML and tAML patients diagnosed between 2013 and 2018 in the Hematology Department of the Medical University in Lodz, Poland. Simultaneously, demographic data, clinical factors, and laboratory findings were collected. For statistical analysis, we used Cox proportional hazard models and log-rank tests.

Results: The study included 110 patients with either MDS/MPN-AML (n = 78) or tAML (n = 32), with a median age of 66 years (range 31–86). The median follow-up was 3.2 months [95% confidence interval (CI): 2.5–5.3]. The median overall survival (OS) for MDS/MPN-AML patients was 4.1 months (95% CI: 2.5–7.0) and for tAML it was 2.8 months (95% CI: 1.6–5.6). In multivariate Cox regression model for OS, factors such as age at diagnosis [hazard ratio (HR) 1.03, 95% CI: 1.00–1.06), higher Eastern Cooperative Oncology Group score (HR 1.85, 95% CI: 1.08–3.15), hypoalbuminemia (HR 3.20, 95% CI: 1.95–5.24) and percentage of bone marrow blasts infiltration (HR 1.01, 95% CI: 1.00–1.03) were independent predictors of poor survival for the whole cohort. On the other hand, the intensive treatment approach was related to longer survival (HR 0.42, 95% CI: 0.21–0.82). There were no differences in OS between MDS/MPN-AML and tAML (p = 0.81).

Conclusion: The poor treatment outcomes for sAML consist of a combination of low response rate and high early mortality. The positive influence of intensive chemotherapy should be highlighted, but nevertheless, optimizing treatment for this high-risk subpopulation remains crucial.

Key words: acute myeloid leukemia, secondary AML, treatment-related AML, MDS/MPN AML, overall survival

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Introduction

Secondary acute myeloid leukemia (sAML) is a term given to AML developing out of preceding myeloid malignancies i.e. myelodysplastic syndromes or myeloproliferative neoplasm (MDS/MPN-AML). However, in the literature this term also includes AML arising after prior exposure to cytotoxic therapy and/or radiotherapy for malignant or

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non-malignant disease, which corresponds to the World Health Organization (WHO) 2016 definition of therapy-related AML (tAML) [1]. Classically, tAML is divided according to the causative agent into type 1, which is associated with prior treatment with alkylating agents or ionizing radiation, and type 2, which follows treatment with topoisomerase II inhibitors [2, 3].

Type 1 tAML usually appears 4–7 years after treatment, and approximately two-thirds of patients have a preceding MDS. High frequency of abnormalities involving the long arm of chromosome 5 [del(5q)], the long arm of chromosome 7 [del(7q)], or loss of chromosome 7 (del7) is also characteristic of this type.

In type 2 tAML, the latency period is shorter, and the disease usually develops 2–3 years after treatment, without a preceding myelodysplastic phase and with common balanced chromosomal translocations involving 11q23 (*MLL*) or 21q22 (*RUNX1*) [2, 4]. The most frequently mutated genes in sAML are those related to DNA methylation (46%), chromatin modification (42%), RAS signaling (42%), RNA spliceosome machinery (55%), transcriptional regulation (34%), and those related to proteins that regulate the three-dimensional organization of chromatin in the nucleus (22%) [5]. Analyzing the molecular findings for MDS/MPN-AML, the presence of mutations in the *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR* or *STAG2* genes is highly specific [6].

Although the latest classification for AML, according to European LeukemiaNet (ELN) in 2022, removed sAML from the main classification categories, the features of that subtype are still clinically important and have been applied as diagnostic qualifiers to the AML-defining category [7]. Similarly, the International Consensus Classification (ICC) of Myeloid Neoplasms and Acute Leukemias 2022 emphasizes the role of prior therapy as well as antecedent myeloid neoplasms in the development of AML and also distinguishes them as diagnostic qualifiers [8]. The reason for exclusion as an independent entity is the current emphasis on categorizing AML based on genetic alterations. Simultaneously, in 2022 the WHO's Classification of Hematolymphoid Tumors was published. However, this maintained the AML myelodysplasia related (AML-MR) categorization, although changing the name to AML "with myelodysplasia-related changes" (AML-MRC) and updating the diagnostic criteria. The key changes are the removal of morphology as the sole factor determining the diagnosis of AML-MR, the updating of the cytogenetic criteria, and the introduction of mutations of eight genes that mandate the diagnosis i.e. *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR,* and *STAG2*. MDS/MPN-AML continues to be defined under AML-MR in view of the broader unifying biological features, while tAML categorization, referred to myeloid neoplasms post cytotoxic therapy (MN-pCT), also remains classified after slight modifications [9].

sAML is characterised by a poor prognosis with an estimated survival of 6–12 months and it is considered to be a risk factor for early death in some prognostic models compared to *de novo* AML [2]. However, it is still a challenge to describe in detail the mechanisms and reasons for unsatisfactory treatment outcomes. There have been many population-based analyses characterizing possibly crucial factors. Nevertheless, the independent prognostic value of sAML itself has been questioned, as the diagnosis is often associated with older age, frequent comorbidities/organ dysfunction, and an unfavorable cytogenetic and molecular profile [10].

In this study, we aimed to characterize MDS/MPN-AML and tAML patients treated at our center and to evaluate relevant prognostic factors in these subtypes of AML.

Material and methods

We conducted a comprehensive analysis of adult patients diagnosed with AML in the Department of Hematology at the Medical University in Lodz, Poland, between 2013 and 2018. We developed a database of AML patients to search for significant prognostic features and to compare and characterize particular subtypes of sAML in our region. We based our study on the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) classification and searched for data via the medical records. Patients were assigned to either an MDS/MPN-AML group or a tAML group, according to the WHO 2016 classification [1] (MDS/MPN-AML and tAML were defined as previously described).

The cytogenetic risk profile was classified as favorable, or intermediate, or adverse according to the ELN 2017 criteria [11]. Patients eligible for intensive chemotherapy were treated according to Polish Acute Leukemia Group (PALG) protocols, while the unfit or elderly population was given low-intensity treatment or best supportive care. The intensive treatment included induction chemotherapy based on daunorubicin (DNR 60 mg/m^2 i.v., days 1–3) and cytarabine (Ara-C, 200 mg/m^2 i.v., days 1–7) — DA or DA with cladribine (5 mg/m^2 i.v., days 1–5) (DAC) or for patients aged over 60 — DA/DAC with reduced dose of Ara-C (100 mg/m^2 i.v., days 1–7). The post-remission therapy was based on high/intermediate-dose cytarabine regimens with a subsequent allogeneic hematopoietic stem cell transplantation (allo-HSCT). The non-intensive treatment included repeated courses of azacitidine (AZA) 75 mg/m^2, days 1–7, low-dose of Ara-C (LD-Ara-C) 20 mg/m^2, days 1–10, cytoreduction with hydroxyurea or 6-mercaptopurine, or best supportive care (BSC) alone. The functional status of patients was assessed according to the Eastern Cooperative Oncology Group (ECOG) scale. The patients' characteristics are set out in Table I.

Table I. Patients' characteristics

Variable	MDS/MPN-AML	tAML
Patients, n	78	32
Gender, n [%]:		
• female	32 (41)	19 (59)
• male	46 (59)	13 (41)
Age at diagnosis, median (IQR)	66 (60–71.75)	67.5 (62.75–72)
Age distribution, n [%]:		
• <60 years	19 (24)	5 (16)
• ≥60 years	59 (76)	27 (84)
Primary disease, n [%]	MDS 48 (62) MPN 30 (38)	Breast 9 (28) Prostate 6 (19) Other 17 (53)
ECOG, n [%]:		
• 0	36 (46)	11 (34)
• 1	25 (32)	9 (28)
• 2	11 (14)	10 (31)
• 3	2 (3)	2 (6)
• 4	3 (4)	0
• no data	1 (1)	
Cytogenetic risk profile, n [%]:		
• 1	2 (3)	2 (6)
• 2	28 (36)	5 (15)
• 3	16 (21)	13 (41)
• no data	32 (41)	12 (37)
WBC at dgn. [G/L], median (IQR)	6 (1.9–42)	6.21 (1.98–66.66)
ANC at dgn. [G/L], median (IQR)	1.25 (0.5–11.2)	1.22 (0.27–9.91)
PB blasts at dgn. [%], median (IQR)	14 (4–45)	2.5 (0–30)
PLT at dgn. [G/L], median (IQR)	45.5 (21.2–107)	45 (21.5–80)
Hb at dgn. [g/dL], median (IQR)	8.3 (7.4–9.2)	8.4 (6.9–9.6)
LDH at dgn. [U/L], median (IQR)	282 (230–559)	350.5 (222.7–506)
Uric acid [mg/dL], median (IQR)	6.1 (4.6–8.2)	5.5 (4.7–7.2)
Albumin [g/dL], mean ± SD	3.6 ± 0.67	3.8 ± 0.61
BM blasts at dgn. [%], median (IQR)	35 (25–55)	45 (27.5–63.2)
Dysplasia (lines), n [%]:		
• 1	9 (11.5)	7 (22)
• 2	24 (31)	14 (44)
• 3	39 (50)	9 (28)
• no data	6 (7.5)	2 (6)
Intensive treatment, n [%]:		
• all age groups	22 (28)	5 (15.5)
• <60 years	16 (84)	3 (60)
• ≥60 years	6 (10)	2 (7)
Non-intensive treatment, n [%]:		
• LD-Ara-C	22 (28)	15 (47)
• AZA	16 (21)	4 (12.5)
• cytoreduction or BSC	18 (23)	8 (25)
Response rates, n [%]:		
• CR	13 (17)	5 (15.5)
• PR	18 (23)	7 (22)
• NR	8 (10)	5 (15.5)
• PD	20 (26)	12 (38)
• ED	19 (24)	3 (9)

→

Table I (cont.). Patients' characteristics

Variable	MDS/MPN-AML	tAML
Time since primary disease dgn. to AML dgn., median (IQR) [months]	13.5 (4.3–30)	90 (38.5–112.0)
OS since primary disease dgn., median (IQR) [months]	26 (11–48.7)	94 (44.0–119.0)
OS since AML dgn., median (IQR) [months]	4.1 (1.1–13.7)	2.8 (1.4–8.2)

MDS/MPN-AML — acute myeloid leukemia secondary to myelodysplastic syndrome/myeloproliferative neoplasm; tAML — therapy-related AML; n — number; IQR — interquartile range; ECOG — Eastern Cooperative Oncology Group scale; WBC — white blood count; dgn. — diagnosis; ANC — absolute neutrophil count; PB — peripheral blood; PLT — platelets; Hb — hemoglobin; LDH — lactate dehydrogenase; SD — standard deviation; BM — bone marrow; LD-Ara-C — low-dose of cytarabine; AZA — azacytidine; BSC — best supportive therapy; CR — complete remission; PR — partial remission; NR — no response; PD — progressive disease; ED — early death; AML — acute myeloid leukemia; OS — overall survival

Table II. Univariate analysis of overall survival in both therapy-related acute myeloid leukemia and acute myeloid leukemia secondary to myelodysplastic syndrome/myeloproliferative neoplasm patients

