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40 YEARS OF HEMATOPOIETIC STEM CELLS TRANSPLANTATIONS IN THE CLINICAL CENTER OF VOJVODINA: EXPERIENCES, POSSIBILITIES AND THE FUTURE

40 GODINA TRANSPLANTACIJE MATIČNIH ĆELIJA HEMATOPOEZE U KLINIČKOM CENTRU VOJVODINE : ISKUSTVO, MOGUĆNOSTI I BUDUĆNOST

Novi Sad, October 20, 2017.

HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) INTRODUCTION

TRANSPLANTACIJA MATIČNIH ĆELIJA HEMATOPOEZE UVOD

Hematopoietic stem cell transplantation (HSCT) is one of the most unique medical procedures, which has experienced a spectacular rise in the second half of the twentieth century and became a successful and standard method of treatment for many hematologic malignancies, congenital and acquired disorders of the hematopoietic system.

Analysis of the survey data spanning over 25 years has shown a continued and constant increase in the annual numbers of HSCTs and transplant rates for both allogeneic and autologous HSCT. According to the European Society for Blood and Marrow Transplantation (EBMT) data in 2015, more than 40 000 transplantations were performed, 43% allogeneic and 57% autologous HSCTs, worldwide, primarily for the treatment of hematologic malignancies. Main indications for HSCT were myeloid malignancies (25% overall; 96% allogeneic), lymphoid malignancies (67% overall; 20% allogeneic), solid tumors (4% overall; 3% allogeneic) and non-malignant disorders (6% overall; 90% allogeneic). The allo-HCT practice has changed with the introduction of stem cell collection from peripheral blood, cord blood, and introduction of haploidentical transplantations and reduced-intensity conditioning regimes. However, both acute and chronic GvHD still remain a serious barrier to successful allo-HCT implementation. Peripheral blood stem cell transplantation (PBSCT) induced in 1986, almost completely replaced bone marrow (BM) as a stem cell source (100% in the autologous and approximately 75% in the allogeneic transplant setting).

This year marks the 40th anniversary of the first, successful syngeneic allogeneic stem cell transplantation at the Clinical Center of Vojvodina, the former Medical Faculty in Novi Sad. Long experience and clinical practice have established an experienced and successful team for stem cell transplantation, and up to now, we have performed 35 allogeneic and 90 autologous stem cell transplantations at the Clinical Center of Vojvodina. Based on all of the above, we considered it necessary to mark this anniversary, to remind ourselves of our teachers and the participants in the first stem cell transplantation in Novi Sad, and present our experience and at the same time, to once again draw attention to the increased need for SCT in our center. We hope that in the coming years, the number, performance, and introduction of new types of stem cells transplants will grow and that our institution, Clinical Center of Vojvodina, will be one of the leading centers in the implementation of this procedure in Serbia and beyond.

Associate Professor Ivana Urošević

Medical Sciences Academy Branch
of the Serbian Medical Society, Novi Sad

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DEVELOPMENT OF HEMATOPOIETIC STEM CELL TRANSPLANTATION AT THE CLINIC OF HEMATOLOGY, CLINICAL CENTER OF VOJVODINA IN NOVI SAD

RAZVOJ TRANSPLANTACIJE MATIČNIH ĆELIJA HEMATOPOEZE NA KLINICI ZA HEMATOLOGIJU KLINIČKOG CENTRA VOJVODINE U NOVOM SADU

Dušan PEJIN

Summary

Hematology underwent substantial development in the second half of the 20th century. Research into the possibility of treating affected experimental animals with bone marrow transplantation was initiated at that time. Successful treatment of leukemia in animals by giving them chemo- and radiation therapy and subsequent administering of the bone marrow of a healthy animal showed to the clinicians the way to apply transplantation in humane medicine. The first allogeneic bone marrow transplantation in a patient suffering from acute leukemia was done in the United States by Prof. E. D. Thomas in 1957 after radiation of the entire body and chemotherapy with cytostatic agents. The patient's destroyed bone marrow was successfully recovered by a voluntary HLA identical donor. This first experience encouraged numerous hematologists so they prepared themselves for a new era of treating malignant and benign hematological diseases. The Clinic for Hematology in Novi Sad gained its first experiences in 1977 when a patient with severe aplastic anemia successfully received, for the first time in our country, the bone marrow transplant from his twin brother. After extensive preparations at the Clinic for Hematology, in 1990, bone marrow and hematopoietic stem cell transplantation from blood became a standard method of treating malignant and benign hematological diseases. By 1999, the transplant team successfully performed 20 transplantations, 4 in patients with severe aplastic anemia, and 16 in patients with malignant hematological diseases which were unresponsive to standard therapy such as leukemia, myelodysplastic syndrome, multiple myeloma. Donors were HLA identical relatives, twins in two patients. The best results were achieved in all patients with aplastic anemia and in four patients with malignant hematological diseases, while in others the transplantation had a months-long transient effect. Our experiences, as well as experiences across the world and Europe, were invaluable. New recommendations were given, narrowing down indications for allogeneic transplantation and the treatment has been focused on autologous transplantation and new anti-tumor drugs.

Key words: History of Medicine; Hematopoietic Stem Cell Transplantation; Transplantation, Autologous; Treatment Outcome

Sažetak

Hematologija je doživela značajan razvoj u drugoj polovini XX veka. U tom periodu su započeta ispitivanja mogućnosti lečenja obolelih eksperimentalnih životinja transplantacijom koštane srži. Uspeh u lečenju leukemija životinja primenom hemio i radioterapije sa naknadnim davanjem koštane srži zdrave životinje bio je kliničarima putokaz za primenu transplantacije u humanoj medicini. Prvu alogenu transplantaciju koštane srži kod obolele osobe od akutne leukemije obavio je u Sjedinjenim američkim državama prof. E.D. Tomas (Thomas) 1957. godine, nakon zračenja čitavog tela i hemiotetapije citostaticima. Uništena koštana srž bolesnika bila je uspešno obnovljena od dobrovoljnog HLA identičnog davaoca. Ovo prvo iskustvo ohrabrilo je mnoge hematologe, te su se prepremili za novu eru lečenja malignih i benignih hematiloških oboljenja. Klinika za hematologiju u Novom Sadu prva iskustva stekla je 1977. godine kada je bolesniku sa teškom aplastičnom anemijom uspešno, prvi put u našoj zemlji, transplantirana koštana srž od brata blizanca. Nakon obimnih priprema na Klinici za hematologiju, 1990. godine, transplantacija koštane srži i matičnih ćelija hematopoeze iz krvi postala je standardna metoda lečenja malignih i benignih hematoloških obolenja. U periodu do 1999. godine tim za transplantaciju obavio je uspešno 20 transplantacija, četiri kod bolesnika sa teškom aplastičnom anemijom, i 16 kod bolesnika sa malignim hematološkim bolestima koje nisu reagovale na standardnu terapiju kao što su leukemije, mijelodisplastični sindrom, multipli mijelom. Davaoci su bili HLA identični rođaci, kod dva bolesnika blizanci. Najbolji rezultati postignuti su kod svih bolesnika sa aplastičnom anemijom i kod četiri bolesnika sa malignim hematološkim bolestima, dok je kod ostalih transplantacija imala prolazni višemesečni efekat. Naša iskustva, kao i iskustva u svetu i Evropi bila su dragocena. Donete su nove preporuke u kojima su sužene indikacije za alogenu transplantaciju a terapija usmerena na autolognu transplantaciju i nove antitumorske lekove.

Ključne reči: istorija medicine; transplantacija hematopoetskim stem ćelijama; autologna transplantacija; ishod lečenja

The second half of the 20th century is known for progress in medicine, in particular in hematology, transfusiology and immunology. In addition to innovative diagnostic possibilities, including the typing of HLA antigens through agglutination tests in 1954 [1] by J. Dausset, the French Nobel Prize winner in

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This work is dedicated to Prof. Kosta Popović, PhD, an excellent hematologist, a researcher and a teacher. Grateful associates and students proudly preserve his image and his work.

Ovaj rad posvećen je Prof. dr Kosti Popoviću vrhunskom hematologu, istraživaču i pedagogu. Zahvalni saradnici i učenici sa ponosom čuvaju njegov lik i delo.

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Abbreviations

HLA – human leucocytes antigens
SMS – Serbian Medical Society
GvHD – graft versus host disease
ACD – anticoagulant citrate dextrose solution
RBC – red blood cell
AML – acute myeloid leukemia
EBMT – European Sosiety for bone and blood transplantation

1980, in 1966 Terasaki introduced HLA antigen serotyping [2]. Discoveries of a variety of antibiotics, cytostatic agents, *immunosuppressives* and other drugs assume a very important place in a demanding procedure such as bone marrow transplantation.

Successful experimental trials regarding treatment possibilities of bone marrow transplantation in mice and dogs suffering from leukemia, following chemotherapy with cytostatic agents and total body irradiation (TBI), were done by JM Main in 1955 [3]. Experimental transplantation in animals showed the importance of irradiation of the entire body as well as the phenomenon of leukemia relapse in syngeneic, and the lethal aGvHD ("wasting syndrome") in allogeneic transplantation. The first allogeneic transplantation in patients with acute leukemia, who underwent TBI and received chemotherapy was performed in 1957 by E. D. Thomas [4], the Nobel Prize winner in 1990. Professor Thomas' team continued their work with allogeneic transplantations in patients with aplastic anemia and acute leukemia types in 1977 [5], and survival in the group of patients with acute leukemia in first complete remission was 54% in their report from 1979 [6].

In this period of allogeneic transplantation development, it is also important to highlight urgent allogeneic transplantation in five physicists from Vinča lethally and in one sublethally, accidentally irradiated with gamma rays and neutrons. For the purpose of treating bone marrow aplasia, Professor G. Mathe in Paris gave to the most irradiated physicist, through infusion, the cells with cell nuclei from the spleen and the liver of dead and prematurely born fetuses but the acceptance did not occur. Using knowledge of allogeneic transplantation from the experimental period when it was evident that TBI results suppression of the recipient's immune system, Mathe decided to carry out bone marrow transplantation in all five irradiated physicists from voluntary unrelated donors of the same blood type with minimal phenotypic differences. Hematological response indicated acceptance of stem cells. One of the patients, the most irradiated of all, died due to severe damage of visceral organs. After 12 weeks, transplanted bone marrow ceased to function, with recovery of its own hematopoietic. A patient, a sublethally irradiated physicist, who was not transplanted, recovered spontaneously, yet slowly. It was concluded that the effect of allogeneic transplantation was of transitory character [7].

After this period, aided by new discoveries, including, most importantly, the method of DNA serotyping, hematological transplantation in big medical centers across the world became standard of care for malignant and non-malignant hematological diseases. Transplantations were carried out by taking the bone marrow from related, matched donors (allogeneic transplantation) or from identical twins (syngeneic transplantation).

Precisely at the time when bone marrow transplantation in our country had not yet been given a place in the treatment of hematological diseases, in 1976, the Clinic for Hematology in Novi Sad was faced with the problem of urgent treatment of a patient with severe idiopathic aplastic anemia, a 19 year-old man BS from Subotica, a worker at a printing house. Due to severe anemia and bleeding, he was receiving blood transfusions and concentrated platelets. Bone marrow stimulation with testosterone and administration of cortisone and B-vitamins did not give results. Taking into account the fact that the patient had a healthy twin brother, BF, the head of the Clinic, Professor Kosta Popović, carried out the immunogenetic testing at the Blood Typing Centre of the Blood Transfusion Institute in Belgrade. The serologic testing of HLA antigens confirmed the presence of HLA antigens: 3,-/w 14,- in both patients, while Rh phenotype differed minimally: in BS patient CcDee was inherited from their mother, and in his brother, BF, CCDee was inherited from their father. The twin brothers had the same blood type A that they inherited from their mother. The cross-reaction of the patient's serum and the donor's lymphocytes was negative. At the Department of Applied Immunology of the Institute for Experimental Medicine at the Military Medical Academy in Belgrade, the testing of their mixed lymphocyte culture was carried out. It was concluded that no stimulation occurred between the potential donor and the recipient of the bone marrow in the mixed two-way and in both cases mixed one-way culture of lymphocytes, indicating high similarity of their genes. The twins were viewed as identical and so Prof. K. Popović made a decision for the bone marrow syngeneic transplantation to be done at the Clinic for Hematology in Novi Sad, since there were no bone marrow transplantation centers in the country at that time **(Figure 1)**. Prof. K. Popović asked Prof. Božidar Radojičić, the head of Hematology Department at Military Medical Academy in Belgrade, who was performing the first transplantation attempts and had certain experience, for advice and technical assistance. Considering compatibility of the donor and the recipient it was decided according the then data from Cammita studies from 1975 [7] not to apply immunosuppression in the recipient for the purpose of preventing graft rejection. With technical assistance of Prof. B. Radojičić, on July 5, 1976, in the operating room of the Surgical Clinic in Novi Sad only $0.14x10^9$/L of bone marrow cells with cell nuclei, i.e. $0{,}028x10^8$/kg BW in 300 ml were collected from the donor under general anesthesia. The collected bone marrow was administered within ACD and heparin solution through a filter to the patient in-

travenously without any problem. It is necessary to underline that the recommendation of an experienced author, such as Prof. Bernar [8], was to administer a much higher number of cells with nuclei, more than $5x10^9$/L of cells with nuclei in particular, i.e. more than $1x10^8$/kg BW. After this syngeneic transplantation only for a brief period of time reticulocytes were increased in number, but following their decline and the progression of pancytopenia, it was evident that the potential acceptance of the transplantat did not occur completely. This patient's case was presented at the meeting of the Hematology and Transfusiology Section of the SMS in Belgrade where in discussions on transplantation failure they highlighted the difference in Rh factor phenotype, the recipient's CcDee against the donor's CCDee, indicating the so-called third kind of twins, namely that those were monozygotic twins with minor genotypic divergences. This discussion pointed out the facts that this was a variant of monozygotic twins with Rh phenotype differences, that an insufficient number of cells with nuclei was administered through transplantation, and that the patient was a polytransfunded person who received 20 L of blood. We saw the way out of this situation in a second transplantation including immunosuppressive preparation of the recipient and transplantation of the sufficient number of bone marrow cells. As part of the preparations, considering pancytopenia and the forthcoming immune suppression, the patient was isolated in a disinfected single room at the Clinic for Hematology with only nurses and doctors in sterile uniforms having access to it (the so-called reverse isolation of the patient). The patient was examined bacteriologically, and he was perorally administered tetracycline antibiotics and nystatin. The recipient's immunosuppression protocol included full blood transfusion from the bone marrow donor four days prior to transplantation for the purpose of desensitization, and then for four days he was administered cyclophosphamide 40-60 mg/kgBW i.v. per day, 7000 mg in total, and after 48h following the final immunosuppression the second transplantation was done on August 30, 1977, with allogeneic transplantation characteristics. 500 ml of the recipient's bone marrow, containing $6.294x10^9$ cells with nuclei, or $1.234x10^8$/ per kilogram of BW was aspirated. Both the patient and the donor underwent the surgery well. On the tenth day following the transplantation the patient's body temperature increased due to staphylococcus infection of tonsils which was cured with a combination of antibiotics. Due to worsening pancytopenia, the patient received 1,200 ml of irradiated fresh blood and it was the last transfusion to our patient. On the thirty-fifth day following transplantation, reticulocytes increased to $178.830x10^9$/L. With further substantial increase in number of reticulocytes, on the 44th day erythrocytes increased in number to $3.61x10^{12}$/L and Hb increased to 112 g/L, as well as leucocytes to $3.950x10^9$/l with $2.528x10^9$/L granulocytes and $120x10^9$/L platelets. Bone marrow was slightly hypocellular, with erythroblastosis or RBC and dysplastic granulocyte cell count. On the twenty-eighth day following the transplantation, it was concluded at the Institute of Blood Transfusion in Belgrade, thus confirming chimerism and the success of the transplantation. In the further post-transplantation period hematopoiesis was stabilized and the blood count was normalized. Additional blood analyses did not prove the previously increased erythropoietic activity, while the values of IgA and IgG, as well as of C3c complement, were moderately lowered.

We presented this case of our successful bone marrow transplantation in the patient with aplastic anemia from his homozygous brother with the minimum discrepancy in Rh genotype in 1978 at the 17th Congress of the International Association of Hematologists and Transfusiologists in Paris where the work attracted attention and was discussed [9].

The patient was dismissed from the clinic on December 27, 1977. He underwent regular examinations in the next six-year period, and the last one was in 1997. He felt fine; his blood count and bone marrow findings were within the limits of normal with slight fluctuations. In the magazine Medical Overview numbers 5 and 6 from 1984 Prof. Kosta Popović published his work titled Aplastic anemia cured through bone marrow transplantation from a non-identical twin brother, followed by changes to the recipient's Rh phenotype [9].

Since 1979, BS was working as a full-time employee at the printing house. Our former patient was diagnosed with cholelithiasis in Subotica in 2014 after a general medical examination; he underwent surgery and in the same year ultrasonography of his neck detected underactive thyroid gland and hypothyreosis that he has been treating with Letrox and Rosuhol tablets. He retired in 2016 at the age of 60.

On the 40th anniversary of the first successfully performed bone marrow transplantation, the Clinic for Hematology invited its former patient for a medical examination and he gladly accepted so we arranged additional examinations. Blood count was normal. This involved immunological as well as immunogenetic examinations with Rh genotype identification **(Figure 2).**

After 1980, one more allogeneic transplantation was carried out in a patient with AML who had not been responding to therapy. It was decided to carry out the bone marrow transplantation from her HLA identical sister. The patient received TBI which resulted in bone marrow aplasia. Unfortunately, she died due to gastrointestinal perforation caused by irradiation. After Prof. Kosta Popović's retirement, bone marrow transplantation was given a place of considerable importance in the new work organization. All details in professional literature were indicating a constant increase in the number of transplant patients and the forming of new centers across the world, and in Yugoslavia as well (Belgrade – Military Medical Academy, Zagreb, Ljubljana). Considering Vojvodina's population of 2,000,000 and constant increase in the number of people suffering from malignant diseases, with absolute understanding of Vo-

The tables below show, details on patients and donors, as well as the effect of HSCT
Na priloženim tabelama prikazane su, podaci o bolesnicima i davaocima, kao i efekat TMĆH

Table 1. Allogeneic bone marrow and bsc transplantation
Tabela 1. *Alogena transplantacija kostne srži i mćk*

Patient/*Bolesnik*	Dg	Dg-TKS	Blood type	Donor/*Donor*	Blood type/*K.G.*	D>P
1.BS 19 y	AA	6 m	A, Rh+	Twin/*Blizanci*	A, Rh+	M>M
2 SI 18 y	AA	3 m	A, Rh-	Sister/*Sestra*	A, Rh+	F>F
3.BD 23 y	AA	2 m	A, Rh-	Sister/*Sestra*	A, Rh+	F>M
4.NS 19 y	AA	4 m	A, Rh+	Twin/*Blizanci*	A, Rh+	F>F
5.BM 44 y	AML, res	3 m	B, Rh+	Brother/*Brat*	B, Rh+	M>F
6.PS 38 y	AML,1.R	5 m	0, Rh+	Sister/*Sestra*	0, Rh-	F>F
7.JJ 44 y	ALL,1.R	7 m	B, Rh+	Brother/*Brat*	0, Rh+	M>M
8.ME 41 y	MM-III	31 m	AB, Rh-	Sister/*Sestra*	AB, Rh-	F>F
9.DB 16 y	ES-dis.	9 m	A, Rh+	Father/*Otac*	A, Rh-	M>M
10.BS 36y	RAEB	12 m	AB, Rh+	Sister/*Sestra*	B, Rh+	F>F
11.JS 44 y	sAML-M7	6 m	A, Rh+	Sister/*Sestra*	A, Rh+	F>M
12.SD 29y	CML-acc	18 m	A, Rh+	Brother/*Brat*	A, Rh-	M>M
13.NS 37y	CMML-BT	22 m	A, Rh+	Sister/*Sestra*	A, Rh+	F>F
14.BŠ 26y	CML-CP	13 m	0, Rh+	Sister/*Sestra*	0, Rh+	M>M
15.MV34y	CML-CP	12 m	A, Rh+	Brother/*Brat*	A, Rh+	M>F
16,TD 36y	CML-BT	16 m	A, Rh+	Brother/*Brat*	AB, Rh+	M>M
17.GM32y	CML-CP	12 m	0, Rh+	Sister/*Sestra*	A, Rh+	F>F
18,ŽJ 34 y	CML-CP	10 m	A, Rh+	Sister/*Sestra*	A, Rh+	F>M
19.GR32y	CML-BT	6 m	0, Rh+	Sister/*Sestra*	0, Rh+	F>M
20.KM32y	CML-CP	8 m	A, Rh+	Sister/*Sestra*	0, Rh+	F>M

Legend: AA - aplastic anemia;AML - acute myeloid leukemia, ALL - acute lymphoid leukemia; RAEB - refractory anemia with excess blast; MM - multiple myeloma; CML - chronic myeloid leukemia, CP - chronic phase, AP - acceleration phase, BT - blast transformation; ES - Ewing sarcoma; D - donor; P - patients. M - male; F - female.
Legend: AA - aplastična anemija; AML - akutna mijeloidna leukemija, ALL - akutna limfoidna leukemija; RAEB - refraktarna anemija sa viškom blasta; MM - multipli mijelom; CML - hronična mijeloidna leukemija, CP - hronična faza, AP - faza akceleracije, BT - blastna transformacija; ES - Ewing sarcom; D - donor; P - pacijent; M - muški pol; F - ženski pol

jvodina's authorities and funds for science, health care and education, the needed Sterile Unit has been formed at the Clinic for Hematology in Novi Sad **(Figures 3 and 4)**, nurses have increased in number and a professional transplant team has been formed and given the opportunity for professional development at well-known centers and for participation in professional congresses. A broader team was formed

Figure 1. Prof. Kosta Popović and the Bošnjak brothers
Slika 1. *Prof. dr Kosta Popović i braća Bošnjak*

Figure 2. Prof. Ivana Urošević, the head of the Clinic for Hematology, the Bošnjak brothers and Prof. Dušan Pejin
Slika 2. *Prof. Ivana Urošević upravnica Klinike za hematologiju, braća Bošnjak i prof. Dušan Pejin*

Table 2. Allogeneic transplantation effects
Tabela 2. *Efekat alogene transplantacije*

Patient *Bolesnik*	Dg	Hematological status *Hematološki status*	Cytogenetic *Citogenetika*	Survival *Preživljavanje*	Complications/cause of death *Komplikacije – uzrok smrti*
BS	AA	Remission/*Remisija*	Normal/*Normalna*	>40 g	
SI	AA	Remission/*Remisija*	Normal/*Normalna*	>27 g	
BD	AA	Remission/*Remisija*	Normal/*Normalna*	>20 g	
NS	AA	Remission/*Remisija*	Normal/*Normalna*	>25 g	
SD	CML	Remission/*Remisija*	Ph- > Ph+	9 m	Relaps in ALL/*Relaps u ALL*
NS	CML	Remission/*Remisija*	PCR negative *PCR negativna*	13 m	Rhabdomyosarcoma
BS	CML	Remission *Remisija*	Ph negative *Ph negativna*	48 m	Extensive hGvHD *Prošireni cGvHD*
MV	CML	Remission *Remisija*	Ph negative *Ph negativna*	47 m	Relapse >12 m AML *Relaps>12 m AML*
GM	CML	Remission *Remisija*	Ph negative *Ph negativna*	>42 m	
ŽJ	CML	Remission *Remisija*	Ph negative *Ph negativna*	49 m	Relapse> 4 y in AML *Relaps > 4 g u AML*
GR	CML	Remission *Remisija*	Ph negative *Ph negativna*	8 m	Relapse>6 m>DLI>Remission 2m *Relaps>6 m>DLI>Remisija 2m*
JJ	ALL	Remission/*Remisija*	Normal/*Normalna*	>21 g	
PS	AML	Remission/*Remisija*	Normal/*Normalna*	12 m	cGvHD - Lungs/*cGvHD - Pluća*
JS	AML	Remission/*Remisija*	Normal/*Normalna*	8 m	Relapse after 5 m, 2 IDL *Relaps nakon 5 m, 2 IDL*
BS	MDS	Remission/*Remisija*	Normal/*Normalna*	>18 g	hGvHD
ME	MM	Partial remission *Parcijalna remisija*		6 m	Extramedullare relapse, AIM *Ekstramedularni relaps, AIM*

Legend: AA - aplastic anemia; CML - chronic myeloid leukemia; MDS - myelodysplastic syndromes; MM - multiple myeloma; AIM - acute myocardial infarction; DLI- donor lymphocytic infusion; PCR- polymerase chain reaction.
Legenda: AA - aplastična anemija; CML - hronična mijeloidna leukemija; MDS - mijelodisplastični sindrom; MM - multipli mijelom; AIM - akutni infarkt miokarda; DLI - donorska limfocitna infuzija; PCR - polimeraza lančana reakcija.

to monitor transplantations with experts in the fields of transfusiology, immunology, microbiology and virology, radiology, histopathology, pathophysiology, etc. taking active part in it. Healthcare policy as such soon proved to be correct and necessary for our Province. Since 1990 the Unit for Transplantation has been constantly working, taking active part in the work of the European Bone Marrow Transplantation Group (EBMT) as its member, and with other centers in the country and abroad as well. In our work we used the

Figure 3. Sterile Unit for hematopoietic stem cell transplantation
Slika 3. *Sterilna jedinica za transplantaciju matične ćelije hematopoeze*

Figure 4. Clinic for Hematology. Transplant Team of the Clinic for Hematology – Clinical Center of Vojvodina
Slika 4. *Klinika za hematologiju, Transplantacioni tim Klinike za hematologiju Kliničkog centra Vojvodine*

Table 3. New options in HSCT
Tabela 3. *Nove mogućnosti u TMĆH*

Molecular testing of HLA antigenes A, B, C, DR, DQ/*Molekulsko ispitivanje HLA antigena*
Family donors, unrelated donors/*Porodični davaoci, nesrodni davaoci*
Revised indications for HSCT: CML (Glivek et al.), MM (Lenalomid, anti CD38 etc). *Revidirane indikacije za TMĆH: HGL (Glivek i dr.), MM (Lenalomid, anti CD38 i dr).*
Risk factors and optimal timing/*Faktori rizika i optimalno vreme*
Mobilisation of BSC/*Mobilizacija matičnih ćelija iz periferne krvi*
Submyeloablative conditioning/*Submijeloablativno kondicioniranje*
Prophylaxis and treatment of aGvHD/*Profilaksa i lečenje aGvHD*
Increase in GvL effect and relapse therapy/*Povećanje GvL efekta i terapija relapsa*
Register of voluntary donors of HSC/*Registar dobrovoljnih davalaca MĆH*

known work protocols from large world centers and from EBMT as well. We were presenting our results at domestic and foreign congresses and meetings and we also participated in scientific and research projects on hematopoietic stem cell transplantation.

We initiated a new series of allogeneic stem cell transplantation from bone marrow, and from peripheral blood (BSC) as well, in cooperation with the Institute of Blood Transfusion in 1990 and we finished the demanding work in this field prior to the NATO bombing of Novi Sad in 1999.

