Vol. 44 1-2 2018. ISSN 0352-194X

ČASOPIS UDRUŽENJA ANESTEZISTA REANIMATORA I TRANSFUZISTA SRBIJE

# ANESTEZIJA REANIMACIJA TRANSFUZIJA

B E O G R A D
2 0 1 8

**Vol. 44 1-2 2018. ANESTEZIJA, REANIMACIJA, TRANSFUZ IJA ISSN 0352-194X**

**Časopis Udruženja anestezista, reanimatora i transfuzista Srbije**



**Časopis izlazi kao dvobroj:** Tiraž : 350

**Štampa:** ACTION PRO , Beograd

**Predsednik Udruženja:** Draga Udovica

**Izdaje:** Udruženje anestezista, reanimatora i transfuzista Srbije
Beograd, Svetog Save 39

# S A D R Ž A J


194
Београд


СРБИЈА
ВОЈНОМЕДИЦИНСКА АКАДЕМИЈА • БЕОГРАД
1844


INSTITUT ZA TRANSFUZIJU KRVI SRBIJE
BEOGRAD


КМСЗТС
2006

# APHERETIC CYTOREDUCTION: THROMBOCYTODEPLETION – A CRITICAL REVIEW AND OUR APPROACHES/EXPERIENCE

**Bela Balint[1,2,3,4], Milena Todorovic[5,6], Gordana Ostojic[1,2], Dusan Vucetic[1,2], Vanda Balint[7], Mirjana Pavlovic[8]**

[1]Institute for Transfusiology and Hemobiology, MMA, Belgrade, Serbia;
[2]Institute for Medical Research, University of Belgrade, Serbia;
[3]Serbian Academy of Sciences and Arts;
[4]Faculty of Medicine of Military Medical Academy, University of Defence, Belgrade, Serbia;
[5]Clinic for Hematology, Clinical Center of Serbia, Belgrade, Serbia;
[6]Faculty of Medicine, University of Belgrade, Serbia;
[7]Faculty of Molecular Biology, University of Belgrade, Serbia;
[8]Department of Computer and Electrical Engineering and Computer Science, FAU, FL, USA.

## Summary

*Beside supportive transfusions, hemotherapy involves also performance of apheretic procedures due to removal of accumulated (extreme) quantities and/or replacement of pathologically changed blood constituents (cells and plasma contents, pathogens, metabolic products). Hemotherapy also involves collection/application of stem cells (SCs) with a goal of reconstitution of affected hematopoiesis by conventional transplantation within the frame of regenerative medicine. Thus, the basic application of hemotherapy is reconstitution and maintenance of the hemobiological equilibrium (homeostasis) by supplementation of content/components in deficiency, through performance of apheretic and other "hemo-modulation-related" procedures. By doing this, the influence upon the volume is created, but also the blood capacity for binding/transport of the oxygen, hemostasis, and activity of mediators of immune response. The rational and timing-related chemo therapy is contributing to increased survival of the patients with certain diseases.*

*During the application of hemotherapy, it is necessary to perform the most reliable intervention in the most appropriate manner: optimization of indications, a real-time choice of the most efficient version and the therapeutic protocol. Thus, to the patient Hemotherapy - in which is realistic to expect satisfactory clinical response - is needed to approach as early as possible. The application of the most efficient therapeutic modalities, in the most appropriate manner means at the same time consideration of today's all approachable therapeutic procedures and techniques, e.g. medical culture, which enabled Hemotherapy to such a high degree of complexity and efficiency today. Also, the most sensitive point during planning and performing of therapeutic Apheretic procedures, especially cytapheresis is determination of the most optimal approach to the treatment – or even better, "time-frequency-intensity-continuum" of the procedure. The introduction of cytapheresis (among others), requires determination of total quantity of blood cells within organism, sometimeseven sometimes determination of the ratio between cells in extra - and intra-vascular space (for example, the reserve*

*in hematopoietic tissues), as well as the estimation of the intensity of cell production (kinetics of cytogenesis) in periapheretic period.*
*This paper will treat briefly the knowledge linked to apheretic therapy (without description of procedures for plasma exchange) obtained within period 1981-2017 with particular accent upon apheretic cytoreduction or thrombocytodepletion – and with a short presentation of our own results as well as comparison of efficiency different versions and generations of the blood cell separators of with discontinued and continued blood flow (Hemonetics M-30, IBM-2997, Cobe–Spectra and Spectra-Optia) during performance of therapeutic thrombocytapheresis.*

**Key words:** Hemotherapy, cytapheresis, plateletapheresis, thrombocytodepletion.

## Apheresis – general considerations

Apheresis is the exfusion of the entire blood, its separation into components, removal/collection of some constituents and reinfusion back into circulation of remaining and/or "reconstituted" part of the blood. From therapeutic point of view, they can be performed with the goal to: 1) collect particular blood constituents in the concentrated form (donor apheresis) and 2) due to achievement of the certain therapeutic effect in the patients in whose circulation there is a "pathogenic substrate" or extreme quantities of some blood constituents (therapeutic apheresis). Based upon the criterion of what was the removed substrate, it is possible to differentiate them into plasmapheresis or therapeutic plasma exchange (TPE) and cytaphereses (1–5).
During the performances of specific apheretic procedures, the patient is reinfused with his own (autologous) "cleaned" plasma or blood cells after *ex vivo* modulation of their functions. Thus, by using of these TPE procedures the selective reduction of pathogen is achieved – for example, lipoproteins of small density (LDL-apheresis) or auto - or autoantibodies by means of extracorporeal immunoadsorption (i.e. TPE by ECIA). Selective cytaphereses also involve photopheresis, as well as adsorption granulocytapheresis. Photopheresis is cytapheresis approach of selective separation of mononuclear cells (MNC), along with their *ex vivo* inactivation by ultraviolet light A (UVA) and use of the psoralens (PUVA) or some other "photosensitizer" substance. With respect to the fact that granulocytes in the tissues may result with damages (*in situ* release of immunoinflamatory cytokines and different mediators), their removal from circulation is applied by selective cytapheresis – e.g. "adsorption granloctapheresis" (4–14).
At a larger sense of the word, apheretic immunomodulation is possible to be achieved by removal some of normal constituents or pathogenic substrate from plasma (by mentioned TPE procedures), e.g. by reduction of the number and/or by changing percentage of particular types of cells in the patient blood, but also my *ex vivo* modulation of their activity, followed by reinfusion of such changed blood. It is necessary to adjust the definition of such introduced methods – such, therapeutic apheresis involves excursion of patient′s blood ex *vivo* modulation of its content and/or come functions and (re) infusion of thus "elaborated" (*ex vivo* manipulated) blood. These therapeutic procedures are designated as "extracorporeal immunomodulation" (5, 10, 11). Similar therapeutic effects, was earlier possible to achieve only by applying corticosteroids or

immunosuppressive, eventually immunostulatory medicaments.

By collecting stem cells (SC) with apheresis, some potential side effects of general anesthesia were avoided: the taking of the bone marrow (BM) cells by, multiple aspirations, inevitability of autologous transfusions due to support of the removed red blood cells (RBCs) – large aspirate volume. Not only that apheresis of SC supplies with a superior number of SCs for transplants, but it also substantially reduces the volume of cell suspension, along with smaller percentage participation of RBCs (important for AB0-incompatible transplants and cryoconservation of SCs) (15–18).

As far as of a short historical review on apheresis as is concerned, it is necessary to say that about the separation of the plasma in animals, as well as reinfusion of the remaining part of the blood the first time was reported in 1941 – this approach is designated as "plasma-pheresis" (3). In humans, the beneficial effect of TPE in curing patients with macroglobulinemia and serum-hyperviscosity syndrome was described in 1960 (4). A year after that, there were reports about beneficial effects of cytapheresis in patients with leukemia (5). From the middle seventies, TPE is performed by using separators of the blood cells in the treatment of patients with Good pasture syndrome and myasthenia gravis (3–5). In our country the first TPE was performed in MMA 1966 (19) within the treatment of the patients with multiple myeloma and serum-hyperviscosity syndrome, while the treatment with therapeutic cytapheresis started from 1971 – first time in the treatment of pregnancy with hyperleukocyte leukostasis (in collaboration of CCS and MMA) (20).

It is important to emphasize that apheresis (especially therapeutic procedures) are aggressive methods, by which there is a direct inte--rvention in circulation of patients and therefore they might be followed by side effects. In order to minimize them, the special criteria for choice of donors and patients are applied, and if necessary, the follow up of certain biological parameters is performed (such as hemogram – i.e. complete blood count examination – bioche mistry, screening tests for coagulation), along with clinical follow up of the individuals for which the apheretic treatment is predicted. The performance of the apheresis, especially therapeutic procedures, is not possible without good understanding of hemobiology, physiology, and disturbances in the blood and blood stream, fundaments of immunology, biochemistry, hemorheology and cardiopulmonal reanimation (1–5).

The condition for successful performance of apheresis is also proficiency of the team members for the work with extracorporeal circulation and manipulation with entire modern equipment. For that reason, therapeutic apheresis is applied only in medical centers with tertiary level of health protection (in the countries with developed medical service).

**Purpose of the therapeutic apheresis**

Therapeutic apheresis is a procedure by which is possible to eliminate the part of patient's blood (plasma or its specific content, and/or blood cells) which is changed (qualitatively/quantitatively) or in which there is some "pathogenic substrate". During performance of standard or conventional apheresis, the essential goal is the achievement of the decrease of the total content of "pathogen" in circulateon by using non-selective removal of plasma or blood cells to the level which would not further have damaging effects upon the course of disease. The mechanism of actions of this apheresis could be achieved by the change in ratio of antigen and antibody, by modification of acti-vity of the specific mediators/modulators of inflammation, by "deblocking" of mononucle-ar-phagocytic system (MNFS), by decrease of extreme quantities of blood cells

and by improvement of rheological features of the blood. There are specific TPE, the target of which is the support of that constitutes of patient's plasma which is deficient in the blood – the replacement liquid is allogeneic plasma (1–5).

By means of therapeutic cytaphereses the extreme quantities of blood or pathologically altered blood cells are removed from patient's circulation with intention to reduce their number in organism and prevent or tune signs and symptoms cause by particular cytemias. It is about the cure of the patient with accumulation of particular varieties of blood cells. Relative to the type of blood cells which are to be removed, therapeutic cytapheresis can be classified into: leukapheresis, thrombocytapheresis or plateletapheresis, erythrocytapheresis and RBC-exchange.(6, 9).

Briefly, the goal of leukaphereses is the reduction of the number of leukocytes in the, prevention and/or cure of the hyperleukocyte leukostasis syndrome, regression of organo- and lymphadenomegaly and improvement in patient's general status. It is induced when the number of leukocytes in the circulations is $\geq 150x10^9$/L, followed by manifested leukostasis syndrome. It is worth to emphasize that leukapheresis presents an efficient "cytoreductive" therapy during leukostasis crisis, however, they have no influence upon the course and outcome of leukemia. By usingthrombocytapheresis, the intention is to achieve to reduce the increased number of circ-ulating thrombocytes in the case of over production (prevention of the "thrombohemorrhagic syndrome"). Thrombocytaphereses are indicated when the number of thrombocytes is $\geq 1500x10^9$/L of peripheral blood – although the degree of thrombocytosis and the difficulty of clinical status of patient do not have always to be causally connected. Beside removal of the excess of RBCs from the patient's blood and normalization of hematocrit, by erythrocytapheresis it is possible to reach hematological recovery. (reduction of "cellular-hyperviskosity") with consecutive improvement and intensification of tissue perfusion/oxygenation. Specifically, and relatively new type of apheresis is the RBC - exchange. It is applied in patients with pathologically altered RBCs and consecutive hemorheological abnormalities, anemic syndrome and other disturbances (4–6, 9, 14).

In the case of hematological disturbances with excessive number of leukemic cells in the circulation, their removal by cytapheresis (leukapheresis) would have been adequate to "partial excision" of tumor mass even over one kg mass (Figure 1).

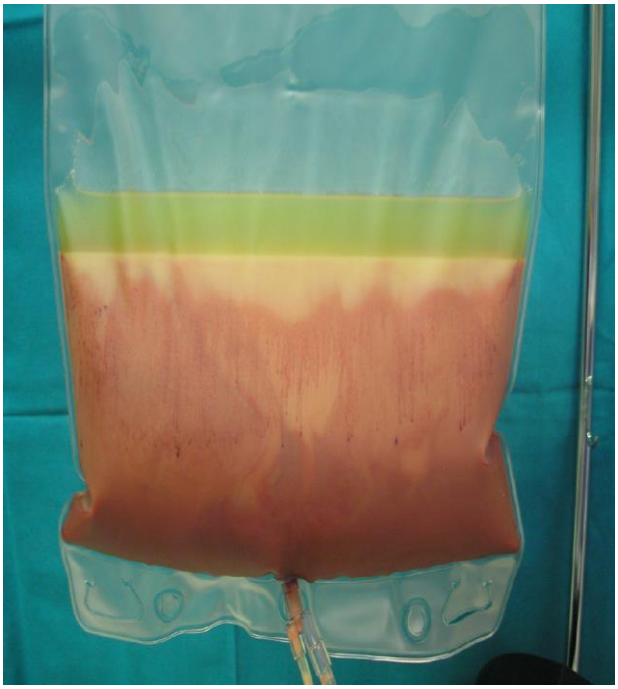


**Figure 1.** Leukocyte suspension removed by one therapeutic leukapheresis by using separator Cobe-Spectra.

By using therapeutic cytaphereses it is also possible to change ex *vivo* the function of particular, in the first place, immunocompetent cells. (5, 12–14). They are typically performed using blood cell separators, while in a therapy of the patients with policytemia or secondary eritrocytosis with manual technique – using multiple plastic bags. Generally, the removal of blood cells by means of separator is faster, and for the patient more appropriate, because at the same time it is synchronized with regulation of intravascular volume and replacement with normal blood cells – in the case it is needed. Anticoagulation is the most frequently achieved by the use of solutions of ACD (formula B, USP XX) and sodium-citrate, as needed, along with the use of "rouleaux-forming" agent – solution of hydroxyethil starch (HES; MM = 450 kD) – in the case of some types of leukaphereses (granulocytepheresis) (1–6).

**Thrombocytosis/thrombocythemia and thrombocytapheresis**

Before presentation of plateletapheresis and estimation of its efficacy, there will be briefly described ehtiopatogenesis, classification and principals/approaches of the theory of the therapy in the diseases and disturbances with thrombocytosis/thrombocythemia where aphereses are needed, and sometimes might result in resque of the life of patient in acute critical situation.

All pathological statements with number of thrombocytes $\geq 400–450x10^9$/L were designated as thrombocytosis. If thrombocytosis (often with enormous number of thrombocytes in blood) appears within myeloproliferative neoplasm, we would talk about essential thrombocytopenia. Thus, the states with increased number of thrombocytes can be: 1) primary (essential thrombocythemia – which

are the consequence of clonal disease of pluripotent SC of hematopoiesis) and 2) secondary (reactive thrombocytoses – which might evolve within individuals with some malignancies, chronic inflammatory proce-sses, iron deficit, after splenectomy, etc (2, 21–23).

Existence of thrombocytes in patient's circulation in essential thrombocythemia is not altered/changed/protracted, but results in accelerated and/or intensive production of thrombocytes from megacariocytes. The cause of this pathological manifestation is not completely known. Yet, it is supposed that some states and/or phenomenons at the hemobiological level might cause thrombocytosis/thrombocythemia – such are, for example, autonomous production, increased sensitivity of certain categories of SC upon the cytokines with hematopoietic activity (Interleukin-3 [IL-3]), lower sensitivity upon factors/inhibitors of thrombocytopoiesis, TGF-β (Transforming Growth Factor β). The patients with thrombocytosis/thrombocythemia have physiological, and sometimes even lower content of thrombopoietin (TPO) – which is known also as MGDF (Mega-karyocyte Growth and Development Factor) (21, 24). It is determined that in most of patients there is a mutation of the following three oncogenes: JAK2 (Janus kinaza 2), CALR (calreticulin) or MPL (Myeloproliferative Leukemia Protein – Thrombopoietin receptor). In rare cases including mutations of the genes TPO – which are linked to autosomal dominant inherited thrombocytosis and mutations TET (Ten-Eleven Translocation, more correct TET2 or Tet Methylcytosine Dioxygenase 2) (21–23).

Mechanisms, by which thrombocytosis lead to thromboses/hemorrhages, are also not comple-tely clarified. Different hemostatic defects/abnormallities are described, – including altered aggregability of thrombocytes, intracellular accumulation of specific active substances, decreased activity of " cofactors von Willebrand ristocetin" and "multimer von Willebrand factor" of highmolecular weight (3–5).

Diagnosis of thrombocytosis/thrombocythemia is established on the basis of the existence of following criteria of World Health Organization (WHO 2008 – World Health Organizatiоn): 1) continual number of thrombocytes $\geq 450x10^9$/L in blood; 2) proliferation of big and mature megakaryocytes, without synchronous proliferation of granulocytes and erythrocytes; 3) differential-diagnostic exclusion (according to WHO criteria) of the following disorders/diseases: CML (Chronic Myelogenous Leukemia), PV (Polycythemia Vera), PMF (Primary Myelofibrosis), MDS (Myelodysplastic syndrome); 4) presence of JAK2-V617F or some other clonal marker, along with lack of confirmation of reactive thrombocytosis, called as secondary thrombocytosis or thrombocythemia, too (2, 21, 24).

Thrombocytapheresis (in combination with medicaments) is performed with the goal of urgent therapy of the patient with the clinical symptoms, which are the consequence of extreme number of thrombocytes in circulation. Typical clinical manifestations of thrombocytosis are headaches, vertigo, transient disturbance of the vision, modest or intense pain in the chest and/or in the belly, acrocyanosis, paresthesia and some other alterations (priapism) (5, 21). In patients with essential thrombocythemia (myeloproliferative neoplasm) or with difficult case of above mentioned secondary or reactive thrombocytosis (especially in some carcinomas or asplenia) it is not rare appearance of thro-mbosis, with consecutive hemorrhages (epistaxis and other minor bleedings) – thus we are talking about "thrombohemorrhagic syndrome". However, as already mentioned, in patients with essential or secondary thrombocytosis/thrombocytemia it is not proved undoubtedly that there is a correlation between the degree of increased number

of thrombocytes in blood and complexity of clinicalcourse of the disease (1–5).

Therapy of these disorders includes the application of cytostatic and antiaggregational medicaments – and if needed, performance of therapeutic plateletapheresis – the use of thrombocytodepletion. Hydroxyurea is considered the drug of the first line for medicamentous cytoreductive therapy in the case of thrombocytoses. Second line therapeutic drugs are busulfan (myleran), Interferon alfa (IFN–α), ruksolitinib (inhibitor of JAK2) (5, 21–25).

The performance of thrombocytapheresis regularly results in beneficial effects in the treatment of most patients with life-threatening therapy is dependent on (beside others), the clinical situation due to extreme thrombocytosis. Efficacy of this urgent cytoreductive initial number of thrombocytes in circulation, dynamics of thrombocyte production, the volume pf blood processed, but also on efficacy of the blood cell separator used.

By using cell separators mentioned above (older generations) therapeutic apheresis are performed usually every second day (seldom everyday), followed by application of standard medicamentous therapy. Thus, by applying thrombocytapheresis using separators of the first and second generation (Haemonetics M30, IBM-2997 and Cobe-Spectra) it was possible to remove about $3x10^{12}$ thrombocytes per liter of cell suspension, e.g. total of $9,6x10^{12}$ cells during one total therapeutic cure.(Figure 2).


**Figure 2.** Thrombocyte suspension removed by one therapeutic thrombocytapheresis by using Cobe-Spectra (with labeled cell values).

The performance of one session of thrombocytepheresis resulted in *in vivo* decreased number of circulating thrombocytes for about 30–50%, while the performance of the total therapeutic cure (total of 3–11 procedures, dependent on the cause and version of thrombocytosis) was leading to reduced thrombocyte count for 55–85% in peripheral blood of the patient. The results of cytoreduction reached in the Center for hemotherapy and apheresis of MMA are comparable to literature data (26).

However, by means of introduction of novel separators and programmed software's of the newest generation (Spectra-Optima, IDL-System), along with application of our own modification of original protocol for thrombocytapheresis (Figures 3 and 4) statistically more significant apheretic cytoreduction is achieved.