Variable	Coefficient	*p*	HR	95% CI	
				Lower	Upper
Age at dgn.	0.04	0.00	1.04	1.02	1.06
Age at dgn. ≥60 years	0.78	0.00	2.18	1.28	3.69
ECOG 0/1 vs. 2/3/4	0.90	0.00	2.47	1.56	3.92
Cytogenetic risk low/intermediate vs. high according to ELN 2017	1.02	0.00	2.76	1.61	4.73
WBC at dgn.	0.00	0.27	1.00	1.00	1.01
WBC at dgn. >20 G/L	0.21	0.33	1.23	0.81	1.87
WBC at dgn. >50 G/L	0.12	0.63	1.12	0.70	1.80
ANC at dgn.	0.01	0.14	1.01	1.00	1.01
PB blasts at dgn.	0.01	0.01	1.01	1.00	1.02
PLT at dgn.	0.00	0.48	1.00	1.00	1.00
Hb at dgn.	–0.09	0.07	0.91	0.83	1.01
LDH at dgn.	0.00	0.01	1.00	1.00	1.00
LDH norm vs. above norm	0.06	0.81	1.06	0.66	1.71
Uric acid at dgn.	0.06	0.14	1.06	0.98	1.15
Uric acid norm vs. above norm	0.35	0.10	1.41	0.94	2.13
Albumins at dgn.	–0.38	0.04	0.68	0.47	0.99
Albumins norm vs. below norm	0.54	0.02	1.72	1.08	2.74
BM blasts at dgn.	0.02	0.00	1.02	1.01	1.03
BM blasts ≥50%	0.64	0.00	1.90	1.26	2.88
BM blasts ≥60%	0.69	0.01	2.00	1.23	3.25
Dysplasia in 1 vs. 2/3 lines	0.33	0.25	1.40	0.79	2.47
Intensive vs. non-intensive treatment	–1.11	0.00	0.33	0.20	0.55
Time from primary disease dgn. to AML dgn.	0.00	0.05	1.00	1.00	1.01

HR — hazard ratio; CI — confidence interval; dgn. — diagnosis; ECOG – Eastern Cooperative Oncology Group scale; ELN — European LeukemiaNet; WBC — white blood count; ANC — absolute neutrophil count; PB — peripheral blood; PLT — platelets; Hb — hemoglobin; LDH — lactate dehydrogenase BM — bone marrow; AML — acute myeloid leukemia

Statistical analysis

We performed a survival analysis and created a Cox proportional hazards model. We calculated the median overall survival (OS) for the MDS/MPN-AML and tAML groups, as well as the median OS from diagnosis of primary cancer. We performed a comparison of survival in groups divided according to the selected variables using the log-rank test. Initially we created a univariate analysis of OS for all patients (Table II) and selected the variables with the highest level of statistical significance. Next, we created a Cox proportional hazards regression model including covariates with $p < 0.15$. As a result, this included quantitative variables such as albumin level, percentage of blasts in the bone marrow (BM) and age, and qualitative variables such as cytogenetic risk, ECOG grade, and type of treatment. We considered variables with $p < 0.05$ as

Table III. Cox proportional-hazard regression for overall survival in both therapy-related acute myeloid leukemia and acute myeloid leukemia secondary to myelodysplastic syndrome/myeloproliferative neoplasm patients

Variable	Coefficient	*p*	HR	95% CI	
				Lower	Upper
Albumin (norm vs. below norm)	1.16	<0.00	3.20	1.95	5.24
Blasts BM [%]	0.01	0.02	1.01	1.00	1.03
Cytogenetic risk (low/intermediate vs. high)	0.39	0.11	1.48	0.92	2.38
ECOG (0/1 vs. 2/3/4)	0.62	0.02	1.85	1.08	3.16
Intensive vs. non-intensive treatment	–0.87	0.01	0.42	0.21	0.81
Age at AML diagnosis	0.03	0.04	1.03	1.00	1.06

HR — hazard ratio; CI — confidence interval; BM — bone marrow; ECOG — Eastern Cooperative Oncology Group scale; AML — acute myeloid leukemia

significant independent prognostic factors (Table III). Confidence intervals for the hazard ratio were set at 95%. The software used was Statistica 13.1 (TIBCO Software Inc.) and MedCalc Software Ltd.

Results

A total of 110 patients from the database [78 with MDS/MPN-AML and 32 with tAML; women 46% (n = 51); men 54% (n = 59)] was included. The median age at diagnosis was 66 years [interquartile range (IQR) 60–71.75] for MDS/MPN-AML and 67.5 years (IQR 62.75–72) for tAML (p = = 0.51). In the MDS/MPN-AML group, 76% (n = 59) of patients were aged 60 years or above, while in the tAML group, they accounted for 84% (n = 27). The baseline clinical and laboratory parameters are detailed in Table I. Among MDS/MPN-AML patients, myelodysplastic syndrome was the most common antecedent disorder (n = 48; 62%), and myeloproliferative diseases accounted for 38% (n = 30). Regarding patients with tAML, the most common primary solid tumors were breast cancer (n = 9; 28%) and prostate cancer (n = 6; 19%) (Table IV).

As for laboratory parameters, there were no differences in peripheral blood morphology values, nor in biochemical exponents, between the groups (p >0.05). The median percentage of blasts in peripheral blood was 14% for MDS/MPN-AML and 2.5% for tAML (p = 0.007), while the percentage of blasts in BM was 35% vs. 45%, respectively (p = 0.22).

Overall, the proportion of patients referred to intensive chemotherapy was only 25%. In the MDS/MPN-AML group, intensive treatment was administered to 28% of patients (n = 22), of whom 16 were <60 and six patients were ≥60. For the tAML group, only 16% of patients (n = 5) were intensively treated, comprising three patients <60 and two ≥60. Considering patients treated non-intensively, LD-Ara-C therapy was given to 33.5% of patients (n = 37), AZA to 18% (n = 20), and 23.5% of patients were qualified for cytoreduction or BSC alone (n = 27). The division into MDS/MPN and tAML groups is included in Table I.

Allo-HSCT was performed in nine patients, of whom five received myeloablative (MAC) and four reduced-intensity (RIC) conditioning. One patient underwent two allo-HSCTs with an interval of two years. The mean age in this group was 47 years [standard deviation (SD) ± 11.6], and two patients were >60. Median OS for patients undergoing allo-HSCT was 18.8 months (95% CI: 4.4–35.6 months).

Complete remission (CR) was achieved in 16% (n = = 18) of patients, 23% (n = 25) had partial remission (PR), 12% (n = 13) had no response to the applied treatment (NR), 29% (n = 32) of patients experienced disease progression despite therapy, and 20% (n = 22) suffered early death (ED), defined as death within 28 days from the start of treatment. Considering intensively treated patients, CR was achieved by 56% (n = 15) and PR by 22% (n = 6) (78% CR + PR, n = 21). Among patients not treated intensively, 4% achieved CR (n = 3; two patients treated with AZA and one treated with LD-Ara-C), and 23% achieved PR (n = 19). The division into MDS/MPN and tAML groups considering each response rate is included in Table I.

The median time from primary disease diagnosis to AML was 13.5 months (IQR 4.2–30) for MDS/MPN-AML, and 90 months (IQR 38.5–112.0) for tAML. The median OS from primary disorder diagnosis was 26 months (IQR 11–48.7, 95% CI: 21–32) and 94 months (IQR 42.0–119.0, 95% CI: 66–114) for MDS/MPN-AML and tAML, respectively. Survival in the whole sAML group was very poor, with median OS of 3.1 months (IQR 1.4–13, 95% CI: 2.5–5.3) consisting of 4.1 months (IQR 1.1–13.7, 95% CI: 2.5–7.0) for MDS/MPN-AML and 2.8 months (IQR 1.4–8.2, 95% CI: 1.6–5.6) for tAML patients; no statistical difference was observed between the groups (p = 0.81) (Figure 1).

Median OS for the entire cohort was significantly longer for patients with low (median not reached) versus intermediate (13.2 months) versus high (2.8 months) cytogenetic risk, respectively, (p = 0.0001) (Figure 2A). Moreover, patients with an initial BM blast level below 50% had longer survival (5.5 vs. 1.6 months) (p = 0.001) (Figure 2B). A comparison of survival in patients treated with intensive versus non-intensive therapeutic approaches

Table IV. Distribution of primary disorders/cancers among patients with secondary acute myeloid leukemia

Variable	Primary disorder/cancer	Number of cases	Percentage of MDS/MPN-AML/tAML	Percentage of all cases
MDS/MPN-AML	MDS	48	61.5%	43.6%
	PV	7	9.0%	6.4%
	PMF	7	9.0%	6.4%
	CMML	7	9.0%	6.4%
	CML	5	6.4%	4.5%
	ET	3	3.8%	2.7%
	CNL	1	1.3%	0.9%
tAML	Breast	9	28.1%	8.2%
	Prostate	6	18.8%	5.5%
	Colon	5	15.6%	4.5%
	Ovaries	3	9.4%	2.7%
	Hodgkin lymphoma	3	9.4%	2.7%
	Stomach	1	3.1%	0.9%
	Endometrium	1	3.1%	0.9%
	DLBCL	1	3.1%	0.9%
	Lung	1	3.1%	0.9%
	Thyroid	1	3.1%	0.9%
	Sarcoma	1	3.1%	0.9%

MDS/MPN-AML — acute myeloid leukemia secondary to myelodysplastic syndrome/myeloproliferative neoplasm patients; tAML — therapy-related acute myeloid leukemia; MDS — myelodysplastic syndrome; PV — polycythemia vera; PMF — primary myelofibrosis; CMML — chronic myelomonocytic leukemia; CML — chronic myeloid leukemia; ET — essential thrombocytopenia; CNL — chronic neutrophilic leukemia; DLBCL — diffuse large B-cell lymphoma

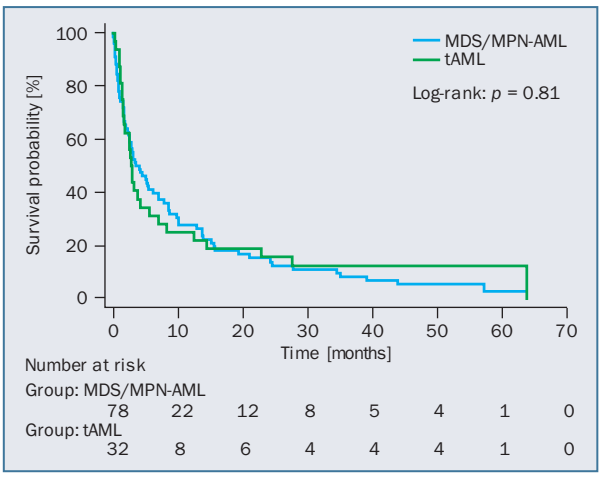

Figure 1. Kaplan-Meier curves for overall survival in secondary acute myeloid leukemia patients, acute myeloid leukemia secondary to myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN-AML) versus therapy-related acute myeloid leukemia (tAML) patients

showed a significant advantage in OS for the intensively treated group (13.9 vs. 2.5 months, p <0.0001) (Figure 3A). Moreover, OS analysis showed longer survival in patients <60 vs. ≥60 (7.9 vs. 2.8 months, p = 0.002) (Figure 3B). Comparing OS among patients with overall performance status classified as 0 or 1 in the ECOG scale versus 2–4 showed significantly longer survival in the former group than the latter (4.3 vs. 1.2 months, p = 0.001). Taking each ECOG grade separately, a trend toward longer survival was proven for patients with lower grades, with the longest survival being for grade 0 (7.0 months) and the shortest for grade 4 (0.2 months) (Figure 4A). Comparative OS analysis for the albumin level showed shorter survival in patients with hypoalbuminemia (defined by albumins concentration <3.5 g/dL) (6.2 vs. 2.5 months, p = = 0.012) (Figure 4B). The median follow-up was 3.2 months (95% CI: 2.5–5.3).

Regarding the univariate Cox proportional hazards model for OS, significant factors with potential prognostic importance for shorter survival were: age ≥60 years [hazard ratio (HR) 2.18, 95% CI: 1.28–3.69], ECOG >1 (HR 2.47, 95% CI: 1.56–3.92), high-risk cytogenetics (HR 2.76, 95% CI: 1.61–4.73), higher percentage of blasts in peripheral blood (HR 1.01, 95% CI: 1.00–1.02), higher LDH (HR 1.00, 95% CI: 1.00–1.00), hypoalbuminemia (HR 1.72, 95% CI: 1.08–2.74), higher BM infiltration (HR 1.02, 95% CI: 1.01–1.03), blasts in BM >50% (HR 1.90, 95% CI: 1.26–2.88) and >60% (HR 2.00, 95% CI: 1.23–3.25), and longer time to sAML diagnosis calculated from the primary disease diagnosis (HR 1.00, 95% CI: 1.00–1.01).