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ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION AT THE CLINICAL CENTER OF VOJVODINA

ALOGENA TRANSPLANTACIJA MATIČNIH ĆELIJA HEMATOPOEZE U KLINIČKOM CENTRU VOJVODINE

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Summary

Introduction. Allogenic hematopoietic stem cell transplantation is the best therapeutic option for the treatment of some inherited and acquired diseases of the hematopoietic system as well as various hematological malignancies. **Material and Methods.** The study was conducted as a retrospective analysis of 35 patients who underwent allogenic hematopoietic stem cell transplantation at the Clinic of Hematology, Clinical Center of Vojvodina. **Results.** In a group of 35 patients with median age 33 years, 13 patients had acute myeloid leukemia, one patient had acute lymphoblastic leukemia, nine had chronic myeloid leukemia, five had aplastic anemia, five myelodysplastic syndrome, one had multiple myeloma and one had Ewing sarcoma. Nine patients (26%) had an advanced, resistant disease at the time of transplantation. The majority of patients had a matched related transplantation - 89% (31/35) - three patients had syngeneic transplantation, and one patient had a haploidentical transplantation. Out of 35 patients, 16 (45.7%) are alive. The European Bone Marrow Transplantation score ≥3 and the presence of advanced disease at the time of transplant were unfavourable prognostic factors for survival ($p<0.01$). If we exclude the cases with advanced, resistant disease at the time of transplantation, the probability of 5-year survival rate was as follows: 100% in patients with aplastic anemia, 75% in patients with acute leukemia, 34% in myelodysplastic syndrome, and 14% in chronic myeloid leukemia patients. **Conclusion.** The outcome of allogeneic hematopoietic stem cells transplantation in our patients is generally comparable with previously reported results. The main prognostic factors for survival were European Bone Marrow Transplantation risk score and disease status at the time of transplantation.

Key words: Hematopoietic Stem Cell Transplantation; Survival Rate; Graft vs Host Disease; Treatment Outcome

Sažetak

Uvod. Alogena transplantacija matičnih ćelija hematopoeze je metoda izbora za lečenje raznih urođenih i stečenih poremećaja hematopoeznog sistema, kao i raznih hematoloških maligniteta. **Materijal i metode.** Retrospektivna studija, koja je obuhvatila 35 pacijenata lečenih alogenom transplantacijom matičnih ćelija hematopoeze na Klinici za hematologiju Kliničkog centra Vojvodine u Novom Sadu. **Rezultati.** Od ukupno 35 pacijenata, medijane životnog doba od 33 godine, 13 pacijenata je bolovalo od akutne mijeloidne leukemije, jedan od akutne limfoblastne leukemije, devet od hronične mijeloidne leukemije, pet od aplastične anemije, pet od mijelodisplastičnog sindroma, jedan od multiplog mijeloma i jedan je bolovao od Juingovog sarkoma (*Sarcoma Ewing).* Devet bolesnika (26%) imalo je uznapredovalu, rezistentnu bolest u vreme transplantacije. Većina bolesnika je imala transplantaciju od podudarnog srodnog davaoca 89% (31/35); kod tri bolesnika sprovedena je singena transplantacija i kod jednog haploidentična transplantacija. Od 35 pacijenata, 16 (45,7%) su živi. *European Bone Marrow Transplantation* skor ≥ 3 i prisustvo uznapredovale bolesti predstavljali su nepovoljne prognostičke činioce za preživljavanje ($p < 0,01$). Nakon isključenja bolesnika sa uznapredovalom, rezistentnom bolesti u vreme transplantacije, petogodišnje preživljavanje iznosilo je 100% kod bolesnika sa aplastičnom anemijom, 75% kod bolesnika sa akutnom leukemijom, 50% kod bolesnika sa mijelodisplastičnim sindromom i 14% sa hroničnom mijeloidnom leukemijom. **Zaključak.** Rezultati alogene transplantacije matičnih ćelija na Klinici za hematologiju Kliničkog centra Vojvodine u korelaciji su sa rezultatima drugih svetskih istraživanja. *European Bone Marrow Transplantation* skor kao i status bolesti pre transplantacije glavni su prognostički činioci.

Ključne reči: transplantacija hematopoetskih stem ćelija; preživljavanje; bolest kalema protiv domaćina; ishod lečenja

Introduction

Over the last half-century, allogenic hematopoietic stem cell transplantation (AHSCT) has evolved from an idea to a well-established therapy used in the treatment of tens of thousands of individuals annually [1]. AHSCT is still the best therapeutic option for the treatment of inherited and some acquired diseases of the hematopoietic system as well as various hematological malignancies [2]. AHSCT requires the harvest of an adequate number of hematopoietic stem cells (HSC) from a histocompatible donor and their infusion into a patient following a conditioning regimen [3]. HSC could be collected from the bone marrow (BM), peripheral blood (PB) after chemotherapy and/or recombinant hematopoietic growth factors, as

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Abbreviations

AHSCT – allogenic hematopoietic stem cell transplantation
HSC – hematopoietic stem cells
BM – bone marrow
PB – peripheral blood
HLA – human leukocyte antigen
GvHD – graft versus host disease
EBMT – European Bone Marrow Transplantation
HCT-CI – hematopoietic cell transplantation -specific comorbidity index
BW – body weight
AL – acute leukemia
CR – complete remission
CML – chronic myeloid leukemia
MDS – myelodysplastic syndrome
PR – partial remission
MM – multiple myeloma
AA – aplastic anemia
AML – acute myeloid leukemia
ES – ewing sarcoma
TKI – tyrosine-kinase inhibitor

well as from the umbilical cord blood [2]. In the past two decades, peripheral blood AHSC replaced the BM as a HSC source due to faster engraftment and practicability. As transplant indications and conditioning regimens continue to change, whether the choice of the stem cell source has an impact on transplant outcomes remains to be determined [4]. The AHSCT is multi-staged and begins with identifying the HSC donor. The donor can be a Human leukocyte antigen (HLA)-matched relative (most often a brother or a sister), HLA-matched unrelated donor, HLA miss-matched family member (haploidentical donor) or HSC from unrelated umbilical cord blood [5]. Then an adequate conditioning regimen needs to be applied. The conditioning regimens have been classified as high-dose (myeloablative), reduced-intensity and non-myeloablative. The conditioning regimens are administered as part of the procedure to achieve two goals: provide sufficient immunoablation to prevent graft rejection and reduce the tumor burden. The intensity of conditioning regimens can vary substantially, and when selecting the optimal conditioning regimen for any given patient, disease-related factors such as diagnosis and remission status, as well as patient-related factors including age, donor availability, and presence of comorbid conditions, need to be considered [6]. HSCT performed in the early phases of the disease and in young patients offers more than a 50% cure rate. The transplant-related mortality still represents the greatest obstacle, ranging from 20–30%, despite the less toxic conditioning regimens, high-resolution HLA typing, and better supportive care. Graft versus host disease (GvHD) and infections remain the main causes of morbidity and mortality. As for disease relapse, it correlates with the disease status at the time of transplantation [3].

The aim of this study was to analyze the medical data, prognostic factors, survival rate and transplant-related complications of 35 patients who underwent AHSCT at the Clinic of hematology, Clinical Center of Vojvodina.

Table 1. Clinical and transplant characteristics of the study patients
Tabela 1. *Kliničke i transplantacione karakteristike ispitivanih bolesnika*

Variable/*Varijabla*	Values/*Vrednosti*
Age Median, min-max, years/*Životno doba, Medijana, min-maks, godine*	33, 15-56
Sex Male/Female, N (%)/*Pol Muški/Ženski N (%)*	15 (43)/20 (57)
EBMT score < 3 / ≥ 3 N (%)/*EBMT skor < 3 / ≥ 3 N (%)*	22 (63)/13 (37)
HCT-CI score < 2 / ≥ 2 N (%)/*HCT-CI skor < 2 / ≥ 2 N (%)*	30 (86)/5 (14)
Donor sister to brother Yes/No N(%)/*Davalac sestra bratu Da/Ne N(%)*	7 (20)/28(80)
Time from diagnosis to transplant, Median, min-max, months *Vreme od dijagnoze to transplantacije, Medijana, min-max, meseci*	10, 2-146
Stem cell source BM/ PB N(%)/*Izvor matičnih ćelija periferna krv/koštana srž N (%)*	16 (45.7)/19 (54.3)
Major ABO incompatibility* N (%)/*Major ABO inkompatibilija* N (%)*	7 (20)
Minor ABO incompatibility* N (%)/*Minor ABO inkompatibilija* N (%)*	8 (23)
MNCx10^8/kg BW - Median, min-max/*Mononuklearne ćelije x10^8/kg TM – Medijana, min-maks*	6.1, 3.2-12
CD34+ cells x10^6/kg BW - Median, min-max/*CD34+ ćelije x10^6/kgTM – Medijana, min-maks*	6.23, 2.21-11.7
Time to engraftment - Median, min-max, days/*Vreme do engraftmenta – Medijana, min-maks, dani*	12, 8-21
Acute GvHD N(%)/*Akutni GvHD N(%)*	13 (37%) (grade 3/4 6%)
GvHD chr. N(%)/*Hronični GvHD N(%)*	11 (31%) (5 (16%) extensive)
Relapse N(%)#/*Relaps N(%)#*	9 (26%)
Non-relapse mortality N(%)/*Mortalitet koji nije u vezi sa relapsom N(%)*	10 (28.6%)

*In one case both major and minor ABO incompatibility was present
**U jednom slučaju bila je prisutna i major i minor ABO inkompatibilija*

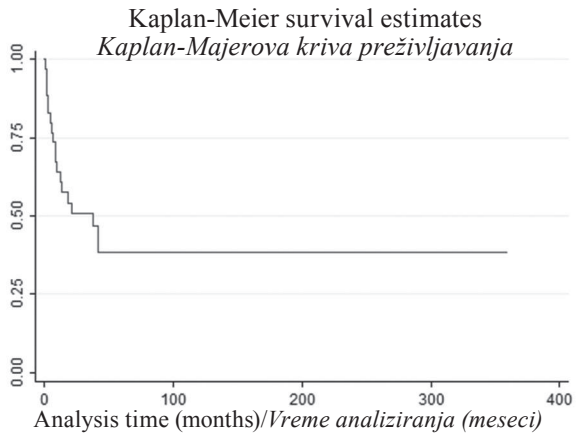
Material and Methods

We retrospectively analysed 35 patients who underwent transplants at the Clinical Center of Vojvodina in Novi Sad, Serbia. The study was conducted in accordance with the Helsinki Declaration.

Data on clinical outcome (death, survival, relapse) and other clinical and laboratory characteristics were collected from patients' medical files.

The following parameters were assessed: age, sex, time from diagnosis to transplantation, transplantation date, diagnosis, European Bone Marrow Transplantation-EBMT score [7], Hematopoietic cell transplantation - specific comorbidity index - HCT-CI [8], the presence and grade of acute and chronic GvHD, HSC source (PB vs BM), the number of apheresis, number of mononuclear cells per kg/bodyweight (BW) of the recipient, the number of CD34+ cells per kg/BW of the recipient, the type of transplantation (matched related, syngeneic, haploidentical), the conditioning regimen, the presence of ABO incompatibility, the presence of graft failure, survival and relapse status. We defined the advanced disease similar to definition of the late disease in EBMT score [7]: AL not in complete remission (CR), chronic myeloid leukaemia (CML) in blast crisis, myelodysplastic syndrome (MDS) not in CR or partial remission (PR) previously treated with more than 10% of blasts, lymphoma and multiple myeloma (MM) not in CR, PR nor in stable state, previously treated. Staging was not applicable for aplastic anemia.

Numerical variables are presented in median values and ranges. Categorical variables are shown as counts and relative frequencies. Overall survival was determined as the time between the date of transplantation and the date of death or last follow-up for censored patients. The survival was analyzed using Kaplan–Meier plots. The survival curves were compared using the log-rank test. A p-value of less than 0.05 was considered to be statistically significant. Analyses were performed using Stata 12 (Stata Corp LP, College Station, USA).



Graph 1. Survival of transplanted patients
Grafikon 1. *Preživljavanje bolesnika kojima je urađena transplantacija*

Results

The first transplantation was syngeneic and performed in 1977 in a patient with AA. The patient recovered after second transplantation. The second transplantation was done in 1980 in a patient with resistant acute myeloid leukemia (AML) but without success. From 1990 to 1999 eighteen transplantations were performed (four AA, three AML, nine CML, one MDS, one MM and one Ewing sarcoma-ES). From 2004 to 2017 fifteen transplantations were done, predominantly in patients with AL (ten AML, four MDS, one AA).

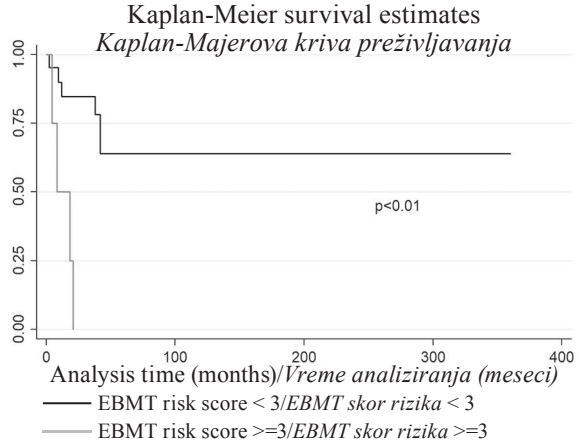
Out of 35 patients, 20 (57%) were female and 15 (43%) male. The median age of the patients was 33. **Table 1** shows the clinical and transplantation characteristics of the study patients.

In relation to the diagnosis of the underlying disease, the distribution of the patients was as follows: acute leukemia in 40% of the patients (13 patients had AML - including 3 cases of secondary AML not in CR nor PR, one patient had acute lymphoblastic leukemia), CML in 26% (9 patients, two were in resistant, blast transformation phase, two in acceleration phase), AA in 14% (five patients), MDS in 14% (five patients), MM in one patient as well as one case of Ewing sarcoma (ES).

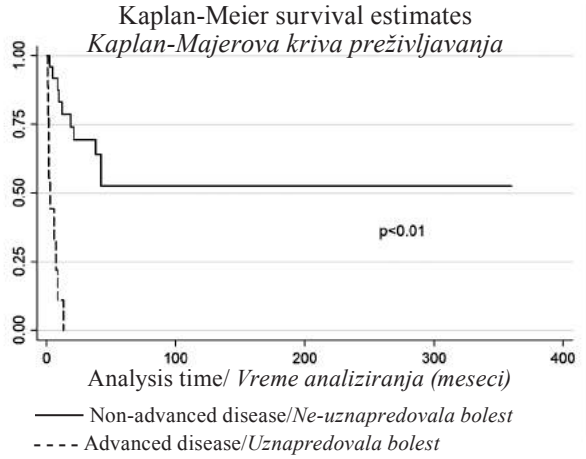
Nine patients (26%) had an advanced, resistant disease at the time of transplantation.

The majority of patients had a matched related transplantation, 89% (31/35), three patients with AA had syngeneic transplantation, and only one patient with ES had a haploidentical transplantation.

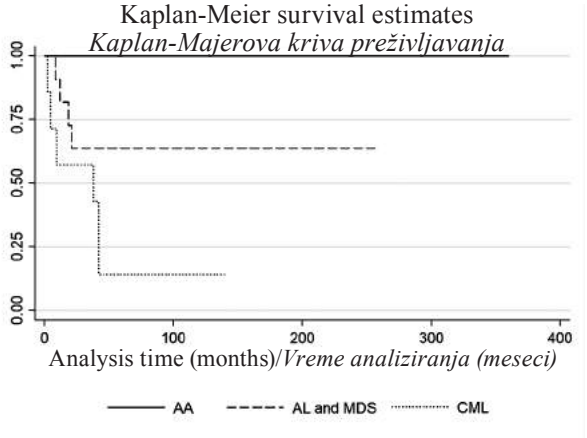
The conditioning regimes were as follows: cyclophosphamide in four patients with AA, ATG only in hepatitis associated AA in the case of syngeneic transplantation, cyclophosphamide + busulphan in 23 patients, busulphan+ fludarabine in three cases with reduced conditioning, busulphan + cyclophosphamide + ATG in one patient, busulphan + cy-



Graph 2. Survival of patients according to EBMT risk score
Grafikon 2. *Preživljavanje bolesnika prema EBMT skoru rizika*



Graph 3. Survival of patients according to disease status at time of transplantation
Figure 3. *Preživljavanje bolesnika prema statusu bolesti u vreme transplantacije*



Graph 4. Survival of patients according to diagnosis (patients with advanced disease are exluded from analysis)
Figure 4. *Preživljavanje bolesnika u odnosu na dijagnozu (bolesnici sa uznapredovalom bolešću su isključeni iz analize)*

clophosphamide + etoposide in one patient, fludarabine + melphalan in one case, and TBI in one patient.

Thirteen patients (37%) had an EBMT score ≥3 while five patients (14%) had a HCT-CI score ≥2.

In 7 (20%) cases, the donor was female (sister) to a male recipient (brother).

The GvHD prophylaxis was cyclosporine A + methyl-prednisolone in 19 patients (18/19 were transplanted before 2000), cyclosporine A + methotrexate in 11 patients and cyclosporine A + mycophenolate mofetil in three patients (all patients transplanted after 2004), and methotrexate and corticosteroids in two patients transplanted till 1980.

The acute GvHD was present in 37% of patients, chronic GvHD in 31% of patients **(Table 1)**.

Relapse occurred in nine (26%) of patients. Non-relapse mortality was present in 10 (28.6%) of patients.

Out of 35 patients, 16 (45.7%) are alive. The median follow-up time of censored patients was 49 months (range from 3 to 480 months). The median survival rate was 18 months (range from 1 to 480 months). Survival of all patients is presented in **Graph 1.** The EBMT score ≥3 and the presence of advanced disease at the time of transplant were unfavorable prognostic factors for survival ($p<0.01$, **Graph 2 and graph 3**). The other factors, age, sex, time from diagnosis to transplantation, HCT-CI, the presence of acute and chronic GvHD, HSC source (peripheral blood vs bone marrow), the presence of ABO incompatibility did not show prognostic significance. If we exclude the cases with advanced, resistant disease at the time of transplantation, projected 5-year survival rate would be as follows: 100% in patients with AA, 75% in patients with AL, 34% in MDS patients, and 14% in CML patients (**Graph 4**, AL and MDS are grouped together because of the small number of patients in the MDS group; log rank tests were not done considering the limited number of patients).

Discussion

We presented the results of allogeneic hematopoietic stem cell transplantation from our institution. The study group is very heterogeneous considering the time of transplantation, diagnosis, conditioning regimens, and GvHD prophylaxis. In the period before 2000, the main indications for AHSCT were CML and AA. After 2000, the majority of patients had AL or MDS. The outcome of transplantation in AA patients was excellent. All patients had engraftment with long-term survival of 100%. These results are in concordance with published reports with long-term survival in more than 80% of patients younger than 40 years old [9, 10].

However, the long-term results of transplantation in CML patients were not satisfactory. These transplants were all performed in pre-tyrosine-kinase inhibitor (TKI) era and without real time PCR monitoring which could have had an impact on the outcome. Almost half of the CML patients were in blast transformation or acceleration phase of the disease, both phases known as prognostically unfavorable [11]. According to EBMT results, the probability of survival at 20 years was 34% for transplanted CML patients which is higher than in our group [7] but still seems to be not very satisfactory. These results have a historical value considering the fact that the indications for transplantation in CML in TKI era is restricted to patients who didn't respond to at least two lines of TKI treatment [12].

The outcome of transplantation in AL and MDS is in correlation with the published results. The probability of five-year survival in AML patients transplanted in any remission is 75% which is similar to the published results [13, 14]. The projected five-year survival rate in MDS patients after AHSCT is 34% if we exclude the patients with advanced, late disease. Published results reported long-term survival in MDS at the level of 30 to 40% for a similar group of patients [15, 16].

In our study, considering the limited number of transplanted patients our primary aim was to present the main results of AHSCT, and not to conduct a detailed prognostic analysis. However, we found the EBMT risk score and disease status have a strong prognostic value which is in concordance with the published results [11, 17].

Conclusion

The outcome of allogeneic HSC transplantation in our patients is generally comparable with the previously reported results. The main prognostic factors for survival were European Bone Marrow Transplantation risk score and disease status at the time of transplantation.

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AUTOLOGOUS STEM CELL TRANSPLANTATION FOR THE TREATMENT OF HEMATOLOGICAL MALIGNANCIES IN THE CLINICAL CENTER OF VOJVODINA

AUTOLOGNA TRANSPLANTACIJA MATIČNIH ĆELIJA HEMATOPOEZE U LEČENJU HEMATOLOŠKIH MALIGNITETA U KLINIČKOM CENTRU VOJVODINE

Ivana UROŠEVIĆ[1,2], Ivanka SAVIĆ[1], Amir EL FARRA[1,2], Borivoj SEKULIĆ[1,2], Aleksandar SAVIĆ[1,2] and Bela BALINT[3,4,5]

Summary

Introduction. Autologous stem cell transplantation combined with high dose chemotherapy is an effective and safe approach in the treatment of different hematological malignancies. Nowadays, autologous stem cell transplantation represents a standard therapeutic option in the treatment of multiple myeloma, lymphomas and other hematological malignancies. Aim **i**s to analyze the available medical data of patients with multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma acute myeloid and lymphoblastic leukemia, who underwent autologous stem cell transplantation, and to compare the results with published data from other similar studies. **Material and Methods.** A retrospective study included 90 patients with multiple myeloma, acute myeloid and lymphoblastic leukemia, non-Hodgkin's and Hodgkin's lymphoma who underwent autologous stem cell transplantation in the period from 2004 to August 2017. **Results.** In relation to the underlying disease, the distribution of the respondents was as follows: 39 patients had multiple myeloma, 25 non-Hodgkin's lymphoma, 20 Hodgkin's lymphoma and 6 had acute leukemia. 75 patients (89.3%) had the large volume apheresis procedure, while 9 patients (10.7%) had the conventional two-day apheresis procedure. The average number of the mononuclear cells in the apheresis product was $7,8x10^8$/kg, and the number of the CD34 + cells was about $12,11x10^6$ kg. After applying the conditioning regimen, depending on the underlying disease, neutrophils engraftment mainly occurred on the 11th while the platelet engraftment occurred on the 14th post-transplant day. Transplant-related mortality was low, and the mortality rate was 3.57%. **Conclusion.** Autologous stem cell transplantation is an efficient method of treatment for patients with hematological malignancies. It is associated with a low rate of complications as well as low rate of transplant-related mortality.

Key words: Transplantation, Autologous; Hematopoietic Stem Cell Transplantation; Hematologic Neoplasms; Survival Rate; Graft vs Host Disease; Treatment Outcome

Sažetak

Uvod. Visokodozna hemioterapija sa potporom autolognom transplantacijom matičnih ćelija hematopoeze prihvaćena je kao efikasan terapijski izbor kod bolesnika sa hematološkim malignitetima. Cilj istraživanja bio je da se prikažu rezultati i iskustvo sprovedene visokodozne hemioterapije sa potporom autolognim matičnim ćelijama hematopoeze kod bolesnika sa malignim hemopatijama na Klinici za hematologiju Kliničkog centra Vojvodine. **Materijal i metode.** Ispitivanjem su obuhvaćeni bolesnici sa hematološkim bolestima (multipli mijelom, akutne mijeloidne i limfoblastne leukemije, nehočkinski i hočkinski limfomi) kod kojih je sprovedena autologna transplantacija matičnih ćelija hematopoeze iz periferne krvi. **Rezultati.** Istraživanjem je obuhvaćeno 90 ispitanika kod kojih je u periodu od 2004. do avgusta 2017. godine sprovedena autologna transplantacija matičnih ćelija hematopoeze. U odnosu na osnovnu bolest, distribucija ispitanika bila je sledeća: 39 ispitanika sa multiplim mijelomom, 25 ispitanika sa nehočkinskim limfomima, 20 sa hočkinskim limfomom i šest ispitanika sa akutnim leukemijama. Kod 75 ispitanika (89,3%) urađena je aferezna procedura velikog volumena, a kod devet ispitanika (10,7%) konvecionalna dvodnevna aferezna procedura. Prosečni broj u afereznom produktu iznosio je $7,8 \times 10^8$/kg tm, a $CD34^+$ ćelija je iznosio $12,11 \times 10^6$/kg tm. Nakon primenjenog kondicionog režima u zavisnosti od osnovne bolesti, engraftment neutrofilnih granulocita je zabeležen 11. dana, a trombocita 14. dana. Mortalitet povezan sa transplantacionom procedurom bio je nizak i iznosio je 3,57%. **Zaključak.** Analizom ispitanika sa hematološkim malignitetima i sprovedenom autolognom transplantacijom matičnih ćelija hematopoeze, možemo zaključiti da je pomenuta procedura uspešna metoda lečenja bolesnika sa malignim hemopatijama, sa niskim transplantacionim mortalitetom i komplikacijama nastalim zbog pomenute procedure.