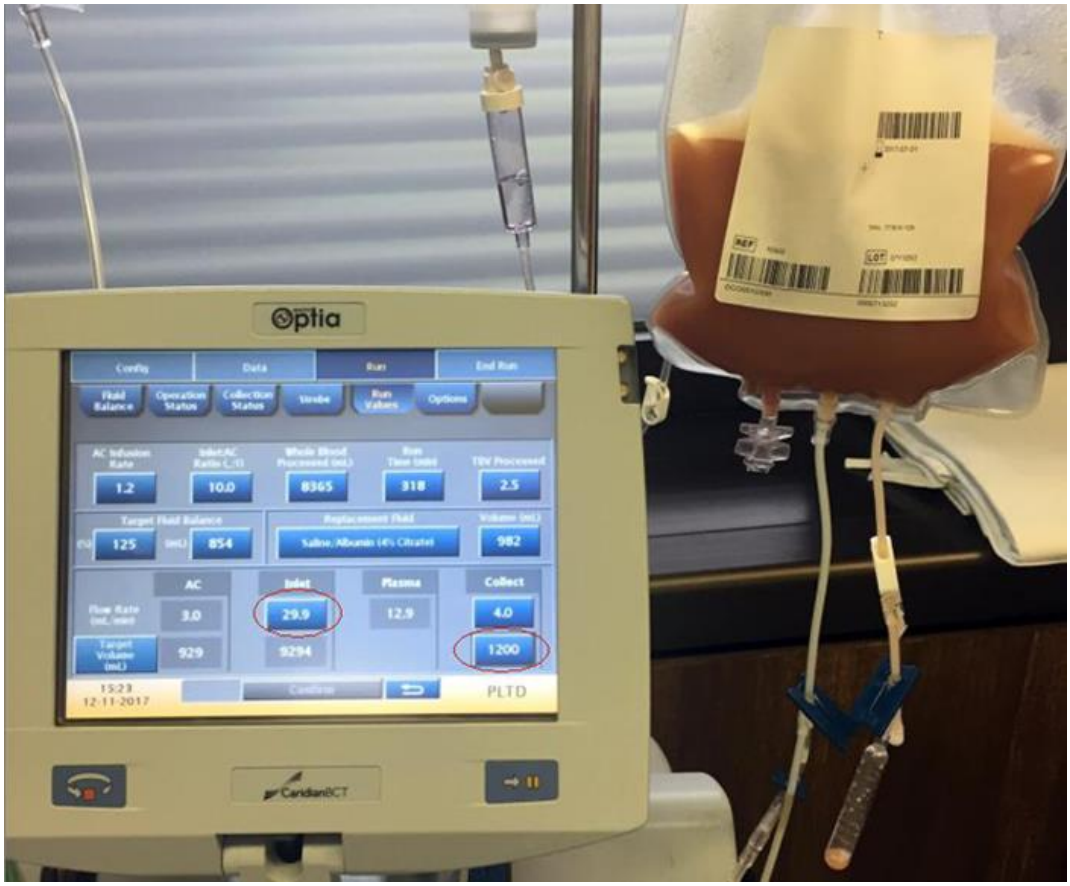



**Figure 3.** Modified parameter ("blood flow and removed cell suspension volume") of thrombocytapheresis using separator Spectra-Optia.

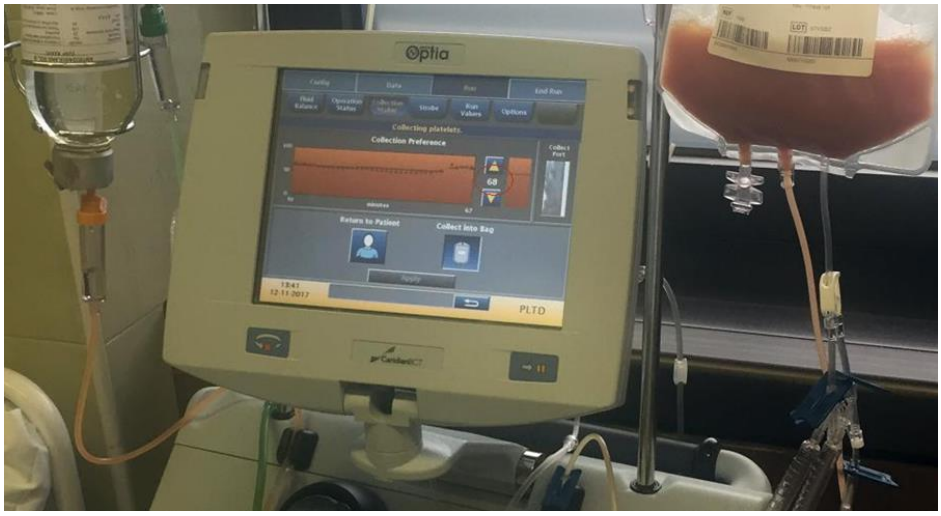



**Figure 4.** Modified parameters ("preferent collection") thrombocytapheresis Using Spectra-Optia.

.

Namely, by using Spectra-Optia (along with our own modifications) it was possible achie-ve similar therapeutic effect to that one which was realized after the application of one total therapeutic apheretic cure by using sepa-rators of earlier generations.

In fact, by applying one singular thrombocyte-pheresis it was possible to remove about $7{,}5x10^{12}$ cells (thrombocytes) in about 1200 mL of cell suspension. This thrombocyte-depletion is comparable with the degree of removed thro-mbocytes ($9{,}6x10^{12}$) after the performance of at least three cytaphereses (which assumed the separation of about 3500 mL of cell suspen-sion) – see Figure 5.

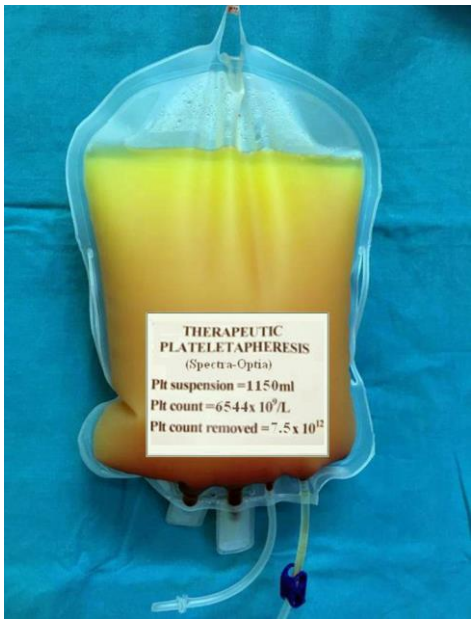



**Figure 5.** Thrombocyte suspension removed by one therapeutic thrombocytapheresis (application of Spectra-Optia and modified apheretic protocol).

In accordance with the data obtained ex *vivo* thrombocytodepletion, a high degree of in *vivo* apheretic cytoreduction in the patient circulation was achieved. The results of thrombocyte-depletion and cytoreduction are confirmed by quantification of the cells in the removed cell product of apheresis (Figure 6).

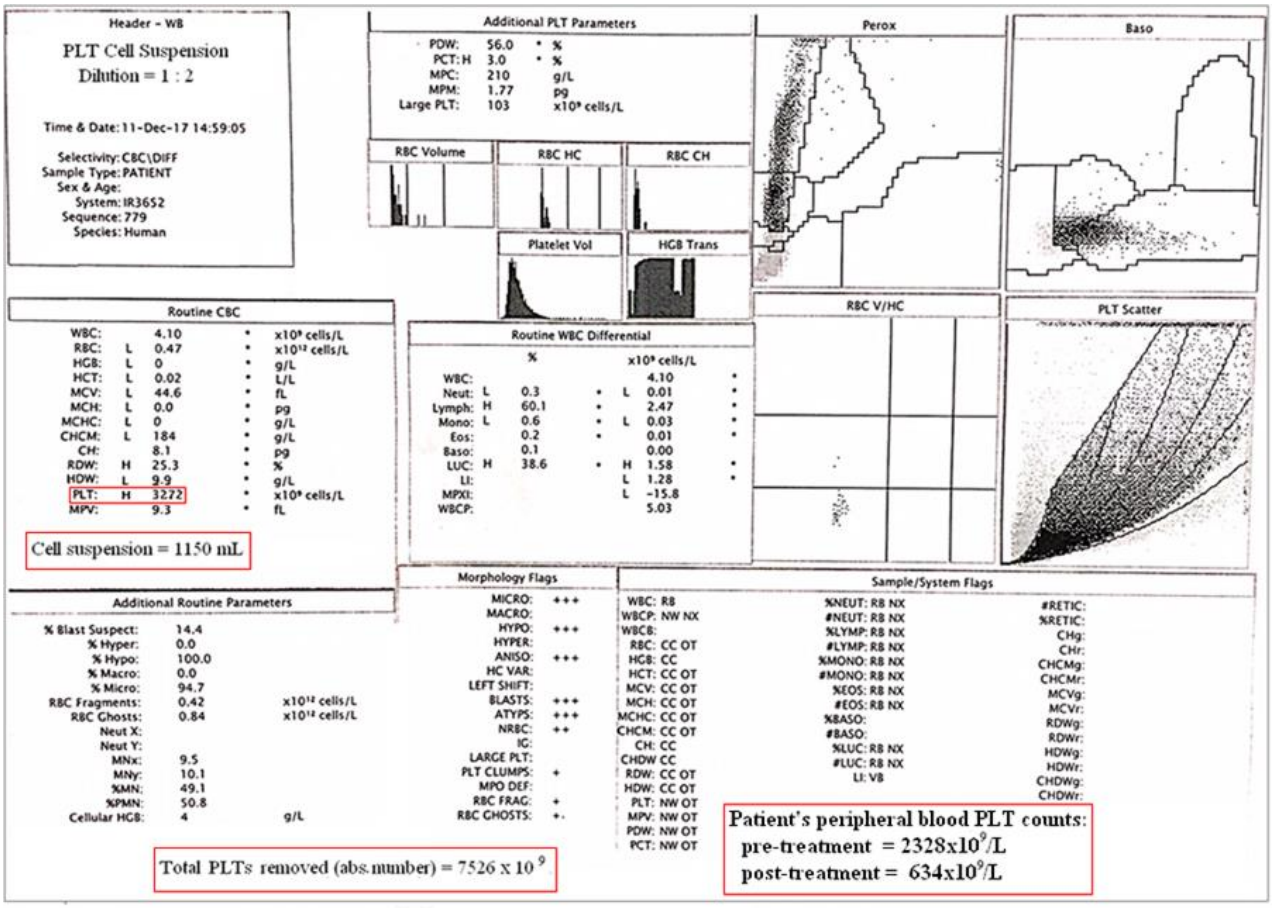



**Figure 6.** Quantitatification of cells in thrombocyte suspension removed by one therapeutic thrombocytapheresis (Spectra-Optia applied).

Finally, the number of thrombocytes in the patient's blood before apheresis was 2328x $10^9$/L, and after the treatment it was 634x $10^9$/L – accordingly, the degree *in vivo* of apheretic cytoreduction was 73%.

Based on literature data and our own results it is possible to conclude that the performance of the apheresis in optimized time and appropriate intensity – along with most favorable choice of the equipment and apheretic protocol – results in undoubtedly clinical benefit and presents efficient procedure in the treatment of most patients with lifethreatening crisis due to extreme thrombocytosis. However, it is worth to say, that thrombocytodepletion – although very efficient cytoreductive treatment – cannot result in bone marrow remission, neither can they be of influence upon the course and outcome of essential myeloproliferative disease.

## References


1. McLeod BC. Apheresis: principles and practice, 2nd edition. Bethesda: AABB Press; 2005.
2. Klein HG, Anstey DJ. Mollison's Blood transfusion in clinical medicine. 12th ed. Oxford: Wiley and Blackwell; 2014.
3. Romack JD, Grossman BJ, Harris T, Hillyer CD. Technical Manual, 17th ed. Bethesda: AABB Press; 2011.
4. Balint B, Bogdanović G, Dimitrijević G. Hemafereze. In: Balint B, editor. Transfuziologija. Beograd: Zavod za udžbenike i nastavna sredstva; 2004. p. 455–77.
5. Balint B, Vucetic D, Ostojic G, Ljubenov M. Osnovi transfuziologije sa hemobiologijom. Beograd: Medicinski fakultet VMA – Medija centar Odbrana; 2015.
6. Radovic M, Balint B, Milenkovic Lj, Tiska–Rudman Lj, Taseski J. Therapeutic leukapheresis. Transf Sci 1991; 12: 193–6.
7. Radovic M, Balint B, Tiska–Rudman Lj, Milenkovic Lj, Taseski J. Application of therapeutic Plasma Exchange in Hematology. Transf Sci 1994; 15: 481–5.
8. Balint B, and the National Apheresis Group. Apheresis in donor and therapeutic settings: recruitments vs. possibilities – a multicenter study. Transf Apher Sci 2005; 33(2): 229–37.
9. Balint B, Ostojic G, Pavlovic M, Hrvacevic R, Pavlovic M, Tukic Lj. et al. Cytapheresis in the treatment of cell–affected blood disorders and abnormalities. Transf Apher Sci 2006; 35(1): 25–31.
10. Balint B, Pavlovic M, Jevtic M, Hrvacevic R, Ostojic G, Ignjatovic Lj, Mijuskovic Z, Blagojevic R, Trkuljic M. Simple "closed–circuit" group–specific immunoadsorption system for ABO–incompatible kidney transplants. Transf Apher Sci 2007; 36: 225–33.
11. Balint B, Pavlovic M, Todorovic M, Jevtic M, Ristanovic E, Ignjatovic Lj. The use of simplified *ex vivo* immunoadsorption and "multi–manner" apheresis in ABO/H–mismatched kidney transplant setting – phase II clinical study. Transf Apher Sci 2010; 43(2):141–8.
12. Todorovic M, Balint B, Suvajdzic N, Pavlovic M, Petrovic M, Jevtic M, et al. Triple–way therapeutic approach for paraganglioma–dependent erythrocyte-sis: drugs plus surgery plus "multi–manner" apheresis. Med Oncol 2008; 25(2): 148–53.
13. Balint B, Todorovic–Balint M, Petakov M, Ostojic G, Vucetic D. Effectively "cross–bridged" hemostatic and blood screening test defects due to glycogenosis type 1 associated extremely hyperlipidemia. Transf Apher Sci 2014; 50: 314–5.
14. Balint B, Todorovic-Balint M, Ljubenov M, Tarabar O, Pavlovic M, Kanjuh V. Apheretic "rescue-protocol" designed for treatment of CLL associated life-threatening hemolytic crisis. Transfus Apher Sci 2015; 52(2): 256–8.
15. Balint B, Ljubenov M, Stamatović D, Todorović M, Pavlović M, Ostojić G, et al. Stem cell harvesting protocol research in autologous transplant setting: large volume vs. conventional cytapheresis Vojnosanit Pregl 2008; 65(7): 545–51.
16. Balint B, Stanojevic I, Todorovic M, Stamatovic D, Pavlovic M, Vojvodic D. Relative frequency of immature CD34+/CD90+ subset in peripheral blood following mobilization correlates narrowly and inversely with the absolute count of harvested stem cells in mulTPEle myeloma patients. Vojnosanit Pregl 2017; 74(11): 1071–7.
17. Balint B, Todorovic M, Urosevic I, Pavlovic M. Stem cell transplat: from cell harvesting to cryopreservation. Med Pregl 2017; 70(Suppl 1): 41–45.
18. Todorovic Balint M, Balint B, Bila J, Vujic D, Andjelic B, Mihaljevic B. Autologous stem cell transplants - single center standpoints for lymphoma patients. Med Pregl 2017; 70(Suppl 1): 47–50.
19. Balint B, Radovic M, Malesevic M, Jovicic A, Stamatovic D, Ostojic G, et al. The rationale for therapeutic apheresis in the treatment of hematologic and immune–mediated neurologic disorders. Blood Banking Transf Med 2003; 1: 73–80.
20. Radović M, Balint B, Đurić D, Taseski J, Milenković Lj, Krstić Č, Tiška–Rudman Lj, Ciko Z, Malešević M. Clinical application of therapeutic cytapheresis. Libri Oncol 1990; 19: 15–19.
21. Rumi E, Cazzola M. How we treat essential thrombocythemia. Blood 2016; DOI: 10.1182/blood-2016-05-643346
22. Tefferi A, Barbui T. Polycythemia vera and essential thrombocythemia: 2015 update on diagnosis, risk-stratification and management. Am J Hematol 2015;90(2) :162–73.
23. Aruch D, Mascarenhas J. Contemporary approach to essential thrombocythemia and polycythemia vera. Curr Opin Hematol. 2016; 23(2):150-60.
24. Bleeker JS, Hogan WJ. Thrombocytosis: Diagnostic evaluation, thrombotic risk stratification, and risk-based management strategies. Thrombosis, 2011; doi: 10.1155/2011/536062PMCID: PMC3200282
25. Kiladjian JJ, Besses C, Griesshammer M, Gugliotta L, Harrison C, Coll R, et al. Efficacy and safety of with essential thrombocythaemia aged >80 years: an interim analysis of the EXELS

study. Clin Drug Investig 2013; 33(1): 55–63cytoreductive therapies in patients
26. Prakash S, Hans R, Sharma Malhotra P, Marwaha N. Therapeutic Thrombocytapheresis for Symptomatic Thrombocytosis in Hemato-Oncology Patients. Therap Apher Dialysis 2017; doi: 10.1111/1744-9987.12635.

Corresponding author: Prof. Bela Balint, MD, PhD, SASA- member,
Institute of Transfusiology and Hemobiology of MMA, Belgrade and Serbian Academy of Sciences and Arts
Knez Mihajlova 35,
11 000 Belgrade, Serbia
e-mail: **balintbela52@yahoo.com**

# MOLECULAR TESTING FOR BLOOD-BORNE PATHOGENS IN TRANSFUSION MEDICINE – A REVIEW AND OUR TEN-YEAR'S EXPERIENCE

**Nemanja Borovcanin[1], Dusan Vucetic[1,2], Elizabeta Ristanovic[3], Milena Todorovic[4,5], Mirjana Pavlovic[6], Bela Balint[1,2,7,8]**

*[1]Institute for Transfusiology and Hemobiology, Military Medical Academy, Belgrade, Serbia;*
*[2]Faculty of Medicine of Military Medical Academy, University of Defense, Belgrade, Serbia,*
*[3]Institute for Microbiology, Military Medical Academy, Belgrade, Serbia;*
*[4]Clinic for Hematology of CCS, Belgrade, Serbia;*
*[6]Department of Computer and Electrical Engineering and Computer Science, FAU, FL, USA;*
*[7]Institute for Medical Research, University of Belgrade, Serbia;*
*[8]Serbian Academy of Sciences and Arts, Belgrade, Serbia;.*

## Summary

*For detection of blood-borne pathogens in transfusion medicine various serological testing (ST), i.e. Enzyme or Chemiluminescent Immunoassay (EIA/CIA) and molecular analysis (MA), i.e. Polymerase Chain Reaction or Nucleic Acid Testing (PCR/NAT) are in routine use.*
*PCR/NAT technology is very important for pre-transfusion testing for blood-borne diseases, genotyping of Human Leukocyte Antigens (HLA) and antigens of human blood groups and also for discovering and clinical diagnostic of genetic diseases. The most important use is in pre-transfusion testing of blood units on nucleic acid of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). Molecular screening is currently widely conducted with commercial HBV/HCV/HIV multiplex and West Nile Virus (WNV) Nucleic Acid Testing (NAT) systems performed on highly automated instrument platforms that ensure reliable results with high sensitivity and specificity levels.*
*We present briefly our results of testing of blood donors in Institute of Transfusiology and Hemobiology using complementary EIA/CIA and PCR/NAT for period of 2007–2017. We found five ST-negative/MA-positive donations; one of them was in "window period" (WP).*

**Key words:** molecular and serological testing, blood-borne pathogens, HBV, HCV, HIV, WNV.

## General considerations

Watson and Crick identified molecular structure of Deoxyribonucleic acid (DNA) in 1953. Since that time molecular biology and genetics are present with their achievements in many different parts of medical sciences. In Transfusion Medicine we use them for diagnostic and therapeutic reasons.DNA amplification or Polymerase Chain Reaction (PCR) was discovered in 1987. This technology is very important for pre-transfusion testing for

bloodborne diseases, genotyping of Human Leukocyte Antigens (HLA) and antigens of human blood groups and also for discovering and clinical diagnostic of genetic diseases (1).

Hepatitis B Virus (HBV) was discovered by Blumberg in 1963. Epidemiological data show that more than 300 millions of people are carriers of this virus (2). The frequency of HBV infection is from 8 to 15% (Far East, Middle East, Africa and parts of South Ame-rica), less than 2% (USA, Canada, North Europe and parts of South America). There are 4 million reported cases of acute HBV per year. Annually million people die from com-plications of HBV (3, 4).

After discovery of Hepatitis A Virus (HAV) and HBV, significant number of patients with clinical hepatitis had unknown cause, so this hepatitis was called, non–A, nAon–B (NANB) hepatitis. NANB hepatitis was connected with intravenous drug users, therapy with derivates of pooled plasma and with blood transfusion. After the genome of this virus was cloned it got name hepatitis C virus (HCV) (5). There are 1 million people infected by HCV per year. The prevalence is from 2% in North America, Europe and Australia to 5% in Africa (6).

Human Immunodeficiency Virus (HIV) was discovered in the middle of 1980s, in the same time by Gallo in USA and by Montagnier in France. There are two types of this virus: HIV–1 and HIV–2. HIV–1 has two subtypes: main (M) and outlier (O). M subtype has 11 variants (from A to K) (4).

The most significant use of MA is in screening of blood units for nucleic acid of HCV, HIV and HBV. Namely, Nucleic Acid Testing (NAT) of these viruses uses PCR for amplification of virus genetic material so we can detect deoxyribonucleic or ribonucleic acid (DNA or RNA). This method is assigned as PCR/NAT investigation.At the end of 1990s blood banks started to use this method for detection HCV-RNA, HIV-RNA and HBV -DNA. In Japan, since 1999 all blood units have to be tested by PCR/NAT before transfusions because of relatively high prevalence of HBV and HCV. Also, since1999 PCR/NAT is mandatory for HCV and HIV in USA (1).