It is worth emphasizing that variables such as albumin level (HR 0.68, 95% CI: 0.47–0.99) and intensive therapeutic approach (HR 0.33, 95% CI: 0.20–0.55) were important

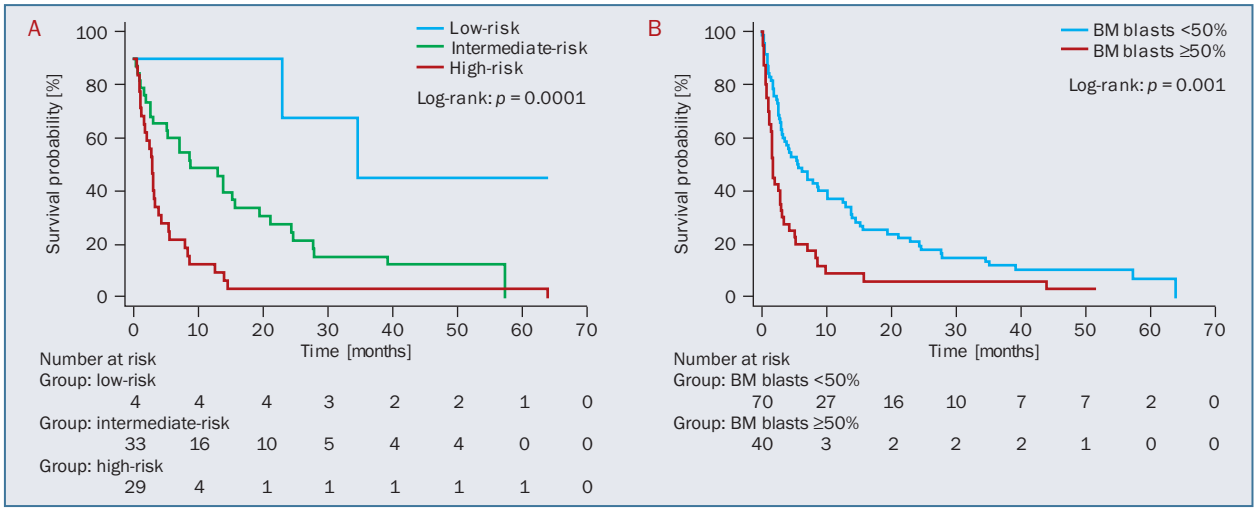

Figure 2. Kaplan-Meier curves for overall survival in secondary acute myeloid leukemia patients: **A.** Comparing cytogenetics risk, low versus intermediate versus high; **B.** Comparing bone marrow (BM) blasts infiltration, <50% versus ≥50%

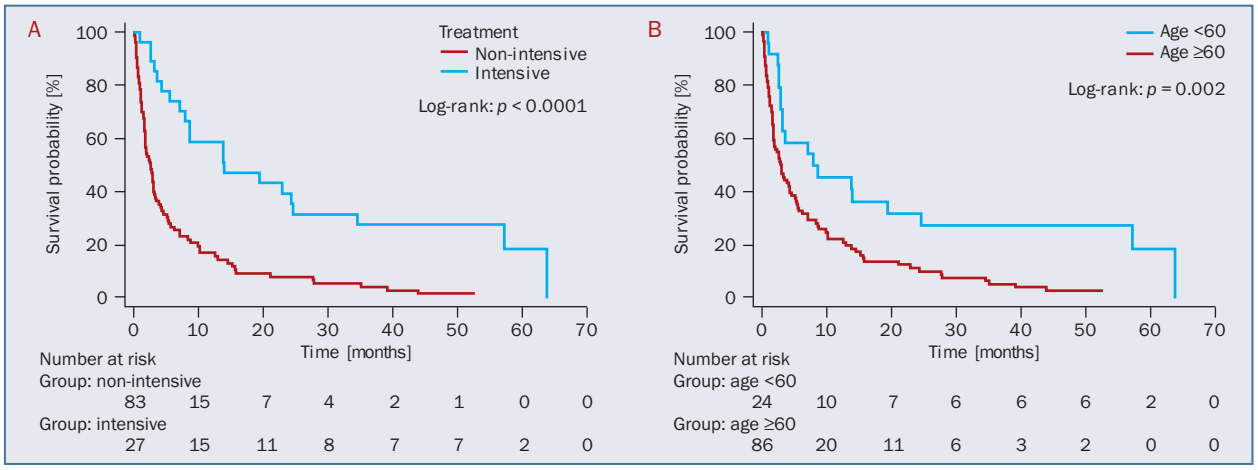

Figure 3. Kaplan-Meier curves for overall survival in secondary acute myeloid leukemia patients: **A.** Comparing patients treated intensively versus non-intensively; **B.** Depending on age at diagnosis, <60 versus ≥60 years

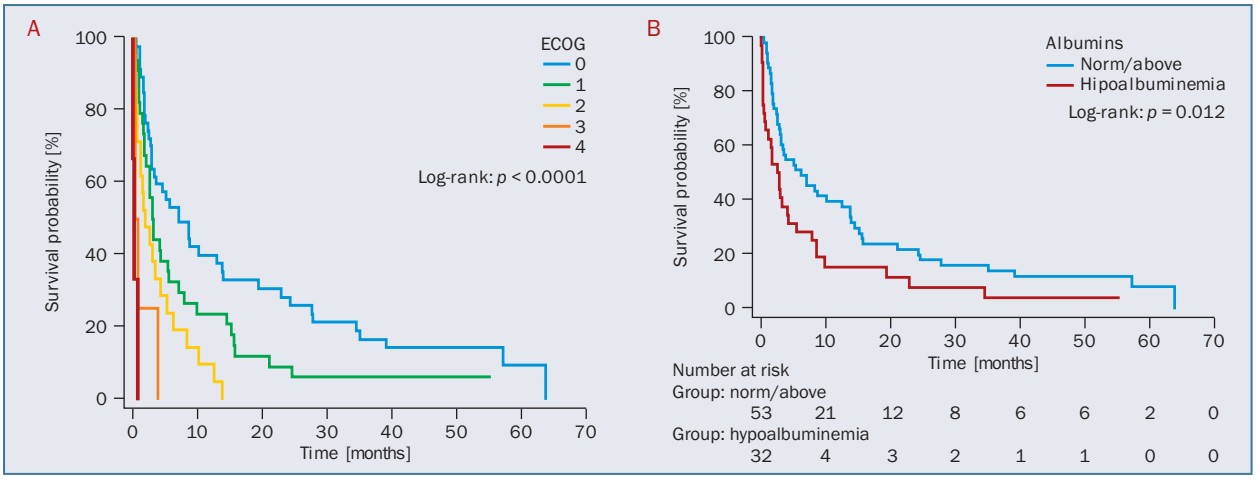

Figure 4. Kaplan-Meier curves for overall survival in secondary acute myeloid leukemia patients: **A.** Comparing patients stratified by Eastern Cooperative Oncology Group (ECOG) score; **B.** Comparing patients with and without hypoalbuminemia

for improving prognosis. The analysis in detail, including other studied factors, is presented in Table II.

Multivariate Cox proportional hazards model showed age at AML diagnosis (HR 1.03, 95% CI: 1.00–1.06), ECOG >1 (HR 1.85, 95% CI: 1.08–3.16), percentage of BM infiltration by blasts (HR 1.01, 95% CI: 1.00–1.03), and hypoalbuminemia (HR 3.20, 95% CI: 1.95–5.24) as independent prognostic factors for worsening OS. On the other hand, an intensive therapeutic approach was found to be an independent prognostic factor acting favorably for OS (HR 0.42, 95% CI: 0.21–0.81). High cytogenetic risk seemed to negatively affect survival (HR 1.48, 95% CI: 0.92–2.38), but remained without statistical significance ($p = 0.11$). Details of the multivariate Cox proportional hazards model for OS are set out in Table III.

Discussion

The incidence of sAML appears to have increased in recent years, due to the more widespread use of radio-chemotherapy and the greater long-term survival of cancer patients. sAML accounts for 15–30% of AML cases, with 18–20% being MDS/MPN-AML and 6–8% tAML [2, 12, 13]. In the PETHEMA registry study, 2,310 patients with sAML were analyzed. Of them, MDS-AML accounted for 44%, tAML for 25%, MPN-AML for 11%, MDS/MPN-AML for 10%, and antecedent neoplasia without prior chemo/radiotherapy (neo-AML) for 9% [13]. In our analysis, MDS-AML accounted for 44% of sAML cases, MPN-AML for 27%, and tAML for 29%. Our frequency of recognizing particular types of sAML was also similar to data that has been reported by other centers [14, 15].

sAML is associated with a lower rate of complete responses; according to our results, only 16% of patients achieved CR and 23% PR, with the majority receiving non-intensive treatment. Among the intensively treated, the percentages were 56% and 22%, respectively. These results were similar to those obtained by Schuler et al. [16], in patients with high-risk MDS and MDS-AML, where CR after the first induction was 50% and PR 22%.

The prognosis in sAML remains poor, with frequent resistance to conventional chemotherapy and OS shorter than 12 months [17]. Considering tAML and MDS/MPN-AML together often blurs the clinical and prognostic differences between them. In our study, OS for all sAML patients was 3.1 months, with the worst result being for the group of patients with tAML, at 2.8 months, whereas for MDS/MPN-AML it was 4.1 months. These results are relatively consistent with the observations of the PETHEMA registry, in which the median OS for sAML was 5.6 months. In analysis performed on 95 sAML patients by Koh et al. [15], median OS for MPN-AML, MDS-AML, and tAML was 3.9, 6.6, and 8.7 months, respectively. Meanwhile, Lalayanni et al. [18] analyzed 149 patients with sAML and found no difference in median overall survival (mOS) between MDS/MPN-AML and tAML patients. The shorter survival in our study compared to those mentioned above may be due to the more advanced age and more comorbidities, and as a result the lower percentage of patients who were eligible for intensive treatment, with few patients receiving allo-HSCT.

The 2-year survival of sAML patients after allo-HSCT ranges from 20–30% [19, 20] and is shorter than in the entire AML population of patients undergoing this procedure (as salvage therapy in AML, the procedure achieves a 3-year OS of 44%, while in CR2 59%) [21]. However, Lalayanni et al. [18] showed that allo-HSCT recipients in CR1 had superior median OS compared to patients without HSCT (24 vs. 8 months, respectively). They proved allo-HSCT to be an independent predictor of outcome, although we must bear in mind that only a relatively small percentage of sAML patients can undergo the procedure. In our study group, only nine patients underwent allo-HSCT, but their median OS (18.8 months) was significantly higher compared to the rest of the patients, and comparable to that in the abovementioned literature [18].

Nevertheless, it remains controversial as to whether sAML is an independent prognostic factor on its own, or only through its correlation with other risk factors [5, 18]. Many studies have shown that the prognosis of sAML is similar to *de novo* AML with an equal cytogenetic risk [22–24]. Also, ELN 2022 emphasized the importance of cytogenetics and mutational profile of AML cells, rather than the clinical history of antecedent disorders or chemo/radiotherapy, when considering prognostic factors and treatment approach [7].

Consistent with the results of the PETHEMA study, in our analysis, clinical and laboratory variables such as age, higher ECOG score, greater percentage of blasts infiltration, and hypoalbuminemia have proven to be independent prognostic factors for poorer survival [13].