Ključne reči: autologna transplantacija; transplantacija hematopoetskih stem ćelija; hematološki maligniteti; preživljavanje; bolest kalema protiv domaćina; ishod lečenja

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Abbreviations

ASCT – autologous stem cell transplantation
HGF – hematopoietic growth factor
rHGF-CSF – recombinant human growth factor of granulocytopoiesis
GM-CSF – granulocytic-monocyte growth factor
G-CSF – granulocytic growth factor
PB – peripheral blood
MM – multiple myeloma
HL – Hodgkin lymphoma
NHL – Non-Hodgkin lymphoma
AML – acute myeloid leukemia
ALL – acute lymphoblastic leukemia
DMSO – 10% dimethyl sulfoxide
7-AAD – 7- Aminoactinomycin - D
BW – body weight
BEAM – Carmustine, Etoposide, Cytarabine, Melphalan
DHAP – Dexamethason, High dose Cytarabine, Platinum

Introduction

Autologous stem cell transplantation (ASCT) is considered one of the best therapeutic methods for treating many hematological malignancies, and the number of ASCTs has increased significantly in recent decades [1, 2]. Contemporary research has made an enormous progress in understanding the hematopoietic stem cells (HSCs) biology and their great therapeutic potential, which has led to the development of new therapeutic approaches, primarily in regenerative medicine and cellular therapy. HSCs are defined as the cell population that has the ability to self-regenerate by mitosis and to differentiate into specialized blood cells [3]. From the early 1980s to the mid-1990s, the only source of HSCs was the bone marrow. Based on the results obtained by experimental research, it was confirmed that the number of HSCs is significantly higher in the stage of bone marrow recovery after applying a high-dose chemotherapy regimen, also known as the mobilization chemotherapy protocol. By introducing the hematopoietic growth factor (HGF) – usually the recombinant human growth factor of granulocytopoiesis rHGF-CSF and the granulocytic-monocyte growth factor GM-CSF – peripheral blood (PB) became the dominant source of the HSCs [4]. The ASCT process consists of: mobilization, collection, cryopreservation, and the application of a high-dose chemotherapy regimen and/or radiotherapy with the support of prior collected HDCs. The basic precondition of a successful ASCT is chemo/radiosensitivity of the underlying disease, satisfactory mobilization and apheresis of the HSCs (optimum number and quality). Chemotherapeutic mobilization protocols generally depend on the underlying disease, but mostly consist of cyclophosphamide alone or in combination with Vepeside, with the addition of HGF [4]. Apheresis of the HSCs is performed using a special apparatus, a blood cell separator. The quality of the apheresis product depends on a number of parameters: age and sex of patient, nature and stage of the underlying disease, degree of the hematopoietic tissue damage, mobilization protocol, apheresis procedure and the number of CD34 + cells before the start of apheresis [5]. The collected HSCs are re-suspended in special substances, cryoprotectants,

Table 1. The patients' characteristics in relation to the underlying disease
Tabela 1. *Karakteristike ispitanika u odnosu na osnovnu bolest*

Disease *Bolest*	Number of patients n (%) *Broj pacijenata n (%)*
Multiple myeloma/*Multipli mijelom*	39 (43,3%)
IgG kappa	22
IgG lambda	5
IgA kappa	1
Bence Jones kappa	7
Bence Jones lambda	4
Non Hodgkin lymphoma/*(Nehočkinski limfom)*	25 (27,8%)
Diffuse large B cell (DLBCL) CD20+/*Difuzni krupnoćelijski B-limfom CD20+*	8
Diffuse large B cell (DLBCL) CD20-/*Difuzni krupnoćelijski B-limfom CD20-*	1
Primary mediastinal B cell lymphoma/*Primarni medijastinalni B-limfom*	2
Burkitt lymphoma/*Burkitov limfom*	1
B cell unclasified/*B-ćelijski neklasifikovani*	1
Peripheral T cell lymphoma/*Periferni T-ćelijski limfom*	1
Anaplastic large cell lymphoma/*Anaplastični limfom velikih ćelija*	2
T cell unclassified/*T-ćelijski neklasifikovani*	1
Follicular lymphoma/*Folikularni limfom*	5
Mantle cell/*"Mantle" ćelijski limfom*	3
Hodgkin lymphoma/*Hočkinski limfom*	20 (22,2%)
Nodular sclerosis type/*Tip nodulske skleroze*	16
Mixed cellularity type/*Tip mešane celularnosti*	4
Acute myeloid leukemia/*Akutna mijeloidna leukemija*	5 (5,6%)
Acute lymphoid leukemia/*Akutna limfoblastna leukemija*	1 (1,1%)

Table 2. The patients' characteristics and transplant disease condition before ASCT
Tabela 2. *Karakteristike pacijenata i stanje osnovne bolesti pre ATMĆH*

Characteristics/*Karakteristike*	Patients number n (%)/*Broj bolesnika n (%)*
Age/*Starost*	51 (20-65)
Sex M/F/*Pol M/Ž*	47/43
CS for MM/*KS za MM*	
II	9 (23.1%)
III	30 (76.9%)
CS for HL and NHL/*KS za HL i NHL*	
II	4 (8.9%)
III	11 (24.4%)
IV	30 (25.6%)
Patients status before AHSCT (MM)/*Stanje bolesti pre ASCT (MM)*	
CR + VGPR	13 (33.3%)
PR	16 (41.1%)
RD	10 (25.6%)
Patients status before AHSCT (HL+NHL)/*Stanje bolesti pre ASCT (HL+NHL)*	
CR	23 (51.1%)
PR	15 (33.3%)
RD	7 (15.6%)

Legend: CS – clinical stage, MM – multiple myeloma, HL and NHL– Hodgkin lymphoma and Non-Hodgkin lymphoma, VGPR – very good partial response, PR – partial remission, RD – resistant disease
Legenda: CS – klinički stadijum, MM – multipli mijelom, HL i NHL – Hočkinov i nehočkinski limfomi, VGPR – vrlo dobar parcijalni odgovor, PR – parcijalna remisija, RD – rezistentna bolest

which preserve their vitality during the freezing process, after which they are lowered to a certain temperature (from -80 to -196°C), depending on the required length of storage. In the second phase, a high-dose chemotherapy regimen and/or radiotherapy, i.e. the conditioning regimen is applied, which is adapted to the nature of the underlying disease, followed by the reinfusion of the quickly thawed suspension of the previously frozen HSCs.

The aim of this paper was to show the results of treatment of patients with hematological malignancies (multiple myeloma, non-Hodgkin's and Hodgkin's lymphoma and acute leukemia) with ASCT at the Clinic for hematology, Clinical Center of Vojvodina.

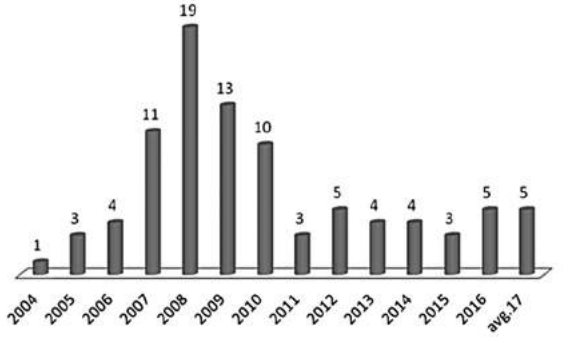
Material and Methods

The research involved 90 patients treated with ASCT at the Clinic for Hematology, Clinical Center of Vojvodina, in the period from 2004 to August 2017.

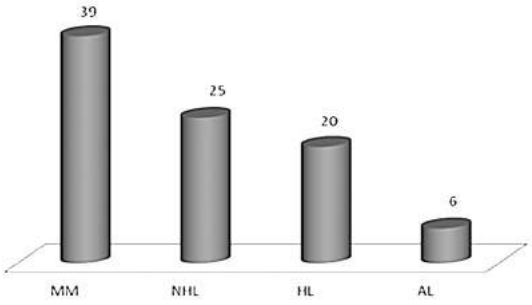
Prior to the transplantation procedure itself, the appropriate mobilization protocol was applied, depending on the underlying disease, after which the HGF was applied at a dose of 5-16 μg/kg body-weight (BW). When the leukocyte count reached planned number, an apheresis procedure of HSCs was performed on the blood cell separator (Gambro - BCT Spectra, version 7.0 and Spectra Optia from 2016). The collected HSCs were suspended in 10% dimethyl sulfoxide (DMSO) as a cryoprotectant, and stored at a temperature of -140 ± 5 °C. A conditioning regimen was applied accordingly (depending on the underlying disease), after which the HSCs were returned. The rate of hematopoiesis recovery after the return of HSCs was estimated based on the detected engraftment of neutrophils and thrombocytes (when the neutrophils count was over $0.5x10^9$/l and platelets count over $20x10^9$/l).

Results

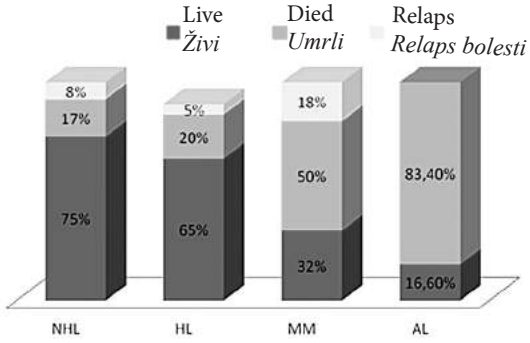
In the period from 2004 to August 2017, the total number of autologous stem cells transplanta-



Graph 1. The number of ASCT performed annually from 2004 to August 2017
Grafikon 1. *Broj autologih transplantacija u periodu od 2004. do avgusta 2017. godine*



Graph 2. Distribution of the patients who underwent AHSCT according to the underlying disease
***Grafikon 2**. Distribucija bolesnika u odnosu na osnovno oboljenje*



Graph 3. The outcome of ASCT in relation to the underlying disease
***Grafikon 3**. Ishod ASCT u odnosu na osnovnu bolest*

tions, obtained from peripheral blood was 90 **(Graph 1)**.

In relation to the underlying disease, the distribution of the respondents was as follows: 39 patients had multiple myeloma (MM), 25 had non-Hodgkin's lymphoma (NHL), 20 had Hodgkin's lymphoma (HL) and 6 patients had acute leukemia (AL) **(Graph 2)**.

The characteristics of the patients in relation to the underlying disease are shown in **Tables 1 and 2**.

The most commonly used mobilization protocol in patients with MM was cyclophosphamide (Cy) (22.73%) and Cy in combination with vepeside (9.1%), in NHL and HL patients DHAP protocol (21.21%), ICE and dexa-BEAM (6.2%) were used, while the most commonly used mobilization protocol in patients with AL was a high dose of cytosine arabinoside (12.12%).

After the mobilization protocols were applied, HGF was administered and after an adequate increase in leukocyte count was reached, a large-volume apheresis procedure was performed in most cases, while in others a conventional two-day apheresis procedure was performed.

In 75 patients (89.3%) a large-volume apheresis procedure was performed, while in 9 patients (10.7%) a conventional two-day apheresis procedure was performed. The mean volume of processed blood was 14922.96 ml (range 10500-18600 ml), while in the two-day conventional apheresis, an average of 25425 ml (range 19800-35840ml) was processed. The time from the apheresis procedure to ASCT ranged from 15 to 282 days (in average 68 days). The detected number of mononuclear cells (MNC) in the apheresis product ranged from $3x10^8$ to $14x10^8$/kg BW (in average $7.8x10^8$/kg BW). The average number of CD34 + cells was $12,11x10^6$/kg BW. The engraftment of neutrophils was detected in average on the 11th, and platelets engraftment on the 14th post-transplant day.

Stem cells viability was tested by the standard trypan blue method and flow cytometry with the addition of 7- Amynoactinomycin D (7AAD) in 66 patients. A statistically significant difference between the two methods was found when comparing the number of viable cells in the apheresis product pre and post cryoconservation. The standard trypan blue method detected 95.78% viable cells in the apheresis product prior to cryoconservation, and 82.58% after thawing. The flow cytometry with the addition of 7AAD prior to cryoconservation detected a 97.1% viable cells prior to cryopreservation, and 95.42% after thawing.

The type of the conditioning regimen used was based on recommendations in relation to the underlying disease. In patients with MM, melphalan was administered at a dosage of 200 or 140 mg/m^2. In 9 of them a tandem AHSCT was performed, which means that these patients underwent 2 ASCTs in less than 6 months. In patients with HL and NHL the BEAM protocol was administered, while in subjects with AL the Busulfan/Cyclophosphamide (Bu/Cy) regimen was applied. Transplant-related mortality rate was low, as 3 subjects (3.57%) had died in the phase of bone marrow aplasia due to infection.

After the ASCT, 23% of the patients had no complications whatsoever, 40% had a one or more febrile episodes, 17% developed oral mucositis, and 10% had gastrointestinal disorders. Complications such as the engraftment syndrome (7%) and ecthyma gangrenosum (3%) presented in a small number of patients.

Discussion

High-dose chemotherapy regimens with the support of ASCT is the standard of treatment for numerous hematological malignancies and solid tumors [1]. The results of multiple studies point to the high efficacy of this type of treatment in patients with aggressive lymphomas, multiple myeloma, and acute leukemia [6–8]. In most transplant centers, ASCT with SC obtained from the bone marrow was replaced by ASCT with SC obtained from peripheral blood, which requires the application of various mobilization protocols and apheresis procedures. A number of factors, such as age of the patient, previous exposure to chemotherapy and/or radiotherapy and the degree of bone marrow infiltration by the

underlying disease, affect the adequate mobilization of the peripheral blood SCs. Independently of the mentioned factors, the application of different types of collecting procedures, the apheresis time, the type of blood cells separator used, and the volume of the processed blood are other factors that can affect the efficiency of the collection procedure [9].

The most commonly used mobilization protocol was cyclophosphamide (Cy) with the addition of granulocytes growth factor (G-CSF). The dose of cyclophosphamide applied varies in numerous reports and ranges from 1-7 g/m^2. The results of one study indicated that the application of a high dose Cy (7 g/m^2) regimen with G-CSF results in a higher yield of CD34 + cells than the intermediate doses of Cy (3-4 g/m^2) with G-CSF, while the results of other studies have shown that there is no difference between the high and intermediate doses of Cy. In addition, some transplant centers have shown similar efficacy in SC mobilization with the use of low-dose Cy (1-2 g/m^2) in patients with MM. High dose Cy results in a greater incidence of adverse effects, including hemorrhagic cystitis, renal failure, febrile neutropenia and bleeding [10]. In patients with relapsed lymphomas, it was found that chemotherapy protocols with platinum derivatives provided better results and a more favorable mobilization outcome when compared to regimens involving the use of cyclophosphamide as monotherapy [11]. In our study, the majority of patients with MM received cyclophosphamide (Cy) or Cy in combination with vepeside (VP-16) as a mobilization protocol. As for the patients with HL and NHL, they mainly received DHAP + G-CSF as a mobilization protocol. However, an optimal mobilization regimen, with an acceptable toxicity and efficacy has yet to be defined.

The start of G-CSF application after the mobilization protocol had been applied, depends on the transplant center's experience, as there are no recommendations on the most effective term for starting G-CSF administration. Lefrere and associates, on a group of 65 patients, applied G-CSF from +1 or +2 days in some of them and from the +7 or +8 day in the rest, after chemotherapy, but it was found that there was no significant difference between the two groups of patients when comparing the number of conducted apheresis procedures, medians number of cells collected, the recovery of leukocytes and platelets, or the risk of infection [12]. Other researchers applied G-CSF from +4, +5 even +14 days after chemotherapy, but none of them showed a significant difference in the number of CD34 + cells obtained. In subjects where G-CSF was started on the +14 day, there was an increased risk of complications due to prolonged myelosuppression. In our research, in all the respondents, G-CSF was applied between the +7 and +8 day, having applied the mobilization protocol. The recommended dose of G-CSF in ASCT, according to the American Society for the Transplantation of Core and Blood (ASBMT), is 10 μg/day, with variations depending on the intensity of the chemotherapy given and the underlying disease [9].

Predicting the most adequate time for implementing of the apheresis procedure depends on a number of factors, which include the type of the mobilization protocol and the dose and the time of G-CSF application. In some transplant centers, the collection of HSCs begins when the number of leukocytes is 1-3x10^9/l or more than 5x10^9/l, and after recovery from the bone marrow aplasia, while other centers monitor the number of CD34 + cells in the circulation and by that determine the time for the apheresis procedure [9]. Obtaining an adequate number of CD34 + cells by apheresis can be predicted by determining the number of these cells immediately before the procedure, i.e. in the blood circulation, which should be at least 20/μl of blood [9]. The efficacy of HSCs apheresis from peripheral blood can be estimated by quantifying the number of MNCs and the number of CD34 + cells. For a successful and rapid recovery of hematopoiesis after ASCT, it is necessary that the MNCs number is at least 2-5x10^8/kg BW and the number of CD34 + cells is greater than 2x10^6/kg BW, while even better results are achieved if the CD34 + cells number is greater than 5x10^6/kg BW. In our study, the MNCs number averaged 7.8x10^8/kg BW and the number of CD34 + cells was 12.11x10^6/kg BW in average.

The volume of the processed blood is affected by the level of blood flow and the patient's ability to withstand the procedure. More and more centers apply the large volume apheresis (LVA), which processes three or more blood volumes of the organism in order to obtain the desired number of MNCs and CD34 + cells [13]. No significant decrease in the number of CD34 + cells in peripheral blood during LVA was detected, while the number of cells collected was proportional to the volume of processed blood.

In our study, 81 patients (89.3%) underwent the LVA procedure, while 9 patients (10.7%) had the conventional two-day apheresis procedure. Until 2016, the collection of HSCs from peripheral blood was carried out on the Cobe Spectra® (version 7.0). From 2016 the procedure is done on the Spectra Optia® separator. According to published data, there is no difference in the efficiency of collecting mononuclear cells between these two separators [14, 15]. The mean volume of processed blood in the LVA group was 14922.96 ml (range 10500-18600 ml), while in the two-day conventional apheresis group 25425 ml of blood was processed on average (range 19800-35840 ml). The time passed from apheresis to ASCT averaged 68 days (range 15-282 days).

The results of transplant centers with extensive experience in the implementation of the conventional apheresis procedure indicate that the desired number of CD34 + cells is reached in 75% of patients and that there is no need for prolonged apheresis longer than two days, as it was confirmed by our research as well. However, in a certain percentage of patients (10-20%), the first mobilization does not provide an optimal number of CD34 + cells, which requires another apheresis procedure. Ac-

cording to studies conducted on NHL patients, it has been proven that it is more effective to do a second apheresis shortly after the first one, because prolonged waiting leads to a poor mobilization outcome. This approach to apheresis involves: a lower risk of infection, a lower risk of disease progression, and a faster bone marrow recovery after the initial mobilization attempt [16].

For the successful engraftment of neutrophils and thrombocytes, aside from the sufficient number of CD 34+ cells, enough viable precursor cells need to be transfused. It is well known that adding a cryoprotectant such as DMSO alters the biological characteristics of cells leading to their death via apoptosis.

In our study, aside from monitoring the viability of cells by the trypan blue method and flow cytometry with the addition of 7-Aminoactinomycin-D (7AAD), we also determined the number of the necrotic/dead cells. Using the flow cytometry method with the addition of 7AAD, the percentage of viable cells pre and post-cryopreservation was 97.10% and 95.42%, which was significantly higher than the percentage of viable cells detected by the trypan blue method, as it detected 82.58% viable cells post-cryopreservation [3].

The complications associated with ASCT include infections, especially in the period of bone marrow aplasia, as well as complications related to the applied conditioning regimen. Transplant-related mortality rate in our study was low, and was estimated at 3.75%. This low mortality rate can be explained by the adequate selection and pre-transplant treatment of patients undergoing ASCT, and the thorough monitoring of patients during the whole procedure.

In our study, the most common complications of ASCT were: fever, gastrointestinal complications and oral mucositis, which matches the results of other published studies while ecthyma gangrenosum was significantly less common. Although the engraftment syndrome is a pretty common complication in ASCT, only one patient suffered from it in our study.

Conclusion

Autologous stem cell transplantation is an efficient method of treatment for patients with hematological malignancies, with a low rate of transplant-related mortality, as well as low rate of complications related to the procedure itself. It is essential to carefully and thoroughly select the patients who could potentially benefit from autologous stem cell transplantation, bearing in mind the recommendations regarding the stage of the underlying disease.

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OPTIMIZATION OF TIMING FOR HEMATOPOIETIC STEM CELL TRANSPLANTATIONS IN PATIENTS WITH MYELOID LEUKEMIA

OPTIMIZACIJA VREMENA ZA TRANSPLANTACIJU HEMATOPOEZNIH MATIČNIH ĆELIJA KOD PACIJENATA SA MIJELOIDNIM LEUKEMIJAMA

Stevan L. POPOVIĆ

Summary
Hematopoietic stem cells transplantations, allogeneic and autologous are forms of treating acute myeloid leukemia, chronic myeloid leukemia and other forms of malignant hematological diseases. Autologous stem cells trransplantations is the most intense form of chemotherapy with myeloablative doses of cytostatic agents and with the aim of destroying "each and every" leukemic cell in the patient's body. In order for patients to survive myeloablation, infusion of their own stem cells which renew their own hematopoiesis is carried out. Thanks to myeloablative and submyeloablative conditioning regimen and the biological graft-versus-leukemia effect, allogeneic has the best anti-leukemic effect and is considered the only form of therapy which can lead to curing patients with malignant hemopathies. However, the same or similar mechanisms, which are basically graft-versus-leukemia effect, are responsible for the similar reaction of the graft versus host and cause serious deadly illnesses, which is why allo-hematopoietic stem cells transplantations is a risky form of treatment in terms of ethics. Lower mortality of patients makes auto-SCT safer ethical-wise, but its anti-leukemic effect is weaker.
Key words: Transplantation, Autologous; Hematopoietic Stem Cell Transplantation; Precision Medicine; Leukemia, Myeloid; Antineoplastic Agents; Myeloablative Agonists

Sažetak
Transplantacija hematopoeznih matičnih ćelija, kako alogenih tako i autologih, oblici su terapije akutne mijeloidne leukemije, hroničnе mijeloidne leukemije i drugih malignih hematoloških bolesti. Alogena transplantacija matičnih ćelija hematopoeze je najintenzivniji oblik hemioterapije sa mijeloablativnim dozama citostatika i ciljem da se uništi „poslednja" leukemijska ćelija u organizmu bolesnika. Uslov da bolesnik preživi mijeloablaciju je infuzija sopstvenih matičnih ćelija koje obnavljaju bolesnikovu hematopoezu. Zahvaljujući mijeloablativnim ili submijeloablativnim kondicionim režimima i biološkom efektu kalema protiv leukemije, alogenih hematopoeznih matičnih ćelija ima najbolji antileukemijski učinak i važi za jedini oblik terapije koji može dovesti do izlečenja bolesnika sa malignim hemopatijama. Međutim, isti ili slični mehanizmi koji su u osnovi graft verzus leukemija odgovorni su za reakciju kalema protiv domaćina i uzrok su visoke smrtnosti bolesnika, zbog čega je alogena transplantacija matičnih ćelija hematopoeze etički rizičan oblik lečenja. Manja smrtnost bolesnika čini autogena transplantacija matičnih ćelija hematopoeze etički bezbednijom, ali je i njen antileukemijski učinak slabiji.
Ključne reči: autologna transplantacija; transplantacija hematopoetskih stem ćelija; personalizovana medicina; mijeloidna leukemija; antineoplastički agensi; mijeloablativni agonisti

Introduction

Hematopoietic stem cells transplantations (HCT), allogeneic (allo-SCT) and autologous (auto-SCT) are forms of treating acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and other forms of malignant hematological diseases. Auto-SCT is the most intense form of chemotherapy with myeloablative doses of cytostatic agents and with the aim of destroying "each and every" leukemic cell in the patient's body. In order for patients to survive myeloablation, infusion of their own stem cells which renew their own hematopoiesis is carried out. Thanks to myeloablative and submyeloablative conditioning regimen and the biological graft-versus-leukemia effect (GvL), allo-SCT has the best anti-leukemic effect and is considered the only form of therapy which can lead to curing patients with malignant hemopathies. However, the same or similar mechanisms, which are basically GvL, are responsible for the similar reaction of the graft versus host (GvHD) and cause serious deadly illnesses, which is why allo-SCT is a risky form of treatment in terms of ethics [1–3]. Lower mortality of patients makes auto-SCT safer ethical-wise, but its anti-leukemic effect is weaker [3].

The positioning of SCT in treating AML, CML and other malignant hemopathies depends on the results and development, transplant, pace of progress and toxic profile of competitive forms of treatment, quality of clinical studies in which these forms of treatment were compared, and indications for selective application of SCT in optimal time. During the last decades of the previous century, fascination with the anti-leukemic effect of SCT and disappointment with the moderate effects of the competitive forms

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Abbreviations

SCT	– stem cell transplantation
AML	– acute myeloid leukemia
ALL	– acute lymphoblastic leukemia
CML	– chronic myeloid leukemia
GvL	– graft versus leukemia
GvHD	– graft versus host disease
EBMT	– European Society for Blood and Marrow transplantation
RIC	– reduced- intensity conditioning
HLA	– human leucocyte antigen
EFS	– event free survival
ELN	– European leukemia net
FLT3-ITD	– FMS-like tyrosine kinase 3- internal tandem duplication
NPM1	– nucleophosmine 1
CEBPA	– CCAAT/enhancer-binding protein alpha
MRD	– minimal residual disease

of therapy led to SCT being applied non-selectively even in early stages of AML and CML in all patients who met two, essentially technical, conditions, that they are young enough and that they have an HLA-identical related donor, for allo-SCT. The ethical risk of non-selective application of allo-SCT measured by the ratio of the curative/mortality rates was very high and accompanied by the risk of transplanting patients cured by pre-transplant therapy [4,5]. This kind of attitude toward SCT represents absolute confidence in the treatment procedure, probably, unprecedented in medicine [6]. Raising awareness about the ethical risk of applying SCT, better understanding of the pathogenesis of the illness and development of competitive forms of treatment have resulted in the selective application of SCT in optimal time. Selective SCT means it is applied only with those patients and in such stages of the disease when the risk is at its lowest, and the effect of competitive forms of therapy inferior, while the main aim of optimizing timing is to avoid the ethical risk of transplanting potentially healthy persons cured with the prior anti-leukemic therapy. Today, at a time when minimal residual disease is measured, optimization of timing for SCT is a prevailing problem, and standpoints and recommendations for selective application of transplant are dynamically changing.

This summarizing article presents our results of mathematical processing of clinical data with the aim of optimizing the timing of SCT in AML and CML and our decades-long effort invested in selective application of SCT and individualization of anti-leukemic treatment [7,8,9], as well as a comparison of our previous ideas and calculations with contemporary therapeutic standpoints.

Advancement of transplantation in CML and AML

Over the last few decades, major breakthroughs have been made in the development of allo-SCT, while auto-SCT has gradually been outplaced from treating CML and AML. Introduction of reduced intensity conditioning regimens (RIC), better HLA-matching and choice of donor, and more efficient control of GvHD have expanded the indications for application of transplant in older patients and patients without an HLA-identical related donor, and with better quality supportive care, patient mortality has decreased [10].

According to data of EBMT group [11], per 2628 patients two-year survival, mortality and relapse frequency after allo-SCT from HLA-identical related donors in the first chronic stage of CML were 54%, 37% and 11% in the period 1980-1990, 70%, 25% and 12% in the period from 1991-1999 and 74%, 22%, 18% in the period from 2000-2003. Thanks to reduced transplant-related mortality, chances for curing patients have been increased, while the anti-leukemic effect, measured by risk of relapse has been stagnating. Ethical risk related to transplants in the analysed periods, inversely proportional to the cure/mortality ratio [3], has gone down from 1.46 to 3.36. Two-year survival and mortality of patients after allo-SCT from an unrelated donor in the specified periods are 38% and 10%, 56% and 12%, 63% and 19% with a considerable reduction of ethical risk and anti-leukemic effect similar to transplant effect from a related donor. Results of SCT from a haploidentical donor, RICT, SC transplant and transplant of cells from umbilical blood have also been continuously improving. According to the release of the German CML Group and SAKK [12], three-year survival of patients after allo-SCT from an HLA-identical related donor is about 90% in the first chronic stage of CML and 56% in advanced stages of the disease with transplant-related mortality of 8%. Results of developing allo-SCT in CML are significantly lower treatment-related mortality of patients, better chances for curing and lower ethical risk attached to its application.

Trends in developing allo-SCT in AML are similar. According to the analysis of clinical studies, chances for curing patients after allo-SCT in first remission, relapse and secondary remission of AML are 48%, 15% and 26% with risk of transplant-related death of 31%, 28% and 26% and a high ethical risk of 1.5, 0.9 and 1.0 [3]. Clinical studies, from the start of this century, have shown significantly better results of SCT and lower ethical risk. Event free survival (EFS) and early mortality rate (ERM) of patients after allo-SCT in first remission of AML are 55-65% and 7-10%, in secondary remission 30-40% and 10-20%, and in resistant disease 15-20% and 30-40% [13]. Ethical risk related to applying allo-SCT is 3-4 times lower in first and nearly two times lower in second remission of AML. Improvement of results of allo-SCT in childhood AML is impressive, EFS grew from 22.9% in the period from 1980-1990 to 62.4% after 2002 [14].