Molecular screening is currently widely conducted with commercial multiplex (HBV/-HCV/HIV) PCR/NAT systems performed on highly automated instrument platforms that ensure reliable results with high sensitivity and specificity levels. A published international survey on PCR/NAT of blood donations over 10 years (1999–2009) (7), including 33 countries from the five continents, pointed out that approximately 3 000 "viral" blood donations (due to viremia) would have been missed by serological screening methods. PCR/NAT yields vary depending on epidemiological situations, ranging from 0.0 (South America) to 47.3 (South Africa) per million donations for HIV-1, from 0.0 (South America) to 3.1 (North America) per million donations for HCV and from 2.05 (North America) to 43.2 (South Africa) per millions donations for HBV. In countries with medium and high HBV prevalence where anti-HBC is not screened, HBV PCR/NAT detect more often occult B infections than acute phases with proportions depending on local epidemiology and sensitivity of PCR/NAT (55% in South Africa, 96% in Italy). PCR/NAT is subjected to false-negative results due to viral diversity or to extremely low viral loads. In Laperche's (8) 11-year experience of NAT, of the 10 HIV Ab-positive and PCR/NAT-negative donors, three were HIV-2, one was HIV-1gO and six had HIV-1 undetectable RNA. A recent study described a complex HIV-1 recombinant B/F which escaped PCR/NAT detection. In the context of the occurrence of new HIV variants, regulatory agencies in Germany promote the use of dual-target PCR/NAT for HIV-1. Low viral loads explaining the lack of PCR/NAT reactivity have been observed in donors with occult HBV infection or HIV elite

controllers. A repeatedly PCR/NAT reactive sample with multiplexed NAT, especially when serological testing (ST) is negative, is submitted to an additional virus-specific amplification testing to discriminate between the three viruses is performed. However, this procedure cannot be considered a confirmation method since discriminatory assays are using the same methodology and the same reagents as the initial screening assays. This two-step screening systems have introduced the problematic situation consisting of sam-ples reactive with the screening assay but nonreactive with the discriminatory assay. In the absence of serological evidence of infection and sample cross-contamination non reproducible PCR/NAT reactivity may be explained by false-positive results (8).

For HCV compiled data were reported for 29.9 million donations in 2008, of which 60 were HCV PCR/NAT-only positive (7). These HCV PCR/NAT yield donations sorted into 27 HCV PCR/NAT-only positive first-time donations (6.2⁄ million) and 33 HCV NAT-only-positive repeat donations (1.3⁄ million). There was a great difference between rates of HCV RNA detection by PCR/NAT among se-ropositive first-time (71.63%) and repeat (57.96%) donations (7).

There are also two commercial PCR/NAT systems for detection of the West Nile Virus (WNV) RNA. Routine screening of blood donors on WNV RNA was started in 2003 in USA and Canada, as an answer on docum-

ented cases of transmission WNV trough blood transfusion. Combination of "minipool" (MP; 16 donations) and "individual-donation" (ID) testing using PCR/NAT (MP- PCR/NAT and ID-PCR/NAT) was successful on endemic locations in detection 1 500 infected donors in USA from 2003 to 2005. If we suggest that each of these donations were infective and that 1.45 blood products were made, testing on WNV RNA prevented potential/possible infection and clinical disease of 2 175 recipients. During the use of PCR/NAT, WNV transmission by blood transfusion has happened in 7 MP negative/ ID positive units (6 in 2003. and 1 in 2004.) or total number of 30 cases of transfusion tran-smitted WNV since it was identified in North America. All 30 donations were negative on anti-IgM or anti-IgG tests. There is also strategy in Quebec (Canada) that blood donors test by WNV PCR/NAT seasonally, out of season are screened by questionnaire and if screening is positive that WNV PCR/NAT is used to confirm presence of WNV in blood of these donors (9–11).

***Our experience***

We evaluate results of the blood donors ST and MA investigations in the Institute of Transfusiology and Hemobiology of MMA in the period 2007–2017 for blood-borne pathogens. In this period 70 938 donors were investigated by ST and MA for HBV, HCV and HIV. If they were negative using Enzyme or Chemiluminescent Immunoassay (EIA/-CIA) than were investigated using MP-PCR/NAT (24 donations) method. Opposite, all ST-positive samples were examined by ID-PCR/ NAT (Table1).

**Table 1**

**Results of donor investigations by EIA/CIA and PRC/NAT**

| | EIA/CIA | | PCR/NAT | |
|---|---|---|---|---|
| **Pathogens** | **TNT** | **RcT** | **MP-PCR/NAT-pos** | **ID-PCR/NAT-pos** |
| **HBV** | 70 938 | 1 371 | 2 | 224 |
| **HCV** | 70 938 | 1 461 | 3 | 141 |
| **HIV** | 70 938 | 1 408 | / | 9 |
| **WNV** | 3 753 | / | 1 | 1 |

TNR= Total number of tested samples; RcT = Reactive samples

We found five ST-negative/MA-positive donations. One donation was HBV EIA/CIA-negative, confirmatory and PCR/NAT positive and one donation was (by administrative error) EIA/CIA-negative, but was PCR/NAT positive. Also, we had four donors HBV ST-positive/MA-negative. Finally, there was three EIA/CIA-negative, but PCR/NAT-positive samples for HCV. However, two of them were negative because of the use of EIA/CIA with a low/insufficient sensitivity. However, the third donor was in the "window period" (WP) for HCV ($HCV_{WP}$). Namely, during 2012 one unit of whole blood (450 ± 45 mL) from a 23-year-old male first-time donor was collected into a quadruple blood bag system (Terumo, Japan) – containing CPD anticoagulant-preservative and SAGM additive solutions. The blood sample was nonreactive for serological screening testing. Then, qualitative MP-PCR/NAT checking was performed by COBAS Ampliprep and COBAS Amplicor (Roche, USA). Since HCV positivity was obtained, all blood donations from this MP were temporarily blocked. Samples from MP were then resolved to the single donations and retested by ID-PCR/NAT. One of them was positive, thus all components were definitely blocked from that donation (12). Novel additional information appeared in donor's medical-history: data of intravenous drug usage two month prior to donation (not assigned in the initial questionnaire).

Thus, blood samples of this donor underwent further investigations once monthly, i.e. – at five time points (tP). Precisely, donor's serum was then retested using Architect Anti-HCV (Abbott, Germany), Vitros Anti–HCV (Ortho, UK), Bioelisa HCV 4.0 (Biokit, Spain) and Monolisa Anti-HCV Plus (Bio-Rad, France) STs (Table 2). Samples were confirmed by Western Blot Technique, as previously presented (12).

**Table 2**

**Serological testing and molecular analysis data**

| Investigations/Samples | | Initial | 2nd tP | 3rd tP | 4th tP | 5th tP |
|---|---|---|---|---|---|---|
| EIA/CIA | ST–1. | NR | NR | NR | NR | RcT |
| | ST–2. | NR | NR | NR | RcT | RcT |
| | ST–3. | NR | RcT | RcT | RcT | RcT |
| | ST–4. | NR | NR | RcT | RcT | RcT |
| MP-PCR/NAT and ID-PCR/NAT | | | positive | (3.966; range 0.2 – 4.0) | | |

ST = serological testing; NR= Non-reactive; RcT = Reactive; tP= time point (i.e. once mountly).

As shown in Table 2, anti-HCV was detected as reactive by different EIA/CIA tests and confirmed serologically beginning with the 2nd to 5th time points.

Total 18 donations were HCV ST-positive/MA-negative because donors had old infection with HCV (14). There were no discrepancies between HIV EIA/CIA or PCR/NAT results. Nine samples were HIV ST-positive/MA-positive. We tested 3 753 donations on WNV using firstly MP-PRC/NAT(16 samples) and found one positive donor – she was confirmed by ID-PRC/NAT.

In conclusion, the choice of blood screening strategy should be adapted to local epidemiology and medical/economic limitations. Basic advantages of using PCR/NAT in pre-transfusion testing are in higher sensitivity and specificity of testing, as well as in a shortage of WP. However**,** despite an improved donor selection and the use of more sensitive serological blood STs – infectious complications could even now represent a risk in

blood component therapy. The complementary application of STs and MP-PCR/NAT or ID-PCR/NAT undoubtedly decreases WP-hazard in each one, especially in high-infectious-risk donors – reducing infectious risk-rate (primarily for $HCV_{WP}$-donations) in transfusion practice.

**Acknowledgements:** This work was supported by the Ministry of Defense of the RS (Project MF/VMA 9/17-19) and by the Ministry of Education, Science and Technological Development of the RS (Project No III41030).

## References

1. Borovčanin N., Trkuljić M., Balint B. Testiranje nukleinskih kiselina (NAT).ART. 2006;34:203–12.
2. Zarski JP, Ganem D, Wright TL. Hepatitis B virus. In: Richman DD, Whitley RJ, Hayden FG, Eds. Clinical Virology. Washington, DC: ASM Press; 2002. p. 623–57.
3. Andre F. Hepatitis B epidemiology in Asia, the Middle East, and Africa. Vaccine 2000; 18: 20–2.
4. Borovčanin N, Vučetić D, Jocić M, Jovičić D, Balint B. Osnovni pricipi NAT tehnologije sa osvrtom na rezultate rada. Bilt Transfusiol 2011; 56: 99–107.
5. Choo QL, Kuo G, Weiner AJ Overby LR, Bradley DW, Houghton M et al. Isolation of a cDNA clone derived from a blood–borne non–A, non–B viral hepatitis genome. Science 1989; 244: 359–62.
6. Quer, J, Mur, JIE. Hepatitis C virus: epidemiology. In: Thomas HC, Lemon S, Zuckerman AJ, editors. Viral Hepatitis. Malden, MA: Blackwell; 2005. p. 407–25.
7. Roth WK, Busch MP, Schuller A, Ismay S, Cheng A, Seed CR, el al. International survey on NAT testing of blood donations: expanding implementation and yield from 1999 to 2009. Vox Sanguinis. 2012; 102: 82–90.
8. Laperche S. Testing for established viruses: from the screening to the confirmation. ISBT Science Series. 2013; 8: 58–64.
9. Borovčanin N, Balint B. Virus Zapadnog Nila (WNV) – etiopatogeneza, klinička slika i transfuziološki aspekti. ART. 2014; 41: 77–81.
10. Peterson LR, Brault AC, Nasci RS. West Nile Virus: Review of the Literature.JAMA2013;3:-308–15
11. Stramer SL. Current Risks of Transfusion-Transmitted Agents. Arc Path Lab Med 2007; 131: 702–7.
12. Balint B, Vucetic D, Todorovic-Balint M, Borovcanin N, Jovanovic-Cupic S, Mandusic V. Safety improving by complementary serological and molecular testing combined with pathogen reduction of the donated blood in window period (Letter). Transfus Apher Sci 2013; 49: 103–4.
13. Busch M, Glynn S, Stramer S, Orland J, Murphy E, Wright D, et al. Correlates of hepatitis C virus (HCV) RNA negativity among HCV– seropositive blood donors. Transfusion 2006; 46: 469–75.

Correspondence to: Nemanja Borovcanin MD,
Institute of Transfusiology and Hemobiology, Military Medical Academy,
Crnotravska 17,
11 000 Belgrade, Serbia.
Tel.: +381 11 3609135; +381 64 6131303.
E-mail address: **drsky2@gmail.com.**

# STEM CELLS USED IN DENTISTRY – STATE OF THE ART

**Kristie Lagalante[1], Milena Todorovic-Balint[2], Bela Balint[3,4] and Mirjana Pavlovic[1]**

[1]Department of Computer and Electrical Engineering and Computer Science, FAU, FL, USA;
[2] Clinic for Hematology, Clinical Center of Serbia, Belgrade, Serbia;
[3]Institute for Transfusiology and Hemobiology, Military Medical Academy, Belgrade, Serbia;
[4]Serbian Academy of Sciences and Arts;

## Abstract

*Stem cells are undifferentiated unit-multi-pluripotent cells that have the ability to differentiate into multiple specialized cell types with a more specific functions. They are essential component of regenerative biomedical engineering and key player in stem cell therapy in a wide spectrum of diseases. Stem cell therapy has become an interest within the regenerative dental medicine field as they can be helpful in treating different diseases of oral cavity, including teeth.Both oral and maxillofacial defects are a kind of disease that can benefit from the development of stem cell therapies. This paper focuses on and discusses of the various oral and maxillofacial defects that can benefit from the uses of stem cell therapy, the different types or oral stem cells, induced pluripotent stem cells (iPSC), and possible challenges for applying these therapies to treat oral disease.*

**Keywords:** dentistry, stem cells, induced pluripotent stem cells, cellular therapy,oral diseases

## Introduction

There are many dental problems that are caused by periodontal disease, dental caries, and tooth injury. These dental problems compromise oral health leading to general health issues. The development for regenerative therapy has been influenced by the understanding of stem cell biology, embryonic development, and technology within tissue engineering. Stem cells have the ability to regenerate tooth tissue because of their potential to differentiate into all layers of the tooth tissue both in vivo and in vitro. Replacing the whole tooth as a therapy is considered to be an appealingconcept for regenerative therapy as a form of organ replacement within bioengineering [1].

Stem cells are unspecialized, immature cells that can develop into many and/or all cell lineages by differentiation into more speci-alized cells with specific functions, such as; skin cells, muscle cells, bone cells, and blood cells [3]. Stem cells possess the ability to divide indefinitely by self- renewing [2]. Sources of stem cells include: embryonic stem cells, adult stem cells, and induced pluripotent stem cells (iPSCs). Research within the stem cell engineering field has advanced since the invention of the iPSC technology [4]. Stem cells have

been shown effective in therapies for lymphoma, leukemia, spinal cord injury, hearing loss, vision impairment, teeth missing, and various other degenerative diseases [5-14]. It is promising that oral and maxillafacial efects can be improved or cured by stem cell therapies.

**Examples of Oral and Maxillofacial Defects**

Both the oral and maxillofacial areas of the face consist of several different types of tissues. Each differs from one another and can affect bodily functions, such as; chewing, breathing, smell, and speech. Esthetics can also influence the patient's psychological health and even lead to depression and dementia [16, 17]. Defects in the oral area can ultimately be divided into four major groups: periodontal disease, pulpal disease, hard tissue defects, and maxillofacial defects [18].

**Periodontal Disease**

The most common type of periodontal disease is periodontitis: the inflammation of the periodontium. Periodontitis is a public healthproblem worldwide [23-25].The type of periodontal disease that arises from inflammation and expansion from the root canal is called periodical periodontitis. Often times, periodontal disease is called by the stimuli or diverse bacteria in the mouth [21]. It can cause lesions of the gingiva, the attachment, and the alveolar bone and eventually lead to tooth loss [19, 23]. In the review, "Definitions and Epidemiology of Endodontic Infections," Persoon describes recent findings gathered from epidemiological studies on endodontic infections. It is shown that the dental health of periodical tissues has not been improved throughout the general population[20]. Periodontitis can lead to pain and discomfort in the mouth and also causes a significant impact on oral health related quality of life as well as overall quality of life [26]. Fig.1 presents the concept in stem cell treatment of periodontal diseases.

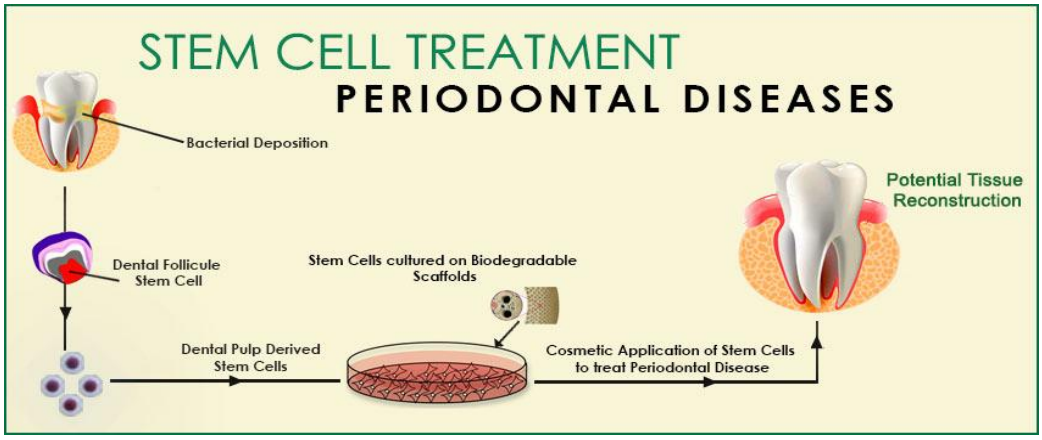



**Figure 1.** Stem cell treatment of periodontal diseases:concept

**Pulpal Disease**

The inflammation at the site of the dental pulp is referred to as pulpitis. Pulpitis is described as being either irreversible or in some cases reversible disease. Clinically, pulpits can be acute, chronic, or hyperplastic [27]. This condition usually occurs after experiencing trauma or dental caries [28]. Root canal therapy can be applied to treat pulpitis [29].

During a root canal procedure, inflammatory tissue is extracted, preparation of the root ca

nals are done, and then it is filled with materialsin order to stop bacteria from reaching the root canal system [27].Removing all the plural tissue leaves the teeth becoming insensitive, prone to nutrient loss, and the properties of the enamel and dentin is altered (Fig.2).

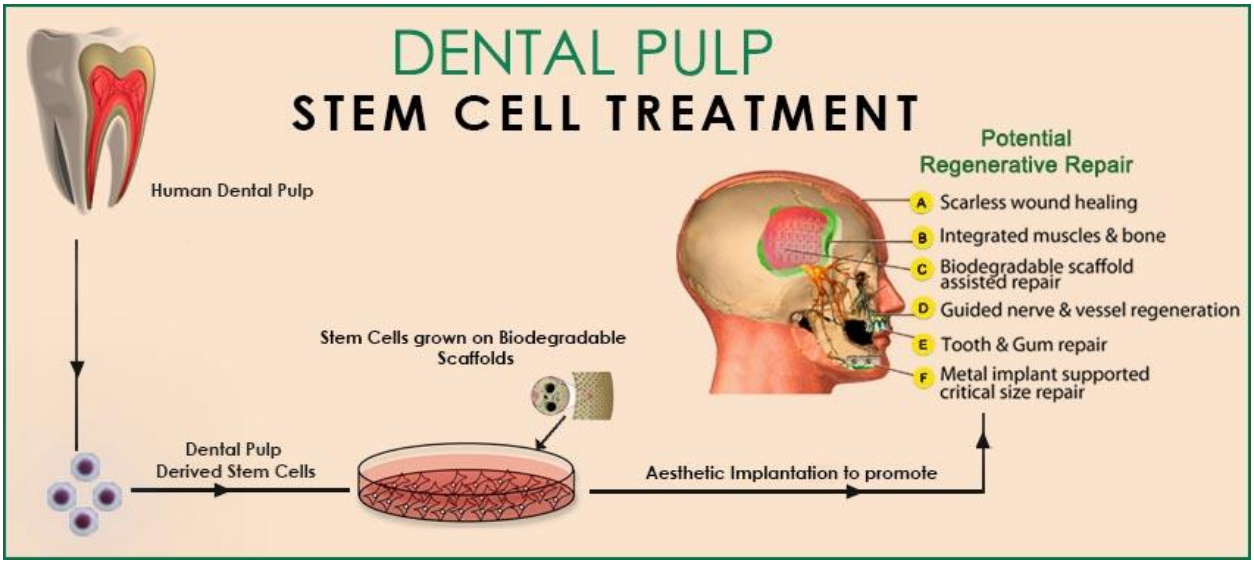



**Figure 2.** Stem cell concept of cellular treatment of pulpal/dental pulp diseases

**Hard Tissue Defects**

Dental caries or cavities are the most common reason causing defects in dental hard tissue. Dental hard tissue defects are a multifactorial disease that is caused by demineralization of the tooth enamel surface due to oral bacteria. Demineralization leads to a weakened structure [30]. Lesions that are caused by either normal or abnormal wear, leads to erosion, abrasion, and degradation via chemicals to the dental tissues [31]. Traumatic injuries to the hard tissue are typically caused by external impacts on the surrounding tissues of the tooth [32]. Currently the most common method of treating these defects to the hard tissue to the teeth is filling it with wither composite resins or amalgam. This restore the defects that are caused [33]. When using amalgam there is the problem of having to partially remove health tissues in order to gain retention of the amalgam [34].

Problems that arise when using composite resins are, easy wear and tear, shrinkage, and aging of the material [35].

**Maxillofacial Defects**

Maxillofacial tissues are important to the patients overall psychological wellbeing. People tend to have difficulty living confide-ntly when they are suffering from maxilla-facial defects. These defects are usually caused by infections, tumor section, trauma, and congenital abnormalities. Effective rege-neration or reconstruction of the damaged maxillofacial tissues would benefit the patient psychologically and physiologically [36].

The main therapy to treat maxilla-facial defects are currently flap transplants in combination with dental implants [37, 38].

## Potential use of stem cells for the treatment of oral and maxillofacial defects

Stem cells are typically described as cells that have the ability to proliferate in order to achieve self-renewal and differentiate into different cell lineages. Stem cells are divided into four main types; embryonic stem cells (ES), adult stem cells, perinatal stem cells, and induced pluripotent stem cells (iPSC) [39].
Embryonic stems cells are the most pluripotent, meaning they can divide into the most types of stem cells, but they are ethically controversial. They controversy is due to them being derived from the embryo [40]. Amniotic stem cells need to be further studied for the understanding of their full potential. Currently, adult stem cells and iPSC are not as ethically controversial and can be used to treat the previously mentioned oral and maxilla-facial defects.