Cytogenetic abnormalities like complex karyotype, 5q deletion, 7q deletion, and trisomy 8 have been reported as independent prognostic factors for worsening OS in sAML patients [15]. In our study, low-risk cytogenetics resulted in the longest survival (mOS not reached), and high-risk cytogenetics implied the poorest outcomes (mOS 2.8 months). However, this did not turn out to be a significant independent prognostic factor ($p = 0.1$, HR 1.48, 95% CI: 0.92–2.38), the main reason for which may be missing data (n = 44).

Recently, conventional 3 + 7 therapy has given way to CPX-351, which was approved by the Food And Drug Administration (FDA) in 2017 [25] and recommended by the European Society for Medical Oncology (ESMO) 2020 guidelines for the treatment of acute myeloid leukemia with myelodysplasia-related changes (MRC-AML) and tAML ≥60 years [26]. A randomized phase III trial involving a cohort of 309 patients comprising 54% with MDS/MPN-AML, 21% tAML, and 25% with myelodysplasia-related

cytogenetic abnormalities, demonstrated the superiority of that therapy over a 3 + 7 regimen (mOS 9.6 vs. 5.9 months, composite complete remission (CRc) 47.7% vs. 33.3%, respectively) [27].

In practice, CPX-351 gives many clinical benefits, yet nevertheless is associated with prolonged cytopenia as well as longer hospitalization [28]. There is a need for further therapeutic improvements, including ongoing clinical trials that are testing the combination of CPX-351 with other drugs, such as cladribine, or targeted therapies, like FLT3 or IDH inhibitors. In our analysis, only 28% of MDS/MPN-AML and 16% of tAML patients were able to receive intensive treatment, yet it significantly improved their survival (13.8 vs. 2.4 months, p <0.00) and turned out to be a favorable prognostic factor.

We must acknowledge the fact that these patients were predominantly younger (only 30% were 60 or older) and this, alongside better ECOG, certainly had an additional positive impact on the prognosis.

For unfit patients, the use of hypomethylating agents (HMA) is usually the preferred therapy. Azacitidine seems to have a good impact, even in poor-risk cytogenetics or *TP53* mutations (encountered in as many as a third of tAML and MPN-AML cases) [29, 30]. In one single-center cohort study, HMA had an advantage over cytarabine-based regimens in terms of CR rates and CR duration and significantly extended mOS (9 vs. 5 months, p = 0.019) [30]. HMA might overcome some of the chemoresistance in the hypomethylating mechanism of tumor suppressor genes, leading to their re-expression, but the incomplete destruction of blast cells results in short responses and frequent relapses. A combination therapy of HMA and venetoclax has been proven to accelerate the achievement of treatment response and prolong survival in patients with unfavorable cytogenetic prognoses [31]. Another study conducted on a population of 145 unfit patients evaluated the efficacy and safety of venetoclax and HMA. This showed beneficial effects in patients with sAML and high cytogenetic risk. The median OS for all patients was 17.5 months, while the overall response rates [CR + CR with incomplete blood count recovery (CRi) + partial remission (PR)] were 76% for venetoclax + azacitidine (VEN/AZA) and 71% for venetoclax + decitabine [32, 33]. DiNardo et al. [34] also confirmed that VEN/AZA was superior to AZA alone by improving both median OS and CRc in sAML patients.

There are also reports of the effectiveness of venetoclax with low-dose cytarabine. A multicenter phase Ib/II study of 82 patients ineligible for intensive chemotherapy, of whom 49% were diagnosed with sAML, showed the efficacy of such therapy, with 54% of patients achieving CR/CRi, while the median OS was 10 months [35].

Taking into consideration the two main therapeutic approaches in sAML, Matthews et al. [36] compared CPX-351 versus VEN/AZA in a study of 656 AML patients (439 in the VEN/AZA arm and 217 in CPX-351). In the VEN/AZA group, 49% of patients had a diagnosis of sAML (n = 213), while in the CPX-351 group the figure was 71% (n = 154). Median OS for all patients was 12 months; 13 months for CPX-351 versus 11 months for VEN/AZA (p = 0.22). However, VEN/AZA patients were significantly older (median age 75 vs. 65-years-old; p <0.01) and fewer VEN/AZA patients received allo-HSCT compared to CPX-351 (10% vs. 28%, respectively; p <0.0005) [36]. This does not conclusively resolve the superiority of one therapy over the other, especially considering only patients with sAML, mainly due to differences between study groups, lack of randomization, and inconsistent inclusion criteria. However, differences in the primary endpoint have not been demonstrated, and thus the therapeutic approach should be individualized for each patient.

In our study, unfit patients were treated with AZA, LD-Ara-C, or qualified only to cytoreduction or BSC alone. In the analyzed period, venetoclax and CPX-351 were not approved and available. It is possible that the addition of venetoclax might improve the treatment results in that group, as both drugs are now available in Poland for AML patients.

Nevertheless, new therapeutic options are on the horizon. There are some histone deacetylase inhibitors tested in AML, such as panobinostat and vorinostat, yet there is no data for specific advantages of their usage in sAML [31]. As reported in one study, a combination of vorinostat with cytarabine and etoposide did not result in an increased response rate in a cohort of patients with relapsed/refractory (r/r) AML or sAML [37, 38]. Another agent in the early investigational phase is pinometostat, which may play a role in indirectly inhibiting the oncogenic effects of *KMT2A* — a common mutation in tAML. However, the hypothetical advantages of these drugs' application in sAML remain to be tested [31].

Some novel agents that could have an impact on sAML are currently being studied in clinical trials in combination or alone. Great expectations rest on the use of immune check-points [nivolumab (NCT02532231), pembrolizumab (NCT04284787)], *IDH* inhibitors — ivosidenib (NCT03173248, NCT02632708), enasidenib (NCT02632708), and targeted drugs for spliceosomes (NCT04278768) or bromodomain and extra-terminal domain (BET) proteins — NCT02698189, which unfortunately was terminated due to limited efficacy [2]. The literature indicates that therapy with chimeric antigen receptor-T (CAR-T) may also play a role in sAML [39–41].

Conclusions

Regardless of the increasing understanding of AML biology and the more accurate description of prognostic factors based on genetic mutations, the prognosis for patients with MDS/MPN-AML and tAML remains poor. Contributing

factors include unfavorable cytogenetic risk, a specific dysplasia-related mutational profile, and older age in the MDS/MPN-AML group, as well as the effects of preceding malignancy or prior treatment in patients with tAML.

Although the positive impact of intensive chemotherapy with subsequent allo-HSCT is marked in sAML patients, a therapeutic approach based on more personalized treatment may provide a better outcome. Therefore, there is no doubt that further progress is needed in optimizing treatment and in better understanding the biology of sAML, both at the clinical and molecular levels.

Authors' contributions

PS, MC, AP — creating the study protocol. PS, KMK and SS — patient enrolment and data acquisition. PS, DM — data analysis and statistics. PS and KMK — writing the manuscript. AW, AP and MC — manuscript revision and proofreading.

Conflict of interest

The authors declare no conflict of interest.

Financial suport

None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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Very late disease relapse in patient with B-common acute lymphoblastic leukemia treated with allogeneic stem cell transplantation: does clonal evolution play a role?

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Introduction

BCR::ABL1-like acute lymphoblastic leukemia (*BCR::ABL1*-like ALL) is a high risk disease subtype characterized by a gene expression profile similar to Philadelphia positive (Ph+) B-cell ALL, but lacking the distinguishing molecular fusion gene — *BCR::ABL1* [1, 2]. *BCR::ABL1*-like ALL has been incorporated as a separate entity into the 5th edition of the World Health Organization's classification of Hematolymphoid Tumors due to its therapeutic and prognostic significance [3]. The nature of the underlying genetic features of *BCR::ABL1*-like ALL is heterogenous, and therefore diagnosis remains challenging [4].

BCR::ABL1-like ALL patients often relapse early after initial treatment, but late and very late recurrences also occur [5, 6]. They are predominantly reported in the pediatric population, while in adults they are seldom observed [5, 7]. The relapse rate within the first three years from achieving complete remission (CR) among adults with B-cell ALL (B-ALL) varies from 35% in standard risk patients to 50% among high risk subjects [5]. The relapse rate beyond three years and five years in patients who achieve CR is estimated at approximately 3.8% and 2.8%, respectively [5, 8].

Herein, we present a case of a very late disease relapse with features of *BCR::ABL1*-like ALL which were absent at the first manifestation of the disease.

Material and methods

The presented case was identified during a study of immune, molecular and cytogenetic characteristics of *BCR::ABL1-negative, KMT2A*-rearrangement negative patients with B-cell ALL admitted to the Department of Hematology and Bone Marrow Transplantation of Poznań University of Medical Sciences in Poznań, Poland (study funding: Poznań University of Medical Sciences Doctoral School Large Research Grant No. DGB 3/2021).

The expression of thymic stromal lymphopoietin receptor (TSLPR), predictive of the rearrangement of the *CRLF2*, with an anti-TSLP antibody (Invitrogen™) was performed using the 10-color multiparameter flow cytometry method (FCM; BD FacsCanto II Ilyric™).

The karyotype analysis was performed using G banding (GTG). Fluorescent *in situ* hybridization (FISH) studies were performed on the interface nuclei using break-apart probes for CRFL2, JAK2, EPOR, ABL1, ABL2 (CytoCell®, Cambridge, UK) and for BCR::ABL1, KMT2A, and PDGFRβ (Vysis, IL, USA) and, additionally, for IGH and P2RY8 in the *CRLF2* rearranged (*CRLF2*-r) cases (CytoCell®, Cambridge, UK). At least 100 interphase nuclei were scored for each probe by two independent examiners. Analysis of the *JAK2* exon 16 sequence was conducted using DNA extracted from whole-blood leukocytes at the time of the

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diagnosis with the use of a QIAmp DNA Mini Kit (Qiagen) and high-resolution melt analysis (HRMA). For the variant type identification screened by HRMA, Sanger sequencing was applied using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific) and the following primers: forward: 5'-TGCTCCAGTACTTGTGGACTGA-3' and reverse: 5'-CCACTGCCCAAGTAAAGCTTAG-3'.

To identify *BCR::ABL1*-like cases, we implemented an algorithm combining multiparameter flow cytometry (FCM), karyotype analysis using G banding and FISH. In a prospective analysis, the expression of TSLPR was evaluated by FCM. Patients expressing TSLPR on leukemic blasts were enrolled for the FISH analysis with a CRLF2 break-apart probe. Patients lacking the TSLPR expression were recognized as non-*CRLF2*-rearranged (non-*CRLF2*-r) and, subsequently, proceeded to analysis with the remaining FISH probes for other *BCR::ABL1*-like ALL features (JAK2, EPOR, ABL1, ABL2, PDGFRβ).

In retrospective analysis, the patients were primarily examined for the presence of *CRLF2* rearrangement with a FISH probe. In *CRLF2*-r cases, the next step included an analysis with IGH and P2RY8 FISH probes to identify the fusion gene. Non-*CRLF2*-r cases proceeded to the analysis with the remaining FISH probes. This study was approved by the Poznań University of Medical Sciences Bioethical Committee (Resolution No 705/20).

Results and case medical history

A 32-year-old male was diagnosed with a relapse of B-ALL 13 years after treatment with chemotherapy and an allogeneic hematopoietic stem cell transplantation (allo-HSCT). He was diagnosed at the age of 19 with B-common ALL in October 2008. Intensive polychemotherapy according to the Polish Adult Leukemia Group 2007 protocol was given immediately after disease diagnosis. The treatment resulted in the first complete remission (CR1) with negative measurable residual disease (MRD). Allo-HSCT from a matched unrelated donor (MUD) was performed in August, 2009. The pre-transplant conditioning regimen consisted of total body irradiation (TBI) and cyclophosphamide (120 mg/m^2). Thereafter, the patient remained in long-term remission for 13 years.