Allogeneic SCT and competitive forms of treatment

Position of SCT in treating malignant hematological diseases depends, primarily, from the poten-

tial and toxicity of competitive forms of treatment. Reduction of ethical risk with the preservation of the anti-tumor effect enabled allo-SCT to resist new molecule drugs and save its place in treating most hematological diseases. The only exception are tyrosine-kinase inhibitors which have pushed allo-SCT out of the forefront when it comes to chronic stage of CML with the remark of the authors that such a standpoint requires new and better quality clinical evidence [15]. Early analysis of the results of the IRIS study [16] showed that imatinib mesylate has a better effect than interpheron, that it changes the course of CML and gives a real chance for operative treatment [17] with acceptable toxicity. Analysis of treatment results in 1569 patients with CML treated since 1965 has shown an increase of eight-year survival in patients in the first chronic stage with less than 15% in the period up to 1983, to 42-65% in the period 1983-2000 and to 87% in the era of imatinib, after 2001 [18]. Nilotinib, dasatinib and ponatinib have proved efficient in CML resistant to imatinib mesylate. Tyorosine-kinase inhibitors have drastically changed the position of allo-SCT in treating CML despite its improved results. In the era of imatinib, CML was the most frequent indication for allo-SCT, while now its share is down to 2%. Absolute trust in allo-SCT in CML has been replaced by almost absolute trust in treatment with tyrosine-kinase inhibitors.

Development of competitive forms of treatment in AML was less dynamic. Results of induction treatment have slightly improved, mostly thanks to the development of supportive care which reduced the patient mortality [19]. Intensifying of induction therapy [20] and consolidation of remission with high-doses of cytosine-arabinoside improved the prognosis only in certain patient categories [21]. Insufficient development of chemotherapy and transition to selective and early application of allo-SCT, which improved its results, made AML the most frequent indication for allo-SCT.

Analysis of clinical studies

Medicine based on evidence assumes the existence of quality clinical studies which justify the position and ranking of some forms of treatment. What are the clinical evidence on which the place of allo-SCT in treating CML and AML rested in the past and on which it rests today?

Busulfan and hydroxyurea had moderate effects in the chronic stage of CML, very poor effects in the advanced stages of the disease and they did not offer chance of curation. Interferon alfa extended the survival of patients in the chronic stage of CML, but did not improve the prognosis in advanced stages of the disease [22], and the fact that this drug could cause molecule remission in a smaller number of patients was not accompanied by clinical evidence that it can lead to curing the patient. The advantage of allo-SCT over chemotherapy and interferon has not been proven in any prospective clinical study or meta-analysis. However, convincing results of transplants are sufficient for the leading authorities to conclude that allo-SCT is the treatment of choice in CML and that it should be applied within the first year following diagnosis [23, 24]. Retrospective comparison of data from the International Bone Marrow Transplant Registry for 548 patients with CML under 55 years of age who underwent allo-SCT from HLA-identical donors in the period from 1983-1991against treatment results for 196 patients of the German CML Study Group treated with hydroxyurea and interferon revealed inconsistencies: transplant patients were six years younger, they had lower percentage of blasts and were more often without splenomegaly [25]. Though seven-year survival is significantly higher after allo-SCT than in the group of non-transplant patients (58% vs 32%), in the first four years following diagnosis, non-transplant patients survive longer, and after 5.5 years, transplant patients survive longer [25]. Results of the IRIS study were sufficient to confirm the undoubted advantage of imatinib mesylate over interferon alfa [16] and sufficient to oust allo-SCT from the first treatment line [26] with the occurrence of new tyrosine-kinase inhibitors. Comparison of allo-SCT and drug therapy was done in only three prospective studies: German CML Group and SAKK who had different conclusions [27, 28, 29]. In the first study [27], allo-SCT from HLA-identical related donor and best drug treatment (43% of pts treated with imatinib) were compared over a period from 1995 to 2001. Drug treatment induced longer survival of patients than allo-SCT in the first eight years from diagnosis, the difference was the biggest in the first three years and maintained the longest in the low-risk group according to Euro Score. Complete cytogenetic remission and large molecule response was achieved in 91% and 81% of transplant pts and 48% and 45% of pts treated with competitive therapy. Conclusion of the study is that contemporary medicament care should replace allo-SCT in the first treatment line [27]. Conclusion of another study [28], in which allo-SCT (all pts were administered imatinib prior to transplant) was compared to treatment with imatinib, is that both treatments achieve equal survival in patients and that allo-SCT should be applied only in pts resistant to imatinib. Two years after allo-SCT, 79% of pts is in complete molecule remission. The last study [12] compared allo-SCT and drug therapy (84% treated with tyrosine-kinase inhibitors) and showed that the effect of therapy depends on the combination of prognostic risks according to the EBMT score in the transplant group and according to Euro score in non-transplant pts. In a Mexican study, the choice between allo-SCT and treatment with imatinib depended on non-medical, financial reasons [29]. Six-year survival is equal after allo-SCT and imatinib treatment (77% vs 84%) with equal risk of acceleration and blast transformation.

The position of auto-SCT in treating myeloid leukemia is not clearly defined. Meta-analysis of

studies with comparison of auto-SCT and interferon treatment in the chronic stage of CML did not show any difference in survival, but authors leave the possibility of applying auto-SCT in patients resistant to drug treatment [30]. Prospective comparisons of auto-SCT with imatinib mesylate or allo-SCT in the chronic stage of CML have not been made.

Conclusion from reviewing the above named studies could be that the attitude of absolute trust toward allo-SCT in the era of imatinib and conversion of positions into almost absolute trust toward tyrosine-kinase inhibitors were not founded on clear and solid clinical evidence.

Comparison of allo-SCT from HLA-identical related donor and consolidation chemotherapy in the first remission of AML was done in a larger number of prospective clinical studies and a few meta-analyses [31, 32]. Our analysis of older studies with non-selective application of allo-SCT in first remission of AML showed that they are encumbered with statistical and other irregularities [3]. Patients envisaged for allo-SCT who died or went through early relapse during consolidation chemotherapy were included in the chemotherapy group, which improved results of allo-SCT and disrupted results of the competitive treatment. Though the upper age limit was equally set for both groups of subjects, transplant pts were, on average, five years younger than pts treated with chemotherapy which points to "preselectivity" of the allo-SCT group. Application of allo-SCT after consolidation chemotherapy bears the risk of transplanting in patients in whom leukemia may have been eradicated and who are potentially healthy. Result of irregularity in studies of allo-SCT versus chemotherapy is such that over 35% of pts have an HLA-identical donor which does not correspond to the demographic reality. In more recent studies, donor and no-donor groups were compared in first remission of AML regardless of whether the donor group underwent allo-SCT or not. Analysis of data of three HOVON/SAKK studies, designed in this way, revealed "preselectivity" of the group of transplant patients because DFS (disease free survival) in no-donor group (37%) is significantly higher than in non-transplant pts in the donor group (22%).

Controlled clinical studies and meta-analysis defined the place of selective application of allo-SCT in the first remission of AML: it is not recommended to apply it in the cytogenetic group of good risk, and in high-risk group transplant of HLA-identical related donor and unrelated donor is recommended, while the place of allo-SCT in consolidation of first remission in the group of intermediate risk is not uniquely defined [33–35]. Auto-SCT is a competitive form of treatment for both consolidation chemotherapy and allo-SCT in first remission of AML. Meta-analyses of clinical studies have not proven the advantage of auto-SCT over consolidation chemotherapy or over allo-SCT in first remission of AML [36–38]. Current indication for applying auto-SCT is second remission of acute promyelocitic leukemia where it gives better results than allo-SCT [39]. Data on high frequency of secondary primary neoplasm in late post-transplant period contributed to the replacement of auto-SCT in AML treatment [40, 41], which has not been confirmed in other studies which showed that the life expectancy of transplant patients is no different than the life expectancy of the general population [40, 42]. Maybe it is time to expand indication for applying auto-SCT in first remission of AML in some categories of pts who do not have an adequate donor for allo-SCT [39, 43].

Optimal timing of transplantation in CML

SCT from HLA-identical related donor was, in the era prior to imatinib, the treatment of choice in the first chronic stage of CML with the lowest mortality rate and the best results in the first year following diagnosis [25]. At the time of absolute trust in allo-SCT, the biggest issue was to make waiting lists for transplantation according to medical criteria. According to our cumulative risk models [44–46], allo-SCT from HLA-identical related donor should be applied no later than the second, third and fourth year following diagnosis in low-, intermediate- and high-risk groups according to the Sokal score. Through similar modelling of CML treatment results with interferon, we found that in low-risk patients according to the Euro score, allo-SCT from an HLA-identical donor may be delayed until year nine of the disease, and that allo-SCT from an unrelated donor should not be applied [46]. Our models suggest that treatment with interferon in a group of low-risk may replace allo-SCT and perhaps lead to cure. Such possibility has been confirmed by later clinical studies and it is believed that interferon may induce long-term molecule remissions. Analysis of survival curves in transplant CML patients and patients treated with hydroxyurea and interferon revealed that seven-year survival is significantly different (58% vs. 32%) and that allo-SCT is gaining an advantage over the competitive therapy after 5.5. years from diagnosis [25]. In a low-risk group, the difference in seven-year survival is not as high, 58% vs. 49% and allo-SCT takes the lead only after eight years. However, the specified study does not deny the advantage of applying allo-SCT in the first year of the disease and in low-risk patients. An important conclusion of this study is that prognosis of risk groups affect the results of drug treatment of CML, but it does not affect the results of allo-SCT which may be basis for individual optimization of timings for applying transplantation.

Tyrosine-kinase inhibitors (TKI) have been replacing allo-SCT at the chronic stage of CML and absolute trust in allo-SCT has been replaced with absolute trust in contemporary drug treatment. Today, allo-SCT is recommended in patients resistant or intolerant to at least one TKI of the second generation in advanced stages of the disease [26]. The German CML Study IIIA [12] showed that there is no difference in ten-year survival between allo-SCT and the "best drug treatment" (84% pts treated with

imatinib) and that results of therapy depend on the disease risk measured according to EURO score and the transplant risk measured through the modified EBMT score. Patients with EURO score 0-1 have longer survival than patients undergoing drug treatment with high and intermediate risk according to the Euro score, while EMBT score higher than one makes no difference in terms of survival. Such results justify a personalized approach to optimization of timing of allo-SCT in the chronic stage of CML, with the inclusion of data that TKI have excellent results in post-transplant relapse [47]. It seems that adolescents and younger adult patients (AYA) with CML with whom the results of imatinib treatment are relatively poor [48, 49] and transplant risk low are prime candidates for the individual approach and that a prospective study which would define the value and place of allo-SCT in their treatment.

Optimal timing for transplantation in AML

Over the last couple of decades of the last century, at a time of absolute trust, allo-SCT was applied non-selectively in first remission of AML after finishing consolidation chemotherapy, with high risk of applying transplantation in potentially healthy persons in whom leukemia had been eradicated with previous therapy. First mathematical analysis of clinical data [50] showed that cumulative results of allo-SCT applied non-selectively in first remission of AML are identical to the results of non-selective transplants delayed until the second remission. The specified mathematical conclusions implied the possibility that SCT in first remission of AML should be applied selectively, only in patients with whom the competitive forms of therapy do not provide chance of curing patients, and in other patients, it should be delayed for possible relapse or secondary remission. Our calculations [3, 51–53] are also among the pioneer papers. They show that selectively applied allo-SCT in first AML remission significantly increases chances of curing patients (61% vs. 48%, $p<.01$), reduces the mortality risk in patients (22.7% vs 31%, $p<.01$) and reduces the ethical risk of applying transplantation measured by the cure/death ratio by nearly two times. Similar calculations have proven the advantages of selective application of auto-SCT in first remission of AML [3, 52]. Nowadays, selective application of allo-SCT is the official position in treating AML.

The issue with selective application of SCT in first remission is good prognosis of cure chances, i.e. relapse risk. Candidates for the position of best predictor are the cytogenetic changes (chromosome anomalies and gene mutations), minimal residual disease and speed of inducing remission. Results of meta-analysis of clinical studies have provided good basis for selective transplantation in cytogenetic groups with good and bad prognosis, but they have not provided basis for a clear position in the group of intermediate risk, i.e. patients with normal karyotype. According to ELN recommendations [33] in patients with high cytogenetic risk, therapy of choice in first remission is allo-SCT from an HLA-identical related or unrelated donor, but it is not applied in the group with good prognosis, except maybe in patients with present c-KIT mutation or measurable minimal residual disease (MRD) with low risk of transplant-related mortality. Patients with acute promyelocytic leukemia are not candidates for allo-SCT, and auto-SCT is recommended in second remission of the disease. In patients with normal karyotype, genetic mutations may be good basis for deciding on treatment [54, 55]. The best known prognostic significance is the mutation of three genes, FLT3-ITD, NPM1 and double CEBPA mutation and their combinations [56]. FLT3-ITD is the bearer of bad prognosis after chemotherapy, but also allo-SCT and is candidate for transplant in first remission [56]. Presence of minimal residual disease (MRD) is a sign of bad prognosis in any stage of remission of AML and for each form of post-induction therapy [57, 58]. Lack of this prognostic parameter is insufficient precision of measurement, indefinite limit values and still, limited prognostic significance because in about one third of MRD-negative remissions, relapse develops, and in one third of MRD-positive remissions the patient is cured without therapy. Because of this measurement of MRD in remission of AML before allo-SCT, risk of transplanting potentially healthy persons is not eliminated. Contrary to this, presence of MRD in remission of AML is considered a counter indication for applying auto-SCT [35]. Speed of inducing remission has a considerable impact on the effects of all forms of post-induction therapy because it correlates with the depth of remission and MRD, initial WBC count and cytogenetic anomalies [59]. Acute promyelocytic leukemia, remission induced by the first therapy and initial count of leukocytes are predictors of other remissions in our patients treated with standard induction in our programs for individualized therapy ANLL-NS [7, 8, 60] acute promyelocitic leukemia and remission caused by first induction treatment are criteria for giving up on allo-SCT in first remission of AML. Prognostic significance of speed of inducing remission in the decision about applying SCT in first remission of AML has changed the therapeutic regimes which assess the effect of first induction therapy one week after its completion in order to prevent resistance by quickly applying other induction therapies. Comparison of remissions caused in first and second line of treatment in such therapeutic regimes has proven that they are of equal duration and quality [61]. Since such therapeutic protocols are applied in a large number of American and European facilities, with the inclusion of their data in the clinical studies about predictors of SCT outcomes, speed of induction has lost its place in the decision-making about the selective application of transplantation in the first remission of AML. Advantages of therapeutic protocols with the assessment of early effects of the first induction treatment give good basis for preemptive application of allo-SCT immediately after the first unsuccessful induction treatment and this

gives better results from applying allo-SCT as salvage therapy in resistant AML.

According to last ELN recommendations, allo-SCT needs to be done in refractory AML and in first remission in patients with high cytogenetic risk, and auto-SCT in MDR- negative remission. In other patients, the decision on applying SCT needs to be tailor-made based on the dynamic prognosis and assessment of risk of relapse and risk of transplant-related mortality.

The biggest ethical risk which accompanies SCT is the risk of its application in potentially healthy persons in whom leukemia has been eradicated with prior treatment. Such a risk is the highest in non-selective application of SCT after finishing consolidation chemotherapy. Some thirty years ago, the EBMT group [62] published data that LFS, patient mortality and risk of relapse are 23%, 13% and 64% if auto-SCT is applied in the first three months of first remission of AML, and that the same parameters are 48%, 6% and 46% if auto-SCT is applied after six months of remission. The specified results were the basis for recommendations that auto-SCT and allo-SCT should be applied in later stages of remission. If we exclude patients who die or relapse during consolidation chemotherapy which "artificially" improves results of auto-SCT, the assumption that transplant patients include patients in whom leukemia has been eradicated with the previous therapy is realistic. Using a mathematical model of cumulative risks, we calculated that pure anti-leukemic effect of auto-SCT is 23% in all stages of remission, that late transplant "re-cures" 22.6% of patients and that 1.4% patients who die after auto-SCT have been cured with previous treatment. Such risks would be considerably higher today when modern consolidation potentially cures twice as many patients, and especially in late application of allo-SCT. Fortunately, today, allo-SCT is usually applied after first consolidation treatment, rarely after second or immediately after induction therapy considerably decreasing the risk of transplanting potentially healthy persons.

Mathematics is farsighted and accurate. Through mathematical processing of results from renowned clinical studies which we did a quarter of a century ago, we proved that SCT in AML should be applied selectively and in early stages of remission, as well as that AML should be individualized based on the dynamic prognosis, and not protocol type and equally for all patients. Back then this was heresy, today selective early application of SCT is standard clinical practice, and personalized medicine is a very current term and a wide field for further research and progress.

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THE HUMAN LEUKOCYTE ANTIGEN SYSTEM AND TRANSPLANTATION IMMUNOLOGY

SISTEM HUMANIH ANTIGEN LEUKOCITA I TRANSPLANTACIONA IMUNOLOGIJA

Svetlana VOJVODIĆ

Summary

Introduction. The antigens primarily responsible for the rejection of genetically different tissues are known as histocompatibility (i.e. tissue compatibility) antigens and the genes coding for these antigens are referred to as histocompatibility genes. **History.** The groundwork for the new science of transplantation immunology was laid by Medawar in the 1940s who described the rejection of tissue transferred from one person or animal to another, except for grafts between identical twins. In the 1950s, it was shown that this tissue rejection was mediated by major histocompatibility complex group of antigens which cause a strong immune response and are in humans known as human leucocyte antigens. **Polymorphism and inheritance of human leucocyte antigens.** According to the most recent human leucocyte antigens nomenclature, there are currently 17,344 human leucocyte antigens and related alleles, of which 12,544 are I human leucocyte antigens class alleles, 4,622 are II human leucocyte antigens class alleles and 178 other non-human leucocyte antigens alleles. Due to close location at the short arm of the sixth chromosome, the genes of the human leucocyte antigens system are inherited as haplotypes or alleles pairs. **Biological role.** The primary role of the human leucocyte antigens molecule is to present a peptide to the T-cells which recognize both the human leucocyte antigens molecule and the presented peptide, distinguishing the own peptides from the foreign. The ability to allow an immune response which is directed against the "foreign", makes human leucocyte antigens antigens the main immunological barrier in the transplantation. **Conclusion.** The immunobiology of transplantation is important for many reasons: in terms of both its impact on our understanding of immunological processes and its application in the development of clinical transplantation. Advances in immunogenetics and histocompatibility have facilitated the clinical transplantation of solid organs and tissues.

Key words: Transplantation Immunology; HLA Antigens; Histocompatibility Testing; Graft Rejection; Organ Transplantation; Polymorphism, Genetic; T-Lymphocytes

Sažetak

Uvod. Antigeni prvenstveno odgovorni za odbacivanje genetski različitih tkiva poznati su kao histokompatibiliti (npr. kompatibilnost tkiva) antigeni a geni koji kodiraju ove antigene nazivaju se histokompatibilnim genima. **Istorijat.** Osnov za razvoj oblasti transplantacione imunologije dao je Medavar (*Medawar*) 1940. godine koji je opisao odbacivanje tkiva presađenog sa jedne osobe ili životinje na drugu, osim kod presađivanja između identičnih blizanaca. Pedesetih godina prošlog veka je pokazano da je ovo odbacivanje tkiva posredovano antigenima glavnog kompleksa tkivne podudarnosti koji izazivaju jaku imunoreakciju i koji su kod ljudi poznati kao humani leukocitni antigeni (***H**uman **L**eukoycte **A**ntigen*). **Polimorfizam i nasleđivanje humanih leukocitnih antigena.** Prema najnovijoj nomenklaturi humanih leukocitnih antigena, trenutno postoje 17.344 humanih leukocitnih antigena i srodnih alela, od čega je 12,544 alela I klase humanih leukocitnih antigena, 4.622 alela II klase humanih leukocitnih antigena i 178 drugih non-humanih leukocitnih antigena alela. Zbog bliske lociranosti na kratkom kraku 6. hromozoma, geni sistema humanih leukocitnih antigena se nasleđuju kao parovi alela ili haplotipovi. **Biološke uloge humanih leukocitnih antigena.** Primarna uloga humanih leukocitnih antigena molekula je prezentovanje peptida T-ćelijama, pri čimu T-ćelija prepoznaje i molekul humanih leukocitnih antigena i peptid koji je prezentovan, razlikujući sopstvene peptide od stranih. Sposobnost razlikovanja sopstvenih od stranih peptida i dozvoljavanje imunoreakcije koja je uperena protiv „stranog", čini antigene humanih leukocitnih antigena glavnom imunobarijerom u transplantaciji. **Zaključak.** Imunobiologija transplantacije je važna iz više razloga, pre svega u smislu njenog uticaja na naše razumevanje imunoprocesa kao i njene primene u razvoju kliničke transplantacije. Napredak u oblasti imunogenetike i histokompatibilnosti ubrzali su razvoj kliničke transplantacije solidnih organa i tkiva.

Ključne reči: transplantaciona imunologija; HLA antigeni; tipizacija tkiva; odbacivanje graftova; transplantacija organa; genetski polimorfizam; T-limfociti

Introduction

One area of medicine in which human leucocyte antigens (HLA) proved to be of great importance is transplantation. Although transplantation of organs or tissues from one human being to another had been tried for many centuries, it was only accomplished over the past four decades. Success in this endeavour followed the discovery of the human major histocompatibility complex (MHC) in 1967. Identification of this genetic region launched the field of clinical organ and tissue transplantation. In 1968, the World

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Abbreviations

HLA – human leucocyte antigens
MHC – major histocompatibility complex
WHO – World Health Organization
CDC – complement dependent cytotoxicity
PCR – polymerase chain reaction
TRALI – transfusion related acute lung injury
vCJD – variant Creutzfeldt-Jakob disease

Health Organization Nomenclature Committee designated that the leukocyte antigens controlled by the closely linked genes of the human MHC be named HLA (for *human leukocyte antigens*). HLA are the group of tissue antigens, controlled by the chromosomal region, bearing a number of genetic loci, each with multi alleles, that have relevance to transplantation rejection reaction & other immunological phenomena [1, 2]. The essential role of the HLA antigens lies in the control of self-recognition and thus defence against microorganisms. HLA antigens are the major determinants used by the body's immune system for recognition and differentiation of self from non-self (foreign substances). There are many different major histocompatibility (HLA) proteins, and individuals possess only a small, relatively unique set that is inherited from their parents. It is unlikely that two unrelated persons will have the same HLA set of genes. A person, on the average, will have one-half of the HLA antigens that match with one-half of their mother's antigens; the other half of the antigens will match with one-half of their father's antigens. This is particularly important in identifying good "matches" for tissue grafts and organ transplants, such as a kidney or bone marrow transplant [3]. Matching the donor and the recipient for MHC antigens has been shown to have a significant positive effect on graft acceptance. The roles of the different components of the immune system involved in the tolerance or rejection of grafts and in graft-versus-host disease have been clarified. These components include: antibodies, antigen presenting cells, helper and cytotoxic T-cell subsets, immune cell surface molecules, signalling mechanisms and cytokines that they release. The development of pharmacologic and biological agents that interfere with the alloimmune response and graft rejection has had a crucial role in the success of organ transplantation [4]. This overview provides some background information about the biologic role of HLA in relation to issues of histocompatibility testing for transplantation.

History

Three papers appeared in 1958 by Jean Dausset, Jon van Rood and Rose Payne, respectively, which laid the foundation of what was later to become the HLA complex. All three papers described antibodies in human sera from multitransfused patients or multiparous women, sera that reacted with leucocytes from many but not all individuals who were tested. Thus, antibodies in these sera detected a polymorphic system of antigens on human leucocytes. The credit for discovery of the first HLA antigen goes to Dausset. Studying sera from patients who had received multiple blood transfusions, he found seven sera that behaved quite similarly, in that they agglutinated leucocytes from 11 of 19 individuals tested. Because leucocytes from the donor of the sera were also not agglutinated, the antisera obviously detected an alloantigen present on human leucocytes. He gave the name MAC to this antigen to honour three individuals who had been important volunteers for his experiments and whose names began with the initials M, A and C, respectively. Antigen MAC (later to become HLA-A2) was present in approximately 60% of the French population. For his discovery, Dausset received the Nobel Prize in 1980 (shared with Snell and Baruch Benacerraf).

Both van Rood and Payne followed up their initial findings of alloantigens on human leucocytes. Using (at that time) a sophisticated computer analysis of the reaction patterns of 60 sera from multiparous women against leucocytes from a panel of 100 donors, van Rood found some sera that apparently detected a diallelic system of leucocyte antigens, which he called 4a and 4b (later to become HLA-Bw4 and -Bw6, respectively). The results were reported in his PhD thesis from 1962. Two years later, Rose Payne, together with Julia and Walter Bodmer, also using sera from multiparous women, not only detected two leucocyte antigens, LA1 (later HLA-A1) and LA2 (later HLA-A2), apparently controlled by alleles, but also postulated at least one additional antigen, LA3, determined by an additional allele at the same locus [5, 6].

Following the discovery of the first histocompatibility antigen "MAC" in 1958, numerous independent laboratories began identifying HLA specificities with alloantibodies. During the span of about a decade, 1958-1970, virtually all of the common HLA-A and HLA-B antigens were identified by various names and presented at the International Histocompatibility Workshops by means of different serological methods. Because many of the independently discovered specificities were found to be reactive to similar determinants on lymphocytes, it became necessary to classify the antigens by means of a standard method. The micro lymphocytotoxicity test was chosen to be the basis for future antigen testing, thereby allowing the various laboratories to confidently exchange sera. At the 1968 WHO Nomenclature Meeting, the naming of the first antigen ultimately gave rise to the designation "HLA" in the human MHC Class I antigen system [7].

Genetics and inheritance of HLA

The antigens of the HLA system are determined by genes present in the major histocompatibility complex which is located on the short arm at the 6th chromosome (6p21.3) in humans and extends over some 4 centimorgans of DNA, about 4×10^6 base pair. The HLA complex contains more than 220 genes, more than 40 of which encode leukocyte antigens. They and their cell surface and soluble protein products are divided into three classes (I, II, and III) on the basis of their tissue distribution, structure, and function. MHC

class I and II genes encode co-dominantly expressed HLA cell surface antigens, and class III genes encode several components of the complement system; all share important roles in immune function. Class I MHC antigens are present on all nucleated cells and are each composed of a 45-kd α heavy chain encoded by genes of the HLA-A, HLA-B, or HLA-C loci on chromosome 6 and associated non-covalently with a 12-kd protein, β2-microglobulin encoded by a gene on chromosome 15. MHC class II antigens have a more limited tissue distribution and are expressed only on B lymphocytes, activated T lymphocytes, monocytes, macrophages, Langerhans cells, dendritic cells, endothelium, and epithelial cells. Each is a heterodimer composed of non-covalently associated α and β chains of approximately 230 amino acids encoded by genes of the HLA-D region. Class III genes are located between the HLA-B and HLA-D loci and determine the structure of three components of the complement system: C2, C4, and factor B.

HLA antigens are inherited in a Mendelian dominant manner. Because of the closeness of the different loci of the MHC and the resultant low crossover frequency, however, HLA genes are almost always inherited together. This fixed combination of genetic determinants is referred to as a *haplotype*. Because chromosome 6 is an autosome, all individuals have two HLA haplotypes (one for each chromosome), and there are only four possible combinations of haplotypes among the offspring of any two parents [1, 8–10].