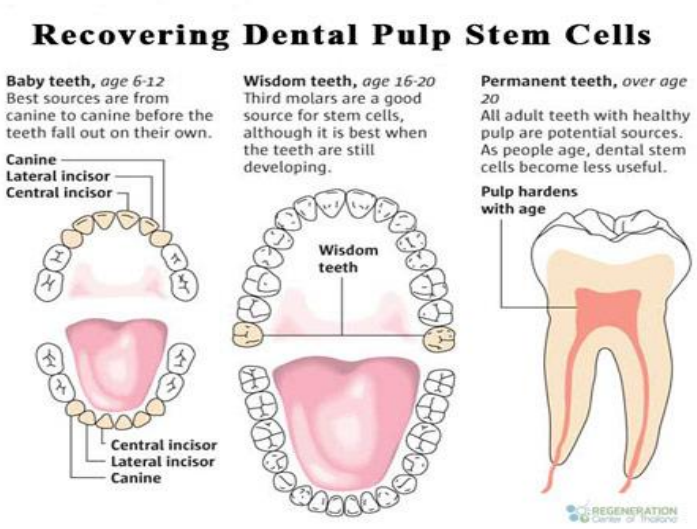



**Figure 3.** Sources of adult stem cells for cellular therapy

## Adult stem cells, oral-derived

Adult stem cells can also be referred to as somatic stem cells. These cells are directly harvested from adults [41]. Adult stem cells can be sourced from various dental tissues, including; dental pulp, bone marrow, dental follicle, gingiva, apical papilla, and perio-dontal ligament.They are multipotent cells, meaning they differentiate into multiple, but not all cell types [42]. The cells can then express specific molecular markers to be isolated. The oral cavity has been a primary focus on research for gaining somatic cells because it is more easily harvested by surgeons without causing unnecessary trauma to patients [43].
With oral and maxillofacial compartments there are different types of somatic stem cells. Examples includes; inflammatory periapical progenitor cells (iPAPCs), apical papilla (SCAP), dental follicle stem cells (DFSCs), periodontal ligament stem cells (PDLSCs), dental pulp stem cells (DPSCs), bone marrow stem cells (BMSCs), salivary gland stem cells (SGSCs), tooth germ progenitor cells (TGPCs), gingival derived mesenchymal stem cells (GMSCs), oral epithelial stem cells

(OESCs), human exfoliated deciduous teeth (SHED), and periosteal derived (PSCs) [44, 45]. Most adult stem cells that can be taken from oral and maxillofacial regions are mesenchymal stem cells (MSCs). There are many pre-clinical animal model studies that show MSCs being successfully used in trea-ting several diseases ( Fig.3.).

**Induced pluripotent stem cells (iPSC), oral derived**

iPS cells are stem cells with pluripotent ability. They are induced via reprogramming of somatic cells by introducing defined factors [46]. IPSC are the least controversial among the four different categories of stem cells. It eliminates the ethical concerns of using embryonic stem cells and the limited material source problems with adult stem cells. Many studied have shown that introducing defined transcription factors will directly induce the stem cell into one cell type [47-51].

Since the discovery of iPSC, biomedical research has focused on, clinical application, method optimization, and the mechanisms behind reprogramming iPS cells [52]. iPS cell technology has progressed the regenerative medical field to replace lost and damaged organ parts by creating normal functioning cells, tissues, and whole organ parts [53].

The regeneration of alveolar bone, periodontal tissues, and tooth are greatly needed within the dentistry field. iPS cell technology has the ability to transform the regenerative dental medicine world. Currently, researchers are looking into dental tissue derived iPS cells and their application in dental tissue regeneration. There are studies showing that three kinds of dental stem cells derived from human can induce iPS cells by the introduction of 3 or 4 factors with an increased efficiency than human fibroblasts. These three dental stem cells are; DPSCs, SHED, and SCAP [54]. It has also been shown that mesenchymal stroll cells that are derived from third molars in humans can also be reprogrammed into iPS cells with a high efficiency [55]. Gingival fibroblasts are a great source for iPS cells because they can be acquired effectively and easily from patients without needing pulp or tooth extraction surgeries. Gingival fibroblasts have also been shown to have a high reprogramming efficie-ncy [56-58].

**Using somatic stem cells in both oral and maxillofacial repair and regeneration**

The aim of regenerative dental medicine is to regenerate or replace damaged dental tissue in order to regain tooth physiology and function. Stem cells can be used as an alternative therapy within the dental medicine field. Stem cell therapy can lead to a more effective rehabilitation because it can provide more exact physiological structures leading to increased function [66]. Several studies have aimed to treat periodontal disease via ADSCs, MSCs, PDLSCs, or DPSCs. There are also many clinical trials that show bone reparation by nasal stem cells, allogeneic MSCs, ADSCs, and bone marrow stromal cells. Other clinical trials look at tooth extractions, graft versus host disease, and facial tissues. We shall discuss some of the clinical trial results in this review.

**Somatic stem cells with scaffolds used in oral and maxillofacial repair and regeneration**

Various studies have shown that stem cells will respond differently based on the type of scaffold they are in [59-64]. Scaffold within oral and maxillofacial repair and regeneration are three dimensional biomaterials that mimic the extracellular matrix which facilitates cell survival by cell scaffold interactions, proliferation, and eventually differentiation. Designed scaffolds can improve both oral and maxillofacial repair and regeneration. They

need to be comprised of degradable material that has a low toxicity level [67].

The main types of scaffolds are; synthetic polymers, natural polymers, composite scaffolds, and calcium phosphate based ceramic scaffolds. These scaffold can be combined with bioactive compounds or factors and stem cells to get the desired effect [68]. Examples of factors that are combines with scaffolds includes; vascular endothelial growth factor, bone morphogenetic proteins, platelet derived growth factors, and SDF-1 [69-72].

As previously mentioned, the stem cells will respond differently based upon the type of scaffold they are implemented in. Zinc modified titanium can be engineered to release zinc which is used in dental and maxillofacial implants and can stimulate DPSCs to differentiate into osteoblasts [65]. DPSCs are capable of moving into cavities and differrentiating into osteoblasts after attaching onto biocoral scaffolds [73]. PLGA micro scaffolds are very porous and have been shown to increase the adhesion of DPSCs. This leads to increased stemness, viability, and plasticity of the dental pulp mesenchymal stem cells that are cultured [74]. Bone reiteration kinetics are also greatly increased due to scaffold morphology [75].

Stem cells and the scaffolds they are a part of can be transferred to the dental canal systems in order to regenerate vital pulp and allow for root formation [76]. VEGF can be used in scaffolds to increase optimization and be used in endodontic regenerative procedures [77]. Studies show that DPSCs with treated dentine matrix scaffolds associate significantly with bone formation when it was used to repair furcation perforations of the premolar teeth in dogs [78]**.**(Fig.4.)

The combination of stem cells with scaffolds can regenerate bone tissue effectively [79]. Collagen scaffolds seeded with DPSCs were shown to enhance osteogenic differentiation of DPSCs when plastically compressed. This causes an increase in the collagen fibrillary density in rat models with calvarial defects [80].

There is a clinical study that demonstrates how DPSC and collagen sponge biocomplex can completely restore the human mandible bone. The study also indicates how the cell population can repair or regenerate tissues and organs [81].

## The application of iPSC within dentistry

### Generation of iPS cells from fibroblasts

The first step of the application of iPS cells within disease treatment is to get the iPS cells with defined factors. There are four factors that can be used to induce pluripotent stem cells from fibroblasts. These factors are Lin28, Nanog, Sox2, and Oct4 [82]. It has also been shown that a set of family proteins of these four factors Oct3/4, Klf4, c-Myc, and Sox2 can reprogram mouse embryonic fibroblasts of MEFs to iPS cells. It was also shown that iPS cells could be generated without Myc [83]. iPS cells are able to be induced by primary human fibroblasts containing Sox2 or Oct4. Only one factor, Oct 3/4 needs to be introduced to derive iPS cells from neural stem cells in mice [84, 85]. These studies together show that there are defined factors that are essential to the reprogramming of cells to generate iPS cells.

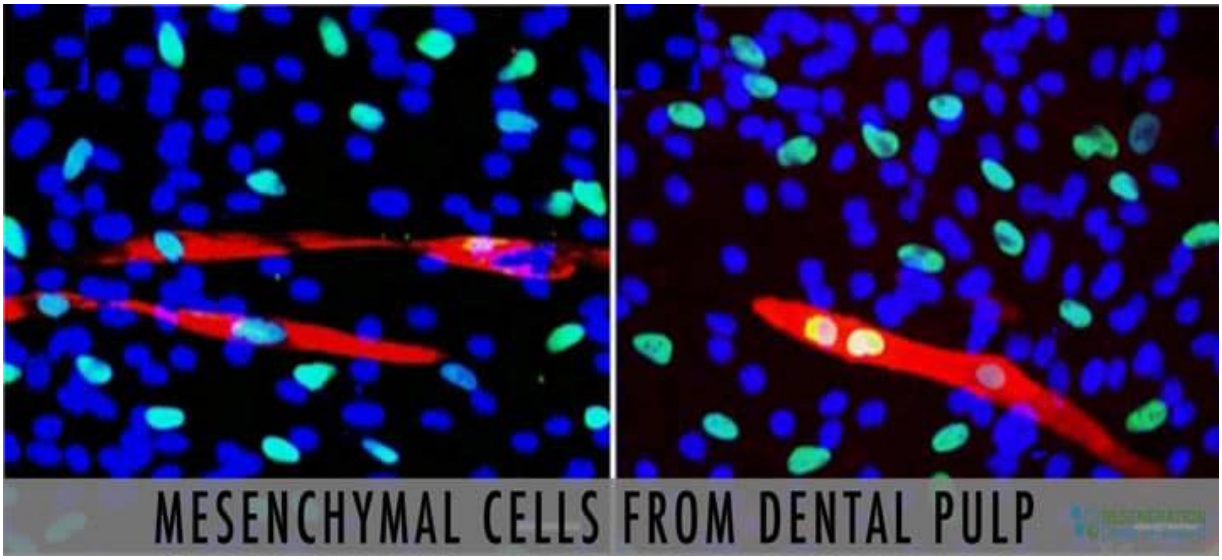



**Figure 4.** Generation of mesenchymal stem cells from dental pulp.

**Generating dental stem cells via iPS cells**

The four factors previously discussed; Nanog, Sox2, Oct4, and Lin28 or Oct3/4, Sox2, Klf4, and c-Myc are carried by viral vectors that are able to reprogram different dental stem cells. They have been shown to reprogram SCAP, SHED, and DPSCs [86]. Three factors without the factor c-Myc are able to reprogram gingival fibroblasts adult wild type mouse. The generation of iPS cells from human gingival fibroblasts requires the presence of all 4 defined factors [87, 88].

MSC like populations are highly present in dental tissues and have the potential to be reprogramed into different tissues using transcription factors that are cultured in specific conditions. Studies should be done to understand the technology behind the int-roduction of defined factors to generate iPS cells.

**Regenerating dental tissues form iPS cells**

With the increased understanding of iPS cells and the technology needed to regenerate them, much research has focused on the regeneration of alveolar bone, ameloblasts, cementum, etc [89]. There is an increased interest in whole tooth reiteration by using iPS cell technology. One of the first trials that aimed at regenerating a whole tooth from iPS cells was not successful [90]. Since then, tooth like structures containing dental pulp like areas and bone areas were generated from mouse derived iPS cells. This was achieved by mixing the iPS cells with mesenchymal and dental epithelial cells [91].

To generate a tooth germ there is a need for the integration between neural crest mesenchymal cells and ectoderm epithelial cells. This means that iPS cells need to differentiate into two different lineages and one of the lineages must be odontogenic [92]. It has been shown that iPS cells derived from dental epithelial cells do not have odontogenic potential [93].

**iPS cell challenges in dental applications**

Human iPS cell lines have not been shown to be as effective in the differentiation as those of mouse embryonic cells [94]. Human iPS cells differentiated from neural cells have been shown to have a lower efficiency than ESCs [95].

## Conclusions

There have been thousands of clinical trials focused around stem cells. This review foc used on several clinical trials that were correlated with different oral diseases. Although these studies have promising results, more research needs to be done to show the stem cells having homogeneity, delivery methods that can be used, the quality of the tissue that is regenerated or repaired, and most importantly if they could be integrated into a host successfully. The main problems in stem cell therapy is how to get the intended stem cells and how to differentiate them into the specific functional cells and whole tissues that are needed.

## Reefrences


1. Oshima M., Tsuji T. (2015) Whole Tooth Regeneration as a Future Dental Treatment. In: Bertassoni L., Coelho P. (eds) Engineering Mineralized and Load Bearing Tissues. Advances in Experimental Medicine and Biology, vol 881. Springer, Cham
2. J.M. SlackOrigin of stem cells in organogenesis Science, 322 (2008), pp. 1498-1501
3. Weissman I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell 100, 157–168. 10.1016/S0092-
4. 8674(00)81692-X
5. Takahashi K., Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676. 10.1016/j.cell.2006.07.024
6. Uskokovic V. (2015). Amelogenin in enamel tissue engineering. Adv. Exp. Med. Biol. 881, 237–254. 10.1007/978-3-319-22345-2_13
7. Hargreaves K. M., Berman L. H., Rotstein I. (2016). Cohen's Pathways of the Pulp, 11th Edn. St. Louis, MO: Elsevier.
8. Mari-Beffa M., Segura-Egea J. J., Diaz-Cuenca A. (2017). Regenerative endodontic procedures: a perspective from stem cell niche biology. J. Endod. 43, 52–62. 10.1016/j.joen.2016.09.011
9. Menicanin D., Hynes K., Han J., Gronthos S., Bartold P. M. (2015). Cementum and periodontal ligament regeneration. Adv. Exp. Med. Biol. 881, 207–236. 10.1007/978-3-319-22345-2_12
10. Wang Y., Zhou L., Li C., Xie H., Lu Y., Wu Y., et al. . (2015). Bone marrow-derived cells homing for self-repair of periodontal tissues: a histological characterization and expression analysis. Int. J. Clin. Exp. Pathol. 8, 12379–12389
11. Baba S., Yamada Y. (2016). Phase I/II Trial of autologous bone marrow stem cell transplantation with a three-dimensional woven-fabric scaffold for periodontitis. Stem Cells Int. 2016:6205910. 10.1155/2016/6205910
12. Chen F. M., Gao L. N., Tian B. M., Zhang X. Y., Zhang Y. J., Dong G. Y., et al. . (2016). Treatment of periodontal intrabony defects using autologous periodontal ligament stem cells: a randomized clinical trial. Stem Cell Res. Ther. 7, 33. 10.1186/s13287-016-0288-1
13. Panduwawala C. P., Zhan X., Dissanayaka W. L., Samaranayake L. P., Jin L., Zhang C. (2016). *In vivo* periodontal tissue regeneration by periodontal ligament stem cells and endothelial cells in three-dimensional cell sheet constructs. J. Periodont. Res. [Epub ahead of print]. 10.1111/jre.12405
14. Rajan A., Eubanks E., Edwards S., Aronovich S., Travan S., Rudek I., et al. . (2014). Optimized cell survival and seeding efficiency for craniofacial tissue engineering using clinical stem cell therapy. Stem Cells Transl. Med. 3, 1495–1503. 10.5966 /sctm.2014-0039
15. Smith B. T., Shum J., Wong M., Mikos A. G., Young S. (2015). Bone tissue engineering challenges in oral and maxillofacial surgery. Adv. Exp. Med. Biol. 881, 57–78. 10.1007/978-3-319-22345-2_4
16. Mele L., Vitiello P. P., Tirino V., Paino F., De Rosa A., Liccardo D., et al. . (2016). Changing paradigms in cranio-facial regeneration: current and new strategies for the activation of endogenous stem cells. Front. Physiol. 7:62. 10.3389/ fphys. 2016.00062
17. Mao J. J., Prockop D. J. (2012). Stem cells in the face: tooth regeneration and beyond. Cell Stem Cell 11, 291–301. 10.1016/j.stem.2012.08.010
18. B. Daly, A. Thompsell, J. Sharpling, Y. M. Rooney, L. Hillman, K. L. Wanyonyi, S. White &J. E. Gallagher (2017) Evidence summary: the relationship between oral health and dementia. Nature. doi:10.1038/sj.bdj.2017.992
19. Yang, B., Qiu, Y., Zhou, N., Ouyang, H., Ding, J., Cheng, B., & Sun, J. (2017). Application of Stem Cells in Oral Disease Therapy: Progresses and Perspectives. *Frontiers in Physiology*, *8*, 197. http://doi.org/10.3389/fphys.2017.00197

20. Caton J., Bostanci N., Remboutsika E., De Bari C., Mitsiadis T. A. (2011). Future dentistry: cell therapy meets tooth and periodontal repair and regeneration. J. Cell. Mol. Med. 15, 1054–1065. 10.1111/j.1582-4934.2010.01251.x
21. Persoon, I. F., &Özok, A. R. (2017). Definitions and Epidemiology of Endodontic Infections. *Current Oral Health Reports*, *4*(4), 278–285. http://doi.org/10.1007/s40496-017-0161-z
22. Persoon IF, Buijs MJ, Özok AR, Crielaard W, Krom BP, Zaura E, et al. The mycobiome of root canal infections is correlated to the bacteriome. Clin Oral Investig. 2017;21:1871–1881. doi: 10.1007/s00784-016-1980-3.
23. Alazawi W, Bernabe E, Tai D, Janicki T, Kemos P, Samsuddin S, et al. (2017) Periodontitis is associated with significant hepatic fibrosis in patients with non-alcoholic fatty liver disease. PLoS ONE 12(12): e0185902. https://doi.org/10.1371/journal.pone.0185902
24. Batchelor P. Is periodontal disease a public health problem? Br Dent J. 2014;217(8):405–9. pmid: 25342346.
25. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ, Cdc Periodontal Disease Surveillance workgroup: James Beck GDRP. Prevalence of periodontitis in adults in the United States: 2009 and 2010. J Dent Res.2012;91(10):914–20. pmid: 22935673.
26. White DA, Tsakos G, Pitts NB, Fuller E, Douglas GV, Murray JJ, et al. Adult Dental Health Survey 2009: common oral health conditions and their impact on the population. Br Dent J. 2012;213(11):567–72. pmid:23222333.
27. Dumitrescu AL. Editorial: Periodontal Disease—A Public Health Problem. Front Public Health. 2015;3:278. pmid:26779473; PubMed Central PMCID: PMCPMC4705816.
28. Hargreaves K. M., Berman L. H., Rotstein I. (2016). Cohen's Pathways of the Pulp, 11th Edn. St. Louis, MO: Elsevier.
29. Trowbridge H. O. (1981). Pathogenesis of pulpitis resulting from dental caries. J. Endod.7,52–60.10.1016/S0099-2399(81)80242-7
30. Holland, Roberto, Gomes Filho, João Eduardo, Cintra, Luciano Tavares Angelo, Queiroz, Índia Olinta de Azevedo, & Estrela, Carlos. (2017). Factors affecting the periapical healing process of endodontically treated teeth. *Journal of Applied Oral Science*, *25*(5), 465-476. https: //dx.doi.org/10.1590/1678-7757-2016-0464
31. Soares T. R., Fidalgo T. K., Quirino A. S., Ferreira D. M., Chianca T. K., Risso P. A., et al. . (2016). Is caries a risk factor for dental trauma? A systematic review and meta-analysis. Dent. Traumatol. 33, 4–12. 10.1111/edt.12295
32. Mjor I. A. (2001). Pulp-dentin biology in restorative dentistry. Part 5: clinical management and tissue changes associated with wear and trauma. Quintessence Int. 32, 771–788.
33. Feliciano K. M., de Franca Caldas A., Jr. (2006). A systematic review of the diagnostic classifications of traumatic dental injuries. Dent. Traumatol. 22, 71–76. 10.1111/j.1600-9657.2006.00342.x
34. Sequeira-Byron P., Fedorowicz Z., Carter B., Nasser M., Alrowaili E. F. (2015). Single crowns versus conventional fillings for the restoration of root-filled teeth. Cochrane Database Syst Rev. 16:CD009109. 10.1002/14651858.cd009109.pub3
35. Ozcan M., Schoonbeek G., Gokce B., Comlekoglu E., Dundar M. (2010). Bond strength comparison of amalgam repair protocols using resin composite in situations with and without dentin exposure. Oper. Dent. 35, 655–662. 10.2341/10-091-L
36. Tantbirojn D., Pfeifer C. S., Braga R. R., Versluis A. (2011). Do low-shrink composites reduce polymerization shrinkage effects?" J. Dent. Res. 90, 596–601. 10.1177/0022034510396217
37. Mertens C., Freudlsperger C., Bodem J., Engel M., Hoffmann J., Freier K. (2016). Reconstruction of the maxilla following hemimaxillectomy defects with scapular tip grafts and dental implants. J. Craniomaxillofac. Surg. 44, 1806–1811. 10.1016/j.jcms.2016.08.010
38. Okay D., Al Shetawi A. H., Moubayed S. P., Mourad M., Buchbinder D., Urken M. L. (2016). Worldwide 10-year systematic review of treatment trends in fibula free flap for mandibular reconstruction. J. Oral Maxillofac. Surg. 74, 2526–2531. 10.1016/j.joms.2016.06.170
39. Wijbenga J. G., Schepers R. H., Werker P. M., Witjes M. J., Dijkstra P. U. (2016). A systematic review of functional outcome and quality of life following reconstruction of maxillofacial defects using vascularized free fibula flaps and dental rehabilitation reveals poor data quality. J. Plast. Reconstr. Aesthet. Surg. 69,1024–1036. 10.1016/j.bjps.2016.05.003
40. Weissman I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell 100, 157–168. 10.1016/S0092-8674(00)81692-X
41. Murray P. E., Garcia-Godoy F., Hargreaves K. M. (2007). Regenerative endodontics: a review of current status and a call for action. J. Endod. 33, 377–390. 10.1016/j.joen.2006.09.013