In July 2022, leukocytosis, anemia and thrombocytopenia were noted. FCM of bone marrow nuclear cells revealed 84% of lymphoblasts expressing B-common phenotype with high expression of TSLPR (99%). A subsequent GTG cytogenetic analysis revealed complex karyotype. Analysis with FISH probes showed the presence of rearrangement of the *CRLF2* (*CRLF2*-r) gene. An additional study with FISH probes identified *CRLF2-IGH* fusion (Figure 1). Due to the frequent co-occurrence of *JAK2* mutation encountered in *CRLF2*-r positive *BCR::ABL1*-like cases, the *JAK2* exon 16 sequence was analyzed using Sanger sequencing. The study confirmed the presence of single nucleotide variant LRG_612:c.2049A>C(p.Arg683Ser) with a variant allele frequency (VAF) of approximately 26%. Interestingly, a study of the samples collected at the initial disease manifestation for the presence of *CRLF2-r* was negative. Moreover, other cytogenetic aberrations characteristic of *BCR::ABL1*-like ALL were absent (Figure 1).

Due to the disease relapse, intensive chemotherapy according to the Polish Adult Leukemia Group ALL7 protocol for newly diagnosed patients was given. The patient achieved CR2 with negative MRD after induction therapy. The MRD was still undetectable after consolidation chemotherapy. In fact, the patient is proceeding to allo-HSCT in CR2.

Discussion

The prognosis for relapsed/refractory B-cell ALL remains dismal, with historical median overall survival (OS) of approximately six months. However, the advent of novel therapies, including monoclonal antibodies (inotuzumab, blinatumomab) and chimeric antigen receptor T-cell therapy (CAR-T) has significantly improved OS in these patients [9].

Although some studies have indicated that late relapses might result in better outcomes than early relapses, the study by Ganzel et al. on 1,909 patients revealed that the outcomes of late recurrences of ALL, i.e. beyond three years of CR, were associated with a poor prognosis, with a five-year OS of 20% [5, 10]. To the best of our knowledge, only one case report of a relapse of *BCR::ABL1*-like ALL after prolonged remission has previously been reported, although the presence of *BCR::ABL1*-like signature at the initial diagnosis was not examined [6].

The background to late B-cell ALL relapses remains a matter of debate since the mutational mechanisms leading to relapse are yet to be thoroughly investigated. It is currently believed that an ancestral leukemic clone or subclones might undergo evolution, leading to disease recurrence [5, 11]. The analysis by Waanders et al. [11] of 92 cases of relapsed pediatric ALL documented a pattern of clonal evolution, indicating that relapse-driving clones most commonly existed at initial diagnosis as minor clones, and less frequently as major clones. Most of the relapses derived from previously existing clones harboring, or acquiring, drug-resistant mutations [11]. Similar observations were made by Sayyab et al. [12], who reported different patterns of clonal evolution, which supports the hypothesis that relapses originate from evolving clones existing at the initial diagnosis.

Data reporting outcomes of allo-HSCT in *BCR::ABL1*-like ALL is scarce. A retrospective analysis of 56 patients by Aldoss et al. showed that patients with *BCR::ABL1*-like ALL had comparable post-allo-HSCT results to those of other B-cell ALL subjects, with 3-year OS reaching 51% and 50%,

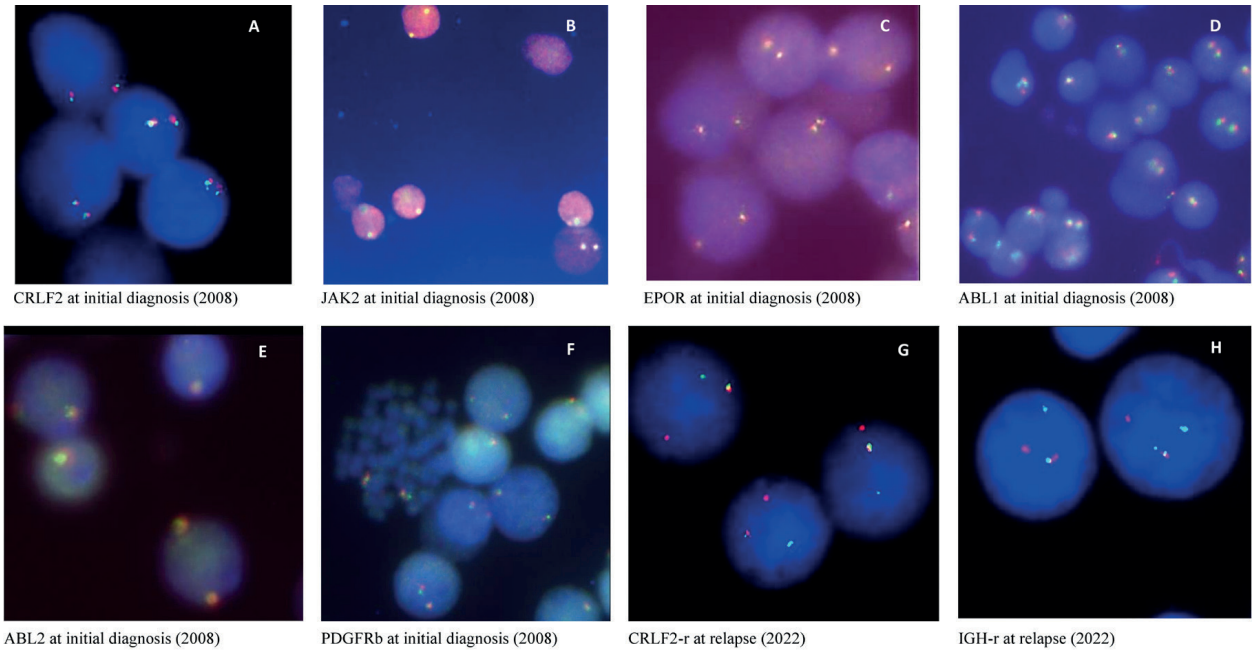

Figure 1. Fluorescent *in situ* hybridization (FISH) analysis of *BCR::ABL1*-like rearrangements in presented patient. In normal cell, 2 red/ /green signals (2R/2G) or yellow (2Y) are observed: **A–F.** FISH analysis with cytokine receptor-like factor 2 gene (*CRLF2*), Janus kinase 2 gene (*JAK2*), erythropoietin receptor gene (*EPOR*), tyrosine-protein kinase ABL1 gene (*ABL1*), tyrosine-protein kinase ABL2 gene (*ABL2*), and platelet-derived growth factor beta gene (*PDGFR*β) break-apart probes on leukemic blasts at initial diagnosis; **G.** FISH analysis with CRLF2 break-apart probe at relapse. A translocation resulting in 1R, 1G, 1Y; **H.** Second step analysis with immunoglobulin heavy *locus* gene (*IGH*) probe. Cells with 1R, 1G, 1Y are indicative of IGH rearrangement

respectively [13]. In most *BCR::ABL1*-like ALL cases, genetic alterations which activate kinases and cytokine receptors signaling pathways are present. An excellent example of this are *JAK2* alternations which are present in 1–7% of *BCR::ABL1*-like ALL cases, and are most commonly detected in *CRLF2*-r cases [4, 14, 15].

In our presented case, the single nucleotide variant LRG_612:c.2049A>C(p.Arg683Ser) of *JAK2* gene was detected. The mutation significantly decreases the stability of JAK2 structure, leading to constitutive activation of JAK2, and is implicated in the pathogenesis of B-cell ALL via cooperation with CRLF2 [16, 17]. According to recently published data, this might serve as a potential target for precision therapy, something which has been confirmed by initial reports of the successful application of targeted therapy in combination with chemotherapy and immunotherapy in *BCR::ABL1*-like ALL patients [18].

Conclusions

Our observation confirmed the clonal evolution of B-cell ALL, even in a patient successfully treated with allo-HSCT many years ago. The background of late relapses remains a matter of debate, although several observations have indicated a role played by the clonal evolution of previously existing minor clones.

We suggest that patients with B-cell ALL should be monitored for a long time after allo-HSCT to provide early detection of disease relapse. We further suggest, in relapsed patients, a detailed immunophenotypic, cytogenetic and molecular analysis should be performed, including screening for *BCR::ABL1*-like features to identify the potentially targetable molecular aberrations acquired during disease clonal evolution. The incorporation of widely available techniques including FCM and FISH enables swifter identification of *BCR::ABL1*-like patients, who may benefit from targeted therapy.

Authors' contributions

AP, KL, AP-Ch, ZK — methodology; AP, KL, ZK, AP-Ch, MK, JK-P, AM — investigation; AP, KL, ZK, MK, AP-Ch, MJ-S, LG — analysis and interpretation of clinical and laboratory data; AP, KL — writing, original draft. All authors read and agreed to published version of manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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Severe factor V deficiency in infants

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Introduction

Hemostasis is a set of physiological processes that ensure the inhibition of bleeding after breaking the continuity of the blood vessel wall, the tightness of the vascular bed, and the fluidity of the circulating blood [1]. The interaction of platelets and plasma coagulation factors with vascular endothelial cells and subendothelial tissues plays an important role in hemostasis.

Most plasma bleeding disorders, apart from hemophilia and von Willebrand disease (platelet-plasma diathesis), can be classified as orphan diseases, i.e. as occurring with a frequency of less than 5/10,000 people in the general population [2]. Rare bleeding disorders are inherited in an autosomal recessive manner and they do not cause any clinically significant bleeding symptoms in heterozygotes [3–5].

Congenital factor V (FV) deficiency is a very rare bleeding disorder, occurring with a frequency of 1:1,000,000 in the general population, and is inherited in an autosomal recessive manner [3, 4].

Methods and results

Case history

Parents brought their 4-month-old daughter to the hospital because of her reluctance to eat, which had lasted for several days, vomiting after each feeding, and increasing apathy.

The child was of Polish descent, born GV PIII on time, spontaneous delivery, Apgar 10, with a body weight of 4,040 g, and normal postnatal development. The family history revealed the death of the patient's brother on the fourth day of life; the autopsy examination showed a subcapsular hematoma of the left lobe of the liver, and kidney bleeds; the mother had had two spontaneous miscarriages.

Results

Cranial ultrasound (CrUSS) and computed tomography (CT) of the head showed a focal lesion in the form of an intracerebral hematoma with a mass effect in the right frontal lobe.

Basic coagulation parameters were urgently determined, showing platelet count 257.0 K/μL (N), activated partial thromboplastin time (APTT) 449.6 s (reference values: 27.2–53.3 s), prothrombin time (PT) 60.7 s (reference values: 9.1–12.1 s), prothrombin index 18.2% (reference values: 88–120%), international normalized ratio (INR) 5.71 (reference values: 0.9–1.39), and bleeding time 8 s (reference values: 4–8 s). Other laboratory test results were normal. The child had blood group 0 Rh (+).

After a fresh frozen plasma (FFP) transfusion, the girl was urgently operated on; a right frontal craniotomy with the removal of the hematoma was performed. The diagnostics of coagulation disorders was extended to include the activity of coagulation factors. The first measurement was performed 48 hours after the FFP transfusion. Significantly decreased factor V (FV) activity (5.4%; reference values: 62–139%) and decreased ristocetin cofactor (vWFR:Co) activity (34.4%; reference values: 53–148%) were found.

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The next measurement was performed 72 hours after the FFP transfusion; the test confirmed severe FV deficiency (0.9%), reduced vWFR:Co activity (37.4%), mild von Willebrand factor (vWF) deficiency (44.7%; reference values: 53–148%), and mild factor VIII (FVIII) deficiency (42.5%; reference values 50–150%). The activity of the above-mentioned coagulation factors, checked many times during the follow-up period which lasted for several months, showed a tendency to normalize vWF:Co, vWF, and FVIII, and persistent, extremely low, FV activity (<1%).