Nomenclature of HLA

Two systems of nomenclature are applied to HLA. The first system is based on serological recognition. In this system, antigens were eventually assigned letters and numbers (e.g., HLA-B51 or, shortened, B51). Modern HLA nomenclature begin with HLA- and the locus name, then * and even number of digits specifying the allele. The first two digits specify the group of alleles. Older typing methodologies often could not completely distinguish alleles and so stopped at this level. The third through fourth digits specify a synonymous allele. Digits five through six denote any synonymous mutations within the coding frame of the gene. The seventh and eighth digit distinguish mutations outside the coding region. Letters such as L, N, Q, or S may follow an allele's designation to specify an expression level or other non-genomic data known about it. Thus, a completely described allele may be up to nine digits long, not including the HLA-prefix and locus notation, (e.g., HLA- A*24:02:01 N N=Null) to designate a specific allele at a given HLA locus. Every two years, a nomenclature is put forth to aid researchers in interpreting serotypes to alleles [11].

Polymorphism

Early in their study, it was recognised that the genes encoding the HLA molecules were highly polymorphic and that there was a need for a systematic nomenclature. The naming of new HLA genes, allele sequences, and their quality control is the responsibility of the WHO Nomenclature Committee for Factors of the HLA System. The committee meets regularly to discuss issues of nomenclature and has published 19 major reports documenting the HLA antigens and, more recently, the genes and alleles. The standardisation of HLA antigenic specifications has been controlled by the exchange of typing reagents and cells in the International Histocompatibility Workshops.

By June 2017, 17,344 HLA and related alleles had been described by the HLA nomenclature and included in the IPD-IMGT/HLA Database. It is now established procedure for authors to submit the sequences directly to the IPD-IMGT/HLA Database for checking and assignment of an official name prior to publication. This avoids the problems associated with renaming published sequences and the confusion of multiple names for the same sequence. The dissemination of new allele names and sequences is of paramount importance in the clinical setting and through the work of the HLA Informatics Group and in collaboration with the European Bioinformatics Institute, we are able to provide public access to the data through the websites http://www.ebi.ac.uk/ipd/imgt/hla/ and here at http://hla.alleles.org. Regular updates to these websites ensure that new and confirmatory sequences are dispersed to the HLA community. The need for reasonably rapid publication of new HLA allele sequences has necessitated an annual meeting of the WHO Nomenclature Committee for Factors of the HLA System. Additionally, we now publish monthly HLA Nomenclature Updates, both in journals and online, to provide quick and easy access to new sequence information [12].

Methods for HLA typing

HLA typing by serology: During the last 40 years HLA typing to detect this polymorphism was undertaken by the serological method better known as the complement-dependent cytotoxicity assay (CDC). A CDC test or microlymphocytotoxicity assay for HLA typing has been developed in the 60s. Serology is still the method of choice in many laboratories for low resolution typing of HLA-A and HLA-B (discrimination between groups of related alleles only), owed to its simplicity and low cost. By contrast, molecular typing has almost entirely replaced the CDC for the other HLA-loci (HLA-C, DR and DQ) for which serology is not precise enough.

The technique: Lymphocytes are tested with a panel of sera containing well-characterized HLA-specific alloantibodies. Each serum is placed in a microtiter well of a Terasaki plate (60-72 wells/plate). After a short incubation, rabbit serum is added as a source of complement and the cells that have bound the alloantibody are lysed making them permeable to the fluorochrome ethidium bromide. The wells containing the lysed cells are easily discriminated by microscopy.

HLA typing by molecular techniques: Molecular polymerase chain reaction (PCR) based HLA typing techniques allow the determination of DNA sequence variations, either by hybridisation with sequence-specific oligonucleotide probes (PCR-SSO), or by a combination of sequence specific primers (PCR-SSP), or by direct sequencing (PCR-SBT) of PCR products. Tissue typing by molecular method, utilizing the sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) technologies has been in routine use in many tissue-typing laboratories worldwide for more than 20 years since the development of the polymerase chain reaction. Both methods are very useful for clinical and research purposes and can provide generic (low resolution) to allelic (high resolution) typing results. DNA based method has more sensitivity, accuracy and resolving power than serologic typing methods. Sequencing-based typing (SBT) is a high-resolution method for the identification of HLA polymorphisms. The majority of HLA Class I alleles can be discriminated by their exon 2 and 3 sequence, and for Class II alleles, exon 2 is generally sufficient. Examination of all nucleotides, both at conserved and polymorphic positions, enables the direct identification of new alleles, which may not be possible with techniques such as SSP and SSO typing [11, 13–15].

Although useful in routine clinical practice, these methods are low-throughput, labour-intensive, and expensive. As an alternative, targeted amplicon sequencing (also known as the PCR-NGS approach) was recently developed. This technology uses standard PCR to capture regions of interest, and the resultant amplicons are then subjected to next-generation sequencing (NGS). The method is relatively high-throughput and inexpensive compared to PCR-SSO and PCR-SBT, and enables highly accurate HLA typing by producing hundreds of base pairs of long sequence reads at high coverage depth. Furthermore, over the past few years, genome-wide sequencing data, such as whole-genome sequence (WGS) or whole-exome sequence (WES), became widely available as a result of various genome sequencing projects [16].

Clinical relevance of HLA and transplantation immunology

The HLA system, originally discovered as the result of a transfusion reaction, is now known to play a crucial role in many areas of clinical medicine such as organ and hematopoietic stem cell transplantation, disease association, population and anthropological studies, respectively [17–21].

HLA alloimmunization induced by pregnancy, multiple transfusions or transplantation is responsible for some of the serious complications seen in patients receiving blood and blood products. These complications are primarily the result of antibody and antigen triggering an acute immunological reaction, which in some cases can be fatal (e.g., TRALI). Some adverse reactions are triggered by HLA antibodies present in the patient whereas others are initiated by antibodies or HLA reactive cells present in the transfused product. The introduction of universal leucodepletion for the prevention of vCJD transmission has resulted in a significant reduction in these reactions by eliminating the main source of alloimmunization, but residual cellular components or platelets are still able to activate the immune system and induce the development of HLA reactive antibodies or T cells.

In organ transplantation, the adaptive immunity is considered the main response exerted to the transplanted tissue, since the main goal of the immune response is the MHC molecules expressed on the surface of donor cells. Cell surface molecules that induce an antigenic stimulus cause the rejection immune response to grafted tissue or organ. The sensitization to MHC antigens may be caused by transfusions, pregnancy, or failed previous grafts leading to development of anti-HLA antibodies that are important factor responsible for graft rejection in solid organ transplantation and play a role in post-transfusion complication [11, 22].

Hematopoietic stem cell donor selection has been almost exclusively based on selecting an HLA identical donor or near-identical donor; however, not all patients are able to find a suitable donor. Advances in HLA testing and matching and understanding donor selection factors are therefore important to improve outcomes of unrelated donor (UD) HSCT. HLA disparity has been associated with graft failure, delayed immune reconstitution, graft-versus-host disease (GVHD), and mortality. Since many patients lack HLA-matched donors, current research is focused on identifying permissible HLA mismatches and donor search program development in regional or worldwide bone marrow donor registries [7, 21].

Population studies carried out over the last several decades have identified a long list of human diseases that are significantly more common among individuals that carry particular HLA alleles. For example, more than 90% of Caucasian patients with ankylosing spondylitis carry particular class I HLA alleles (*e.g. HLA-B*27:02, HLA-B*27:05*). Narcolepsy, a brain disorder characterized by sleep abnormality and falling attacks (cataplexy), is an illustrative example of HLA class II-associated disease, in which 90–100% of Caucasian patients carry the *DQB1*06:02* allele. In type I diabetes mellitus, more than 90% of patients carry either *HLA-DRB1*03/DQB1*02:01*, or *HLA-DRB1*04/DQB1*03:02* gene haplotypes, compared to only 40% of controls. Rheumatoid arthritis (RA) is another emblematic HLA class II-associated disease. Approximately 90% of Caucasian seropositive RA patients carry one or two *HLA-DRB1* alleles (*e.g. DRB1*04:01*, *DRB1*04:04, DRB1*04:05, DRB1*01:01*) [18, 23–26].

Previous studies have shown that allele and haplotype distribution in the HLA system differ from one ethnic group to another or between the members of the same ethnic group living in different geographic areas. Also, certain alleles are exclusively found in some ethnic groups. The comparisons between different populations using genetic distances calculated from HLA allele or haplotype frequencies have been used to determine the genetic relationship between different ethnic groups, so the HLA genetic markers are valu-

able tools for tracing ancient human migrations and determining the origins of different ethnic groups [19, 20, 27, 28].

Conclusion

The main function of the human leucocyte antigens molecules is presenting the antigen (protein chain of antigen) to the T Lymphocytes and initiating the specific immune response. Because foreign major histocompatibility complex molecules are recognized as antigens by the graft recipient, it is beneficial to ensure that donor and recipient share human leucocyte antigens alleles. human leucocyte antigens matching at the highest level of molecular resolution appears to be most beneficial to a successful therapeutic outcome.

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STEM CELL TRANSPLANT: FROM CELL HARVESTING TO CRYOPRESERVATION

TRANSPLANTACIJA MATIČNIH ĆELIJA: OD PRIKUPLJANJA DO KRIOKONZERVACIJE

Bela BALINT[1,2,3], Milena TODOROVIĆ[4,5], Ivana UROŠEVIĆ[6,7] and Mirjana PAVLOVIĆ[8]

Summary
Stem cells could be defined as cells capable for self-renewal with high proliferative capacity and extensive potential to differentiate into blood cells or some somatic cell types – "plasticity" due to "trans-differentiation" – such as osteocytes, chondrocytes, hepatocytes, myocytes, cardiomyocytes and even endothelial cells. Recent increasing clinical use of various cell-mediated therapeutic approaches has resulted in amplified needs for both stem cells and operating procedures to get a minimized cell damages during collection, purification and cryopreservation. The aim of cell harvesting procedures is to obtain the best stem cells yield, high purity and good viability/clonogenicity. The goal of optimized cryoinvestigation protocols is to minimize cell injuries during the freeze/thaw process (cryoinjury). Despite the fact that different stem cells collection protocols and cell freezing practice are already in routine use, a lot of questions related to the optimal blood stem cells harvesting, purification and cryopreservation are still unresolved.
Key words: Hematopoietic Stem Cell Transplantation; Cell Culture Techniques; Stem Cells; Cryopreservation; Cell Separation; Transplantation, Autologous; Dimethyl Sulfoxide; Blood Preservation

Sažetak
Matične ćelije se mogu definisati kao ćelije koje imaju sposobnost samoobnavljanja sa visokim proliferativnim kapacitetom i velikim potencijalom diferencijacije u ćelije krvi ili neke vrste somatskih ćelija – „plastičnost usled transdiferencijacije" – kao što su osteociti, hondrociti, hepatociti, miociti, kardiomiociti i endotelne ćelije. Kako je u poslednje vreme značajno povećan broj kliničkih procedura koje koriste izolovane ćelije, povećana je i potreba za razvojem tehnika za smanjenje oštećenja matičnih ćelija tokom kolekcije, prečišćavanja i krioprezervacije. Cilj procedura za prikupljanje ćelija jeST dobijanje najboljeg prinosa matičnih ćelija, visoke čistoće i dobre vijabilnosti/klonogenosti. Zadatak optimalnih protokola za kriokonverzaciju je minimalizacija oštećenja tokom procesa zamrzavanja/odmrzavanja (krioоštećenja). Uprkos činjenici da se različiti protokoli kolekcije i zamrzavanja matičnih ćelija koriste u rutinskoj praksi, ostaju brojna nerešena pitanja u vezi sa optimalnim prikuplja-njem matičnih ćelija, njihovim prečišćavanjem i kriokonzervacijom.
Ključne reči: transplantacija hematopoetskim stem ćelijama; tehnike kultivisanja ćelija; stem ćelije; krioprezervacija; separacija ćelija; autologna transplantacija; dimetil sulfoksid; konzervacija krvi

Introduction

Hematopoiesis is a continuing/steady event through which many different blood cells are produced from a small number of stem cells (SCs) by proliferation and differentiation. A multifactorious network of interactive mediators controls the survival (self-renewal), proliferation and differentiation of SCs in bone marrow (BM), including extracellular matrix and microenvironment provided by stromal cells [1, 2]. These cells – macrophages, fibroblasts, dendritic, endothelial and other cells – stimulate SCs by producing hematopoietic cytokines, such as SC-factor, interleukins, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and other growth factors [1–3]. Immature SCs have a capacity for self-renewal, a high potential for proliferation and differentiation into pluripotent or committed progenitors and mature blood cells. Thanks to described nature, SCs provide complete and durable or late BM repopulation and hematopoietic reconstitution following transplants. The SC transplants involve myeloablation (high-dose chemotherapy), followed by (re)infusion of harvested autologous or allogeneic cells. Similar procedure with reduced-intensity conditioning (RIC) can be offered to patients who are disqualified for intensive conditioning protocol – because of their age or comorbidity [1–4].

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Abbreviations

ISHAGE – International Society for Hematotherapy and Graft Engineering
SC – stem cell
MSC – mesenchymal stem cell
GM-CSF – granulocyte-macrophage colony-stimulating factor
M-CSF – granulocyte colony-stimulating factor
DMSO – dimethyl sulfoxide
HES – hydroxyetil starch
ACD – acid-citrate-dextrose
TNC – total nucleated cell
MNC – mononuclear cell
LVL – large volume leukapheresis
GvHD – graft versus host disease
MRA – marrow repopulating ability
CFU-S – colony-forming unit spleen
CFU-GM– colony-forming unit granulocyte-macrophage

Generally, SCs can be divided into embryonic and adult cell compartment, but several adult SCs have a similar high-level ("unlimited") biological potential to embryonic cells [2, 4]. Thus, some adult SCs are able to develop into a variety of somatic cells by using the features of "plasticity" or "trans-differentiation" [5–9]. Although the term "plasticity" has become very popular, some studies have suggested that BM might contain different types of SCs that can produce non-hematopoietic (somatic) cells. For example, mesenchymal SCs (MSCs)/pericytes (that is contractile cells that wrap around endothelial cells) from BM can switch to osteocytes, chondrocytes, adipocytes and skeletal muscle cells [2, 4, 8]. Consequently, these cells are applicable in the field of regenerative medicine, i.e. organ repair/regeneration.

In practice, allogeneic transplants require both an effective conditioning regimen, as well as optimized mobilization and harvesting protocol to obtain adequate SC yield [10–14]. For autologous transplants, the use of an optimized cryopreservation procedure guarantees the best recovery of thawed cell [15–21]. Despite the fact that cryopreservation is already in routine use, some questions related to the best freezing method and cryoprotective agents (dimethyl sulfoxide – DMSO and hydroxyetil starch – HES) type and their optimized concentration are not precisely answered [2, 4, 22].

Figure 1. Stem cell collection from bone marrow by multiple aspirations
Slika 1. *Prikupljanje matičnih ćelija multiplim aspiracijama*

The intensifying of myelo- (immune-) ablative therapy combined with SC transplant and the introduction of cell-mediated restorative/regenerative methods ("cell-therapy") resulted in increased needs for both SCs conceptual and practical operating procedures inducing minimized cell damages during their harvesting and cryopreservation. In this article, we will briefly review the practical aspects of an optimized harvesting protocol, processing and/or immunomagnetic selection, as well as cryopreservation of the SCs. Our results of the SC investigations will also be summarized.

Stem cell sources and harvesting

Historically, BM was the first source of SCs for transplant in experimental and clinical setting [4, 23–25]. The BM aspirate collection is the same for an allogeneic donor as for an autologous (occasionally) patient. SCs are harvested by multiple aspirations from the posterior and anterior iliac crest **(Figure 1)**.

The procedure is performed under sterile conditions, while the donor is generally anesthetized. Immediately after collection, aspirate should be filtered in order to remove bone and lipid particles and/or cell aggregates. Anticoagulation is provided by acid-citrate-dextrose formula B (ACD-B; 1.8% citrate concentration) and by heparin diluted in saline (5000 IU/500 mL) [2, 4].

In order to obtain a required total nucleated cell (TNC) yield – that is 3×10^8/kg of body mass (kgbm) of recipient – the target/maximal volume of collected aspirate is 10–15 mL per kgbm of donor (total volume = 800–1000 mL). For ABO incompatible (major and/or minor) transplants, red blood cells number (RBC) and/or plasma quantity reduction is required. Cell purification procedures such as processing and selection, enable reduction of the aspirate volume and decrease of RBC quantity (depletion = 80–90%; processing), as well as $CD34^+$ cell enrichment (positive selection) or T-cell depletion (efficacy 3–4 Log10; negative selection) [4, 16, 26]. In the aspirate, approximately 2-4% of TNCs express the CD34 antigen [14]. Shortly, CD34 is the cluster designation given to a transmembrane glycoprotein present on SC surface and some stromal cells. Cells expressing the stated CD34 and CD90 antigens are capable of complete and durable or late reconstitution of hematopoiesis [2, 4].

The collection of SCs from PB (PB-SCs) is an aphaeretic procedure with respect to the standardized protocol and cell yield. The $CD34^+$ cells are documented in peripheral blood (PB) in the "steady-state" hematopoiesis also (in very low percentage: 0.01–0.05% per TNCs) [4, 14]. The first collections of "steady state" PB-SCs were performed using 6 to 9 collections and additional cryopreservation was required [2].

Mobilization by chemotherapy and recombinant G-CSF extensively increases the quantity of circulat-

ing $CD34^+$ cell count in patients or donors. However, just a minor fraction of $CD45^+/CD34^+$ cells, with typical size and specific intracellular granularity – according to the ISHAGE-protocol are "authentic" SCs [14]. The immature $CD34^+$ cells ($CD34^+/CD90^+$ subset) infused can more precisely predict leukocyte and/or platelet (Plt) recovery after SC-transplant. In practice, SC-engraftment is defined as neutrophil number $\geq 0.5 \times 10^9$/L and Plt count exceeding 20×10^9/L (without blood component support) [2, 4, 14].

The main benefits of PB-SC harvestings/transplants are the absence of general anesthesia, higher $CD34^+$ yield, rapid hematopoietic reconstitution and smaller transplant-related morbidity. As a result, the number of patients transplanted by PB-SCs is ever increasing worldwide, especially in autologous SC transplant setting (about 80% of allogeneic and almost of 100% autologous transplants). On the other hand, the main disadvantage of the use of PB-SCs is high-level T-cell quantity with following elevated risk of graft versus host disease (GvHD), as well as possible "contamination" of harvest with tumor cell [2–4].

Nowadays, the typical number of apheresis required is not more than one to three, and for anticoagulation ACD–B or ACD–A (with 2.2% citrate concentration) are used [10–14] **(Figure 2)**.

For allogeneic transplants, vascular access is typically realized through ante-cubical veins. In autologous setting, collection should be performed by the central-venous vascular access across catheters. The use of catheters simplifies harvesting, but may be associated with topical thrombosis. Finally, there is approximately one percent central-venous catheter-related hazard of the local infection, pneumothorax or bleeding [2, 4].

As mentioned, for obtaining adequate SC or $CD34^+$ yield, efficient mobilization protocol is required. Allogeneic donors are given G-CSF 5–10 μg/kgbm daily subcutaneously. The $CD34^+$ cell count in the circulation begins to rise after 2–3 days of G-CSF administration and peaks on the fifth day [2, 4]. When donor mobilization with G-CSF is poor, the only way to improve yields is to increase the blood volume processed or the number of collections. In autologous donors, doses of recombinant grow factor are higher – patients are given 12-16 μg/kgbm G-CSF daily, combined with chemotherapy (cyclophosphamide 4–7 g/m^2) or by poly-chemotherapy in corresponding doses [2–4].

Figure 2. Stem cell harvesting using blood cell separators
Slika 2. *Prikupljanje matičnih ćelija primenom separatora krvnih ćelija*

The determination of optimized collection timing is very important and a critical event for PB-SC harvesting. Usually, in allogeneic setting the first (or single) harvesting is on the fifth day of G-CSF administration [2, 4]. The definition of optimized timing for autologous collection is more complex and controversial. The optimal harvesting timing can be determined based on the leukocyte, mononuclear cell (MNC) counts, as well as the number of circulating $CD34^+$ cells. The optimal time to begin cell collection in is when the leukocyte count is between $5–10 \times 10^9$/L. However, the leukocytes do not correlate strongly with the number of SC or $CD34^+$ yield in the harvest. Opposite, $CD34^+$ count in PB clearly correlates with harvesting timing and the SC or $CD34^+$ quantity in the harvest (as a function of the volume of blood processed also). Precisely, it was demonstrated that for a $CD34^+ \geq 20–40$/μL of patient's PB the possibility of the $CD34^+$ yield in harvest $\geq 2.5 \times 10^6$ cells/kgbm is about 60% or more using one large volume leukapheresis (LVL). Of course, higher $CD34^+$ number in circulation results in superior yield [2–4, 12–14].

Some patients who have previously been treated with high-dose chemotherapy may be "poor responders" for mobilization. The most efficient approach to obtain adequate SCs from "poor-mobilizers" is not resolute still. Simultaneously collection of SCs from BM and PB has not improved the engraftment rate considerably. Increased doses of G-CSF or use of G-CSF together with GM-CSF has also effectively mobilized some autologous donors. Finally, there are data describing the superior effects of some new agents in combination with G-CSF, such as plerixafor or mozobil (antagonist of the alpha chemokine receptor CXCR4) in mobilizing the $CD34^+$ cells – including the immature SCs, capable for durable or late (long-term) BM repopulation with following hematopoietic reconstitution [2, 14].

Our results confirmed high-level efficacy of the LVL. Namely, for the 89.5% patients using one LVL, the mean $CD34^+$ yield was 12.1×10^6/kgbm (allogeneic) and 6.5×10^6/kgbm (autologous), respectively. In our group of patients, the circulating $CD34^+$ count was also relatively high 40–60/μL following mobilizing regiment [3, 10–14]. Our investigations also verified that CD34+ post-selection cell recovery was 70–80%, when CD34+ purity (CD34+ cell percentage in final cell suspension) was around 80–90% [2–4]. In addition, our latest research demonstrated inverse correlation of the $CD34^+/CD90^+$ frequency with the absolute count of total $CD34^+$ cells in PB and the harvest. We considered that poorer $CD34^+/CD90^+$ yield in the harvest is not a outcome of an inferior harvesting efficacy – but most likely result of several even now not fully resolved immature SC cytomorphological and biophysical features [14].

Cryopreservation of stem cells

The use of cryobiology for cell preservation began in 1949 with the freezing of sperm cells, using glycerol as a cryoprotective agent [15]. Afterwards, DMSO and HES techniques were applied for cryopreservation of different blood-derived nucleated cells and/or Plts [16, 18, 19, 22].

Controlled-rate or microprocessor-restricted freezing is a time-consuming process, which requires high-level technical expertise. Uncontrolled-rate or "dump-freeze" technique (without programmed cooling rate) is less expensive because it does not require a programmed freezing-device. However, the controlled-rate method is an advanced alternative to the uncontrolled-rate system due to superior quantitative, morphological, ultrastructural and functional cell recovery [17–21].

The basic goal of cryoinvestigations was to predict the cell response to freeze/thaw processes and cryoprotective agent addition/removal, as well as evaluation of cryobiological variables such as biophysical, physicochemical and other parameters responsible for cryoinjury of living cells. As stated, SC cryopreservation is nowadays in routine practice, but recent cryoinvestigations suggest that freezing strategies should be revised to optimize specific cryobiosystems – to minimize the cryoinjuries and maximize cell recovery. Cryoinjuries can be detected as cell lesions, caused by the decrease of selected functions to the total cell destruction (cytolysis). At present, cryoinjuries are considered to result from the extensive volume reduction (cellular dehydration or solution effect) and/or massive intracellular ice crystallization (mechanical damage). These mechanisms can act together – the first event is expressed primarily at low-rate ($\leq$ 10 °C/min) freezing, and the second one at high-rate ($\geq$ 10 °C/min) freezing [2, 4, 17].

Thus, to establish an optimized cooling rate during cell freezing, specific for each cell type and cryobiological system should be considered. The speed of cooling should be high enough to prevent cell dehydratation and adequately low to enable the efflux of water from the cell. It would be ideal to find a cooling rate just less than the one, which causes intracellular crystallization [4, 17]. An optimal freezing rate is the function of the ratio between cell surface versus volume and cellular membrane permeability for water and its corresponding temperature coefficient – but it also depends on what type of cryopreservation strategy is applied. Last but not least, a higher degree of cell destruction has occurred when transition period from liquid to solid phase (fusion heat releasing) is prolonged. The released heat of fusion – if not considered during controlled-rate freezing – could result in additional temperature fluctuation. That is why the period of transformation from liquid to solid phase will be prolonged, and its duration is directly related to the degree of cryoinjury [2, 4, 18–21].

An optimized (controlled-rate vs. uncontrolled-rate) cell freezing approach cannot solve all problems related to cryoinjuries – post-thaw cell recovery and viability are high only when cryoprotective agents are present in the cryobiological system. They prevent or reduce the degree of cell thermal damages. In brief, cryoprotective agents can demonstrate protective effects by the reduction of cell dehydration, as well as by decreasing the intensity of intracellular crystallization. Cryoprotective agents are classified into intracellular (penetrating) and extracellular (non-penetrating) compounds. Mechanisms of their action are complex and only partially recognized. Due to the differences in its chemical and other properties, it is not possible to determine a cryoprotective mechanism common for all cryoprotective agents. In brief, extracellular agents could protect cells during rapid freezing, reducing the intracellular ice crystal formation. Opposite, intracellular cryoprotective agents could provide protection in the course of low–rate freezing, decreasing the degree of cell dehydration controlled-rate freezing [2–4, 17, 18, 21].

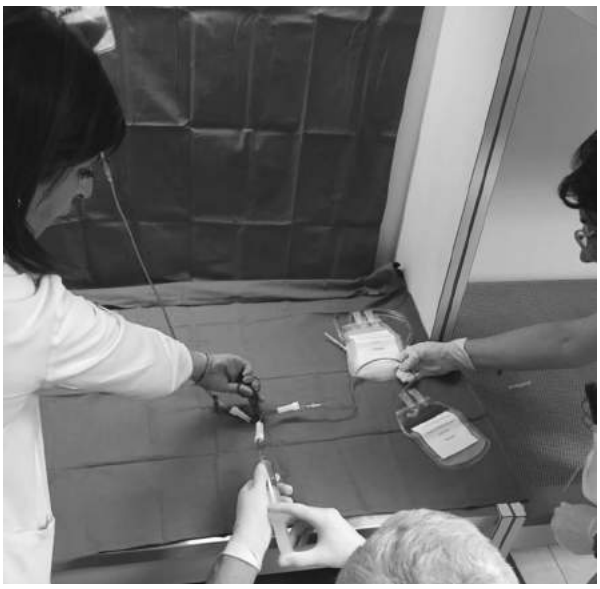

Figure 3. Stem cell cryopreservation with 10% DMSO and autologous plasma
Slika 3. *Kriokonzervacija matičnih ćelija upotrebom 10% DMSO i autologne plazme*

In practice, PB-SC cryopreservation consists of the following steps: 1) equilibration (cell exposure to cryoprotectant – DMSO); 2) freezing process (controlled-rate or uncontrolled-rate system); 3) cell storage at -90±5 °C (mechanical freezer) or at temperature from -120 °C to -150 °C (mechanical freezer/steam of nitrogen) or at -196 °C (liquid nitrogen); and 4) cell thawing in a water bath at 37±3 °C **(Figure 3)** [2–4].