42. Tatullo M., Marrelli M., Paduano F. (2015). The regenerative medicine in oral and maxillofacial surgery: the most important innovations in the clinical application of mesenchymal stem cells. Int. J. Med. Sci. 12, 72–77. 10.7150/ijms.10706
43. Caplan A. I. (1991). Mesenchymal stem cells. J. Orthop. Res. 9, 641–650. 10.1002/jor.1100090504
44. Mao J. J., Prockop D. J. (2012). Stem cells in the face: tooth regeneration and beyond. Cell Stem Cell 11, 291–301. 10.1016/j.stem.2012.08.010
45. Liao J., Al Shahrani M., Al-Habib M., Tanaka T., Huang G. T. (2011). Cells isolated from inflamed periapical tissue express mesenchymal stem cell markers and are highly osteogenic. J. Endod. 37, 1217–1224. 10.1016/j.joen.2011.05.022
46. Egusa H., Sonoyama W., Nishimura M., Atsuta I., Akiyama K. (2012). Stem cells in dentistry–part I: stem cell sources. J. Prosthodont. Res. 56, 151–165. 10.1016/j.jpor. 2012.06.001
47. Takahashi K., Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676. 10.1016/j.cell.2006.07.024
48. Zhou Q., Brown J., Kanarek A., Rajagopal J., Melton D. A. (2008). in vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455, 627–632. 10.1038/nature07314
49. Szabo E., Rampalli S., Risueno R. M., Schnerch A., Mitchell R., Fiebig-Comyn A., et al. . (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468, 521–526. 10.1038/nature09591
50. Vierbuchen T., Ostermeier A., Pang Z. P., Kokubu Y., Sudhof T. C., Wernig M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463, 1035–1041. 10.1038/nature08797
51. Huang P., He Z., Ji S., Sun H., Xiang D., Liu C., et al. . (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475, 386–389. 10.1038/nature10116
52. Kim E. Y., Page P., Dellefave-Castillo L. M., McNally E. M., Wyatt E. J. (2016). Direct reprogramming of urine-derived cells with inducible MyoD for modeling human muscle disease. Skelet. Muscle 6, 32. 10.1186/s13395-016-0103-9
53. Karagiannis P., Eto K. (2016). Ten years of induced pluripotency: from basic mechanisms to therapeutic applications. Development 143, 2039–2043. 10.1242/dev.138172
54. Mason C., Dunnill P. (2008). A brief definition of regenerative medicine. Regen. Med. 3,1–5. 10.2217/17460751.3.1.1
55. Yan X., Qin H., Qu C., Tuan R. S., Shi S., Huang G. T. (2010). iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 19, 469–480. 10.1089/scd.2009.0314
56. Oda Y., Yoshimura Y., Ohnishi H., Tadokoro M., Katsube Y., Sasao M., et al. . (2010). Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. J. Biol. Chem. 285, 29270–29278. 10.1074/jbc.M109.055889
57. Egusa H., Okita K., Kayashima H., Yu G., Fukuyasu S., Saeki M., et al. . (2010). Gingival fibroblasts as a promising source of induced pluripotent stem cells. PLoS ONE5:e12743. 10.1371/journal.pone.0012743
58. Wada N., Wang B., Lin N. H., Laslett A. L., Gronthos S., Bartold P. M. (2011). Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts. J. Periodont. Res. 46, 438–447. 10.1111/j.1600-0765.2011.01358.x
59. Arakaki M., Egusa H., Otsu K., Saitoh K., Miura T., Harada H. (2013). Frontier dental research on iPS cells. J. Oral Biosci. 55, 191–199. 10.1016/j.job.2013.08.002
60. Motamedian S. R., Tabatabaei F. S., Akhlaghi F., Torshabi M., Gholamin P., Khojasteh A. (2016). Response of dental pulp stem cells to synthetic, Allograft, and Xenograft bone Scaffolds. Int. J. Periodontics Restorative Dent. 37, 47–59. 10.11607/prd.2121
61. Jaramillo M, Yeh H, Yarmush ML, Uygun BE. Decellularized Human Liver Extracellular Matrix (hDLM) Mediated Hepatic Differentiation of Human Induced Pluripotent Stem Cells (hIPSc). J Tissue Eng Regen Med. 2017. https: //doi.org/10.1002/term.2627
62. Reddi AH. Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells, and biomimetic biomaterials. Tissue Eng. 2000;6(4):351–9.
63. Gonzalez-Vazquez A, Planell JA, Engel E. Extracellular calcium and CaSR drive osteoinduction in mesenchymal stromal cells. Acta Biomater. 2014;10(6):2824–33.
64. Kutkut A, Andreana S. Medical-grade calcium sulfate hemihydrate in clinical implant dentistry: a review. J Long Term Eff Med Implants. 2010;20(4):295–301.
65. Takanori Eguchi and Takuo Kuboki, “Cellular Reprogramming Using Defined Factors and MicroRNAs,” Stem Cells International, vol. 2016, Article ID 7530942, 12 pages, 2016. doi: 10.1155/2016/7530942

66. Yusa K., Yamamoto O., Takano H., Fukuda M., Iino M. (2016a). Zinc-modified titanium surface enhances osteoblast differentiation of dental pulp stem cells in vitro. Sci. Rep. 6:29462. 10.1038/ srep29462
67. Rada C., Jarvis J. M., Milstein C. (2002). AID-GFP chimeric protein increases hypermutation of Ig genes with no evidence of nuclear localization. Proc. Natl. Acad. Sci. U.S.A. 99, 7003–7008. 10.1073/pnas.092160999
68. Horst O. V., Chavez M. G., Jheon A. H., Desai T., Klein O. D. (2012). Stem cell and biomaterials research in dental tissue engineering and regeneration. Dent. Clin. North Am. 56, 495–520. 10.1016/ j.cden.2012.05.009
69. Mele L., Vitiello P. P., Tirino V., Paino F., De Rosa A., Liccardo D., et al. . (2016). Changing paradigms in cranio-facial regeneration: current and new strategies for the activation of endogenous stem cells. Front. Physiol. 7:62. 10.3389/ fphys. 2016.00062
70. Luu H. H., Song W. X., Luo X., Manning D., Luo J., Deng Z. L., et al. . (2007). Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J. Orthop. Res. 25, 665–677. 10.1002/jor.20359
71. Schipani E., Maes C., Carmeliet G., Semenza G. L. (2009). Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. J. Bone Miner. Res. 24, 1347–1353. 10.1359/jbmr.090602
72. Phipps M. C., Xu Y., Bellis S. L. (2012). Delivery of platelet-derived growth factor as a chemotactic factor for mesenchymal stem cells by bone-mimetic electrospun scaffolds. PLoS ONE 7:e40831. 10.1371/journal.pone.0040831
73. Kitaori T., Ito H., Schwarz E. M., Tsutsumi R., Yoshitomi H., Oishi S., et al. . (2009). Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis Rheum. 60, 813–823. 10.1002 /art.24330
74. Mangano C., Paino F., d'Aquino R., De Rosa A., Iezzi G., Piattelli A., et al. . (2011). Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. PLoS ONE 6:4. 10.1371/journal.pone.0018721
75. Bhuptani R. S., Patravale V. B. (2016). Porous microscaffolds for 3D culture of dental pulp mesenchymal stem cells. Int. J. Pharm. 515, 555–564. 10.1016/j.ijpharm.2016.10.040
76. Giuliani A., Manescu A., Mohammadi S., Mazzoni S., Piattelli A., Mangano F., et al. . (2016). Quantitative kinetics evaluation of blocks versus granules of biphasic calcium phosphate scaffolds (HA/β-TCP 30/70) by synchrotron radiation X-ray microtomography. Implant Dent. 25, 6–15. 10.1097 /ID.0000000000000363
77. Chrepa V., Henry M. A., Daniel B. J., Diogenes A. (2015). Delivery of apical mesenchymal stem cells into root canals of mature teeth. J. Dent. Res. 94, 1653–1659. 10.1177/0022034515596527
78. Yadlapati M., Biguetti C., Cavalla F., Nieves F., Bessey C., Bohluli P., et al. . (2017). Characterization of a vascular endothelial growth factor–loaded bioresorbable delivery system for pulp regeneration. J. Endod. 43, 77–83. 10.1016/j.joen. 2016. 09.022
79. Bakhtiar H., Mirzaei H., Bagheri M. R., Fani N., Mashhadiabbas F., Baghaban Eslaminejad M., et al. . (2016). Histologic tissue response to furcation perforation repair using mineral trioxide aggregate or dental pulp stem cells loaded onto treated dentin matrix or tricalcium phosphate. Clin. Oral Investig. 10.1007/s00784-016-1967-0
80. Kitamura M., Akamatsu M., Machigashira M., Hara Y., Sakagami R., Hirofuji T., et al. . (2011). FGF-2 stimulates periodontal regeneration: results of a multi-center randomized clinical trial. J. Dent. Res. 90, 35–40. 10.1177/0022034510384616
81. Chamieh F., Collignon A. M., Coyac B. R., Lesieur J., Ribes S., Sadoine J., et al. . (2016). Accelerated craniofacial bone regeneration through dense collagen gel scaffolds seeded with dental pulp stem cells. Sci. Rep. 6:38814. 10.1038/srep38814
82. d'Aquino R., De Rosa A., Lanza V., Tirino V., Laino L., Graziano A., et al. . (2009a). Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur. Cell. Mater. 18, 75–83. 10.22203/eCM.v018a07
83. Yu J., Vodyanik M. A., Smuga-Otto K., Antosiewicz-Bourget J., Frane J. L., Tian S., et al. . (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920. 10.1126/science.1151526
84. Nakagawa M., Koyanagi M., Tanabe K., Takahashi K., Ichisaka T., Aoi T., et al. . (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. 26, 101–106. 10.1038/nbt1374
85. Huangfu D., Osafune K., Maehr R., Guo W., Eijkelenboom A., Chen S., et al. . (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat. Biotechnol. 26, 1269–1275. 10.1038/nbt.1502

86. Kim J. B., Sebastiano V., Wu G., Arauzo-Bravo M. J., Sasse P., Gentile L., et al. . (2009). Oct4-induced pluripotency in adult neural stem cells. Cell 136, 411–419. 10.1016/j.cell.2009.01.023
87. Yan X., Qin H., Qu C., Tuan R. S., Shi S., Huang G. T. (2010). iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 19, 469–480. 10.1089/scd. 2009.0314
88. Egusa H., Okita K., Kayashima H., Yu G., Fukuyasu S., Saeki M., et al. . (2010). Gingival fibroblasts as a promising source of induced pluripotent stem cells. PLoS ONE5:e12743. 10.1371/journal.pone. 0012743
89. Wada N., Wang B., Lin N. H., Laslett A. L., Gronthos S., Bartold P. M. (2011). Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts. J. Periodont. Res. 46, 438–447. 10.1111/j.1600-0765.2011.01358.x
90. Yoshida K., Sato J., Takai R., Uehara O., Kurashige Y., Nishimura M., et al. . (2015). Differentiation of mouse iPS cells into ameloblast-like cells in cultures using medium conditioned by epithelial cell rests of Malassez and gelatin-coated dishes. Med. Mol. Morphol. 48, 138–145. 10.1007 /s00795-014-0088-6
91. Otsu K., Kishigami R., Oikawa-Sasaki A., Fukumoto S., Yamada A., Fujiwara N., et al. . (2012). Differentiation of induced pluripotent stem cells into dental mesenchymal cells. Stem Cells Dev. 21, 1156–1164. 10.1089/scd.2011.0210
92. Wen Y., Wang F., Zhang W., Li Y., Yu M., Nan X., et al. . (2012). Application of induced pluripotent stem cells in generation of a tissue-engineered tooth-like structure. Tissue Eng. Part A 18, 1677–1685. 10.1089/ten.tea.2011.0220
93. Ning F., Guo Y., Tang J., Zhou J., Zhang H., Lu W., et al. . (2010). Differentiation of mouse embryonic stem cells into dental epithelial-like cells induced by ameloblasts serum-free conditioned medium. Biochem. Biophys. Res. Commun. 394, 342–347. 10.1016/j.bbrc.2010.03.007
94. Arakaki M., Ishikawa M., Nakamura T., Iwamoto T., Yamada A., Fukumoto E., et al. . (2012). Role of epithelial-stem cell interactions during dental cell differentiation. J. Biol. Chem. 287, 10590–10601. 10.1074/jbc.M111.285874
95. Cai J., Zhang Y., Liu P., Chen S., Wu X., Sun Y., et al. . (2013). Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. Cell Regen. 2:6. 10.1186/2045-9769-2-6
96. Hu B. Y., Weick J. P., Yu J., Ma L. X., Zhang X. Q., Thomson J. A., et al. . (2010). Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc. Natl. Acad. Sci. U.S.A. 107,4335–4340. 10.1073/pnas.0910012107

Corresponding author: Prof. Mirjana Pavlovic, MD, PhD
CEECS, EE-96, #515
Florida Atlantic University, Bioengineering, 33431 Boca Raton, FL, USA
Tel: 561-297-234
e-mail: **mpavlovi@fau.edu**

# ISPITIVANJE FUNKCIJE TROMBOCITA KOD PACIJENATA NA TERAPIJI ACETILSALICILNOM KISELINOM: FAKTORI KOJI UTIČU NA EFIKASNOST LEČENJA

**Ana Antić[1], Zoran Stanojković [1,2], Miodrag Vučić [1,3], Milan Lazarević [4], Nebojša Vacić [3]**

[1]Zavod za transfuziju krvi Niš;
[2]Medicinski fakultet Niš, Univerzitet u Nišu;
[3]Klinika za hematologiju, Klinički centar Niš;
[4]Klinika za kardiovaskularnu i transplantacionu hirurgiju, Klinički centar Niš

## Sažetak

***Uvod:*** *Iako je dokazana nedvosmislena uloga acetilsalicilne kiseline (ASK) u primarnoj i sekundarnoj prevenciji kardiovaskularnih i cerebrovaskularnih neželjenih događaja, postoje slučajevi suboptimalnog odgovora na ASK koji su označeni kao rezistencija, a koji su udruženi sa nepovoljnim ishodom terapije. Cilj ovog ispitivanja je bio da izdvoji faktore koji su od uticaja na promenljivi odgovor ASK na funkciju trombocita, merenjem agregacije trombocita metodom impedantne agregometrije.*

***Pacijenti i metode rada****: Ispitivanje je obuhvatilo 180 pacijenata koji su nakon infarkta miokarda uzimali jedan od tri različita ASK preparata, pojedinačno ili u kombinaciji sa klopidogrelom. Funkcija trombocita merena je na impedantnom agregometru Multiplate (Multiplate Platelet Function Analyzer, Roche) iz uzoraka krvi sa heparinom korišćem ASPI i TRAP testa. Ispitivana je uloga sledećih faktora na vrednosti TRAP i ASPI testa: pol, starost, vrsta ASK preparata, prisustvo šećerene bolesti, pušenje i upotreba lekova (antikoagulansi, inhibitori protonske pumpe, beta blokatori).*

***Rezultati****: Godine starosti i primena antikoagulanasa smanjuje bazičnu funkciju trombocita, dok je inhibitori protonske pumpe povećavaju. Prisustvo šećerne bolesti predstavlja faktor rizika koji smanjuje efikasnost ASK (Beta=0,553; p<0,001), kao i pušenje (Beta=0,543; p<0,001) i primena inhibitora protonske pumpe (Beta=0,536; p<0,001). S druge strane, primena antikoagulanasa pojačava efekat ASK (Beta=-0,529; p<0,001), kao i primena Aspirin protect-a (Beta=-0,435; p<0,001).*

***Zaključak****: Pušenje, prisustvo šećerne bolesti, primena inhibitora protonske pumpe i antikoagulantnih lekova, kao i vrsta primenjenog ASK preparata se izdvajaju kao najvažniji faktori koji utiču na efikasnost ASK na funkciju trombocita, merenu metodom impedantne agregometrije.*

**Ključne reči**: acetilsalicilna kiselina, trombociti, agregometrija

# ASSESSMENT OF PLATELET FUNCTION IN PATIENTS TREATED WITH ACETYLSALICYLIC ACID: FACTORS AFFECTING THE EFFICIENCY OF TREATMENT


## Summary

***Introduction:*** *Altought it is established an unequivocal role of acetylsalicylic acid (ASA) in the primary and secondary prevention of cardiovascular and cerebrovascular adverse events, there are cases of suboptimal response to the ASA, called the resistance, which are associated with adverse outcome of therapy. The aim of this study was to identify the factors that influence the variable response of ASA on platelet function, measuring platelet aggregation by method of impedance aggregometry.*

***Patients and method:.*** *The examination included 180 patients, treated with one of three different ASA preparations after acute myocardial infarction, as a single therapy or with clopidogrel.. Platelet function was measured using impedance aggregometer Multiplate (Multiplate Platelet Function Analyzer, Roche) in blood samples with heparin for ASPI and TRAP tests. We investigated the impact of the following factors on the value of the TRAP and ASPI: gender, age, type of ASA preparation, the presence of diabetes mellitus, smoking and the use of drugs (anticoagulant agents, proton pump inhibitors, beta-blockers).*

***Results****: Age and the use of anticoagulants reduce the basic platelet function, while the proton pump inhibitors increase it. Diabetes is a risk factor that reduces the efficiency of ASA (Beta=0,553; p<0,001), as well as smoking (Beta=0,543; p<0,001) and the use of proton pump inhibitors (Beta=0,536; p<0,001). On the other hand, the use of anticoagulants enhances the effect of ASA (Beta=-0,529; p<0,001), as also the use of Aspirin protect (Beta=-0,435; p<0,001).*

***Conclusion****: Smoking, diabetes, the use of proton pump inhibitors and anticoagulants, as well as the type of the applied ASA preparation stand out as the most important factors that affect the efficiency of ASA on platelet function as measured by the method of impedance aggregometry.*


**Key words:** acetylsalicylic acid, platelets, aggregometry

## Uvod

Aterotromboza koronarnih, cerebrovaskularnih i perifernih arterijskih krvnih sudova je vodeći uzrok morbiditeta i mortaliteta u svetu. Trombociti imaju fundamentalnu ulogu u patofizoiologiji ateroskleroze i postali su glavni cilj terapije u primarnoj i sekundarnoj prevenciji oboljenja udruženih sa aterosklerozom. Brojne kliničke studije podržavaju bezbednost i efikasnost pojedinačne ili dvojne antitrombocitne terapije, a napisani si i različiti vodiči u cilju adekvatne primene antitrombocitne terapije u svakodnevnoj praksi. Acetilsalicilna kiselina (ASK) je vode-ća u antitrombocitnoj terapiji, a brojna ispi-tivanja pokazuju da ASK smanjuje stepen incidence infarkta miokarda, moždanog udara i iznenadne srčane smrti za 25% kod pacijenata sa visokim rizikom (1-3).

ASK ostvaruje svoj pozitivan kardiovaskularni efekat inhibicijom produkcije tromboksana (TxA2), koji je trombocitni agonist i izaziva vazokonstrikciju i proliferaciju glatkomišićnjih ćelija endotela krvnih sudova. Konverzija arahidonske kiseline u TxA2 je reguli-

sana enzimom koji se zove ciklooksigenaza (COX), a koja postoji u dva oblika: COX-1, prisutna u svim tkivima kao konstitutivni enzim, i COX-2, koja se stvara tokon inflamacije (4). Efekat ASK na COX-1 je ireverzibilan i traje koliko i život trombocita, zaviseći od produkcije novih trombocita koji će omogućiti oporavak cikolooksigenazne aktivnosti na približno 10% dnevno kod zdravih ljudi. Male doze ASK su dovoljne da suprimiraju više od 95% sinteze TxA2 preko COX-1, a takva supresija dovodi do inhibicije agregacije trombocita. Ipak, trombociti na koje deluje ASK mogu i dalje da agregiraju u prisustvu nekog potentnog ago-nista, kakav je kolagen ili trombin (5,6). COX-2 se nalazi u aterosklerotičnim plakovima i novostvorenim trombocitima i odgovoran je za sintezu prostaciklina, koji neutrališe efekat TxA2 prevencijom agregacije trombocita i stimulisanjem vazodilatacije. Veće doze aspirina mogu inhibirati COX-2 sintezu prostaciklina u endotelnim ćelijama i izazvati antiinflamatorni efekat. Treba istaći i da selektivna COX-2 inhibicija koja je dovoljna za suprimiranje produkcije prostaciklina može da izazove arterijsku trombozu. Ipak, COX-2 prisutan u endotelnim ćelijama zadržava spo-sobnost da se povrati produkcija prostaciklina nekoliko sati nakon uzimanja ASK zahvaljujući sposobnosti ćelija sa jezgrom da sintetišu proteine (7,8).

Kao i ostale terapijske strategije, terapija ASK ne može da obezbedi preveciju svih kardiovaskularnih dogadjaja, što ne iznenađuje imajući u vidu kompleksnost arterijske tromboze i specifičnosti trombocitne fiziologije. Ovaj nedostatak terapijskog uspeha je izazvao uvođenje brojnih dijagnostičkih testova i platformi testiranja sa ciljem adekvatnog vođenja i optimizacije kliničkog tretmana, a sve u cilju identifikovanja suboptimalnog odgovora na preparate ASK, koji je definisan kao rezistencija (9,10). Iako se termin rezistencija često koristi neadekvatno i mnogi autori se slažu da je bolje reći neadekvatan odgovor ili neuspeh terapije, podaci iz kliničkih studija koji identifikuju tzv. rezistenciju pokazuju povezanost smanjenog odgovora trombocita na ASK sa nepovoljnim ishodom terapije. Identifikovano je nekoliko mehanizama koji objašnjavaju pojavu rezistencije na ASK: neadekvatna podnošljivost leka ili rani prestanak njegove primene, moguće interakcije lekova, primena neadekvatne doze leka, povećana fluktuacija trombocita, komorbiditeti (šećerna bolest, bubrežna slanost, trombocitemija), genetski polimorfizam i potencijalni bypass mehanizmi (11-13).

***Cilj*** ovog ispitivanja je bio da izdvoji faktore koji su od uticaja na promenljivi odgovor ASK na funkciju trombocita, merenjem agregacije trombocita metodom impedantne agregometrije.