Genetic testing performed by next-generation sequencing (NGS) using a custom-made panel [6] revealed two heterozygous missense changes in the *F5* gene (NM_000130.4): a known pathogenic variant c.5419G>A, resulting in an alanine-to-threonine substitution at position 1,807, and a novel, likely pathogenic, change c.6136A>C, resulting in a threonine-to-proline substitution at position 2,046. In order to verify the biallelic nature of the revealed changes, we performed Sanger sequencing of the parents. The results confirmed that the c.5419G>A change was inherited from the father, and the c.6136>C change was inherited from the mother. Coagulation indices and FV activity of the girl's parents and older sister were normal.

The treatment included FFP transfusion from a male donor, initially every other day, then every three days, vasoconstrictors, and tranexamic acid.

The patient underwent control CrUSS several times and magnetic resonance imaging (MRI) of the head twice, which showed the correct evolution of post-hemorrhagic changes, with the formation of malacic cavitation (Figure 1). The image of the abdominal organs in the ultrasound examination was normal.

Currently, the 2-year-old patient remains in a good condition, is developing normally, and shows no abnormalities in neurological examination. She remains under constant hematological care and receives a transfusion of fresh frozen plasma once a week. Hemostasis parameters controlled before transfusion are still several times above the norm, and FFP transfusion only partially corrects the hemostasis.

Discussion

Several different factors are involved in the plasma coagulation cascade, including plasma proteins, tissue factor (TF) contained in cell membranes, cell membrane phospholipids, and calcium ions [5].

Factor V (FV, proaccelerin), one of the plasma coagulation factors, is a glycoprotein discovered in 1943 by Paul Owren while examining women with hemophilia-like bleeding symptoms [3]. The factor V gene (*F5* gene) is located on the long arm of chromosome 1 at position 23 [4, 7]. It is synthesized by hepatocytes. About 80% of the factor in blood plasma is in an inactive form, and 20% is contained in the granules of the platelets. It has both a procoagulant effect i.e. after being activated by thrombin Va it is a cofactor in the reaction of prothrombin activation by factor Xa which is crucial for clot formation, and an anticoagulant effect i.e. it stimulates the inactivation of factor VIIIa by activated protein C [3, 5, 7].

Congenital factor V deficiency, also known as Owren's Disease or parahemophilia, is a very rare bleeding disorder with a frequency of 1:1,000,000 in the general population, and is inherited in an autosomal recessive manner [3, 4]. The disease is diagnosed most frequently in Iranians (approx. 1:100,000), which may result from a tendency towards consanguineous marriages in this population [3]. Two types of the disease are distinguished: type I, characterized by a very low or undetectable FV antigen and factor activity deficiency, which is the form occurring in the majority of patients; and type II, characterized by reduced factor activity with its normal or slightly reduced levels [3–10]. Based on FV activity, the disease can be classified as mild (≥10% activity), moderate (<10–≥1%), or severe (<1%) [7].

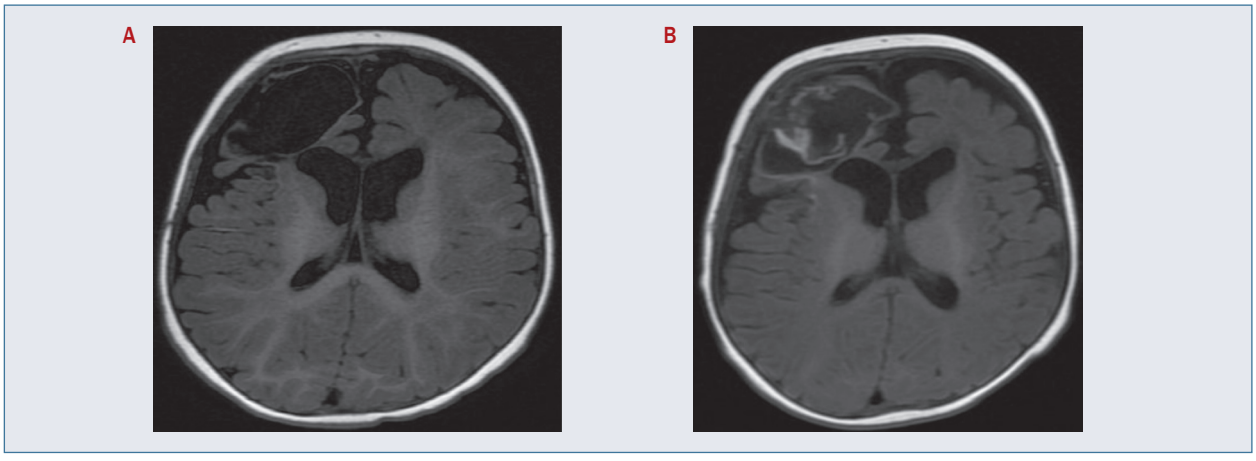

Figure 1A, B. Brain magnetic resonance imaging (MRI): correct evolution of post-hemorrhagic changes

Table I. Characteristics of infants with severe factor V (FV) deficiency (FV <1%) and bleeding into central nervous system

No.	Gender	Age	Clinical manifestation	Imaging studies of brain*	Homozygous/ heterozygous	Family history	Ref.
1	F	4 months	Nausea, vomiting, apathy	Intracerebral hematoma in frontal brain lobe	Heterozygous	Positive, brother died due to generalized bleeding	–
2	F	2 months	Vomiting, twitching left drooping of the left corner of the mounth, poor movement	Fronto-temporal--parietal hemorrhage, subdural hematoma	Heterozygous	Negative	[4]
3	M	Infant	Asymmetric head, tense fontanelle	Subdural hematoma on right side	No data	No data	[13]
4	M	5 weeks	Apathy, pallor, reduced feeding	Hematoma on right hemisphere	Homozygous	Negative	[14]
5	M	3 months	No data	Subdural and intraparenchymal hemorrhage	No data	No data	[11]
6	M	4 months	Vomiting, epileptic attack, reduced feeding	Subdural hematoma and extradural hematoma in frontal area	Heterozygous	Yes	[15]
7	M	2 months	Apathy, reduced feeding	Intraparenchymal hemorrhage	Compound heterozygous	No data	[12]
8	F	1 month	Hemiparesis, worse psychomotor development	Subdural hemorrhage on left hemisphere	No data	No	[16]

*Imaging studies: brain magnetic resonance (MRI), brain computed tomography (CT), or brain ultrasonography

Only a few cases of infants with severe factor V deficiency have been reported in the literature (Table I) [4, 11–16].

Patients with FV deficiency can present with various clinical symptoms, ranging from asymptomatic or oligosymptomatic in mild or moderate deficiency, to life-threatening bleeding in cases of severe deficiency [2, 7–9]. The most common clinical manifestations are mucosal bleeding, excessive menstrual bleeding, a tendency to postoperative and postpartum bleeding, and, less often, spontaneous hematomas, bleeding from the gastrointestinal tract, and recurrent miscarriages. In patients with severe FV deficiency, bleeding occurs in the neonatal period or early childhood as bleeding from the umbilical cord stump, intracranial bleeding, or post-traumatic joint bleeding. According to the literature, only a few infants with severe FV deficiency and spontaneous bleeding into the central nervous system have been described [4, 11–15]. The presented patient is another such case.

In our patient, the symptoms appeared in the first six months of life and resembled life-threatening intracranial bleeding. In addition, the family history of the patient's brother's death aged four days (abdominal bleeding in autopsy, coagulation disorders not diagnosed) and her mother's two miscarriages could indicate the presence of a rare, severe bleeding disorder in the family.

Patients with factor V deficiency have prolonged APTT and PT, and the degree of abnormalities correlates with the activity of factor V determined by the coagulation method [3, 9, 10]. Our patient showed significant deviations in coagulation indices on the day of admission to hospital: APTT increased by almost 10 times, PT increased by five times, and INR increased by four times. Multiple measurements of FV activity (<0.9%) were the basis for the diagnosis of hemorrhagic diathesis with severe factor V deficiency.

The genetic test results confirmed the genetic basis of factor V deficiency. So far, over 150 mutations in the *F5* gene have been identified [3, 7]. In the presented girl, two heterozygous missense mutations were found, including one pathogenic and one likely pathogenic but not yet described in the literature. She inherited these abnormalities from her parents.

Due to the lack of concentrates containing only FV, the treatment of patients with a deficiency of this factor is based on the transfusion of fresh-frozen plasma [3, 4, 7, 9]. The recommended doses, and the frequency of transfusions, depend on the activity of the factor in the patient and the clinical situation. In cases of minor bleeding, topical treatment and antifibrinolytic drugs may be sufficient [2]. In other cases, it is recommended to increase the factor activity to about 10% with prophylactic transfusions, and >15–20% before surgery or in the case of severe bleeding [7]. The half-life of FV is 36 hours, which should be taken into account when planning the frequency of transfusions.

Repeated administration of FFP may cause transfusion-dependent complications, i.e. the risk of viral infections, which is currently reduced to a minimum, plus other challenges such as inhibitor formation, circulatory system overload, and lung damage. Due to the presence of FV in

platelet granules, transfusion of platelet concentrate is suggested as an alternative therapy. Platelet FV in thrombocytes is delivered in an inactive form directly to the bleeding site where it is activated, which increases its local effectiveness, and makes it protected against the inhibitor circulating in the blood [7–9].

Liver transplantation should be considered in patients with severe FV deficiency and coexisting life-threatening bleeding. However, this is a method of treatment which carries the possibility of significant complications [11, 12].

In vitro studies on plasma-derived factor V concentrate are in progress. The results so far indicate a beneficial effect on the correction of PT and APTT, so there is the prospect in the future of targeted supplementation treatment in patients with severe factor V deficiency [3, 7].

The described patient is receiving FFP once a week and so far we have not observed post-transfusion complications; since the introduction of substitution treatment, no spontaneous bleeding has been observed in the child.

Conclusions

1. Severe congenital FV deficiency is a very rare bleeding disorder that can present with life-threatening intracranial bleeding in infancy.
2. Patients with severe FV deficiency and their families require hematological, genetic and molecular diagnostics, and genetic counseling.
3. The presentation of very rare cases is necessary to improve the diagnostic process and future treatment of patients with severe FV deficiency.

Authors' contributions

WS — writing manuscript, concept. AS-C - writing manuscript, collecting literature. OZ-C, AK — collecting literature. MM-S — developing imaging studies. KB-P — developing genetic studies. AM-M — writing manuscript, concept, reviewing.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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Kinetics of CAR-T cells and immunological profile after tisagenlecleucel therapy

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Over the last decade, the use of chimeric antigen receptor (CAR) T cells has emerged as a new strategy in the treatment of relapsed/refractory (R/R) acute lymphoblastic leukemia (ALL). The immune activation plays a pivotal role, both in the therapeutic effect of CAR-T cells and the side effects of the therapy.

The most common toxicities related to CAR-T cell treatment, which are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), are caused by the excessive activation of effector cells and the release of high levels of cytokines [1, 2]. We here report the profile of immunological response in a patient treated with CAR-T cells due to primary refractory ALL.

The patient, a 5-year-old girl, was diagnosed with B-common ALL with co-expression of CD36 in December 2022. After the diagnosis, she received treatment according to the AIEOP-BFM-2017-Poland therapeutic protocol. On the 15th day of treatment, the therapy response was unsatisfactory, with 49.5% blast cells in the bone marrow. On the 33rd day, minimal residual disease (MRD) was measured at 3×10^{-1}. Due to the identification of activating aberrations of the ABL-kinase family in blast cells, the therapy was switched to the imatinib-based EsPHALL-2017 protocol. At that point, a bone marrow aspirate biopsy was repeated, revealing 29.5% blast cells. She was subsequently diagnosed with primary refractory ALL and qualified for CAR-T cell therapy.