Our earlier cryoinvestigations demonstrated that the recovery of pluripotent and committed hematopoietic progenitors (CFU-Sd12 and CFU-GM) in the presence of lower concentration of cryoprotective agent (5% vs. 10% DMSO) was superior. However, it has also been confirmed that the recovery of very primitive pluripotent (Marrow Repopulating Ability – MRA) hematopoietic SCs was better when 10% DMSO used. These results imply a different

"cryobiological request" of MRA cells in comparison with the less primitive progenitors. Moreover, we have demonstrated that differences in cell recovery are not related to the changes in the total number of frozen/thawed cells, regardless of the use of cryopreservation strategy [20]. Finally, our clinical studies showed that therapeutic use of the controlled-rate cryopreserved SCs in treatment of leukemia, multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma resulted with rapid hematopoietic reconstitution [2–4, 8, 12].

Conclusion

Despite the fact that different stem cells collection protocols and cell freezing practice are already in routine use, a lot of questions related to the optimal blood stem cells harvesting, purification and cryopreservation are still unresolved.

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AUTOLOGOUS STEM CELL TRANSPLANTS - SINGLE CENTER STANDPOINTS FOR LYMPHOMA PATIENTS

AUTOLOGNA TRANSPLANTACIJA MATIČNIH ĆELIJA HEMATOPOEZE - STAVOVI JEDNOG CENTRA ZA PACIJENTE SA LIMFOMIMA

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Summary
Autologous stem cell transplant with high-dose chemotherapy is considered to be an effective treatment strategy for outcome improvement in lymphoma patients, especially those with refractory and relapsed disease. Despite the feasibility and efficacy of autologous stem cell transplant, patients with lymphoma still face the risk for relapse, mostly patients who have adverse prognostic features. Salvage chemotherapy followed by high-dose chemotherapy and autologous stem cell transplant is recognized as the most effective strategy for relapsed or refractory Hodgkin lymphoma or aggressive non-Hodgkin lymphoma improving their response rate. First line therapy is ABVD (Hodgkin lymphoma patients), R-CHOP or R-CHOP-like regimens while salvage regimens such as DHAP, ESHAP, ICE represent the standard of care for relapsed/refractory lymphoma patients. Before autologous stem cell transplant, standard condition regimens are BEAM or CBV.
Key words: Transplantation, Autologous; Hematopoietic Stem Cell Transplantation; Lymphoma; Hodgkin Disease; Lymphoma, Non-Hodgkin; Antineoplastic Combined Chemotherapy Protocols

Sažetak
Visokodozna hemioterapija sa potporom autolognim matičnim ćelijama hematopoeze predstavlja efikasnu terapijsku liniju i utiče na poboljšanje ishoda lečenja kod pacijenata sa limfomima, posebno u relapsirajućim i rezistentnim oblicima bolesti. Uprkos izvodljivosti i efikasnosti autologne transplantacije matičnih ćelija hematopoeze, pacijenti sa limfomima još uvek se suočavaju sa rizikom od relapsa bolesti, posebno oni sa lošim faktorima prognoze. *Salvage* hemoterapija praćena visokodoznom hemioterapijom i autolognom transplantacijom matičnih ćelija hematopoeze prepoznata je kao efikasni vid lečenja za relapsirajuće i refraktorne oblike Hočkinovog limfoma ili agresivne nehočkinske limfome i ovim terapijskim pristupom popravlja se nivo terapijskog odgovora. Prva linija je ABVD (pacijenti sa Hočkinovim limfomom), R-CHOP ili R-CHOP-*like* režimi, dok tzv. *salvage* protokoli, kao npr. DHAP, ESHAP, ICE predstavljaju standard lečenja pacijenata sa relapsom ili refraktornim oblikom limfoma. Pre sprovođenja transplantacije matičnih ćelija hematopoeze, kao standardni kondicioni režimi primenjuju se BEAM ili CBV protokol.
Ključne reči: autologna transplantacija; transplantacija hematopoetskih stem ćelija; limfom; Hočkinova bolest; Non-Hočkinov limfom; kombinovani antineoplastični hemoterapijski protokoli

Introduction

High-dose chemotherapy (HDT) combined with autologous stem cell transplantation (ASCT) has been considered a promising treatment option aiming to improve the outcome of lymphoma patients who fail to achieve complete remission after first-line chemotherapy [1–2]. In relapse-refractory disease, salvage regimens are needed to overcome therapeutic resistance with implementation of new drugs. The achievement of remission after salvage regimens, usually platinum-based is of great importance for success of further transplant. Induction treatment of lymphoma is disease-dependent and for Hodgkin lymphoma (HL) it is ABVD or BEACOPPesc/basic according to risk group, while in non-Hodgkin lymphoma (NHL), induction consists of immunochemotherapy (R-CHOP or R-CHOP like regimens). Standard conditioning regimens are BEAM or CBV. However, relapses after ASCT are extremely unfavorable and need innovative therapies with targeted drugs.

The role of ASCT in Hodgkin lymphoma

Concerning HL, patients are usually cured by first-line therapy alone or with combined modality with additional radiation. Unfortunately, about 15% to 30% of patients with HL experience either primary refractoriness or relapse despite modern treatment options

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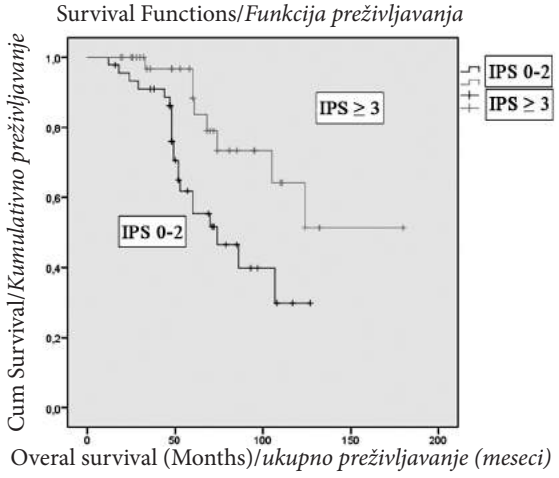
Abbreviations

ABVD	– doxorubicin, bleomycin, vinblastine, dacarbazine
R-CHOP	– rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone
DHAP	– dexamethasone, high-dose cytarabine, cisplatin
ESHAP	– etoposide, methylprednisolone, cytarabine, cisplatin
R-DHAP	– rituximab, dexamethasone, high-dose cytarabine, cisplatin
R-Hyper CVAD	– rituximab, cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate, cytarabine
ICE	– ifosfamide, carboplatin, etoposide
BEAM	– carmustine, etoposide, cytarabine, melphalan
CBV	– cyclophosphamide, carmustine, etoposide
IPS	– international prognostic score
IPI	– international prognostic index
OS	– overal survival
CR	– complete remission
PR	– partial remission
RiC	– reduced intensity conditioning

[3]. For those patients with primary refractory disease, or those experiencing relapse after complete remission, the disease may become life threatening. Still, nearly 50% of these patients can be cured with salvage therapy followed by HDT and ASCT [4], with 5-year PFS rates of approximately 50% [5].

Generally, the prognosis of patients relapsing after ASCT is poor (4) and it is difficult to obtain further clinical response with conventional chemotherapy. Successful outcome depends on chemo-sensitivity at the time of ASCT which is confirmed by pre-transplant-negative FDG-PET imaging providing powerful predictive information on the success of ASCT. Pre-transplant high-dose conditioning regimen has the role to eradicate the disease, and the stem cell collection being tumor free [6]. The most common cause of ASCT failure is disease progression. Management of patients who experience relapse after the ASCT therapeutic approach is palliative with conventional chemotherapy, or can be more successful with targeted-drugs such as brentuximab-vedotin, nivolumab, and pembrolizumab [7].



Graph 1. The impact of International Prognostic Score (IPI) on overall survival (OS) of patients
Grafikon 1. *Uticaj internacionalnog prognostičkog skora (IPS) na ukopno preživljavanje (UP) bolesnika*

The role of ASCT in non-Hodgkin lymphomas

With regard to chemosensitive relapse of NHL, HDT with ASCT is still standard of care. Among NHLs, aggressive diffuse large B-cell lymphoma (DLBCL) is the most common disease. The probability of being cured by the initial treatment is well predicted by the International Prognostic Index (IPI) [8].

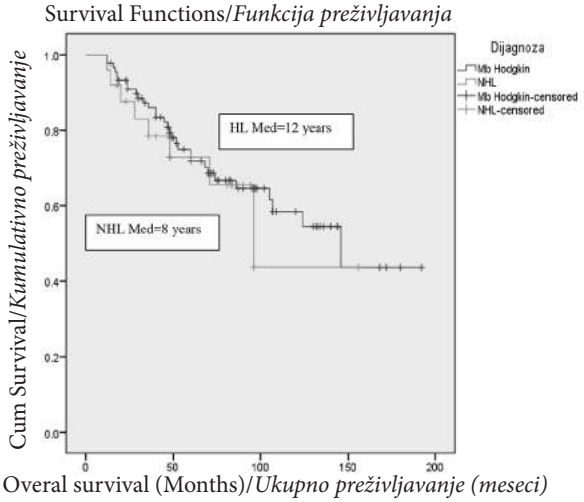
Prior to the introduction of rituximab, the probability of long-term survival varied from 26-73%, [8]. With adding rituximab to standard induction regimens, outcomes got better across all IPI groups [9,10]. For patients with relapsed, chemosensitive DLBCL, the Parma trial established HDT with ASCT as superior compared to conventional salvage chemotherapy alone [11]. However, this study was conducted in the pre-rituximab era, making its relevance in DLBCL patients treated in rituximab era with frontline or salvage regimens uncertain.

Although HDT with ASCT is treatment of relapsed NHL patient, in mantle cell lymphoma, it is therapy for consolidation of first remission. Namely, MCL classically responds to upfront chemotherapy, but it remains incurable with standard approaches. For patients in need of frontline therapy, the initial decision is whether to proceed with an intensive treatment strategy or a non-intensive treatment strategy. In general, younger and fit patients can be considered for intensive strategies. With current high-dose cytarabine-containing immunochemotherapy regimens followed by ASCT, the median PFS has exceeded 7 years [12].

Similarly as mentioned, our results in the treatment of high-risk relapse/refractory lymphomas have also demonstrated the advantage of HDT with ASCT.

Material and Methods

In this study, the results of treating 120 patients with lymphoma were analyzed; HL (90 patients), NHL (30 patients) in whom HDT with ASCT was conducted over a ten-year period (2006-2016). All patients were treated at the Clinic for Hematology KCS. In patients with HL, initial therapy was protocol ABVD, and salvage protocols were DHAP or ICE, which were used for stem cell mobilization. In 3 patients who were poor mobilizers, plerixafor was used. Second ASCT performed in 6 patients. As consolidation of first remission, ASCT performed in MCL patients treated initially with R-CHOP/R-DHAP or R-Hyper CVAD/HDMTX-AraC. In FL and DBKL, patients initially received R-CHOP, and afterward a salvage protocol ESHAP, which was also used for stem cell mobilization. The conditional protocol was BEAM or CBV.



Graph 2. Median survival of patients with Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL)
Grafikon 2. *Medijana preživljavanje bolesnika sa Hočkinovim limfomom (HL) i nehočkinskim limfomom (NHL)*

Results

The average age of patients with HL was 29 (18-51), while in patients with NHL, it was 40 (23-62). The average length of the mobilization was 6 days (5-12), the average number of CD34 + cells in the apheresis product was $8.6x10^6$/kg tm (2-25), the average volume of the apheresis product was 325 (150-900) ml, the average leukocyte engraftment was on day 13 (7-21), platelet engraftment on day 14 (9-30), and transplant related mortality was 0.8%. In HL, the initial Hasenclever index: International Prognostic Score (IPS) ≥ 3 was associated with shorter overall survival and survival after transplant (log rank = 7.128, p <0.008) **(Graph 1)**.

Patients with HL in whom the initial complete remission (CR_1) was > 12 months had two times longer total survival (OS) compared to patients with CR_1 <12 months (161 vs. 83 months). In lymphomas (HL and NHL) treated with VDT-ASCT, post-transplant CR at D+100 was the most powerful predictor of long-term OS (p <0.0001). The use of this type of treatment in HL increased the rate of favorable therapeutic response to D+100 (CR + PR) compared to the initial by more than 30%. However, 46% of patients after transplant have failed treatment either due to a disease-progression, or consecutive relapse. Median survival of patients with HL was 12 years, and for NHL patients it was 8 years **(Graph 2).**

Discussion

Our results showed the importance of HDT with ASCT in terms of increasing the response rate, as well as the satisfactory survival of high-risk patients with lymphoma, overcoming the significant degree of their therapeutic resistance.

For HL, HDT and ASCT represent the standard of care for patients with chemotherapy-sensitive relapsed or primary refractory HL. Certainly, the patients with negative pre-transplant PET/CT have much better post-transplant outcomes. The question whether patients with chemosensitive disease, but persistent positive PET/CT, benefit from additional salvage therapy before ASCT still remains to be determined. There is no consensus concerning the optimal conditioning regimen for ASCT or the role of tandem ASCT. But, patients with primary refractory HL are unquestionably candidates for tandem ASCT, following Morchauser criteria for high risk diseases [13]. The treatment of HL patients with completely refractory disease or those who experienced relapse after ASCT remains a great challenge. The use of RIC allogeneic-SCT (allo-SCT) can be of help for some unfavorable patients, and should be considered, especially if the disease can be controlled to a minimal state pre-transplant. In the allo-SCT setting many questions remain unresolved, regarding the optimal conditioning protocol, the use of DLI, T-cell depletion, and the optimal donor source of stem cells. With each of these treatments, the integration of targeted therapies into conditioning regimens, or as maintenance post-transplant therapy may additionally improve therapeutic outcomes.

In DLBCL patients, HDT followed by ASCT is a potentially curative option for patients with chemosensitive relapse or refractory disease, but more than 50% of patients will ultimately relapse. Many prognostic factors are associated with post-ASCT outcome in patients with relapsed/refractory DLBCL, including pre-ASCT remission status, as assessed by PET/CT, time to relapse, and prognostic indices (e.g. IPI, or revised IPI) [14]. Although these clinical prognostic factors are useful, new tumor-specific biomarkers may allow for improving prognosis prediction. For instance, double-hit lymphomas (DHLs) are a subset of DLBCL with concurrent chromosomal rearrangements involving the MYC and BCL-2 and/or BCL-6 genes, which comprise approximately 2-10% of newly diagnosed DLBCL. Patients with DHLs have dismal therapeutic outcomes with standard induction immunochemotherapy (R-CHOP) [15]. Double-expressor lymphomas are DLBCL with co-expression of the MYC and BCL-2 proteins by immunohistochemistry and encompass 21-34% of newly diagnosed patients with DLBCL.

Conclusion

Although some patients with relapsed/refractory diffuse large B-cell lymphoma had long-term remission after autologous stem cell transplant, the low survival rate in this group point to the fact that alternative transplant strategies, including allogenic stem cell transplantatin or peri-autologous stem cell transplant relapse prevention strategies, should be considered. Therefore, the precise definition of the disease risk degree is necessary before deciding to proceed to autologous stem cell transplantation as a further treatment strategy for these high-risk lymphomas.

The post-transplant complete remission (day+100) is the most important factor in favorable prognosis for long-term survival of patients with lymphomas treated with high dose chemotherapy with autologous stem cell transplantation. Also, the absence of chemo-sensitivity to the application of salvage therapy is an indicative area solely for the application of the so-called "target" therapy modalities.

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THE ROLE OF HIGH-DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION IN THE TREATMENT OF PATIENTS WITH MULTIPLE MYELOMA

ULOGA VISOKODOZNE TERAPIJE I AUTOLOGNE TRANSPLANTACIJE MATIČNIH ĆELIJA U LEČENJU PACIJENATA SA MULTIPLIM MIJELOMOM

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Summary

Introduction. Limited efficacy of standard chemotherapy initiated the introduction of high-dose therapy followed by autologous stem cell transplantation in the treatment of patients with multiple myeloma. The aim of the study was to analyse results of treatment with high-dose therapy followed by autologous stem cell transplantation in 110 newly diagnosed multiple myeloma patients during the period May 2015 – January 2016. **Material and Methods.** Patient group consisted of 51 female and 59 male patients with average age of 57 years. Most of the patients were diagnosed with IgG myeloma (58.2%) and clinical stage III (74.5%, Salmon&Durie). Renal impairment initially existed in 26 patients. By 2008, patients were treated with 4-6 cycles of induction chemotherapy according to the protocol VAD (33 patients, 30%); and afterward according to the CTD protocol (72 patients, 65.5%). Mobilization of the stem cell was performed according to the protocol CAD followed by granulocyte colony-stimulating factor 5-10 μg/kg body weight/day starting on day 9, until the apheresis around day 14 of mobilization (± 1-2 days). Within 4-8 weeks after mobilization, HDT with Melphalan 200 mg/m^2 accompanied with autologous stem cell transplantation was performed. **Results.** Applied induction treatment resulted in the achievement of at least partial remission in 80% patients. The average number of CD34$^+$ in the product of apheresis was 8.1x10^6/kgBW, while during HDT with autologous stem cell transplantation, median was 4x10^6/kg body weight CD34$^+$ cells. Average recovery was registered around +15 days after autologous stem cell transplantation, characterized by a minimal number of febrile days (median 2 days, range 0-10 days). In 95% patients, partial remission was recorded +100 days after autologous stem cell transplantation with average duration of 45 months, and achievement of complete remission in 29% pts with median overall survival of 100 months. This treatment approach resulted in overall survival longer than 45 months in more than 90% patients. The factors found to affect the duration of remission and overall survival are: ISS score ≥2. **Conclusion.** High-dose therapy followed by autologous stem cell transplantation is an efficient and safe treatment approach to multiple myeloma patients. Along with biological characteristics of the disease, complete remission achievement after such treatment is of essential significance for the course and outcome of multiple myeloma patients.

Key words: Transplantation, Autologous; Hematopoietic Stem Cell Transplantation; Multiple Myeloma; Antineoplastic Combined Chemotherapy Protocols

Sažetak

Uvod. Ograničena efikasnost standardne hemoterapije pokrenula je uvođenje visokodozne terapije praćene autolognom transplantacijom matičnih ćelija u lečenju pacijenata sa multiplim mijelomom. Cilj studije bio je da analizira rezultate lečenja visokodoznim terapijama praćenim autolognom transplantacijom matičnih ćelija kod 110 pacijenata sa postavljenom dijagnozom multiplog mijeloma tokom perioda od maja 2015. do januara 2016. **Materijal i metode.** Grupa pacijenata se sastojala od 51 žene i 59 muškaraca prosečne starosti 57 godina. Većini pacijenata je postavljena dijagnoza IgG mijelom (58,2%) i klinički stadijum III (74,5%, *Salmon&Durie*). Renalna insuficijencija je registrovana kod 26 pacijenta. Do 2008. godine, pacijenti su lečeni sa 4–6 ciklusa indukcione hemoterapije u skladu sa protokolom VAD (33 pacijenta, 30%); nakon toga u skladu sa CTD protokolom (72 pacijenta, 65,5%). Mobilizacija matičnih ćelija izvršena je u skladu sa protokolom CAD praćenim granulocitnim faktorom rasta 5–10 μg/kg telesne mase/dan počevši od 9. dana, do afereze oko 14. dana mobilizacije (± 1-2 dana). U roku od 4 do 8 nedelja nakon mobilizacije, sprovedena je HDT melfalanom u dozi od 200 mg/m^2 uz potporu autolognom transplantacijom matičnih ćelija hematopoeze. **Rezultati.** Primenjena indukciona terapija je dovela do postizanja delimične remisije kod 80 pacijenata. Prosečan broj CD34$^+$ u proizvodu afereze bio je 8,10 x 10^6/kg telesne mase, dok je tokom primene HDT sa autolognom transplantacijom matičnih ćelija medijana iznosila 4 x 10^6/kgTM CD34$^+$ ćelija. Prosečan oporavak je zabeležen oko +15 dana nakon autologne transplantacije matičnih ćelija, okarakterisan sa minimalnim brojem febrilnih dana (medijana dva dana, raspon 0–10 dana). Kod 95% pacijenata, delimična remisija je zabeležen +100 dana nakon autologne transplantacije matičnih ćelija, sa prosečnim trajanjem od 45 meseci, i ostvarenjem potpune remisije kod 29% pacijenata sa srednjim ukupnim preživljavanjem od 100 meseci. Ovaj pristup lečenju je doveo do srednjeg ukupnog preživljavanja dužeg od 45 meseci kod preko 90% pacijenata. Faktori za koje je ustanovljeno da utiču na remisiju i srednje ukupno preživljavanje jesu: internacionalni prognostički skor ≥2. **Zaključak.** Visokodozne terapije praćene autolognom transplantacijom matičnih ćelija jesu efikasan i bezbedan pristup pacijentima sa multiplim mijelomom. Uz sve biološke karakteristike bolesti, ostvarenje potpune remisije nakon takvog lečenja je od ključne važnosti za tok lečenja i ishod kod pacijenata sa multiplim mijelomom.

Ključne reči: autologna transplantacija; transplantacija hematopoetskih stem ćelija; multipli mijelom; kombinovani antineoplastični hemoterapijski protokoli

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Abbreviations

VAD – Vincristine, Doxorubicin (Adriamycin), Dexamethason
CTD – Cyclophosphamide, Thalidomide, Dexamethason
CAD – Cyclophosphamide, Adriamycin, Dexamethason
G-CSF – granulocyte colony-stimulating factor
BW – body weight
PR – partial remission
VGPR – very good partial remission

Introduction

Classical start of the majority of publications regarding multiple myeloma (MM) is that despite all the advances in treatment, MM remains an incurable disease [1]. Still, pointing out the state of complete remission (CR) as a predictor of long term survival in transplant eligible patients with ≈15% of curing, the MM landscape is revolutionary changed [2].

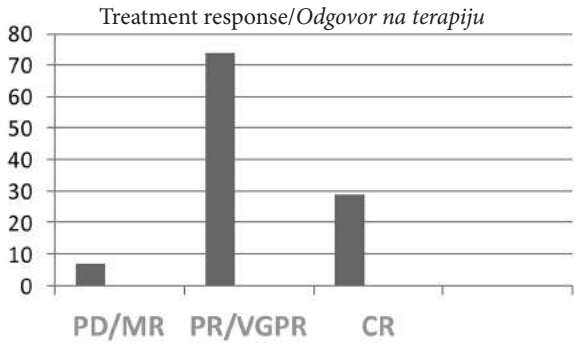
Probably the biggest breakthrough in the treatment of multiple myeloma was achieved with the concept of high-dose therapy followed with ASCT (HDT+ASCT), which is mainly caused by the ability to induce better quality of therapy response as CR [3]. The aim of this study was to evaluate the treatment results in newly diagnosed MM patients treated with HDT+ASCT.

Material and Methods

Our study included 110 newly diagnosed MM patients (age of ≤65yrs: 59 male/51 female, mean age 57yrs, range 35-65). The clinical characteristics were as follows: 64 patients (58.2%) had IgG myeloma, 10 patients (17.3%) IgA, 16 patients (14.5%) light chains, patients (2.7%) IgD 3, and 3 patients (2.7%) non-secretory. According to the clinical stage (Durie&Salmon), the distribution was: I (symptomatic disease) 3 (2.7%); II 25 patients (22.8%); III 82 patients (74.5%). The renal impairment was present in 26 patients (24.0%). Regarding ISS score, 32 patients (29.1%) had ISS 1 score; 17 (15.5%) International Staging System (ISS) 2; and 22 patients (20.0%) had ISS 3. Clinical characteristics of the analysed patient group are summarized in **Table 1**.

Patients diagnosed prior to 2008 were treated with 4-6 cycles of induction chemotherapy according to the protocol VAD (33pts, 30%); and afterwards according to the CTD protocol (72pts, 65.5%). Stem cell mobilization was performed according to the 4-day protocol CAD followed by G-CSF 12-15μgr/kgBW/day starting on day 9, until the apheresis around day 14 of mobilization (± 1-2 days) with harvest >2x10^6/kgBW. Within 4-8 weeks after mobilization, HDT with Melphalan 200mg/m^2 accompanied with ASCT was performed.