## Pacijenti i metoda rada

Istraživanje je obuhvatilo 180 pacijenata koji su bili na pojedinačnoj (samo ASK) ili dvojnoj (ASK i klopidogrel) antitrombocitnoj terapiji nakon akutnog infarkta miokarda u trajanju od dva do šest meseci. Svi pacijenti su imali očuvanu bubrežnu funkciju i broj trombocita u okviru referentnog opsega (150-300 x $10^9$/L). Pacijenti su razvrstani prema polu, starosti, vrsti ASK preparata, prisustvu šećerene bolesti, upotrebi cigareta (pušenje) i konkomitantnih lekova (antikoagulansi, inhibitori protonske pumpe, beta blokatori).Funkcija trombocita određivana je metodom impedantne agregometrije na aparatu *Multiplate* (Multiplate Platelet Function Ana-lyzer, Roche) 12 sati nakon uzimanja leka. Uzorci krvi uzeti su u epruvete od 4 ml sa litijum heparinom (*VenoSafe, Terumo).* Procedura podrazumeva dodavanje 300 µl heparinizirane krvi i 300 µl fiziološkog rastvora u test ćeliju. Nakon inkubacije na 37ºC u trajanju od tri minuta dodaje se 20 µl izabranog agonista, te se postiže finalna koncentracija arahidonske kiseline

(M-AA) od 0,5 mM (ASPI test) i TRAP-a od 3,2 μM (TRAP test). Uzorak krvi sa dodatim agonistom se automatski meša (800 U/min) korišćenjem magnetne mešalice obložene politetra-fluoro-etilenom (PTFE). Aktivirani trombociti adheriraju na elektrode i pojačavaju električnu impedancu između njih koja se registruje u toku 6 minuta, a porast impedance se prevodi u proizvoljne agregacijske jedinice (*arbitary aggregation units* (AU)). Parametri koji se prate u toku testiranja su: površina ispod agregacijske krive (AUC), koja je direktno zavisna od visine krive, a pokazuje ukupnu aktivnost trombocita, visina agregacijske krive, koja pokazuje stepen agregacije trombocita i maksimalni nagib agregacijske krive, koji pokazuje brzinu agregacije trombocita.

Analizirani parametri su prikazani tabelarno, sa srednjim vrednostima i standardnim devijacijama. Statistička signifikantnost je određivana na nivou $p<0,05$, primenom softverskog paketa SPSS, verzija 20 (SPSS Software GmbH).

Normalnost distribucije individualnih vredno-sti ispitivana je Kolomogorov Smirnov test-om. U slučaju normalnog rasporeda distribu-cije korišćen je ANOVA test, dok je u slučajevima nepravilne distribucije podataka korišćen neparametrijski Kruskal Wallis test. Za testiranje statističke značajnosti razlika apsolutnih frekvencija među uzorcima kori-šćen je $\chi^2$-test. Univarijantna i multivarijantna linearna regresiona analiza su korišćene za određivanje prediktivnih faktora za procenu efikasnosti ASK preparata.

**Rezultati**

Od ukupnog broja pacijenata u ispitivanju bilo je 92 (92/180, ili 51,1%) muškarca i 88 (88/180, ili 48,9%) žena. Prosečna starost cele ispitivane populacije je iznosila 55,74±11,21 godina, gde je najmlađi pacijent imao 30 godina, a najstariji 77 godina. Starosna struktura ispitanika prema polnoj distribuciji se nije statistički značajno razlikovala ($t=1,087$; $p=0,278$). Pacijenti su koristili 3 vrste ASK preparata u pojedinačnoj dozi od 100 mg, i to 77 pacijenata (41,1%) su uzimali Aspirin protect (*Bayer),* 56 pacijenata (31,1%) su uzimali Cardiopirin (*GL Pharma GMBH),* dok je 50 pacijenata (27,8%) bilo na Andolu (*Pliva).* Na pojedinačnoj terapiji je bilo 100 pacijenata (100/180, ili 55,55%), dok je na dvojnoj terapiji bilo 80 pacijenata (80/180, ili 44,45%). U Tabeli 1 su date opšte karakteristike pacijenata u ispitivanju.

**Tabela 1**

**Opšte karakteristike pacijenata u ispitivanju**

| | | Aspirin protect | Cardiopirin | Andol | $\chi_{KW}^2/F^*$ | p |
|---|---|---|---|---|---|---|
| ***Pol*** | m | 38(51,4) | 29(51,8) | 25(50,0) | | |
| | ž | 36(48,6) | 37(51,8) | 25(50,0) | 0,037 | 0,982 |
| ***Starost*** | | 55,74±11,21 | 53,66±12,00 | 56,52±11,1 | 0,914 | 0,403 |
| ***Dijabetes*** | da | 6(8,1) | 16(28,6) | 18(36,0) | | |
| | ne | 68(91,9) | 40(71,4) | 32(64,0) | 15,327* | <0,001 |
| ***Pušenje*** | da | 18(24,3) | 29(51,8) | 34(68,0) | | |
| | ne | 56(75,7) | 27(48,2) | 16(32,0) | 25,510 | <0,001 |
| ***Antikoagulansi*** | da | 35(47,3) | 7(12,5) | 9(18,0) | | |
| | ne | 39(52,7) | 49(87,5) | 41(82,0) | 22,649 | <0,001 |
| ***Beta blokatori*** | da | 34(45,9) | 30(53,6) | 25(50,0) | | |
| | ne | 40(54,1) | 26(46,4) | 25(50,0) | 0,750 | 0,687 |
| ***Inhibitori protonske pumpe*** | da | 11(14,9) | 18(32,1) | 18(36,0) | | |
| | ne | 63(85,1) | 38(67,9) | 32(64,0) | 8,441 | 0,015 |

*$\chi_{KW}^2$- Kruskal-Wallis Test F-ANOVA*

Ispitivan je pojedinačni uticaj faktora na bazičnu funkciju trombocita–TRAP test (Tabela 2). Utvrđeno je da starost predstavlja faktor rizika za bazičnu funciju trombocita (Beta=-0,189; p=0,011), odnosno da sa starenjem bazična funkcija trombocita opada. Primena antikoagulanasa smanjuje bazičnu funkciju trombocita (Beta=0,176; p=0,011), dok je inhibitori protonske pumpe povećavaju (Beta=0,169; p=0,023).

**Tabela 2**

**Univarijanta linearna regresiona analiza nezavisnih faktora za procenu bazične funkcije trombocita (TRAP test)**

| | Nestandardizovani koeficijent BSG | | Standardizovani koeficijent Beta | 95%CI | p |
|---|---|---|---|---|---|
| ***Pol*** | 8,447 | 33,905 | 0,019 | -58,460- 75,354 | 0,804 |
| ***Starost*** | -3,756 | 1,459 | -0,189 | -6,635- -0,876 | 0,011 |
| ***Aspirin protect*** | 10,547 | 34,442 | 0,023 | -57,393-78,541 | 0,759 |
| ***Cardiopirin*** | 37,806 | 35,506 | 0,077 | -34,233-109,846 | 0,302 |
| ***Andol*** | -53,149 | 37,635 | -0,105 | -127,418-21,120 | 0,160 |
| ***Dijabetes*** | 34,418 | 40,692 | 0,063 | -45,883-114,718 | 0,399 |
| ***Pušenje*** | -12,779 | 34,060 | -0,028 | -79,988-54,437 | 0,708 |
| ***Antikoagulansi*** | -88,481 | 37,028 | -0,176 | -161,552- -15,410 | 0,018 |
| ***Beta blokatori*** | 31,164 | 33,824 | 0,069 | -35,583-97,911 | 0,358 |
| ***Inhibitori protonske pumpe*** | 87,073 | 38,036 | 0,169 | 12,013-162,132 | 0,023 |

Model multivarijantne višestruke reg-resije (tabela 3) je napravljen kako bi se pro-cenio zajednički uticaj faktora koji su se izdvojili kao statistički značajni za procenu efikasnosti bazične funkcije trombocita mere-ne putem TRAP testa (starost, antikoagulansi i inhibitori protonske pumpe). Ceo model je bio statistički značajan $\chi 2$ (df=3, N =180) =5,245, p=0,002. Model u celini objašnjava 6,6% vari-jance bazične funkcije trombocita.Statistički značajan doprinos modelu je dala samo vari-jabla starost (Beta=0,289, p=0,009).

**Tabela 3**

**Multivarijanta linearna regresiona analiza nezavisnih faktora za procenu bazične funkcije trombocita (TRAP)**

| | Nestandardizovani koeficijent BSG | | Standardizovani koeficijent Beta | 95%CI | p |
|---|---|---|---|---|---|
| ***Starost*** | -3,801 | 1,432 | -0,192 | -6,628- -0,975 | 0,009 |
| ***Antikoagulansi*** | -70,131 | 38,230 | -0,140 | -145,580- 5,318 | 0,068 |
| ***Inhibitori protonske pumpe*** | 64,261 | 39,215 | 0,125 | -13,132-141,654 | 0,103 |

U Tabeli 4. je prikazana univarijantna linearna regresija faktora na efikasnost ASK prema vrednostima ASPI testa.

**Tabela 4**

**Univarijanta linearna regresiona analiza nezavisnih faktora za procenu efikasnosti acetilsalicilne kiseline (ASK) prema ASPI testu**

| | Nestandardizovani koeficijent BSG | | Standardizovani koeficijent Beta | 95%CI | p |
|---|---|---|---|---|---|
| ***Pol*** | 12,702 | 31,352 | 0,030 | -49,167- 74,570 | 0,686 |
| ***Starost*** | 0,568 | 1,374 | 0,031 | -2,143- 3,279 | 0,680 |
| ***Aspirin protect*** | -184,789 | 28,698 | -0,435 | -241,422--128,156 | <0,001 |
| ***Cardiopirin*** | 154,698 | 31,821 | 0,342 | 91,903-217,494 | <0,001 |
| ***Andol*** | 57,732 | 34,737 | 0,124 | -10,817-126,282 | 0,098 |
| ***Dijabetes*** | 278,268 | 31,422 | 0,553 | 216,261-340,275 | <0,001 |
| ***Pušenje*** | 228,492 | 26,457 | 0,543 | 176,282-280,701 | <0,001 |
| ***Antikoagulansi*** | -245,345 | 29,538 | -0,529 | -303,635--187,055 | <0,001 |

Pacijenti koji su uzimali Aspirin protect predstavljaju prediktore nižih vrednosti ASPI testa (Beta=-0,435; p<0,001), dok je kod pacijenata koji su zimali Cardiopirin efikasnost leka merena ASPI testom bila značajno manja (Beta=0,342; p<0,001). Prisustvo šećerne bolesti predstavlja faktor rizika koji smanjuje efikasnost ASK (Beta=0,553; p<0,001), kao i pušenje (Beta=0,543; p<0,001) i primena inhibitora protonske pumpe (Beta=0,536; p<0,001). S druge strane, primena antikoagulanasa predstavlja protektvni faktor, odnosno pojačava efekat ASK (Beta=-0,529; p<0,001). Multivarijantna višestruka regresija je sprovedena kako bi se procenio zajednički uticaj faktora koji su se izdvojili kao statistički značajni za procenu efikasnosti ASK (Aspirin protect, Cardiopirin, dijabetes, pušenje, primena antikoagulanasa i inhibitora protonske pumpe) – tabela 5. Ceo model, sa svim prediktorima je bio visoko statistički značaj $\chi 2$ (df =6, N =180) =47,641, p<0,001. Model u celini objasnjava 61% varijance efik-asnosti ASK. Statistički značajan doprinos modu su dali svi faktori sem primene Aspirin protect-a: Cardiopirin (Beta=0,199; p=0,001), dijabetes (Beta=0,300; p<0,001), pušenje (Beta=0,204; p=0,001), antikoagulansi (Beta=-0,211; p<0,001) i inhibitori protonse pumpe (Beta= 0,284, p<0,001).

**Tabela 5**

**Multivarijanta linearna regresiona analiza nezavisnih faktora za procenu efikasnostiacetilsalicilne kiseline (ASK) prema ASPI**

| | Nestandardizovani koeficijent BSG | | Standardizovani koeficijent Beta | 95%CI | p |
|---|---|---|---|---|---|
| ***Aspirin protect*** | -13,519 | 26,252 | -0,032 | -65,333-38,296 | 0,607 |
| ***Cardiopirin*** | 89,862 | 25,876 | 0,199 | 38,790-140,935 | 0,001 |
| ***Dijabetes*** | 151,017 | 27,518 | 0,300 | 98,820-203,214 | <0,001 |
| ***Pušenje*** | 85,933 | 24,545 | 0,204 | 38,693-133,174 | <0,001 |
| ***Antikoagulansi*** | -97,992 | 25,934 | -0,211 | -147,765 - 48,218 | <0,001 |
| ***Inhibitori protonske pumpe*** | 135,482 | 25,775 | 0,284 | 87,031-183,943 | <0,001 |

## Diskusija

Potencijalna veza između raznolikosti efekta ASK i kliničkog ishoda još uvek nije potpuno razjašnjena, najviše zbog činjenice da još uvek nije opšte prihvaćena i potpuno razjašnjena definicija rezistencije. U farma-kološkom smislu, rezistencija na ASK Podra-zumeva nedovoljnu inhibiciju formiranja $TXA_2$ pod uticajem COX-1, što za posledicu ima smanjenu inhibiciju trombocitne funkcije (10). U kliničkom smislu, rezistencija na ASK se odnosi na pojavu tromboembolijskih doga-đaja uprkos uzimanju preparata ASK (14). Ispitivanja pokazuju vrlo neusaglašene poda-tke o incidenci rezistenije na ASK, koja se kreće od 1,5-9,8% u ispitivanju *Hurlena* i saradnika (15), do čak 55% u ispitivanju *Buchanana* i saradnika (16), mada je najveći broj autora saglasan da približno trećina paci-jenata koji uzimaju neki od preparata ASK pokazuju nedovoljan odgovor trombocita (17-20).

Brojna funkcionalna i biohemijska ispitivanja omogućila su analizu fiziologije trombocita i farmakodimamskih karakteristika koje se menjaju primenom antitrombocitnih lekova. Zahvaljujući novim saznanjima antitrombo-citna terapija se razvija iz dana u dan, a paraleleno sa njom i dodatna testiranja za praćenje trombocitnog odgovora na primenu ovih preparata. Rutinsko testiranje rezistencije na ASK i klopidogrel se ne preporučuje, pre svega zbog nepostojanja po-tpune definicije pojma rezistencije, nera-zjašnjenih uzroka nastanka i neusvojenog terapijskog tertmana. S druge strane, postoji veliki broj ispitivanja koja potvrđuju ulogu kako biohemijskih, tako i funkcionalnih testova, koji mogu da izdvoje pacijente koji su u suštinskom riziku za neželjene događaje dok su na antitrombocitnoj terapiji. Dve velike metaanalize, od kojih je jedna obuhvatila rezultate 15 ispitivanja, a druga ukupno 20 ispitivanja, pokazale su skoro četvorostruko povećani rizik razvoja neželjenog kardiova-skularnog dogadjaja kod pacijenata koji su u ovim testiranjima identifikovani kao reziste-ntni (21,22). Takođe, dve studije koje su se odnosile na primenu ASK, a obuhvatile su preko 3000 pacijenata, pokazale su da je viso-ki nivo 11-dehidrotromboksana $B_2$ prediktor dvostruko povećanog rizika pojave miokar-dnog infarkta, moždanog udara ili iznenadne srčane smrti kod pacijenata koji su na terapiji ASK ili ASK i klopidogrelom (23,24). Varija-bilnost u odgovoru na primenjeni preparat ASK ne iznenađuje, imajući u vidu genetske činioce, faktore sredine, kao i da sama priroda bolesti može uticati na distribuciju leka u organizmu. Poznato je da na promenljiv odgo-vor trombocita na ASK utiču: nepoštovanje uzimanja terapije (loša komplijansa), starost, pol i pušenje, dnevne i sezonske varijacije u statusu trombocitne aktivacije, neodgovara-juća doza leka, interakcije između lekova, alternativni putevi aktivacije trombocita, pro-menjeni odgovor trombocita na ASK usled određenih stanja ili hirurških procedura (npr. CABG- koronarni arterijski by-pass grafting), genetske varijacije COX-1 gena ili trombo-citnih receptora, vasku-larni događaji izazvani drugim mehanizmima, trombocitna reaktivno-st pre terapije i komorbiditeti (11,13,25).

Obzirom da je loša komplijansa glavni i najvažniji kriterijum prilikom interpretacije rezultata ispitivanja promenljivog odgovora pacijenata na ASK, važno je napomenuti da su iz ovog ispitivanja inicijalno isključeni svi pacijenti koji nisu uzimali redovno lek, koji su samoinicijativno menjali dozu leka ili na bilo koji način nisu poštovali propisanu antiagre-gacionu terapiju. Iako hormonske promene kod žena mogu da pojačaju trombocitnu aktivaciju (10), ovo ispitivanje je pokazalo da pol ne utiče na efikasnost ASK, te se ne izdvaja kao faktor mogućeg prome-nljivog odgovora trombocita na ASK. S druge strane, starenjem opada bazična funkcija trombocita, merena putem TRAP testa, ali se efikasnost

ASK u starijoj životnoj dobi ne razlikuje u odnosu na mlađe, iako razgradnja ASK može biti oslabljena kod starijih, pa se time može očekivati i pojačana bioraspoloživost u ovoj grupi pacijenata. Pušenje predstavlja značajan faktor rizika smanjene efikasnosti ASK, jer cigarete pojačavaju aktiv-aciju trombocita i formiranje trombocitnog tromba (26).

Određena klinička stanja, kao što su diabetes mellitus, esencijalna trombocitemija i hronična bubrežna insuficijencija, kao i procedure kao što je CABG, pojačavaju potrošnju trombocita (27,28). ASK ima relativno kratak poluživot i ne dovodi do inhibicije novoformiranih trombocita. Povećanje trombocitnog prometa povećava udeo trombocita koji nisu izloženi dejstvu ASK, čime oslabljuje sveobuhvatni odgovor na uzeti lek. Ovo ispitivanje je potvrdilo da prisustvo šećene bolesti kod pacijenata koji uzimaju preparate ASK smanjuje njihovu efikasnost, odnosno da je šećerna bolest prediktor rizika smanjene efikasnosti ASK sa visokom statisitičkom značajnošću.

Poznato je da konkomitantna terapija može uticati na odgovor na ASK uticajem na kinetiku COX enzima (29,30). Inhibitori protonske pumpe smanjuju efekat ASK zbog lošije apsorpcije odnosno pojačane ina-ktivacije ASK pomoću esteraza gastrointestinalne sluznice, te se ovi lekovi izdvajaju kao faktor rizika smanjene efikasnosti ASK preparata. S druge strane, istovremena primena antikoagulantnih lekova pojačava dejstvo ASK, odnosno predstavlja protektivni faktor efikasnosti ASK preparata. Konačno, ovo ispitivanje je pokazalo i da postoji razlika u efikasnosti različitih ASK preparata, te se primena Aspirin protecta pokazala kao prediktor viših vrednosti ASPI testa odnosno kao faktor bolje efikasnosti preparata na funkciju trombocita u odnosu na druge dve vrste ASK koje su pacijenti uzimali u ispitivanju.

## Zaključak

Mogućnost praćenja trombocitnog odgo-vora na terapiju ASK može imati veliki uticaj na vođenje terapije i izdvajanje pacijenata koji ne dobijaju potrebnu zaštitu sa antitrombocitnom terapijom, ali i da utvrdi moguće uzroke neuspeha terapije.

Pušenje, prisustvo šećerne bolesti, primena inhibitora protonske pumpe i antikoagulantnih lekova, kao i vrsta primenjenog ASK preparata se izdvajaju kao najvažniji faktori koji utiču na efikasnost ASK na funkciju trombocita, merenu metodom impedantne agregometrije.

## Literatura


1. Antić A, Stanojković Z, Stanojević G, Mačukanović-Golubović L. Laboratory monito-ring of the aspirin and clopidogrel therapy effect. Bilt Transfuziol 2011; 57(1-2): 64-9.
2. Guyer KE. The present state of aspirin and clopidogrel resistance. Hämostaseologie 2009; 29: 285–90.
3. Jackson SP, Nesbitt WS, Westein E. Dynamics of platelet thrombus formation. J Throm Haemost 2009; 7 (Suppl. 1): 17–20.
4. Falati S, Gross P, Merrill-Skoloff G, Sim D, Flaumenhaft R, Celi A, Furie B, Furie B. In Vivo Models of Platelet Function and Thrombosis: Study of Real-Time Thrombus Formation. Methods in Molecular Biology 2004; 272: 187-97.
5. Campbell CL, Streinhubl SR. Variability in response to aspirin: do we understand the clinical relevance? J Thromb Hemost 2005; 3: 665-9.
6. Gasparayan AY, Watson T, Lip GY. The role of aspirin in cardiovascular prevention: implications of aspirin resistance. J Am Coll Cardiol 2008; 51: 1829-43.
7. Zimmerman N, Hohlfeld T. Clinical implications of aspirin resistance. Thromb Haemost 2008; 100: 379-90.
8. Lordkipanidze M, Pharand C, Schampaert E, et al. A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. Eur Heart J 2007; 28: 1702-8.