The bridging therapy was based on the FRALL-POST-2004 protocol with the addition of imatinib. Prior to the CAR-T cell infusion, a lymphodepleting regimen consisting of fludarabine and cyclophosphamide was administered. Subsequently, in May 2023 the patient received an infusion of anti-CD19 CAR-T cells (tisagenlecleucel, Novartis). No immediate infusion-related toxic effects were observed. The post CAR-T cell infusion course was complicated by grade I CRS and grade II ICANS which occurred at day +4 after the CAR-T cell infusion and required treatment with tocilizumab and dexamethasone. After a temporary improvement, on day +7 after the infusion, fever and neurological symptoms were observed. The child was diagnosed with grade I CRS and grade III ICANS, with complete remission after treatment with four doses of tocilizumab and dexamethasone. Laboratory test results, including complete blood morphology, C-reactive protein, ferritin, cytokine profiles and flow cytometry of lymphocyte subpopulation, were monitored daily from day –1 to day +14 after the CAR-T cell infusion. Flow cytometry of CAR-T cells was performed on specific days (days 0, +1, +2, +3, +6, +10, and +14). The changes in the cytokine profiles and proinflammatory mediators are set out in Figure 1. Despite the observed toxicities, C-reactive protein (CRP) was

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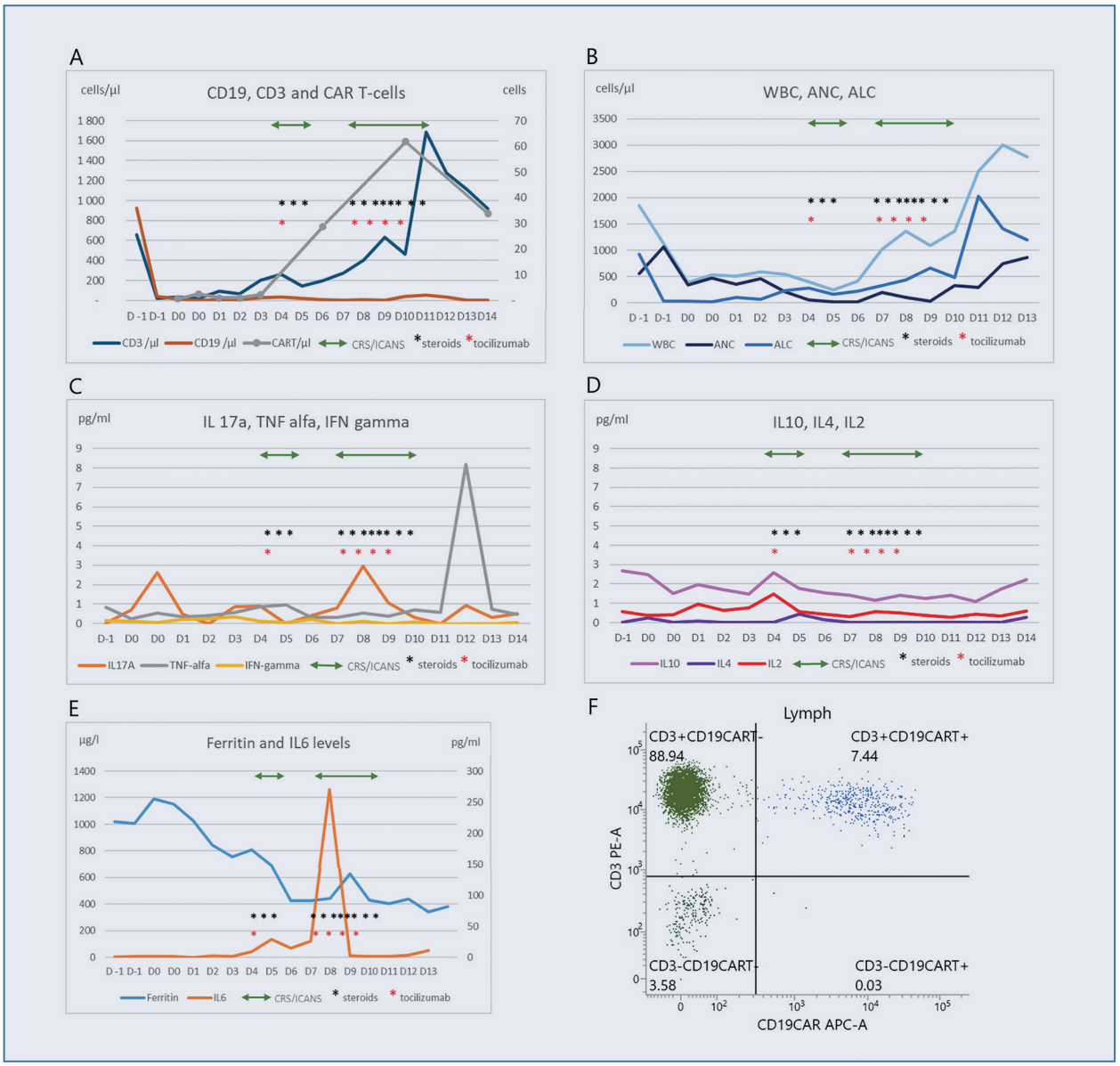

Figure 1. Results of laboratory tests, cytokine profiles, and flow cytometry assessed during observational period, along with their relationship to cytokine release syndrome (CRS)/immune effector cell-associated neurotoxicity syndrome (ICANS) episodes and administered anti-inflammatory treatment: **A.** CD19, CD3 and chimeric antigen receptor (CAR) T cells count; **B.** White blood cells (WBC) count, absolute neutrophil count (ANC), absolute lymphocyte count (ALC); **C.** Interleukin (IL)-17a, tumor necrosis factor (TNF)-alpha, interferon (IFN)-gamma levels; **D.** IL-10, IL-4, IL-2 levels; **E.** IL-6 and ferritin levels; **F.** CAR-T cells in flow cytometry, day +14

<5 mg/L during the entire observation period. The girl was discharged on day +17 after the infusion in good general condition, with scheduled follow-up appointments in the outpatient clinic.

The *in vivo* kinetics of CAR-T cells have provided crucial insights into the therapeutic response and its associated side effects [3]. Although the CAR-T cell count was initially low in the first few days after infusion in our described case, a similar trend has been observed in other studies, with an exponential increase in CAR-T cells levels being observed between days +7 and +11 [4, 5]. Furthermore, the expansion of CAR-T cells happened at the same time as the occurrence of CRS and ICANS. It is still not fully understood whether the peak of CAR-T cells is the cause of the toxicities itself, or an effect of immune-related CAR-T cell expansion [4, 6, 7]. Incidences of those toxicities were associated also with an increase in both proinflammatory mediators (IL-6 and ferritin) and a slight increase in anti-inflammatory cytokines (IL-10). After anti-inflammatory therapy with tocilizumab and steroids, a rapid decrease in cytokine levels, but not CAR-T cells, occurred.

Treatment of CRS (with tocilizumab) and ICANS (with steroids) was successfully applied [8]. However, there is a subset of patients who experience therapy-resistant CRS/ /ICANS, highlighting the need to identify new targets for toxicity treatment [2]. In our patient, the second episode of CRS and ICANS coincided with a significant peak in tumor necrosis factor alpha (TNF-α) levels accompanied by a peak in CAR-T cell count. This finding is in line with the results of early studies of CAR-T cell therapy, where toxicities were related to a notable increase in TNF-α level, making TNF-α a potential target for CRS and ICANS therapy [1, 9]. In some severe cases, TNF-α blockade, in combination with tocilizumab, could effectively reverse CRS [10].

In conclusion, the monitoring of kinetics of CAR-T cells and cytokine profile provided a valuable evaluation of the therapeutic response and its associated adverse effects. Understanding the underlying mechanisms of CAR-T cell-related immune responses is crucial for improving therapy outcomes, and for the early detection of toxicities and their better management. The presence of CAR-T cells might be a good prognostic factor for continuous remission in ALL.

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Authors' contributions

JaS, MRP — design of study. MRP, KC, RD, AM, ED — clinical data. JoS, JaS — writing manuscript. MK, BKR, RD — laboratory analysis. EM, KG, MRP, ŁL — CAR-T handling. JaS, MRP, KC — critical review. All authors — final approval.

Conflict of interest

The authors declare no conflict of interest.

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None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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Infection with *Fusarium solani* presenting as septic shock in child after hematopoietic cell transplantation

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Patients after allogeneic hematopoietic cell transplantation (allo-HCT) belong to a high risk group of invasive fungal disease (IFD). The distribution of pathogens in an allo-HCT setting includes aspergillosis in 55–60%, candidiasis in 25–30%, mycormycosis in 7–8%, and rare species (e.g. fusariosis, scedosporiosis, geotrichosis) in 2–3% [1]. With the widespread introduction of antifungal prophylaxis based on azoles, this epidemiology is tending to change, with the rise of rarer and more sporadic species. No major differences in etiology between children and adults have been reported [2–5], although the incidence of IFD after allo-HCT has also been reported to be significantly higher in children than in adults [5].

Regardless of the age, the following groups of patients are considered as high-risk groups for IFD: acute myeloblastic leukemia (AML), recurrent acute leukemia, allogeneic hematopoietic cell transplantation, and high risk acute lymphoblastic leukemia (ALL) [3, 4, 6, 7].

Clinical symptoms of IFD in immunocompromised patients are dependent on the localization of infection, which in most cases involve the lungs, abdomen, paranasal sinuses, skin or brain. In the majority of cases, general symptoms occur including fever, followed by other systemic symptoms, and laboratory markers of severe infection (e.g. C-reactive protein, procalcitonin). Sometimes symptoms of septic shock can occur.

The objective of this report was to present the case of a pediatric allo-HCT recipient with infection with *Fusarium solani* presenting as septic shock.

A 15-year-old girl treated for refractory relapsing B-precursor ALL (STIL/TAL1-positive) underwent allo-HCT from her sister fully human leukocyte antigen (HLA)-matched, after fludarabine–treosulfan–thiotepa–anti-thymocyte globulin (ATG) conditioning. Due to the persistent growth of minimal residual disease (MRD) after day +60 post-transplant, the patient received two cycles of blinatumomab. However, two months later she had another bone marrow relapse. She underwent a second allo-HCT, this time from an unrelated human leukocyte antigen (HLA)-matched donor, with treosulfan-melfalan-ATG conditioning. For graft-versus-host disease (GvHD) prophylaxis, she received mycofenolate mofetil and methotrexate. Initial anti-infective prophylaxis included intravenous antibiotics, micafungin, and acyclovir. On day +11, she experienced catheter-related septic shock, and skin rash. She was treated with antibiotics and dopamine. Microbiological analysis showed the presence of *Fusarium solani* in the blood, while no bacteria was detected from blood or central venous catheter. The patient was treated with liposomal amphotericin B and voriconazole followed by voriconazole monotherapy for another three months (Figure 1), with no symptoms of fungal infection. Six months later, the patient was diagnosed with a fifth leukemic relapse, with central nervous system (CNS) and bone marrow involvement. After analysis of the previous course of disease, with numerous relapses and the use of all available therapeutic options, the decision was made to switch to palliative treatment.

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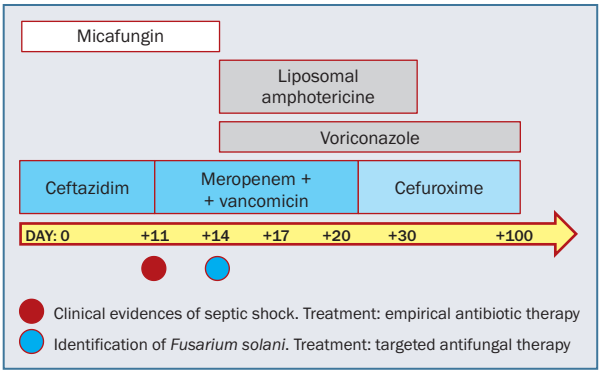

Figure 1. Antifungal and antibacterial prophylaxis and treatment

Septic shock is a very rare presentation of IFD. Literature data describing etiology, age and outcome is limited. Except for Candida species infections, the available pediatric data includes 23 patients, most of them with an underlying non-hematological disease (reviewed in [8]). Only 6/23 are reported to have survived this infection. High mortality after IFD-related septic shock has been reported also in adults (reviewed in [8]). In this context, we have shown the positive effect of therapy of breakthrough invasive fungal infection with *Fusarium solani* presenting as septic shock in a pediatric patient with acute leukemia after allo-HCT. This positive effect was achieved with swift microbiological diagnostics, the quick administration of targeted antifungals, and its continuation for an additional three months.