MM population was described using descriptive statistics to summarize its characteristics. Continuous variables were presented with median values and ranges, while for categorical variables percent-

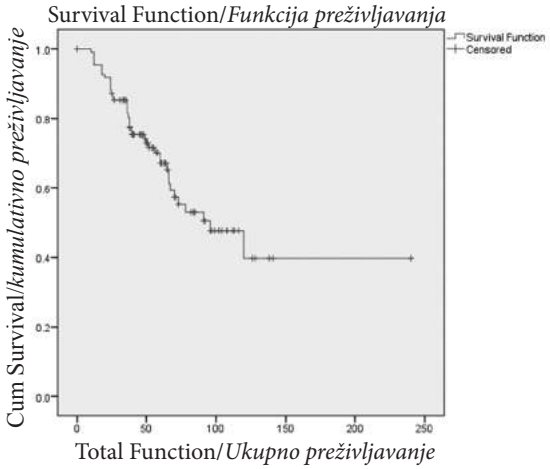


Graph 1. The treatment response in 110 MM patients treated with HDT+ASCT
Grafikon 1. *Terapijski odgovor kod 110 pacijenata sa MM lečenih sa HDT+ASCT*

Table 1. The characteristics of 110 newly diagnosed multiple myeloma patients treated with HDT + ASCT
Tabela 1. *Karakteristike 110 pacijenata sa tek postavljenom dijagnozom multipli mijelom lečenih HDT + ASCT*

Gender (M/F)/*Pol (M/Ž)*	59/51
Age/*Starost*	57 yrs, range 35 – 65yrs/*57 godina (rang 35–65)*
Myeloma multiplex type *Tip multiplog mijeloma*	IgG 64 pts (58.2%), IgA 10 pts (17.3%), *IgG 64 pacijenta (58,2%), IgA pacijenata 10 (17,3%)* BJ 16 (14.5%), IgD 3 (2.7%), non-secretory 3 (2.7%). *BJ 16 (14.5%), IgD 3 (2.7%), nesekretorni 3 (2.7%).*
Renal impairment/*Bubrežno oštećenje*	26 pts (24%)/*26 pacijenata (24%)*
Clinical Stage (CS, Salmon&Durie) *Klinički stadijum (KS)*	I (symptomatic) 3 pts (2.7%), II 25 (22.8%), III 82 (74.5%) *I (simtomatski) 3 pacijenta (2,7%), II 25 (22,8%), III 82 (74,5%)*
ISS score *ISS skor*	1 32 pts (29.1%), 2 17 (15.5%), 3 22 (20%) *1 32 pacijenta (29.1%), 2 17 pacijenata (15.5%), 3 22 pacijenta (20%)*
High-risk cytogenetic abberations (CA) *Citogenetske aberacije visokog rizika (CA)*	8 pts *8 pacijenata*
R-ISS 2015	27/110: 1 6 (22.2%), 2 17 (63.0%) 3 7 (25.9%)

Legend: M- male, Ž- female; R-ISS-Revised International Staging System
Legena: M – muški pol, Ž – ženski pol, ISS – Internacionalni sistem za stadijum bolesti; R-ISS – Revidirani ISS

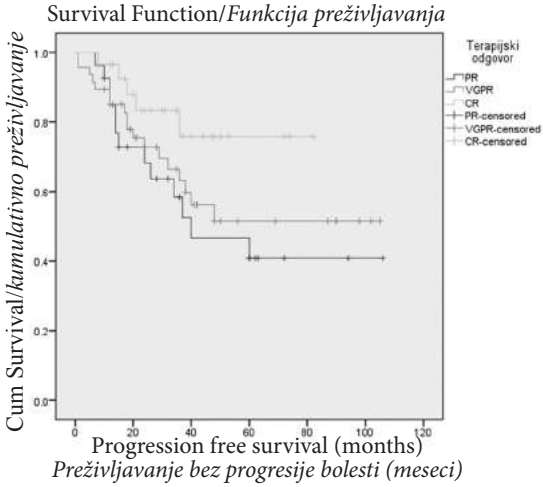


Graph 2. The OS of the MM patients treated with HDT+ASCT
Grafikon 2. *Ukupno preživljavanje (OS) pacijenata sa MM lečenih HDT + ASCT*

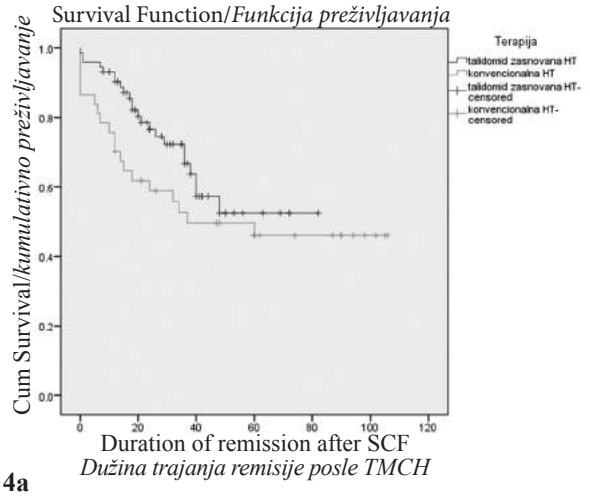
ages and frequencies were used. OS was plotted by the Log Rank estimating method and Kaplan-Meier curves. P value <0.05 was considered to be statistically significant. SPSS version 16.0 was used for all statistical analyses.

Results

The overall positive treatment response (ORR ≥ PR) was achieved in 102 patients (92.7%) with state of complete remission (CR) registered in 29 patients (26.3%) **(Graph 1)**. The median overall survival (OS) of patients treated with HDT+ASCT was 71.4 months (range 10-92) with probability of 5-years survival in 63% patients **(Graph 2)**. The progression free survival (PFS) was significantly longer in patients who achieved CR (Log Rank 6.382, p=0.014) with probability of 5 years duration in 80% of patients **(Graph 3)**. Patients treated with thalidomide-based induction therapy had significantly longer PFS (Log Rank 1.986, p=0.028) in comparison to the patients receiving induction with standard chemotherapy **(Graph 4a)**. There was no significant difference between those two group of patients regarding overall survival (OS, Log Rank 0.182, p=0.670) **(Graph 4b)**. According to the scoring of International Staging System (ISS), patients with ISS score 3 had significantly shorter PFS (Log Rank 8.234, p=0.016) and OS (Log Rank 10.314, p=0.006) in comparison to the patients with ISS score 1 and 2 **(Graph 5a and Graph 5b)**.



Graph 3. The PFS duration in comparison to the treatment response
Grafikon 3. *Dužina preživljavanja bez progresije bolesti (PFS) u poređenju sa terapijskim odgovorom*



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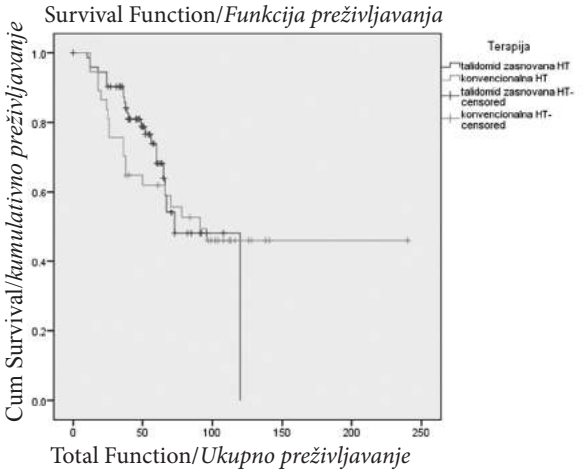
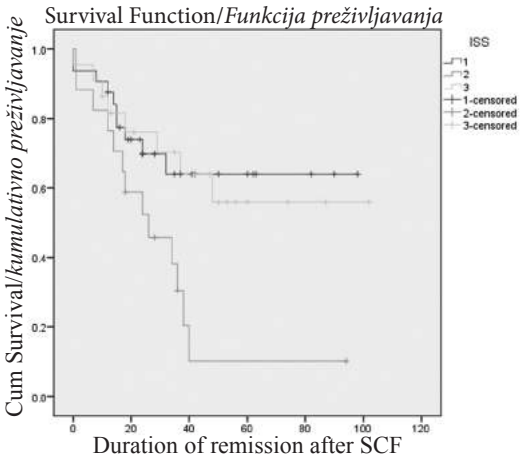
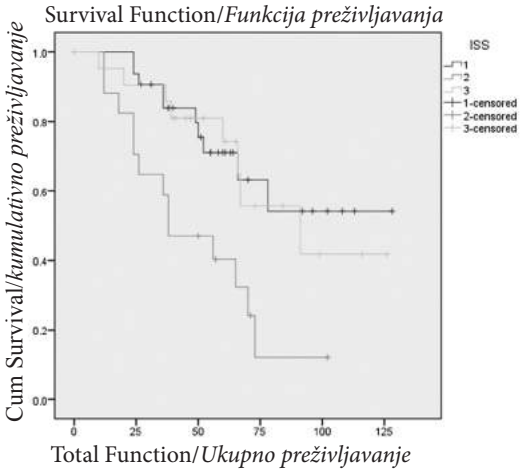


4b

Graph 4a and 4b. The PFS duration and OS length in comparison to the induction treatment
Grafikon 4a i 4b. *Dužina preživljavanja bez progresije bolesti (PFS) i dužine ukupnog preživljavanja u poređenju sa indukcionom terapijom*



5a



5b.

Graph 5a and 5b. The PFS duration and OS length in comparison to the ISS score
Grafikon 5a i 5b. Dužina preživljavanja bez progresije bolesti (PFS) i dužine ukupnog preživljavanja u odnosu sa ISS

Discussion

MM is a disease with a variable outcome, depending on tumour-and patient-related risk factors. Alkylating agents and steroids with novel drugs are the cornerstone of the current standard triple combinations for myeloma treatment. However, high dose Melphalan followed with ASCT has significantly improved the response rate, event-free survival and OS of myeloma patients in comparison with the treatment using conventional chemotherapy [4–6]. Our study has shown that the patients who underwent ASCT had unequivocal benefits from ASCT. During the last decade, various new medications have been introduced in the treatment of MM. Several studies have shown that the introduction of thalidomide, bortezomib and lenalidomid as induction treatment plus ASCT has resulted in high rates of CR and VGPR up to 70% of patients [6, 7]. New agents have improved response rates mainly in younger patients [8, 9]. Since bortezomib has recently been locally approved for the first line of therapy, the majority of patients in the current study, being eligible for the ASCT, have been treated with standard induction therapy based on the thalidomide combinations according to the national guidelines. Induction based on conventional chemotherapy was applied up to 2008, or in patients with comorbidities that excluded the possible use of the thalidomide and bortezomib. In the era of new drugs, aiming at final eradication of the malignant clone, HDT+ASCT retains its importance as standard of care in fit MM patients <70 years [10, 11]. Patients that were treated with HDT+ASCT with ISS score 3 had significantly shorter PFS and OS. Such results are in accordance with the biology of the disease expressed in ISS parameters. This is a major influence over the course and outcome of MM, which implicates the necessity of tailoring the individual therapy, possibly including continuation of treatment in high risk patients with multiple myeloma [12–14]. Still, despite all the advances and introduction of new anti-myeloma drugs, standard conditioning regimen is HDT Melphalan 200 mg/m^2 [15]. Tandem ASCT should be considered in patients that did not achieve complete or very good partial remission after the fisrt HDT+ASCT.[11] Furthermore, current recommendations about the allogeneic stem cell transplantation (AlloSCT) suggest that AlloSCT should be performed only within clinical trials [10].

Conclusion

The concept of high-dose therapy followed with autologous stem cell transplantation still represents the golden standard of treatment in patients with multiple myeloma eligible for such a treatment approach. The efficacy of this treatment mainly depends on the achievement of the complete remission state, along with initial biological characteristics of disease.

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THE ROLE OF PROGNOSTIC FACTORS IN OVERALL SURVIVAL IN PATIENTS WITH BENCE-JONES MULTIPLE MYELOMA - OUR EXPERIENCE

UTICAJ FAKTORA PROGNOZNE NA UKUPNO PREŽIVLJAVANJE BOLESNIKA SA BENS-DŽONS MULTIPLIM MIJELOMOM – NAŠE ISKUSTVO

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Summary

Introduction. Bence-Jones myeloma multiplex is a progressive disease characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of incomplete immunoglobulins, containing only the light chain portion of the immunoglobulins. This type of myeloma occurs 15-20%. The median overall survival is approximately 4 years. Aim of this study was to define prognostic factors affecting overall survival in Bence-Jones multiple myeloma patients. **Material and Methods.** From 1995 to 2015, we treated 75 patients (49 men and 26 female), average age 55.9 years (range 31-85). **Results.** Conventional chemotherapy introductory clinical response was achieved in 54 patients (72%), while in 21 patients (28%) the established disease was resistant. Transplantation was done in 45 patients (60%), while 30 patients (40%) were treated with conventional chemotherapy. In the group of patients with transplantation done, tandem was carried out in 11 patients and secondary stem cell transplantation was done in 5 relapsed patients. With 1 patient with tandem stem cell transplantation allogenic (singen) stem cell transplantation was done. Transplant related mortality is 1.5%. The transplanted patients had significantly longer PFS (mediana 13 months vs 7 months, p<0,05) and longer overal survival (mediana 55 months vs 26 months, p<0,001). Univariate log regression analysis showed that non-transplant patients are 5,1 times more likely to terminate lethal compared to transplant patients (RR 5,1; 95% C.I.43,47-2,52), p<0,001). **Conclusion.** Our study showed autologous stem cell transplantation is a more effective method of treatment of patients with Bence-Jones myeloma compared to the conventional chemotherapy.

Key words: Prognosis; Survival Rate; Mutiple Myeloma; Bence Jones Protein; Risk Factors; Blood Platelets; Cholesterol, HDL; Treatment Outcome

Sažetak

Uvod. Bolest lakih lanaca ili Bens Džouns (Bence Jones) mijelom je progresivna bolest koju karakteriše proliferacija plazma ćelija u koštanoj srži i prekomerna produkcija imunoglobulina lakih lanaca u plazmi i u urinu. Ova vrsta multiplog mijeloma čini 15–20% slučajeva. Medijana preživljavanja iznosi prosečno četiri godine. Cilj istraživanja je da se retrospektivnom analizom definiše uticaj faktora prognoze na ukupno preživljavanje bolesnika sa Bens Džouns multiplim mijelomom i ishod bolesti. **Materijal i metode.** Istraživanjem je od 1995. do 2015. godine obuhvaćeno 75 pacijenata (49 muškaraca i 26 žena), prosečne starosti 55,9 godina (31–85). **Rezultati.** Konvencionalnom hemioterapijom postignut je klinički odgovor kod 54 pacijenta (72%), dok je kod 21 pacijenta (28%) verifikovana rezistentna bolest. Kod 60% pacijenata je sprovedena autologna transplantacija matičnih ćelija hematopoeze, dok je 40% pacijenata lečeno konvencionalnom hemioterapijom. U grupi pacijenata kod kojih je sprovedena autologna transplantacija matičnih ćelija hematopoeze, kod 11 pacijenata je urađena tzv. tandem, a kod pet pacijenata sekundarna transplantacija matičnih ćelija hematopoeze. Kod jednog pacijenta sa sprovedenom tzv. tandem transplantacijom matičnih ćelija hematopoeze, urađena je singena alogena transplantacija matičnih ćelija hematopoeze. Transplantacioni mortalitet je nizak i iznosi 1,5%. Pacijenti kod kojih je urađena autologna transplantacija matičnih ćelija hematopoeze imaju signifikantno duže preživljavanje bez progresije bolesti (medijana 13 meseci vs 7 meseci, p < 0,05) i duže ukupno preživljavanje (medijana 55 meseci vs 26 meseci, p < 0,001). Univarijantna logistička regresiona analiza je ukazala da pacijenti kod kojih nije urađena autologna transplantacija matičnih ćelija hematopoeze imaju pet puta veću verovatnoću za letalni ishod u poređenju sa pacijentima kod kojih je urađena transplantacija (RR 5,1; 95% C. I. 43, 47–2,52), p < 0,001). **Zaključak.** Istraživanje je ukazalo da je autologna transplantacija matičnih ćelija hematopoeze efikasan metod lečenja pacijenata sa Bens Džouns mijelomom u poređenju sa konvencionalnom hemioterapijom.

Ključne reči: prognoza; preživljavanje; multipli mijelom; Bence Jones proteini; faktori rizika; trombociti; HDL holesterol; ishod lečenja

Introduction

Multiple myeloma (MM) is a neoplastic monoclonal plasma cell proliferation with typical restriction in the bone marrow and the clinical pentad (anemia, monoclonal M-protein, bone lesions, hypercalcemia, renal insufficiency) [1]. Light chain deposition disease or Bence-Jones myeloma is a

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Abbreviations

MM – multiple myeloma
SCT – stem cell transplantation
ASCT – autologous stem cell transplantation
MPV – Melphalan, Bortezomib, Pronison
MPT – Melphalan, Thalidomide, Pronison
BP – Bendamustine, Pronison
VCD – Bortezomib, Cyclophosphamide, Dexasone
VTD – Bortezomib, Cyclophosphamide, Dexasone
PAD – Bortezomib, Doxorubicin, Dexasone
RVD – Bortezomib, Lenalidomide, Dexasone
R-ISS – Revised International Staging System
OS – overall survival
PFS – progression free survival
IMiD – immunomodulatory imide drug
LDH – lactate dehydrogenase

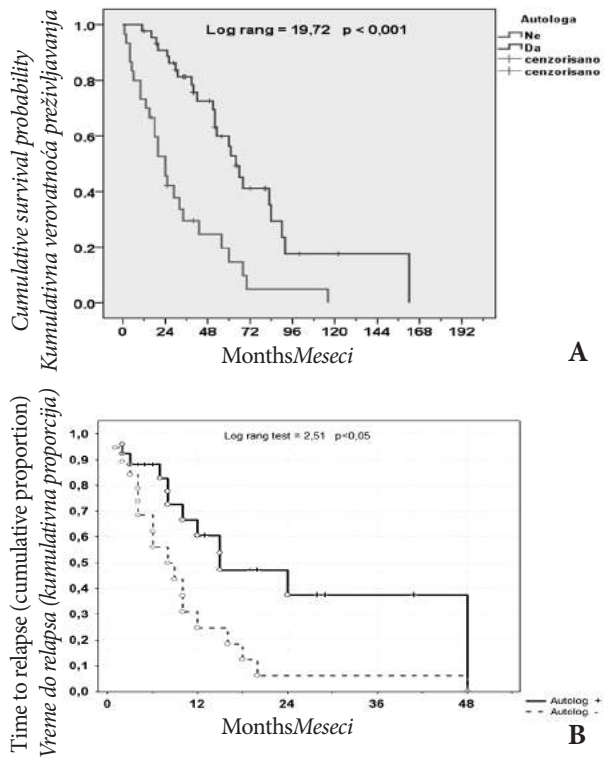
progressive disease characterized by plasma cell proliferation in the bone marrow and overproduction of the light chain portion of immunoglobulins (Bence Jones proteins) in plasma and in urine [2]. Multiple myeloma accounts for 1% of all cancers and for 10% of hematologic malignancies. Incidence is 4.5-6.0 /100,000. It is more common among men [3]. The median age is 72. One fifth of MM patients are diagnosed with a type called light chain deposition disease [4]. Their median overall survival is 4 years [3]. They are diagnosed on the basis of laboratory parameters, bone marrow biopsy, radiographic detection of various sensitivity and specificity levels [3]. After establishing the diagnosis, patients are stratified into risk groups according to the International Prognostic Scoring System (ISS) and the Revised International Prognostic Scoring System (R-ISS) [5]. With patients over 65, the following protocols are applied in the introductory therapy: Melphalan, Thalidomide, Pronison (MPT), Melphalan, Bortezomib, Pronison (MPV), Lenalidomide-Dexasone (Rd), Bendamustine, Pronison (BP). Induction therapy in patients under 65 includes combinations with proteasome inhibitors (Bortezomib, Cyclophosphamide, Dexasone (VCD), Bortezomib, Cyclophosphamide, Dexasone (VTD), Bortezomib, Doxorubicin, Dexasone (PAD), Bortezomib, Lenalidomide, Dexasone (RVD) [1]. A required form of treatment is the application of a "high-dose" chemotherapy supported by autologous cells, with conditioning of 200mg/m^2 of Melphalan. Allogeneic hematopoietic stem cell transplantation was indicated in young high risk and/or resistant myeloma patients [6]. Allogeneic hematopoietic stem cell transplantation is at the stage of clinical trials and

Table 1. Patients' characteristics
Tabela 1. *Karakteristike pacijenata*

Parameters/*Parametri*	N (%)
Age (mediana)/*Starost (medijana)*	55 (31-85)
Sex (M/F)/*Pol (M/Ž)*	26/49 (35/65)
Platelets/*Trombociti* (x10^9/l)	
<130	44 (59%)
>130	31 (41%)
Risk/*Rizik*	
Standard/*Standardni*	17 (23%)
High/*Visok*	58 (77%)
Light chain/Laki lanci	39 (52%)
Kappa/Lambda/Kapa/Lambda	36 (48%)
Renal failure/*Bubrežna insuficijencija*	
Yes/*Da*	42 (56%)
No/*Ne*	33 (44%)
Bence Jones/*Bens Džouns*	
≥2 g/daily/*dnevno*	53 (70%)
<2 g/daily/*dnevno*	22 (30%)
CRP/C-*reaktivni protein* (mg/l)	
≥6	45 (60%)
<6	30 (40%)
LDH (U/l)	
>300	24 (45%)
≤300	41 (55%)
Bone lesions/*Koštane lezije*	
Yes/*Da*	63 (84%)
No/*Ne*	12 (16%)
ISS - International Staging System/*Internacionalni sistem za određivanje stadijuma bolesti*	
I	18 (24%)
II	15 (20%)
III	41 (56%)

Table 2. Multivariate regression analysis: Influence of clinical parameters on OS and PFS
Tabela 2. *Multivarijantna analiza: uticaj kliničkih parametara na OS I PFS*

Parameters/*Parametri*	PFS	OS
Age/*Starost*	p>0,05	p<0,05
Sex (M/F)/*Pol (M/Ž)*	p>0,05	p>0,05
Clinical stage/*Klinički stadijum*	p>0,05	p>0,05
Myeloma type/*Tip mijeloma*	p- 0,05-0,1	p>0,05
Renal insufficiency/*Bubrežna slabost*	p>0,05	p>0,05
Creatinin clearance/*Klirens kreatinina*	p>0,05	p>0,05
Bence Jones protein	p>0,05	p>0,05
Platelets/*Trombociti (x10^9/l)*	p>0,05	p<0,05
CRP/*C-reaktivni protein*	p>0,05	p>0,05
LDH	p-0,05-0,1	p>0,05
Bone lesions/*Koštane lezije*	p<0,05	p>0,05
ISS - International Staging System/*Internacionalni sistem za određivanje stadijuma bolesti*	p>0,05	p>0,05
Bone marrow infiltration/*Infiltracija kostne srži*	p<0,05	p>0,05
Response to primary treatment/*Odgovor na primarnu terapiju*	p-0,05-0,1	p>0,05



Graph 1a and 1b. OS and PFS survival in patients with multiple myeloma (Autologous SCT vs Chemotherapy)
Grafikon 1a i 1b. *Ukupno preživljavanje i preživljavanje bez progresije bolesti kod pacijenata sa multiplog mijeloma (ATMĆH vs hemioterapija)*

it is more efficient if response is achieved before its implementation. The use of consolidated and maintenance therapy is carried out in accordance with disease risk parameters. Application of monoclonal antibodies is considered the treatment of future.

Aim of this study was to define prognostic factors affecting overall survival in Bence-Jones multiple myeloma patients.

Material and Methods

The trial was carried out according to a principle of a retrospective study that included patients with newly diagnosed Bence-Jones MM in the period 1995 – 2015. All patients were diagnosed on the basis of histopathological analysis of tissue samples with immunohistochemistry, based on International Myeloma Working Group (IMWG) criteria.

The patients were treated with the following chemotherapy protocols: Doxorubicin-Vincristine-Dexasone (VAD), Melphalan-Pronison (MP), Cyclophosphamide-Thalidomide-Dexasone (CTD), Thalidomide-Doxorubicin-Dexasone (TAD) and protocols with Bortezomib. Autologous hematopoietic stem cell transplantation (AHSCT) was done in 45 patients (60%), of which primary in 65%, "tandem" in 25%, and secondary in 10% patients. Allogeneic "syngeneic" hematopoietic stem cell transplantation was done in one patient. Maintenance treatment with Thalidomide was applied in 30 patients with average application from 4 to 40 months in accordance with recommendations. Interferon lasting for 6 to 12 months was done in 6 patients. Therapy response after initial therapy and upon completed treatment was assessed on the basis of standard recommendations.

Standard parametric and non-parametric tests were used in data processing: Fisher's, chi-square, Mann-Whitney test. Multivariate Cox regression analysis was applied for the inclusion of variables as prognostic parameters of treatment results in the relative risk assessment.

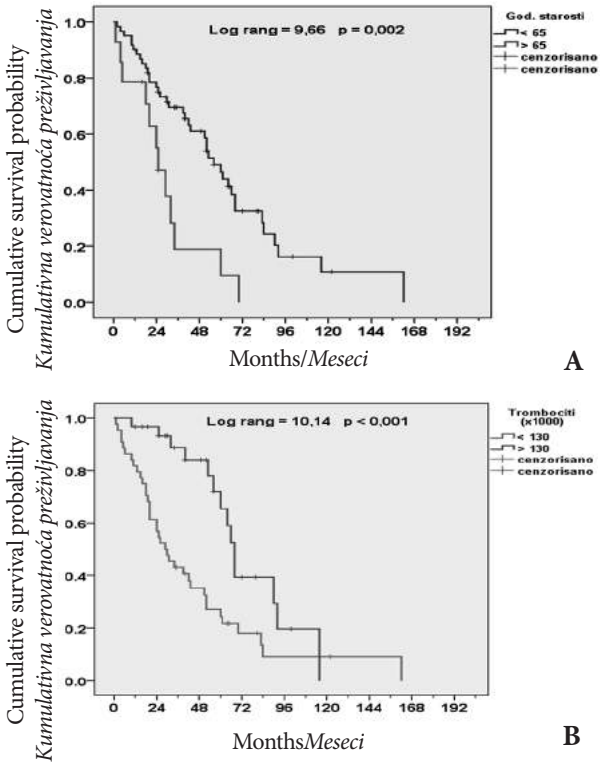
Kaplan-Meier's method was used for the purpose of patients' survival as well as a long-rank test for

the purpose of comparing survivals among tested groups. The value $p<0.05$ is used as the statistical significance criterion in all of the applied analytic methods. Overall survival was established starting from the moment of diagnosis until the time of examination or fatal outcome. Event free survival (EFS) was established starting from the moment of diagnosis until disease progression, death or break in treatment for any reason.

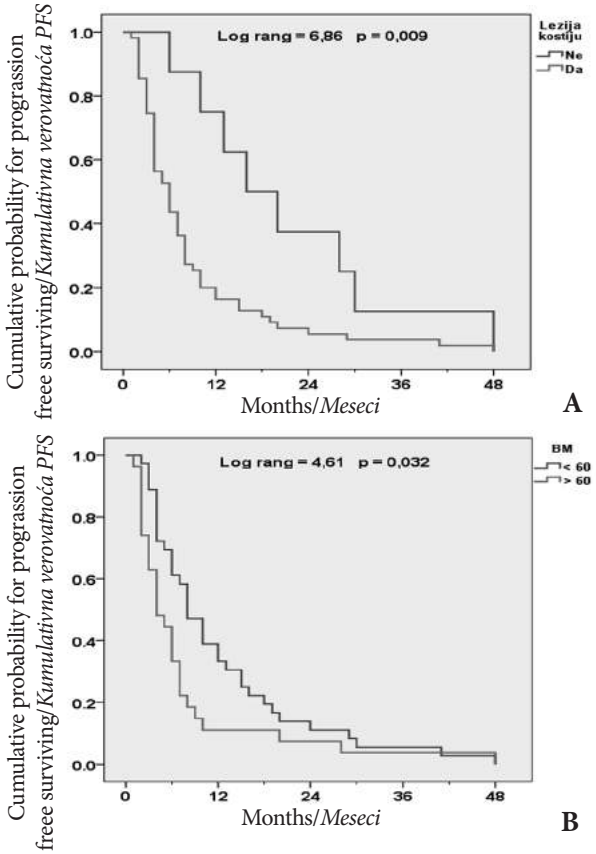
Results

This research included 75 patients with newly diagnosed Bence-Jones multiple myeloma who were treated with various chemotherapy protocols.

Patients' clinical presentations are given in **Table 1.** 54 (72%) patients responded to applied therapy, with a complete remission and a very good partial remission (CR+VGPR) achieved in 34 patients, partial response (PR) in 15 patients, molecular response (MR) in 5 patients, and resistant disease (RD) in 21 (28%) patients. Overall survival (OS) in patients with the done AHSCT amounted to 55 months, whereas in the group of patients treated with chemotherapy protocols OS amounts to 24 months. Transplantation mortality rate is low, amounting to TRM 1.5% **(Graph 1a)**. The median time until disease progression – Progression free survival (PFS) in transplant patients was 13 months while in non-transplant patients it was 7 months **(Graph 1b)**.



Graph 2a and 2b. OS according to age and platelets value in MM patients (Autologous SCT vs Chemotherapy)
Grafikon 2a i 2b. *OS u skladu sa godinama starosti i brojem trombocita kod pacijenata sa MM (ATMČ naspram hemoterapije)*



Graph 3a and 3b. Influence of bone lesions and bone marrow infiltration on PFS
Grafikon 3a i 3b. *Uticaj koštanih lezija i infiltracije srži na PFS*

In the multivariate analysis, we have concluded that OS was affected by the following parameters: age and the number of platelets **(Table 2)**. The following parameters proved to be statistically important against PFS: bone marrow infiltration and the presence of bone lesions **(Table 2)**.