9. Shantsila E, Watson T, Lip GYH. Aspirin resistance: what, why and when?, Thromb Research 2007; 119 (5): 551–4.
10. Mehta JL, Mohandas B. Aspirin resistance: Fact or fiction? A point of view. World J Cardiol 2010; 2(9): 280-8.
11. Krasopoulos G, Brister SJ, Beattie WS, Buchanan MR. Aspirin 'resistance' and risk of cardiovascular morbidity: systematic review and meta-analysis. BMJ 2008; 336:195–8.
12. Awtry E, Loscalzo J. Aspirin. Circulation 2000; 101:1206–18.
13. Tran H, Anand S, Hankey G, Eikelboom J. Aspirin resistance. Thromb Res 2007; 120: 337–46.
14. Rocca B, Patrono C. Determinants of the interindividual variability in response to antiplatelet drugs. J Thromb Haemost 2005; 3: 1597–602.
15. Hurlen, M, Abdelnoor M, Smith P, Erikssen J. & Arnesen H. Warfarin, aspirin, or both after myocardial infarction. New Engl J Med 2002; 347: 969–97
16. Buchanan MR, Verma S. Biological basis and clinical implications of acetylsalicylic acid resistance. The Canadian journal of cardiology 2006; 22(2): 149-51.
17. Liu L, Fan L, Hu G, Hu Y, Zhu B, Li X, et al. Prevalence and Risk Factors for Aspirin Resistance in Elderly Patients With Type 2 Diabetes. Int J Gerontol 2011; 5(2): 112-6.
18. Wang JC, Aucoin-Barry D, Manuelian D, et al. Incidence of aspirin nonresponsiveness using the ultegra rapid platelet function assay-asa. Am J Cardiol 2003; 92: 1492-94.
19. Grundmann K, Jaschonek K, Kleine B, et al. Aspirin non-responder status in patients with recurrent cerebral ischemic attacks. J Neurol 2003; 250: 63–6.
20. Fateh-Moghadam S, Plockinger U, Cabeza N, et al. Prevalence of aspirin resistance in patients with type 2 diabetes. Acta Diabetol 2005; 42: 99–103.
21. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Antithrombotic Trialists' (ATT) Collaboration. The Lancet 2009; 373 (9678): 1849 – 60.
22. De Berardis G, Sacco M, Strippoli GF, Pellegrini F, Graziano G, Tognoni G, Nicolucci A. Aspirin for primary prevention of cardiovascular events in people with diabetes: meta-analysis of randomised controlled trials. BMJ 2009; 339: b4531.
23. Eikelboom JW, Hankey GJ, Thom J et al. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on card-iovascular risk. Circulation 2008; 118: 1705–12.
24. Eikelboom JW, Hirsh J, Weitz JI, et al. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. Circulation 2002; 105: 1650–55.
25. Rocca B, Petrucci G. Variability in the Responsiveness to Low-Dose Aspirin: Phar-macological and Disease-Related Mechanisms. Thrombosis 2012; 2012:376721.
26. Hung J, Lam JY, Lacoste L, Letchacovski G. Cigarette smoking acutely increases platelet thrombus formation in patients with coronary artery disease taking aspirin. Circulation 1995; 92(9): 2432-6.
27. Di Minno MN, Lupoli R, Palmieri NM, Russolillo A, Buonauro A, Di Minno G.
Aspirin resistance, platelet turnover, and diabetic angiopathy: a 2011 update. Thromb Res 2012; 129(3): 341-4.
28. Linden MD, Tran H, Woods R, et al. High platelet reactivity and antiplatelet therapy resistance. Semin Thromb Hemost 2012; 38:200–12.
29. Catella-Lawson F, Reilly MP, Kapoor SC, Cucchiara AJ, DeMarco S, Tournier B, Vyas SN, FitzGerald GA. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N Engl J Med 2001; 345(25): 1809-17.
30. Kurth T, Glynn RJ, Walker AM, Chan KA, Buring JE, Hennekens CH, Gaziano JM. Inhibition of clinical benefits of aspirin on first myocardial infarction by nonsteroidal antiinflammatory drugs. Circulation 2003; 108(10): 1191-5.

Kontakt: Prim. dr sci. Ana Antić
Zavod za transfuziju krvi Niš
Bul. dr Zoran Đinđića 48, Niš
Tel.+381631039045
e-mail: **anaantic@sbb.rs**

# THE ROLE OF CRISPR/CAS9 TECHNIQUE IN BIOENGINEERING

**Robert Rose**[1] · **Bela Balint**[2,3]**, Milena Todorovic and Mirjana Pavlovic**[1]

[1]Department of Computer and Electrical Engineering and Computer Science, FAU, FL, USA;
[2]Institute for Transfusiology and Hemobiology, MMA, Belgrade, Serbia;
[3]Serbian Academy of Sciences and Arts;
[4] Clinic for Hematology, Clinical Center of Serbia, Belgrade, Serbia.

## Abstract

*CRISPR/Cas9 has experienced an explosive surge of interest in recent years for its numerous applications in bioengineering. The basic motivation behind all these efforts is to enhance genome-editing technologies to take less time, be more efficacious, and produce only the designed effects. The diversity of the various applications has led to out-branching into many specific subfields that may be somewhat arduous to navigate. This paper serves as a roadmap of the recent research into CRISPR/Cas9 technology, including discussions on its evolution, characterization, protocols, methodologies, applications, and implications to help connect the broad subfields of interest. The intention is to introduce bioengineers to this increasingly growing technology as well as provide a reference for those who wish to incorporate CRISPR/Cas9 in their experimental designs.*

**Keywords:** CRISPR · Cas9 · Genome Editing · Cancer Therapy

## Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9) is a genome editing technique that utilizes two parts: the enzyme Cas9 which cuts DNA, and a guide RNA which directs the Cas9 to the target DNA and thus contains a sequence that matches the target DNA. Generally, they enter cells together and seek out the target DNA. This is not always the case however; as there exists ample discussion and applications of combining viral delivery of a transgene encoding Cas9 with non-viral delivery of guide RNAs [1].

Cas9 unzips the target DNA and the guide RNA matches up. If the match is compatible, the Cas9 enzyme then uses molecular mechanisms to cut the target DNA and induce double strand breaks. Once cut, the DNA may be disabled or altered, and researchers may edit the gene how they wish.

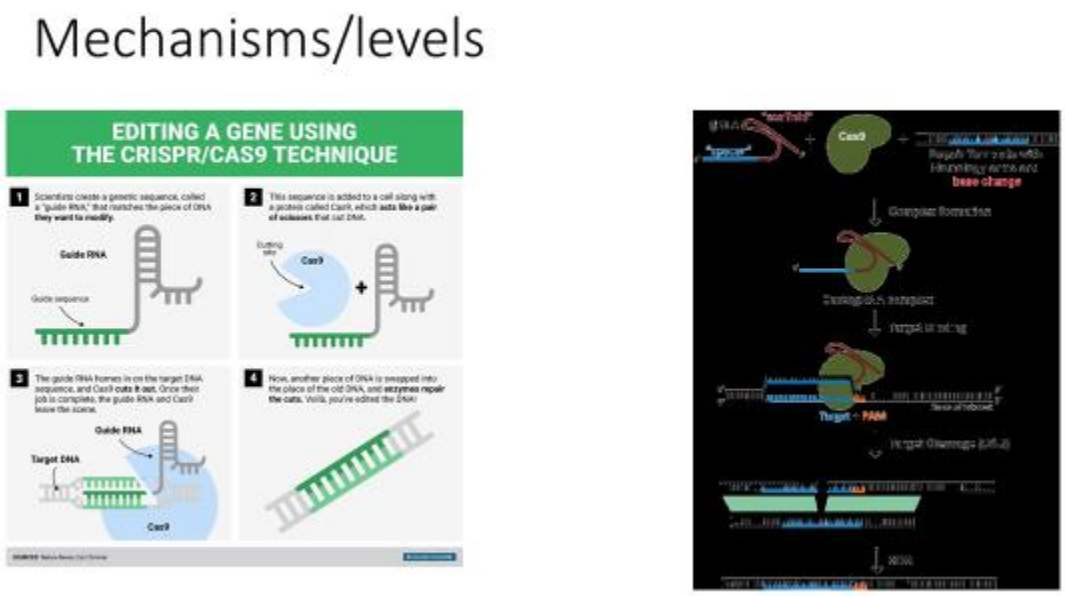



**Figure 1.** 1A and 1B mechanisms of CRISP/Cas9 work

Problems exist in this mechanism in the form of off-target effects and mosaicism. Off-target effects imply that the guide RNA may potentially bind to DNA that is not of its target sequence. This may occur considering the guide RNAs are about 20 base pairs and may be homologous to some off-targets that were unforeseen. Mosaicism refers to the fact when this technique is used on groups of cells, not all of the cells will take the intended genetic change. There are multiple factors that influence how CRISPR/Cas9 is able to reach and edit the target genome.

CRISPR/Cas9 has been used with success in humans, other animals, plants, fungi, bacteria, and archaea. In fact, CRISPR/Cas9 has recently demonstrated its first successful use in a nonvascular plant, the moss *Physcomitrella patens* [2]. The ubiquitous nature of this technique cannot be understated, and many differrent applications exist which are untapped.

**Evolution of CRISPR/Cas9**

The CRISPR system naturally exists as a defense system in bacteria and archaea against bacteriophages [3]. The development of such a system may represent a genetic arms race between prokaryotes and their bacteriophages. DNA in the space between CRISPR's palindromic repeats matches sequences in viral bacteriophage genomes. Certain Cas proteins add these spacer sequences to the genome after exposure of specific viruses and plasmids to bacteria and archaea. RNA made from those spacers directs other Cas proteins to cut invading DNA or RNA that matches the sequence. This is a form of lasting protection that may draw comparisons to our own form of acquired immunity. In turn, bacteriophages mutate in order to bypass forms of CRISPR-based immunity meaning a continual coevolutionary relationship develops [4].

This system is thought to originate from transposons within the prokaryotic genome, which

are genes that may hop from one position to another in the genome. Krupovic et al. have found a class of these mobile genetic elements which encode Cas1 protein that they have named 'casposons' [5]. These may have been the origin of the CRISPR/Cas system of immunity. The contribution of casposons to the evolution of CRISPR-Cas parallels the RAG1 transposase involvement in vertebrate immunoglobulin gene arrangement [5]. This has a broader implication on immune system evolution in general, and suggests that recruitment of endonucleases from mobile ele-ments as readymade tools to edit the genome forms a common thread in the evo-lution of adaptive immunity.

## Characterization

Characterizing CRISPR/Cas9 in terms of conformational changes may be able to highlight structure where Cas9 may be fine-tuned for gene editing. Using single-molecule fluorescence resonance energy transfer, Dagdas et al. identified an intermediate state of *S. pyogenes* Cas9, representing a conformational checkpoint between DNA binding and cleavage [6]. They also found that sequence mi-smatches between the DNA target and guide RNA prevent transitions from the checkpoint intermediate to the active conformation. This provides an avenue for selective avoidance of DNA cleavage at stably bound off-target sites [6].

In terms of structure, a non-catalytic domain within Cas9, REC3, recognizes target complementarity and governs the HNH nuclease domain of Cas9 to regulate overall catalytic competence. Exploiting this observation, Chen et al. designed a new hyper-accurate Cas9 variant (HypaCas9) that demonstrates high genome-wide specificity without compromi-sing on-target activity in human cells [7]. Bothmer et al. demonstrated that single nicks deriving from different Cas9 variants differ rentially activate repair. For instance, D10A but not N863A-induced nicks are repaired by homologous recombination. Design considerations to take from this are that homologous recombination is required for repairing lesions using double-stranded, but not single stranded DNA as a template [8].

## Protocols and Methodology

Various protocols exist for CRISPR/Cas9 technology. Joung et al. have designed a protocol for constructing and packaging customor readymade guide RNAs into lentiviral vectors for delivery into cells for screening [9]. Their protocol suggests a genome-scale screen may be completed in 9-15 weeks, followed by 4-5 weeks of validation. Along the same lines, Baker and Masters have adopted a lentiviral system for delivery of CRISPR/Cas9 components into myeloid cell lines [10]. This is important considering the difficulty in transfecting hematopoietic cells with the necessary components.

Canver et al. have designed a methodology for the production of deletions in mammalian cells, ranging from 1.3 kb to greater than 1Mb in size. Their methodology demonstrates an inverse relationship between deletion frequency and deletion size [11]. When designing a CRISPR/Cas9 experiment, going for smaller or larger genome deletions is sought when considering the frequency of deletions you wish to achieve. There is a balancing act at play here that must be considered.

Another deletion-based methodology exists in the form of ScanDel, a CRISPR-based system described by Gasperini et al. that uses a pair of guide RNAs to program thousands of kilobase-scale deletions that deeply scan across a targeted region in a tiling fashion [12]. This method has the benefit of being able to explore the extent to which non-coding mutations contribute to inheritable diseases.

Additional methodologies include those that take advantage of double-strand breaks indu-

ced by CRISPR/Cas9. Vanioli and Jasin have designed a method using CRISPR/Cas9 technology and homology-directed repair to efficiently isolate cells harboring a chromosomal translocation [13].

Suzuki et al. Developed homology-independent targeted integration (HITI) for CRISPR-Cas9 and non-homologous end-joining (NH EJ) based gene knock-in [14]. The ability for non-homologous ends to be joined for assistance with gene knockin editing means that target gene therapies and basic research may be made less time consuming.

Meyer et al. developed CERES, a computational method to estimate genedependency levels from CRISPR/Cas9 essentiality screens while accounting for the copy number-specific effect [15]. CERES had the benefit of decreesing false-positive results. They estimated single guide RNA (sgRNA) activity across 342 cancer cell lines and previously published screens performed with different sgRNA libraries [15].

A key issue in CRISPR/Cas9 experiments is how to select for cells in which Cas9 is actually active to avoid choosing cells in which no genetic changes have occurred. Niccheri et al. have developed a technique that utilizes a chimeric construct containing the Blasticidin S (an antibiotic) resistance (bsr) gene [16]. End joining of the CRISPR/Cas9-induced double-strand break on the surrogate target may place the bsr gene in frame. This provides temporary resistance to Blasticidin S and thus selection with the antibiotic is used to enrich the cells where Cas9 is active [16].

In undergoing CRISPR/Cas9 screening to identify potential targets, it becomes burdensome when a large number of cells and sequencing reads need to be done to attain a certain statistical significance. To help alleviate this, Schmierer et al. have developed a system that uses random sequence labels (RSLs) incorporated into the guide library [17].These act as unique molecular identifiers (UMIs) to allow massively parallel lineage tracing and lineage dropout screening. RSLs greatly improve the reproducibility of results by increasing both the precision and the accuracy of screens [17].

Chen et al. have designed a CRISPR/Cas9 system for efficient and rapid genome editing in the bacteria *S. aureus* that includes applications such as gene deletion, insertion, and single-base substitution mutations [18]. The designed system is amenable to the assembly of spacers and repair arms by both Golden Gate and Gibson assembly (two methods for the assembly of multiple DNA fragments). This enables rapid construction of the plasmids used for gene editing.

We must consider how to find all the off-target effects that result from CRISPR/Cas9 experiments, as that is the main concern regarding potential human therapeutics. For this, Tsai et al. have designed circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq), which is a sequencing-efficient highly sensitive in vitro screening strategy [19]. CIRCLE-seq may be used to identify off-target mutations associ-ated with cell-type-specific single-nucleotide polymorphisms. The fact this is able to generate personalized specificity profiles demonstrates that CRISPR/Cas9 may be feasibly done in a cell-personalized manner. This is important to the broader application of human therapeutics, since humans will generally have their own genetically distinct cell profiles.

**Implications and applications**

Numerous applications for CRISPR/Cas9 have been developed, and this section will discuss a selection of those applications as well as their implications for future work. The creativity on display here is phenomenal as the genome editing technique has been deployed in unconventional ways. The major cat

egory of work done with CRISPR/Cas9 represents its use in treatment of disease, but there is also a drive to work with understanding cellular machinery as well. The applications are also summarized in Table 1 below.

***Cancer Therapy***

A key focus of CRISPR-Cas9 technology is its application for editing cancer genes. Shen et al. have developed a combinatorial CRISPR/Cas9 genetic interaction mapping technology that successfully identifies many therapeutically relevant genetic interactions in cancer. Their high throughput method shows the great importance of cellular context on the architecture of the genetic interaction network [20].

Young et al. have performed genome-wide CRISPR/Cas9-based lethality (dropout) screens in 8 diffuse large B cell lymphoma lines to discover modulators of B cell receptor signaling. The signaling of B cell receptors is required for cell viability or proliferation [21]. Thus, the therapeutic benefit of using CRISPR/Cas9 for diffuse large B cell lymphoma is an area of interest.

Following along the same lines in editing B cells, Chu et al. have been manipulating genes in germinal center (GC)-like B cells in vitro. Complete gene knockouts have occurred in up to 92 % of activated B cells [22]. This demo-nstrates promising approaches to using CRISPR/Cas9 to target B cells.

Many problems with our current cancer therapies stem from the fact that tumor cells are able to inactivate agents designed to combat them. To overcome suppressed anti-tumor response, Rupp et al. developed a protocol for combined Cas9 ribonucleoprotein (Cas9 RNP)-mediated gene editing and lenti-viral transduction to generate programmed death ligand 1 (PD-1) deficient anti-CD19 chimeric antigen receptor (CAR) T cells [23].

The CAR-T cells are able to effectively combat B cells (which display CD 19) since PD-1 no longer renders them hypo-functional.

Not all CRISPR-Cas9 techniques for cancer therapeutics have demonstrated success though. In an in vitro context, human hepatocellular carcinoma cell lines are able to adapt to decreased rates of amino acid transport despite amino acid transporter silencing by CRISPR/Cas9 [24]. Amino acid transport may not represent the best avenue to target in fighting cancer cells, or an integrative approach may be necessary for hepatocellular carcinoma therapeutics.

Vanoli et al. present a strategy combining CRISPR/Cas9 technology and homology-directed repair. They utilized this strategy to allow for the selection of human mesenchymal stem cells harboring the oncogenic translocation EWSR1-WT1 found in the aggressive desmoplastic small round cell tumor [25]. This method is easily adapted to generate any cancer-relevant rearrangement, which may help identify stem cells that are potentially cancerous.

Neagu et al. engineered CT2A- and GL261-glioblastoma cells bearing Cas9 to express a library of bar-coded single guide RNAs (sgRNAs) [26]. Glioblastoma-bearing mice were treated with vaccination or PD-1 che-ckpoint blockade. They then followed the dropout of sgRNAs targeting reputed immune evasion molecules or the enrichment of sgRNAs mimicking resistance mechanisms with next-generation sequencing (NGS) at the time of harvest post-immunotherapy. They also used NGS at the time of tumor implantation as a baseline. The development of this glioblastoma-specific library may be used to identify novel immunotherapy targets in glioblastoma. In a general sense, this assay represents the first high-throughput method for systematically identifying new candidate targets for immunotherapy and resistance mechanisms in central nervous system cancers. Phelps et al. experimented with pediatric rhabdomyosarco-

ma (RMS) in solid tumor preclinical xenograft mouse models to demonstrate the potential of CRISPR/Cas9 gene therapy [27].

CRISPR/Cas9 in inducible or viral delivery systems was used to target critical exons in the activated RAS or PAX3/FO XO1 oncogenes unique to certain RMS subtypes. Significant regression of tumor xenografts due to a combination of cell death and differentiation was the result. This additional study highlights the effectiveness of CRISPR/Cas9 gene therapy for cancer treatment.

**Table 1**

**Examples of applications in bioengineering for CRISPR/Cas9.**

| Application | Experiments/Findings | Reference |
|---|---|---|
| Cancer Therapy | Combinatorial CRISPR/Cas9 genetic interaction mapping technology that successfully identifies many therapeutically relevant genetic interactions in cancer | [20] |
| | Genome-wide CRISPR/Cas9-based lethality (dropout) screens in 8 diffuse large B cell lymphoma lines | [21] |
| | Manipulating genes in germinal center (GC)-like B cells in vitro. Complete gene knockouts have occurred in up to 92 % of activated B cells | [22] |
| | Protocol for combined Cas9 ribonucleoprotein (Cas9 RNP)-mediated gene editing and lentiviral transduction to generate programmed death ligand 1 (PD-1) deficient anti-CD19 chimeric antigen receptor (CAR) T cells | [23] |
| | Human hepatocellular carcinoma cell lines are able to adapt to decreased rates of amino acid transport despite amino acid transporter silencing by CRISPR/Cas9 | [24] |
| | Selection of human mesenchymal stem cells harboring the oncogenic translocation EWSR1-WT1 found in the aggressive desmoplastic small round cell tumor | [25] |
| | Engineered CT2A- and GL261-glioblastoma cells bearing Cas9 to express a library of bar-coded single guide RNAs; high-throughput method for systematically identifying new candidate targets for immunotherapy and resistance mechanisms in central nervous system cancers | [26] |
| | CRISPR/Cas9 in inducible or viral delivery systems used to target critical exons in the activated RAS or PAX3/FOXO1 oncogenes unique to certain rhabdomyosarcoma subtypes | [27] |
| HIV Therapy | CRISPR/Cas9 mediated CCR5 ablating system in long-term hematopoietic stem cells to confer HIV-1 resistance in vivo | [28] |
| Huntington's disease Therapy | Permanent suppression of endogenous mutant HTT (mHTT) expression in the striatum of mHTT-expressing mice (HD140Q-knockin mice) using CRISPR/Cas9-mediated inactivation | [29] |
| Duchenne Muscular Dystrophy Therapy | CRISPR/Cas9 used to delete exon 45 in the human duchenne muscular dystrophy (DMD) gene in hDMD mice developing a novel dystrophic mouse model | [30] |

| | | |
|---|---|---|
| Transplantation | CRISPR-Cas9 inactivation of all porcine endogenous retroviruses in a porcine primary cell line for xeno-transplantation | [31] |
| Mechanisms for Inactivation | Small bacteriophage-encoded anti-CRISPR proteins (Acrs) which may inactivate Cas9: two Acrs, AcrIIC1 and AcrIIC3, were found to inhibit Cas9 by distinct strategies | [32] |
| | AcrIIA2 and AcrIIA4 prevent target binding by catalytically dead Cas9 in bacteria and inhibit Cas9-mediated gene editing in human cells | [33] |
| | Identified natural regulators of Cas9 in *Neisseria meningitides*, which may provide the key to controlling Cas9 activity | [34] |
| Understanding Cellular Mechanisms | Genome editing of *Drosophila* ovarian somatic cells using the CRISPR/Cas9 system to achieve *l(3)mbt* knockout, resulting in successful induction of the PIWI-interacting RNA (piRnA) amplification machinery | [35] |
| Transcription Activation | Trans-epigenetic remodeling in-vivo: system relies on recruitment of Cas9 and transcriptional activation complexes to target loci by modified sgRNAs | [36] |
| Transcription Blocking | Demonstrated that the level of complementarity between the sgRNA and the target controls the rate at which dCas9 successfully blocks RNA polymerase in bacterial systems | [37] |
| | CRISPR/Cas9 and CRISPRi have different off-target effects for loss-of-function genetic screens | [38] |
| Encoding Information | CRISPR/Cas machinery to encode a black-and-white computer GIF into the genome of living bacteria populations, the computer GIF being that of Eadweard Muybridge's 19th century animation of a running horse | [39] |
| Editing Human Embryos | Corrected heterozygous MYBPC3 mutations (which lead to the development of inherited cardiomyopathy) in human preimplantation embryos with precise accuracy using CRISPR/Cas9 based targeting | [40] |

***HIV Therapy***

Perhaps a promising approach to combating HIV infection comes from work by Xu et al. Their CRISPR/Cas9 mediated CCR5 ablating system is used in long-term hematopoietic stem cells. This use in stem cells would effectively confer HIV-1 resistance in vivo [28].