In conclusion, we here report an unusual presentation of invasive fungal disease with septic shock in an immunocompromised child after hematopoietic cell transplantation. With rapid diagnosis and treatment, the outcome of therapy of IFD-related septic shock was successful.

Authors' contributions

KC — design of study. RD, MRP, KC — provision of clinical data. All authors — analysis of clinical data. PZW — microbiological analysis. TS, JS — literature search, analysis of data, writing manuscript. All authors — critical revision and final approval.

Conflict of interest

The authors declare no conflict of interest.

Financial support

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Ethics

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CD2+ hairy cell leukemia

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Introduction

Hairy cell leukemia (HCL) is an uncommon mature B-cell neoplasm, accounting for only 2% of all lymphoid leukemias [1]. In recent years, clinical and immunophenotypic subtypes of HCL have been described, such as hairy cell leukemia variant (HCL-v) [1] and hairy cell leukemia Japanese variant (HCL-jv) [2, 3]. In the next (5^{th}) edition of the World Health Organization Classification of Hematolymphoid Tumors, the term 'splenic B-cell lymphoma/leukemia with prominent nucleoli' will probably replace the term 'hairy cell leukemia variant' [4].

Here, we present the case of a patient with an abnormal expression of the T-lineage antigen CD2 in HCL cells. To the best of our knowledge, fewer than 10 cases of CD2+ HCL have been described worldwide [5–7].

Case description

A 43-year-old man was referred to our laboratory for bone marrow aspiration and immunophenotyping for leucopenia and thrombocytopenia detected in a complete blood count (CBC), as part of routine medical exams. He had no lymphadenopathy, hepatomegaly, or splenomegaly. Hematological parameters were: hemoglobin (Hb) — 15.0 g/dL; hematocrit (Ht) — 43.1%; white blood cells (WBC) — 2.09×10^9/L (neutrophils — 47.6%, monocytes — 1.4%, lymphocytes — 47.8%, basophils — 0.5%, eosinophils — 2.7%); platelets (PLT) — 90×10^9/L. The blood smear revealed the presence of 3% small to medium-sized lymphoid cells characterized by a nucleus with moderately condensed chromatin without nucleoli, and slightly basophilic cytoplasm devoid of granules and exhibiting thin and elongated hairy projections. Erythrocyte sedimentation rate (ESR) — 3 mm and lactate dehydrogenase (LDH) — 179 U/L (normal range).

A bone marrow aspirate was performed. There were no technical difficulties in the procedure. The bone marrow smear showed the presence of 7% of atypical lymphoid cells characterized by a nucleus with homogeneous chromatin and a pale-blue cytoplasm exhibiting thin and elongated hairy projections, a finding similar to that seen in the blood film. A bone marrow trephine biopsy was not performed. The reason, however, why the trephine biopsy was not performed is not available to us.

The presence of two distinct subtypes of B-lymphoid cells (CD19+) was revealed by flow cytometry analysis of the bone marrow (Figure 1A). The 'green population' ($CD19^{moderate}$, gate 'L') — characterized by SSC^{dim}, CD2–, $CD20^{dim}$, CD22+, CD23+, CD103–, CD200–, and polyclonal kappa+ and lambda+ (Figure 1) — had the phenotype of normal B-lymphocytes. On the other hand, the 'red population' ($CD19^{bright}$, gate 'K') — characterized by SSC^{bright}, CD20+, CD22+, CD23–, CD103+, CD200+, and kappa+ light-chain restriction (Figure 1) — and, moreover, CD10–, $CD11c^{bright}$, CD25+, $CD45^{bright}$, CD79b–, CD123+, and FMC7+ (data not shown) had the typical phenotype of hairy cell leukemia.

Interestingly, the HCL cells exhibited positivity for the antigen CD2 (clone: RPA-2.10) (Figure 1B, C), though the cells did not express other T- and natural killer (NK)-lineage markers (CD3–, CD4–, CD5–, CD7–, CD8–, CD16–, CD56–) (data not shown). The HCL population was practically all positive for CD2, with few HCL cells ('red events') in quadrants 1 and 3 (CD2 negative cells) of the dot-plot 'B' (Figure 1).

Based on clinical and laboratory data, a diagnosis of HCL with atypical expression of CD2 antigen was made. The patient was submitted to monotherapy with chimeric anti-human CD20 antibody (rituximab) 375 mg/m^2 intravenously for three months. After completion of the treatment, a CBC evidenced improvement in leucocyte and PLT counts:

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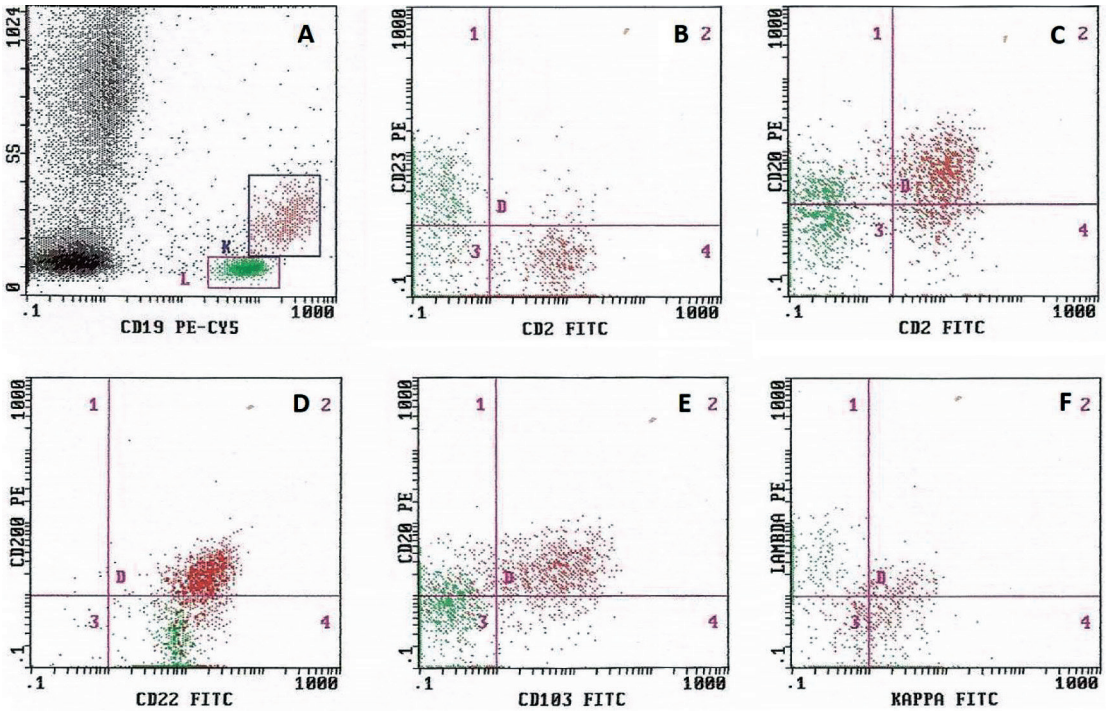

Figure 1. Flow cytometry findings of patient's bone marrow: **A.** The physical parameter (SCC) and CD19 expression show the presence of two distinct populations of B-lymphoid cells (red: HCL cells; green: normal B-lymphocytes); **B, C.** CD2 positivity on HCL cells; **D, E.** HCL cells show positivity for CD20, CD22, CD103, and CD200; **F.** Kappa light-chain restriction of HCL cells

Hb — 15.0 g/dL; Ht — 43.2%; WBC — 4.2 × 10^9/L (neutrophils — 69%, monocytes — 4.3%, lymphocytes — 25.1%, basophils — 0.5%, eosinophils — 1.1%); PLT — 130 × 10^9/L. No atypical lymphoid cells were found on blood smear.

The patient was therefore referred for bone marrow aspiration and minimal residual disease (MDR) evaluation by flow cytometry. The bone marrow aspirate showed 0.3% of atypical lymphoid cells, morphologically similar to those found in the diagnosis. MDR analysis revealed the presence of CD2 once again (0.34% of CD2+ HCL cells in the bone marrow) (data not shown). This data rules out any possibility of eventual technical errors in the identification of CD2 at diagnosis and seems to indicate that the antigen is useful for the detection of minimal residual disease.

Discussion

Preliminary data has shown that the expression of CD2 in HCL could be as high as 25% [6]. However, this was certainly an overestimate of the actual percentage because that data came from a report of only three CD2+ HCL cases out of 12 classical HCL [6]. More recently, Shao et al. [7] studied 169 HCL patients and found only three cases (2%) of CD2+ HCL.

Therefore, given that HCL is a rare disease, accounting for only 2% of lymphoid leukemias, CD2+ HCL is an extremely rare clinical condition, accounting for approximately 0.04% of all lymphoid leukemias. CD2 is also variably expressed in other mature B-cell neoplasms, such as chronic lymphocytic leukemia (16%), large B-cell lymphomas (29%), and follicular lymphoma (55%) [6].

An unusual clinical aspect of this case was the absence of splenomegaly. Kurosawa et al. [5] reported a single CD2+ HCL patient exhibiting only discrete splenomegaly (3 cm below left costal margin). Unfortunately, Kingma et al. [6] and Shao et al. [7] did not report data concerning the presence or absence of splenomegaly in their series.

Another curious feature is that, if it could be shown that the abnormal cells exhibited monoclonality for the T-cell receptor (*TCR*), it could be determined that the leukemic cells belonged to a dual lineage (B and T: 'bilineage' HCL), which cannot be presumed with the isolated presence of the CD2 antigen. However, unfortunately the patient was not submitted to *TCR* rearrangement studies. Kingma et al. [6] employed polymerase chain reaction (PCR) analysis for the determination of *TCR* gamma gene rearrangements in 2/3 CD2+ HCL patients. In both cases, the *TCR* was germ line.

The prognostic significance of CD2 in mature B-cell neoplasms remains to be elucidated. Inaba et al. [9] studied three patients with CD2+ B-cell non-Hodgkin's lymphoma, and showed extranodal involvement at diagnosis in all the cases. Suzuki et al. [10] showed the expression of CD2 in 2.7% of patients with the diagnosis of diffuse large B-cell lymphoma. The expression of CD2 did not appear

to be related to any clinicopathological features or survival [10]. Given the rarity of CD2 expression in HCL, no data exists on the clinical significance of CD2 positivity in this disease.

Significantly, at the present time it also remains unknown whether the abnormal expression of CD2 simply represents a variant phenotype of HCL or, alternatively, whether these cases are truly distinct clinical entities, like the HCL variant that, while resembling classic HCL, exhibits different cytological and immunophenotypic features, as well as diverse sensitivity to therapeutic agents effective in HCL. Following this train of thought, given that previous data showed that CD2+ B-lymphocytes represent a normal cellular population within the peripheral blood (approx. 3.6% of B-cells), it is not far-fetched to speculate that this small population of CD2+ B-lymphocytes could be the benign counterparts of some subtypes of CD2+ mature B-cell leukemias/lymphomas [6, 8].

The identification of more HCL patients with abnormal expression of CD2 — and, hence, the availability of a greater amount of clinical, immunophenotypic, cytogenetic, and histological data — is necessary to address these issues. It seems that the use of an extensive flow cytometry panel, including T- and NK-lineage markers, is appropriate for the immunophenotyping of HCL.

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Author's contributions

DMM — sole author.

Conflict of interest

The author declares no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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