With respect to the age category, patients over 65 have statistically lower survival rate against patients under 65 (p-0.002) **(Figure 2a)**. After the test we concluded that overall survival in patients was also affected by the value of the number of platelets, and the patients with the number of platelets under $130x10^9/l$, have lower survival rate against the patients with the number of platelets above $130x10^9/l$ (p-0,001) **(Graph 2b)**.

Patients with present bone lesions and bone marrow infiltration higher than 60% have statistically lower survival rate without disease progression **(Graph 3a and 3b)**.

Fatal outcome was recorded in 50 patients (66.6%), the cause of death being the primary disease in 40 patients (80%), associated diseases in 7 patients (14%) and infections in 2 patients. Fatal outcome during AHSCT was recorded in one patient.

Discussion

Light chain deposition disease or Bence-Jones myeloma is a progressive disease characterized by proliferation of plasma cells in the bone marrow and overproduction of light chain immunoglobulins (Bence-Jones proteins) in plasma and urine [2]. For the purpose of assessing disease progression, better stratification of patients diagnosed with Bence-Jones multiple myeloma according to parameters, new prognostic parameters have been established over the past few years based on biohumoral and radiographic characteristics of the disease and on cytogenetic analysis as well. Achievement of substantially higher level of complete remissions and longer survival of patients is related to the use of new drugs in clinical practice (proteasome inhibitors, IMiD, monoclonal antibodies) [1]. Despite this, the optimal treatment of patients with MM is not well defined, in part because these patients are underrepresented in clinical studies. Autologous stem cell transplantation (ASCT) after high dose melphalan chemotherapy can result in prolonged response duration and survival in patients under 65 years of age [6]. Having analyzed our group of patients we have concluded that patients with ASCT carried out have longer Overall survival (OS) and Progression-free survival (PFS) compared to patients treated with standard chemotherapeutic protocols **(Graph 1a and 1b)**. OS is also affected by patients' age and the value of platelets. Namely, we have concluded that patients over 60 and with the number of platelets under $130x10^9$/l, are more likely for lower overall survival OS **(Graph 2a and 2b).** A research by the Nordic Myeloma Study group indicated that survival was affected by beta 2 microglobulin values and patients' age.

We have concluded that patients with bone lesions and bone marrow infiltration higher than 60% have less time until disease progression (PFS), but without affecting OS **(Table 2, Graph 3a and 3b)**.

Recent results of published studies indicated significant impact of the number of platelets, LDH and CPR values at the moment of diagnosis on the outcome of the treatment of patients with MM. Also it was verified that low MPV (mean platelet volume) values represent unfavorable prognostic parameter for overall survival in patients with MM.

Conclusion

Our study showed autologous stem cell transplantation is a more effective method of treatment of patients with Bence-Jones myeloma compared to the conventional chemotherapy. Overall survival and progression free survival are affected by the age, the number of platelets, bone lesions and marrow infiltration. It is necessary in the era of new treatment approaches to find additional prognostic parameters for the purpose of projecting survival rate in Bence-Jones multiple myeloma patients.

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MATCHED UNRELATED DONOR HEMATOPOIETIC STEM CELL TRANSPLANTATION - OUR RESULTS

TRANSPLANTACIJA MATIČNIH ĆELIJA HEMATOPOEZE OD NESRODNOG DAVAOCA - NAŠI REZULTATI

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Summary

Introduction. Allogeneic stem-cell transplantation is only potentially curative therapy for variety of hematology malignancies, such as acute and chronic leukemia, myelodisplastic syndrome and aplastic anemia, but also promising treatment option for other disorders. If we know that only 25% of patients have an human leukocyte antigen identical sibling donor, it is obvious that matched unrelated donor hematopoietic stem cell transplantation is an alternative for the rest of the patients. **Material and Methods.** Since 2013, matched unrelated donor hematopoietic stem cell transplantation has been performed routinely in the Military Medical Academy. **Results**. We hereby present the outcome after 77 procedures in 75 patients. Considering primary diseases, 35 patients had acute myeloid leukemia, 25 patients had acute lymphoid leukemia, 5 patients had chronic myeloid leukemia, 9 patients had myelodisplastic syndrome and we performed the transplant on 1 patient with chronic lymphocyte leukemia, 1 patient with aplastic anemia and 1 patient with T lymphoblastic lymphoma. **Conclusion**. It is difficult to make clear conclusions based on this heterogeneous group of patients, but it seems that these results are encouraging. Future research will be performed to evaluate matched unrelated donor and identical sibling hematopoietic stem cell transplantation in the homogenous groups with respect to primary diseases.

Key words: Hematopoietic Stem Cell Transplantation; Unrelated Donors; Treatment Outcome; HLA Antigens; Alleles

Sažetak

Uvod. Alogena transplantacija matičnih ćelija hematopoeze predstavlja jedinu kurativnu metodu za lečenje brojnih hematoloških maligniteta, uključujući akutne i hronične leukemije, mijelodisplastični sindrom i aplastičnu anemiju, ali je i ohrabrujući izbor u brojnim drugim oboljenjima. Poznato je da samo 25% pacijenata ima humani leukocitni antigen identičnog podudarnog davaoca, zbog čega transplantacija matičnih ćelija hematopoeze od podudarnog nesrodnog davaoca predstavlja alternativni pristup za mnoge bolesnike sa hematološkim bolestima. **Materijal i metode.** Transplantacija matičnih ćelija hematopoeze od podudarnog nesrodnog davaoca sprovodi se od 2013. godine na Vojnomedicinskoj akademiji u Beogradu. **Rezultati**. U našem radu ćemo prikazati rezultate pomenute metode kod 75 pacijenata. U odnosu na osnovnu bolest, 35 pacijenata je imalo akutnu mijeloidnu leukemiju, 25 pacijenata akutnu limfoblastnu leukemiju, pet pacijenata hroničnu mijeloidnu leukemiju, devet pacijenata mijelodisplastični sindrom, jedan pacijent hroničnu limfocitnu leukemiju, jedan pacijent aplastičnu anemiju i jedan pacijent T-limfoblastni limfom. **Zaključak**. Na osnovu naših rezultata teško je izvući konkretan zaključak na osnovu pacijenata koji predstavljaju heterogenu grupu. S obzirom na obećavajuće rezultate, dalje istraživanje bi trebalo usmeriti na efikasnost transplantacije od srodnog i nesrodnog podudarnog davaoca u odnosu na primarno oboljenje, odnosno neophodno je homogenizovati grupe ispitanika.

Ključne reči: transplantacija hematopoetskih stem ćelija; nesrodni donori; ishod lečenja; HLA antigeni; aleli

Introduction

We are witnesses of an enormous expansion of all sorts of stem cell transplantations in the treatment of various malignant and non-malignant, hematological and non-hematological diseases.

Allogeneic stem-cell transplantation is only potentially curative therapy for variety of hematology malignancies, such as acute and chronic leukemia, myelodisplastic syndrome and aplastic anemia, but also promising treatment option for other disorders. If we know that only 25% of patients have an HLA (human leukocyte antigen) identical sibling donor, it is obvious that matched unrelated donor (MUD) hematopoietic stem cell transplantation (HSCT) is an alternative for the rest of the patients [1]. The very first one was performed in 1973 in New York for the treatment of a boy with congenital immunodeficiency and now we are facing around 10000 MUD transplants worldwide annually. Currently, around two thirds of all allogeneic HSCT are performed from unrelated donors [2]. More importantly, MUD HSCT has been improved and now

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Abbreviations

HSCT	– hematopoietic stem cell transplantation
MUD	– matched unrelated donors
GvHD	– Graft versus host disease
Bu/Cy	– Busulfan/Cycloposphamide
Flu/Mel	– Fludarabine/Melphalan
Bu/Flu+ATG	– Busulfan/Fludarabine+ Antithymocyte globuline
CMV	– cytomegalovirus
RIC	– reduced intensity conditioning
ANC	– absolute neutrophil count
TRM	– transplant related mortality

we could say that outcomes are equal with those after sibling transplantations. Many advances in MUD HSCT have occurred in the previous two decades. Improvements were achieved due to molecular-based HLA typing, conditioning regimens, prevention of graft-versus-host disease (GvHD) and better supportive care. The primary factor that contributes to better outcome is the level of donor to recipient HLA matching [3]. The overall prognosis with HSCT from donors selected with high resolution (allele level) fully HLA matched at HLA A, B, C and DRB1, preferably young male donors, is excellent and generally equivalent to that of an HLA identical sibling. If a 10/10 allele matched donor is not available, then single mismatches at HLA B or HLA C loci appear to be better than HLA A or HLA DRB1 mismatched. Each additional mismatch is connected with 9-10% reduction in overall survival [4].

Since 2013, MUD HSCT has been performed routinely in the Military Medical Academy. We hereby present the outcome after 77 procedures in 75 patients (pts).

Material and Methods

Median age in our cohort of patients (pts) is 33 (19-57 years), male/ female ratio was 41/ 34. Considering primary diseases, 35 pts had acute myeloid leukemia (AML), 25 pts had acute myeloid leukemia (ALL), 5 pts had chronic myeloid leukemia (CML), 9 pts had myelodisplastic syndrome (MDS) and we performed the transplant on 1 pt with chronic lymphocytic leukemia (CLL), 1 pt with aplastic anemia (AA) and 1 pt with T lymphoblastic lymphoma. Conditioning was myeloablative combination of Busulphane and Cyclophosphamide in 72 cases (93.5%), while 5 pts (6.5%) received reduced conditioning adjusted to the nature of the primary disease and comorbidity status. RIC (FluMel, BuFlu + ATG and FLAMSA-RIC) was given in two cases of secondary HSCT and in pts with CLL, AA and one pt with MDS. Donor search was performed according to valid recommendations based on HLA, ABO, CMV, age and sex of the recipient and donor status. Fully matched 10/10 HLA identical donors were found in 58 pts (75.32%), while in 19 pts (24.67%) MUD HSCT was done with one HLA mismatch 9/10. Peripheral blood was the source of stem cells in 72 cases (93.5%), while 5 pts (6.5%) received stem cells originated from bone marrow. Prevention of GvHD was a combination of ATG (rabbit), Cyclosporine and Methotrexate ("short" course) in 72 pts (93.5%) and those 5 pts with RIC (6.5%) received ATG, Cyclosporine and Mycophenolate Mofetil. Standard of care for all pts implies antimicrobial prophylaxis which consisted of acyclovir, fluconazole, trimethoprim sulphometoxasol and quinolones alternately given for three days and prevention of parenchymal toxicity and mucositis **(Tables 1 and 2)**.

Results

Engraftment according to recommended neutrophil and platelets recovery was obtained in 69 pts (89.61%). Eight pts (10.38%) died in the pre-engraftment period, 7 pts due to sepsis and 1 pt with massive intracranial hemorrhage. Chimerism of marrow cells was routinely determined after 1, 3, 6, 9, 12, 18 and 24 months after MUD HSCT in order to document engraftment and afterward for estimation of remission and for that purpose we use molecular analysis, cytogenetics in the cases of sex mismatched transplantations and ABO in the cases of ABO mismatched transplants. Acute GvHD in our group was 27.27% (21 pts), grade 1-2 had 13 pts (16,88%), while 8 pts (10.39%) had aGvHD grade 3-4. From those pts who are monitored for more than 3 months after MUD HSCT, 24 pts (32,87%) developed chronic GvHD. None of them had severe form of cGvHD, moderate form was diagnosed in 18 pts (24.65%) and mild form in 6 pts (8.22%). Sinusoidal obstruction syndrome, previously known

Table 1. Patients characteristics
Tabela 1. *Karakteristike pacijenata*

Parameter/*Parametar*	N = 75
Age (years)/*Starost (godine)*	33 (19-57)
Sex (male/ female)/*Pol (muški/ženski)*	41/34
Diagnosis/*Dijagnoza*	
Acute myleoid leukemia (AML)/*Akutna mijeloidna leukemija (AML)*	35
Acute lymphoid leukemia (ALL)/*Akutna limfoidna leukemija (ALL)*	25
Myelodisplastic syndrome (MDS)/*Mijelodisplastični sindrom (MDS)*	9
Chronic myeloid leukemia (CML)/*Hronična mijeloidna leukemija (HML)*	5
Chronic lymphocytic leukemia (CLL)/*Hronična limfocitna leukemija (HLL)*	1
Aplastic anemia (AA)/*Aplastična anemija (AA)*	1
T lymphoblastic lymphoma/*T limfoblastni limfom*	1

Table 2. Hematopoietic stem cell transplantation characteristics
Tabela 2. *Karakteristike transplantacije matičnih ćelija hematopoeze*

Parameter/*Parametar*	N= 77
Conditioning/*Kondicioni režim*	
BuCy2	72 (93,5%)
Other/*Drugo*	5 (6,5%)
HLA compatibility/*HLA kompatibilnost*	
10/10	58 (75,32%)
9/10	19 (24,67%)
Stem cells source/*Izvor matičnih ćelija*	
Peripheral blood/*Periferna krv*	72 (93,5%)
Bone marrow/*Koštana srž*	5 (6,5%)
GvHD prophylaxis/*GvHD profilaksa*	
ATG + CsA + MTX	72 (93,5%)
Other/*Drugo*	5 (6,5%)

Table 3. Results
Tabela 3. *Rezultati*

Parameter/*Parametar*	N = 77
Engraftment (days after MUD HSCT)/*Engraftment (dani nakon TMĆH)*	
ANC > 500/mcl/*ABN> 500/mcl*	+21 (16-26)
Platelets > 20 x 10^9/l/*Trombociti> 20 x 10^9/l*	+19 (11-31)
Acute GvHD/*Akutni GvHD*	21 (27,27%)
Grade 1-2/*Gradus 1-2*	13 (16,88%)
Grade 3-4/*Gradus 3-4*	8 (10,39%)
Chronic GvHD/*Hronični GvHD*	24 (32,87%)
Mild/*Blag*	6 (8,22%)
Moderate/*Srednji*	18 (24,65%)
Severe/*Težak*	Ø
TRM/*Mortalitet vezan za transplantaciju*	9 (11,68%)
Sepsis/*Sepsa*	7 (9,09%)
Hemorrhage/*Krvarenja*	1 (1,29%)
Veno occlusive disease/*Venookluzivna bolest*	1 (1,29%)

as veno occlusive disease (VOD), was diagnosed according to clinical criteria in only one pt (1.29%). Relapses in our group of 69 pts with documented engraftment occurred in 16 pts (23.18%). Median time to relapse was 7.6 months (from 2 to 24 months). At follow-up from 1 to 56 months, 44 pts (57.14%) are still alive **(Table 3)**.

Conclusion

It is difficult to make clear conclusions based on this heterogeneous group of pts, but it seems that these results are encouraging. Future research will be performed to evaluate matched unrelated donors and identical sibling hematopoietic stem cell transplantation in the homogenous groups with respect to primary diseases.

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Radovi u časopisima

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∗ Kompjuterska datoteka

Hemodynamics III: the ups and downs of hemodynamics [computer program]. Version 2.2. Orlando (FL): Computerized Educational Systems; 1993.

5. Prilozi (tabele, grafikoni, sheme i slike)

BROJ PRILOGA NE SME BITI VEĆI OD ŠEST!

Tabele, grafikoni, sheme i slike se postavljaju kao posebni dokumenti.

– Tabele i grafikone bi trebalo pripremiti u formatu koji je kompatibilan programu u kojem je napisan tekst rada. Slike bi trebalo poslati u jednom od sledećih oblika: *JPG*, *GIF*, *TIFF*, *EPS*.

– Svaki prilog mora biti obeležen arapskim brojem prema redosledu po kojem se navodi u tekstu rada.

– Naslovi, tekst u tabelama, grafikonima, shemama i legende slika bi trebalo da budu napisani na srpskom i engleskom jeziku.

– Nestandardne priloge označiti u fusnoti uz korišćenje sledećih simbola: *, †, ‡, §, | |, ¶, **, † †, ‡ ‡ .

– U legendi slika trebalo bi napisati korišćeno uveličanje okulara i objektiva mikroskopa. Svaka fotografija treba da ima vidljivu skalu.

– Ako su tabele, grafikoni, sheme ili slike već objavljene, navesti originalni izvor i priložiti pisano odobrenje autora za njihovo korišćenje.

– Svi prilozi će biti štampani kao crno-bele slike. Ukoliko autori žele da se prilozi štampaju u boji, obavezno treba da plate dodatne troškove.

6. Dodatne obaveze

AUTORI I SVI KOAUTORI RADA OBAVEZNO TREBA DA PLATE GODIŠNJU PRETPLATU ZA ČASOPIS *MEDICINSKI PREGLED*. U PROTIVNOM, RAD NEĆE BITI ŠTAMPAN U ČASOPISU.

INFORMATION FOR AUTHORS

Medical Review publishes papers (previously neither published in nor submitted to any other journals) from various fields of biomedicine intended for broad circles of doctors.

Since January 1th, 2013 the Medical Review has been using the service e-Ur: Electronic Journal Editing. All users of the Registration system, i.e. authors, reviewers, and editors have to be registered users with only one e-mail address. Registration should be made on the web address:

http://aseestant.ceon.rs/index.php/medpreg/user/register.

Manuscript submission should be made on the web address:

http://aseestant.ceon.rs/index.php/medpreg/

A SUPPLEMENTARY FILE, WITH THE STATEMENT THAT THE PAPER HAS NOT BEEN SUBMITTED OR ACCEPTED FOR PUBLICATION ELSEWHERE AND A CONSENT SIGNED BY ALL AUTHORS, HAVE TO BE ENCLOSED WITH THE MANUSCRIPT.

Authors may not send the same manuscript to more than one journal concurrently. If this occurs, the Editor may return the paper without reviewing it, reject the paper, contact the Editor of the other journal(s) in question and/or contact the author's employers.

Papers should be written in English language, with an abstract and title page in English, as well as in Serbian language.

All papers submitted to ***Medical Review*** are seen by one or more members of the Editorial Board. Suitable articles are sent to at least two experts to be reviewed, thier reports are returned to the assigned member of the Editorial Board and the Editor. Revision of an article gives no guarantee of acceptance and in some cases revised articles are rejected if the improvements are not sufficient or new issues have arisen. Material submitted to *the Journal* remains confidential while being reviewed and peer-reviewers' identities are protected unless they elect to lose anonymity.

Medical Review publishes the following types of articles: editorials, original studies, preliminary reports, review articles, professional articles, case reports, articles from history of medicine and other types of publications.

1. Editorials – up to 5 pages – convey opinions or discussions on a subject relevant for the Journal. Editorials are commonly written by one author by invitation.

2. Original studies – up to 12 pages – present the authors' own investigations and their interpretations. They should contain data which could be the basis to check the obtained results and reproduce the investigative procedure.

3. Review articles – up to 10 pages – provide a condensed, comprehensive and critical review of a problem on the basis of the published material being analyzed and discussed, reflecting the current situation in one area of research. Papers of this type will be accepted for publication provided that the authors confirm their expertise in the relevant area by citing at least 5 self-citations.

4. Preliminary reports – up to 4 pages – contain scientific results of significant importance requiring urgent publishing; however, it need not provide detailed description for repeating the obtained results. It presents new scientific data without a detailed explanation of methods and results. It contains all parts of an original study in an abridged form.

5. Professional articles – up to 10 pages – examine or reproduce previous investigation and represent a valuable source of knowledge and adaption of original investigations for the needs of current science and practice.

6. Case reports – up to 6 pages – deal with rare casuistry from practice important for doctors in direct charge of patients and are similar to professional articles. They emphasize unusual characteristics and course of a disease, unexpected reactions to a therapy, application of new diagnostic procedures and describe a rare or new disease.

7. History of medicine – up to 10 pages – deals with history with the aim of providing continuity of medical and health care culture. They have the character of professional articles.

8. Other types of publications – The journal also publishes feuilletons, book reviews, extracts from foreign literature, reports from congresses and professional meetings, communications on activities of certain medical institutions, branches and sections, announcements of the Editorial Board, letters to the Editorial Board, novelties in medicine, questions and answers, professional and vocational news and In memoriam.

Preparation of the manuscript

The complete manuscript, including the text, all supplementary material and covering letter, is to be sent to the web address above.

The covering letter:

– It must contain the proof given by the author that the paper represents an original work that it has neither been previously published in other journals nor is under consideration to be published in other journals.

– It must confirm that all the authors meet criteria set for the authorship of the paper, that they agree completely with the text and that there is no conflict of interest.

– It must state the type of the paper submitted (an original study, a review article, a preliminary report, a professional article, a case report, history of medicine).

The manuscript:

General instructions.

Use Microsoft Word for Windows to type the text. The text must be typed in font *Times New Roman*, page format A4, space 1.5 (for tables as well), margins set to 2.5 cm and font size 12pt. All measurements should be reported in the metric system of the International System of Units – SI. Temperature should be expressed in Celsius degrees (°C) and pressure in mmHg.

The manuscript should contain the following elements:

1. The title page.

The title page should contain a concise and clear title of the paper, without abbreviations, then a short title (up to 40 characters), full names and surnames of the authors (not more than 6) indexed by numbers corresponding to those given in the heading along with the full name and place of the institutions they work for. Contact information including the academic degree(s), full address, e-mail and number of phone or fax of the corresponding author (the author responsible for correspondence) are to be given at the bottom of this page.

2. Summary.

The summary should contain up to 250 words, without abbreviations, with the precise review of problems, objectives, methods, important results and conclusions. It should be structured into the paragraphs as follows:

– Original and professional papers should have the introduction (with the objective of the paper), materials and methods, results and conclusion

– Case reports should have the introduction, case report and conclusion

– Review papers should have the introduction, subtitles corresponding to those in the paper and conclusion.

The authors should provide up to 10 keywords below the summary. These keywords will assist indexers in cross-indexing the article and will be published with the summary, but the authors' keywords could be changed in accordance with the list of Medical Subject Headings, MeSH of the American National Medical Library.

The summary should be written in both languages, English as well as Serbian. The summary in Serbian language should be the translation of the summary in English; therefore, it has to contain the same paragraphs.

3. The text of the paper.

The text of original studies must contain the following: introduction (with the clearly defined objective of the study), materials and methods, results, discussion, conclusion, list of abbreviations (if used in the text) and not necessarily, the acknowledgment mentioning those who have helped in the investigation and preparation of the paper.

The text of a case report should contain the following: introduction (with clearly defined objective of the study), case report, discussion and conclusion.

Introduction contains clearly defined problem dealt with in the study (its nature and importance), with the relevant references and clearly defined objective of the investigation and hypothesis.

Materials and methods should contain data on design of the study (prospective/retrospective, eligibility and exclusion criteria, duration, demographic data, follow-up period). Statistical methods applied should be clear and described in details.

Results give a detailed review of data obtained during the study. All tables, graphs, schemes and figures must be cited in the text and numbered consecutively in the order of their first citation in the text.

Discussion should be concise and clear, interpreting the basic findings of the study in comparison with the results of relevant studies published in international and national literature. It should be stated whether the hypothesis has been confirmed or denied. Merits and demerits of the study should be mentioned.

Conclusion must deny or confirm the attitude towards the 0based solely on the author's own results, corroborating them. Avoid generalized and unnecessary conclusions. Conclusions in the text must be in accordance with those given in the summary.

4. References are to be given in the text under Arabic numerals in parentheses consecutively in the order of their first citation. Avoid a large number of citations in the text. The title of journals should be abbreviated according to the style used in Index Medicus (http://www.nlm.nih.gov/tsd/serials/lji.html). Apply Vancouver Group's Criteria, which define the order of data and punctuation marks separating them. Examples of correct forms of references are given below. List all authors, but if the number exceeds six, give the names of six authors followed by 'et al'.

Articles in journals

∗ *A standard article*

Ginsberg JS, Bates SM. Management of venous thromboembolism during pregnancy. J Thromb Haemost 2003;1:1435-42.

∗ *An organization as the author*

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension 2002;40(5):679-86.

∗ *No author given*

21st century heart solution may have a sting in the tail. BMJ. 2002;325(7357):184.

∗ *A volume with supplement*

Magni F, Rossoni G, Berti F. BN-52021 protects guinea pig from heart anaphylaxix. Pharmacol Res Commun 1988;20 Suppl 5:75-8.

∗ *An issue with supplement*

Gardos G, Cole JO, Haskell D, Marby D, Pame SS, Moore P. The natural history of tardive dyskinesia. J Clin Psychopharmacol 1988;8(4 Suppl):31S-37S.

∗ *A summary in a journal*

Fuhrman SA, Joiner KA. Binding of the third component of complement C3 by Toxoplasma gondi [abstract]. Clin Res 1987;35:475A.

Books and other monographs

∗ *One or more authors*

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

∗ *Editor(s) as author(s)*

Danset J, Colombani J, eds. Histocompatibility testing 1972. Copenhagen: Munksgaard, 1973:12-8.

∗ *A chapter in a book*

Weinstein L, Shwartz MN. Pathologic properties of invading microorganisms. In: Soderman WA Jr, Soderman WA, eds. Pathologic physiology: mechanisms of disease. Philadelphia: Saunders; 1974. p. 457-72.

∗ *A conference paper*

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

∗ *A dissertation and theses*

Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [dissertation]. Mount Pleasant (MI): Central Michigan University; 2002.

Electronic material

∗ *A journal article in electronic format*

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 1 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htmArticle

∗ *Monographs in electronic format*

CDI, clinical dermatology illustrated [monograph on CD-ROM]. Reevs JRT, Maibach H. CMEA Multimedia Group, producers. 2nd ed. Version 2.0. San Diego:CMEA;1995.

∗ *A computer file*

Hemodynamics III: the ups and downs of hemodynamics [computer program]. Version 2.2. Orlando (FL): Computerized Educational Systems; 1993.

5. Attachments (tables, graphs, schemes and photographs).

THE MAXIMUM NUMBER OF ATTACHMENTS ALLOWED IS SIX!

– Tables, graphs, schemes and photographs are to be submitted as separate documents, on separate pages.

– Tables and graphs are to be prepared in the format compatible with Microsoft Word for Windows programme. Photographs are to be prepared in JPG, GIF, TIFF, EPS or similar format.

– Each attachment must be numbered by Arabic numerals consecutively in the order of their appearance in the text

– The title, text in tables, graphs, schemes and legends must be given in both Serbian and English languages.

– Explain all non-standard abbreviations in footnotes using the following symbols *, †, ‡, §, | |, ¶, **, † †, ‡ ‡ .

– State the type of color used and microscope magnification in the legends of photomicrographs. Photomicrographs should have internal scale markers.

– If a table, graph, scheme or figure has been previously published, acknowledge the original source and submit written permission from the copyright holder to reproduce it.

– All attachments will be printed in black and white. If the authors wish to have the attachments in color, they will have to pay additional cost.

6. Additional requirements

SHOULD THE AUTHOR AND ALL CO-AUTHORS FAIL TO PAY THE SUBSCRIPTION FOR MEDICAL REVIEW, THEIR PAPER WILL NOT BE PUBLISHED.