***Huntington's disease Therapy***

CRISPR/Cas9 techniques have been designed to help alleviate Huntington's disease and fo cus on targeting the huntingtin gene (HTT). Yang et al. have achieved permanent suppression of endogenous mutant HTT (mHTT) expression in the striatum of mHTT-expressing mice (HD140Q-knockin mice) using CRISPR/Cas9-mediated inactivation in a non-allele specific manner [29]. This effectively depleted HTT aggregates and attenuated early neuropathology. This has the important implycation which suggests that non-allele-specific CRISPR/Cas9-mediated gene editing could be used to efficiently and permanently eliminate polyglutamine expansion-mediated neuronal toxicity in the adult brain [29].

***Duchenne Muscular Dystrophy Therapy***

Young et al. used CRISPR/Cas9 to delete exon 45 in the human duchenne muscular dystrophy (DMD) gene in hDMD mice and thus developed a novel dystrophic mouse model [30]. This placed the DMD out-of-frame. Duchenne muscular dystrophy is caused by mutations in the DMD gene which disrupt the reading frame, thus this represents potential therapeutic use for CRISPR/Cas9. This platform, which targets deletion of human DMD exons 45-55, may be directly applied in vivo to restore dystrophin and alleviate disease.

***Transplantation***

CRISPR/Cas9 is being used for transplantation applications in order to absolve some of the genetic interactions that occur between biologically distinct entities. Niu et al. have used CRISPR-Cas9 to inactivate all porcine endogenous retroviruses (PERVs) in a porcine primary cell line [31]. They then went on to generate PERV-inactivated pigs via somatic cell nuclear transfer. This highlights the value of PERV inactivation to prevent cross-species viral transmission. CRISPR/Cas9 has been used to demonstrate the successful production of PERV-inactivated animals to address the safety concern in clinical xenotransplantation [31].

**Mechanisms for inactivation**

A key issue concerning the CRISPR/Cas9 system is the potentiality that gene editing can go on uncontrolled and produce off-target effects. Potential mechanisms for inactivation need to be explored. Harrington et al. has published work concerning small bacterio-phage-encoded anti-CRISPR proteins (Acrs) which may inactivate Cas9 [32]. These may provide an efficient off switch for Cas9-based applications. Two Acrs, AcrIIC1 and AcrIIC3, were found to inhibit Cas9 by distinct strategies [32].

Rauch et al. have described additional Acrs [33]. They include AcrIIA2 and AcrIIA4, which prevent target binding by catalytically dead Cas9 in bacteria and inhibit Cas9-mediated gene editing in human cells. If an application for editing genes in human cells requires deactivation, this approach may show promising results.

Pawluk et al. identified natural regulators of Cas9 in *Neisseria meningitides*, which may provide the key to controlling Cas9 activity [34]. Controlling Cas9 activity is extremely desired to reduce the effects of mosaicism and off-target effects.

**Understanding cellular mechanisms**

CRISPR/Cas9 has been used to help amplify cellular mechanisms that are less understood. Ishizu et al. performed genome editing of *Drosophila* ovarian somatic cells using the CRISPR/Cas9 system to achieve *l(3)mbt* knockout, resulting in successful induction of the PIWI-interacting RNA (piRnA) amplification machinery [35]. These piRNAS are thought to play a crucial role in maintaining genome integrity by repressing transposons in *Drosophila* ovaries.

**Transcription activation**

CRISPR/Cas9 doesn't have to be used to just edit the genome, it may also influence gene activation through bringing transcriptional activation complexes to their target sequences. This allows regulation of endogenous gene expression without creating double strand breaks. Liao et al. have reported on this system of trans-epigenetic remodeling in-vivo, and their system relies on recruitment of Cas9 and transcriptional activation complexes to target loci by modified single guide RNAs [36]. They were able to use this technology to

effectively treat mouse models of acute kidney disease, muscular dystrophy, and diabetes.

**Transcription blocking**

The catalytically dead Cas9 (dCas9) is guided by single guide RNAs (sgRNAs) to block transcription of target genes. This strategy is commonly known as CRISPRi [37]. Vigouroux et al. have demonstrated that the level of complementarity between the sgRNA and the target controls the rate at which dCas9 successfully blocks RNA polymerase in bacterial systems [37]. This mechanism may be used to precisely reduce gene expression by defined relative amounts. This is an important modality considering experiments may want to achieve differing levels of decreasing gene expression in order to understand transcription gradient effects.

Transcription blocking may also be achieved by cutting the gene with the traditional CRISPR/Cas9 system. It is up to discussion whether this equates to a better or worse approach than CRISPRi. Comparisons by Rosenbluh et al. demonstrate that CRIS PR/Cas9 and CRISPRi have different off-tar-get effects [38]. Combining these approaches is thought to be best for loss-of-function genetic screens as they provide complementary information.

**Encoding Information**

Grayscale pixel values have been encoded into the genetic code much the same way you could encode pixel values in binary. Shipman et al have utilized this with CRISPR/Cas machinery to encode a black-and-white computer GIF into the genome of living bacteria populations, the computer GIF being that of Eadweard Muybridge's 19th century animation of a running horse [39]. The implication here is that the animation has a time component that was successfully retained in the genomes of living organisms. The introduction of a time component in genome editing means that we may use it for other applications such as potentially following stem cell differentiation over time.

**Editing human embryos**

The purpose of editing human embryos is to correct defective genes that lead to disease. Mitalipov of Oregon Health and Science University led the first US effort into this research. Ma et al. were able to correct heterozygous MYBPC3 mutations (which lead to the development of inherited cardiomyopathy) in human pre-implantation embryos with precise accuracy using CRISPR/Cas9 based targeting [40]. They were also able to achieve high homology-directed repair efficiency by activating an endogenous germline-specific DNA repair response.

**Consideration for Electromagnetic Field-based Techniques**

Electromagnetic fields (EMF) may affect cellular reprogramming. Baek et al. demonstrated that EMF exposure induced epigenetic changes that promoted somatic cell reprogramming to pluripotency efficiently [41]. They were able to pinpoint the epigenetic changes resulted from EMF- induced activation of the histone lysine methyltransferase MII2. This reprogramming of cellular development represents a key commonality with CRISPR/Cas9 in its ability to affect cellular machinery, either by genetic changes or epigenetic changes [36].

Although there appear to be no studies exploring the interaction between EMF based techniques and CRISPR/Cas9, the conside-ration must be discussed that they potentially may interact with each other. Reprogramming cells to pluripotency may be better achieved with a combination effort of exogenous transcription factors, EMF based epigenetic changes, and CRISPR/Cas9 based genetic and epigenetic

changes. This suggestion may also be applied to broader applications of therapeutics.

## Concluding Remarks

Trying to target too much with CRISPR/Cas9 may result in diminished effect. Meyers et al. have discussed that genome targeting by CRISPR/Cas9 elicits a gene-independent antiproliferative cell response with a severity proportional to the total number of discrete genomic loci targeted [42]. This effect has important practical implications for interpretation of CRISPR/Cas9 screening data since the more loci targeted means less cell proliferation. This may confound the use of this technology for identification of essential genes in amplified regions [42].

The CRISPR/Cas9 field also seems to generate debate within its community. Schaefer et al generated concerns over the safety of CRISPR/Cas9 after they reported on unexpected mutations in using the technique for treating blind rd1 mice, which they attributed to CRISPR/Cas9 [43]. This generated so much backlash that the editors of Nature Methods had to issue an editorial expression of concern to alert readers about the interpretation of the data. Lareau wrote anopposition article that explains the unexpected mutations after CRISPR-Cas9 editing in vivo are most likely pre-existing sequence variants and not nuclease-induced mutations [44].

The research into human embryos has generated fair concern that CRISPR/Cas9 may be used for so-called "designer babies" implying that genetic traits of human embryos may be controlled and selected for at the whims of the genome editor. The research so far has only been concerned with correcting inheritable diseases and though the embryos are viable, they have not been implanted for fetal development. The National Academy of Sciences study committee has weighed in and affirmed that a careful consideration of benefits vs. risks must be considered, and acknowledged the concern of fairness if this technique would only be available for highpaying individuals.

Despite all these stated issues, there is no doubt the ability of genome editing with CRISPR/Cas9 is incredible in terms of the wide amount of species that may be subjected to targeted genome modification. The numerous applications have demonstrated that there are many potential targets and mechanisms that have benefit for clinical and general science issues, and that there are many exciting opportunities that may build on the work already done.

## References


1. Wang H-X, Li M, Lee CM, Chakraborty S, Kim H-W, Bao G, Leong KW. 2017. CRISPR/Cas9-based genome editing for disease modeling and therapy: challenges and opportunities for nonviral delivery. Chemical Reviews. 117(15): 9874-906.
2. Collonnier C, Epert A, Mara K, Maclot F, Guyon-Debast A, Charlot F, White C, Schaefer DG, Nogue F. 2017. CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and RAD51-independent gene targeting in the moss *Physcomitrella patens*. Plant Biotechnology Journal. 15(1): 122-31.
3. Shabbir MAB, Hao H, Shabbir MZ, Hussain HI, Iqbal Z, Ahmed S, Sattar A, Iqbal M, Li J, Yuan Z. 2016. Survival and evolution of CRISPR-Cas system in prokaryotes and its applications. Frontiers in Immunology. 7: 375.
4. Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, Boyaval P, Romero DA, Horvath P, Moineau S. 2008. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. Journal of Bacteriology. 190(4): 1390-400.
5. Krupovic M, Makarova KS, Forterre P, Prangishvili D, Koonin EV. 2014. Caspos ons: a new superfamily of selfsynthesizing DNA transposons at the origin of proka-ryotic CRISPR-Cas immunity. BMC Biology. 12: 36.
6. Dagdas YS, Chen JS, Sternberg SH, Doudna JA, Yildiz A. 2017. A conformational checkpoint between DNA binding and cleavage by CRISPR-Cas9. Science Advances.3(8):eaao 0027
7. Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, Sternberg SH, Joung JK,

Yildiz A, Doudna JA. 2017. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature. 550: 407-10.

8. Bothmer A, Phadke T, Barrera LA, Margulies CM, Lee CS, Buquicchio F, Moss S, Abdulkerim HS, Selleck W, Jayaram H, Myer VE, Cotta-Ramusino C. 2017. Characterization of the interplay between DNA repair and CRISPR/Cas9-induced DNA lesions at an endogenous locus. Nature Communications. 8: 13905.
9. Joung J, Konermann S, Gootenberg JS, Abudayyeh OO, Platt RJ, Brigham MD, Sanjana NE, Zhang F. 2017. Genome-scale CRISPR-Cas9 knockout and transcriptional
activation screening. Nature Protocols. 12: 828-63.
10. Baker PJ, Masters SL. 2017. Generation of genetic knockouts in myeloid cell lines using a lentiviral CRISPR/Cas9 system. In: De Nardo D, De Nardo C (eds) Innate Immune Activation. Methods in Molecular Biology,vol 1714.Humana Press,NewYork, NY.
11. Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, Maeda T, Paw BH, Orkin SH. 2017. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. J Biol Chem. 292(6): 2556.
12. Gasperini M, Findlay GM, McKenna A, Milbank JH, Lee C, Zhang MD, Cusanovich DA, Shendure J. 2017. CRISPR/Cas9-mediated scanning for regulatory elements required for HPRT1 expression via thousands of large, programmed genomic deletions. AJHG. 101(2): 192-205.
13. Vanoli F, Jasin M. 2017. Generation of chromosomal translocations that lead to conditional
fusion protein expression using CRISPR-Cas9 and homology-directed repair. Methods.121-122:138-45
14. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, et al.. 2016. In vivo genome ediing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. 540(7631): 144-49.
15. Meyers RM, Bryan JG, McFarland JM, Weir BA, Sizemore AE, Xu H, et al. 2017. Computational correction of copy number effect improves specificity of CRISPR-Cas9 essenti-ality screens in cancer cells. Nature Genetics. 49:1779-84
16. Niccheri F, Pecori R, Conticello SG. 2017. An efficient method to enrich for knock-out and knock-in cellular clones using the CRISPR/Cas9 system. Cellular and Molecular Life Sciences. 74(18): 3413-23.
17. Schmierer B, Botla SK, Zhang J, Turunen M, Kivioja T, Taipale J. 2017. CRISPR/Cas9 screening using unique molecular identifiers. Molecular Systems Biology. 13: 945.
18. Chen W, Zhang Y, Yeo W-S, Bae T, Ji Q. 2017. Rapid and efficient genome editing in *Staphylococcus aureus* by using an engineered CRISPR/Cas9 system. Journal of the American
Chemical Society. 139(10): 3790-5.
19. Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ, Joung JK. 2017. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. Nature Methods. 14: 607-14.
20. Shen JP, Zhao D, Sasik R, Luebeck J, Bojorquez-Gomez A, Licon K, Ideker T, Mali P. 2017. Combinatorial CRISPR-Cas9 gene knockout to enable genetic interaction mapping in human cells [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017;
2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res. 77(13 Suppl): Abstract nr 3039.
21. Young RM, Phelan JD, Webster DE, Roulland S, Wright G, Huang D, Schmitz R, Staudt LM. 2017. CRISPR-Cas9 genetic screens uncover a
B cell receptor-MYD88 superpathway in diffuse large B cell lymphoma. Hematological Oncology. 35(S2): 25.
22. Chu VT, Graf R, Rajewsky K. 2017. CRISPR/Cas9-mediated in vitro mutagenesis in GC-like B cells. In: Calado D. (eds) Germinal Centers. Methods in Molecular Biology, vol 1623. Humana Press, New York, NY.
23. Rupp LJ, Schumann K, Roybal KT, Gate RE, Ye CJ, Lim WA, Marson A. 2017. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Scientific Reports. 7: 737.
24. Bothwell P, Bode B. 2017. Human hepatoce-llular carcinoma cells adapt to ASCT2 and LAT1 amino acid transporter silencing by shRNA and CRISPR-Cas9. The FASEB Journal. 31(1S): 942.3.
25. Vanoli F, Tomishima M, Feng W, Lamribet K,Babin L, Brunet E, Jasin M. 2017. CRISPR-Cas9-guided oncogenic chromosomal translocations with conditional fusion protein expression in human mesenchymal cells. PNAS. 114(14): 3696-701.
26. Neagu MR, Manguso RT, Pope H, Speranza MC, Freeman GJ, Doench J, Sharpe AH, Haining WN. 2017. Defining molecular mechanisms of resistance to glioblastoma immunity using a novel CRISPR/Cas9 in vivo loss-of-function screening platform [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res. 77(13 Suppl): Abstract nr 417.

27. Phelps M, Yang H, Chen E. 2017. Targeted ablation of essential oncogenes in rhabdomyosarcoma with CRISPR/Cas9 gene therapy [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res. 77(13 Suppl): Abstract nr 5096.
28. Xu L, Yang H, Gao Y, Chen Z, Xie L, Liu Y, et al. 2017. CRISPR/Cas9-mediated CCR5 ablation in human hematopoietic stem/pro-genitor cells confers HIV-1 resistance in vivo. Molecular Therapy. 25(8): 1782-89.
29. Yang S, Chang R, Yang H, Zhao T, Hong Y, Kong HE, Sun X, Qin Z, Jin P, Li S. 2017. CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Hunting ton's disease. Journal of Clinical Investigation. 127(7): 2719-24.
30. Young CS, Mokhonova E, Quinonez M, Pyle AD, Spencer MJ. 2017. Creation of a novel humanized dystrophic mouse model of duchenne muscular dystrophy and application of a CRISPR/Cas9 gene editing therapy. Journal of Neuromuscular Diseases. 4(2): 139-45.
31. Niu D, Wei H-J, Lin L, George H, Wang T, Lee I-H, et al. 2017. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science.357(6357):1303-07.
32. Harrington LB, Doxzen KW, Ma E, Liu J-J, Knott GJ, Edraki A, et al. 2017. A broad-spectrum inhibitor of CRISPR-Cas9. Cell. 170(6): 1224-33.e15.
33. Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J. 2017. Inhibition of CRISPR-Cas9 with bacteriophage proteins. Cell. 168(1-2): 150-58.e10.
34. Pawluk A, Amrani N, Zhang Y, Garcia B, Hidalgo-Reyes Y, Lee J, et al. 2016. Naturally occurring off-switches for CRISPR-Cas9.Cell.167(7): 1829-38.e9.
35. Ishizu H, Sumiyoshi T, Siomi MC. 2017. Use of the CRISPR-Cas9 system for genome editing in cultured *Drosophila* ovarian somatic cells. Methods. 126: 186-92.
36. Liao H-K, Hatanaka F, Araoka T, Reddy P, Wu M-Z, Sui Y, et al. 2017. In vivo target gene activation via CRISPR/Cas9-mediated trans-epigenetic modu lation. Cell. https: //doi.org/10.1016/j.cell.2017.10.025.
37. Vigouroux A, Oldewurtel E, Cui L, van Teeffelen S, Bikard D. 2017. Engineered CRISPR-Cas9 system enables noiseless, fine-tuned and multiplexed repression of bacterial genes. bioRxiv. 164384.
38. Rosenbluh J, Xu H, Harrington W, Gill S, Wang X, Vazquez F, Root DE, Tsherniak A, Hahn WC. 2017. Complementary information derived from CRISPR Cas9 mediated gene deletion and suppression. Nature Communications. 8: 15403.
39. Shipman SL, Nivala J, Macklis JD, Church GM. 2017. CRISPR-Cas encoding of a digital movie into the genomes of a population of living bacteria. Nature. 547(7663): 345-9.
40. Ma H, Marti-Gutierrez N, Park S-W, Wu J, Lee Y, Suzuki K, et al. 2017. Correction of a patho genic gene mutation in human embryos. Nature. 548: 413-9.
41. Baek S, Quan X, Kim S, Lengner C, Park J-K, Kim J. 2014. Electromagnetic fields mediate efficient cell reprogramming into pluripotent state. ACS Nano. 8(10): 10125-38.
42. Meyers RM, Aguirre AJ, Weir BA, Vazquez F, Zhang C-Z, Ben-David U,et al. 2017. Genomic copy number alterations introduce a gene-independent viability bias in CRISPR-Cas9 knock-out screens of cancer cell lines [abstract]. In: Proceedings of the AACR Precision Medi cine Series: Targeting the Vulnerabilities of Cancer; May 16-19, 2016; Miami, FLPhiladelphia (PA): AACR; Clin Cancer Res. 23 (1 Suppl): Abstract nr B39..
43. Schaefer KA, Wu WH, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. 2017. Unexpected mutations after CRISPR-Cas9 editing in vivo. Nature Methods. 14(6):547-8.
44. Lareau C, Clement K, Hsu JY, Pattanayak V, Joung JK, Aryee MJ, Pinello L. 2017. "Unexpected mutations after CRISPR-Cas9 editing in vivo" are most likely pre-existing sequence variants and not nuclease-induced mutations. bioRxiv. 159707.

Corresponding author: Prof. Mirjana Pavlovic, MD, PhD
CEECS, EE-96, #515
Florida Atlantic University, Bioengineering, 33431 Boca Raton, FL, USA
Tel:561-297-234
e-mail: **mpavlovi@fau.edu**

# ENDOGENOUS THROMBIN POTENTIAL ASSAY IN MONITORING THE EFFICACY OF DIFFERENT PROPHYLAXIS REGIMENS FOR SEVERE HAEMOPHILIA

**Irena Djunic[1,2], Violeta Dopsaj[3], Predrag Miljic[1,2], Ivo Elezovic[1,2]**

*[1]Clinic for Haematology, Clinical Center of Serbia, Belgrade;*
*[2]Medical Faculty of Belgrade University, Serbia; [3]Institute for Medical Biochemistry, Pharmacy Faculty of Belgrade University, Serbia.*

## Abstract

***Background:*** *Endogenous thrombin potential (ETP) assays, provide data about the coagulation capacity of haemophiliac patients and in that way may help in making decisions regarding optimal dose-regimen prophylaxis.* ***Aims:*** *The objective of study was to estimate the validity of laboratory assays – ETP, in monitoring and evaluating different prophylactic treatment regimens in patients with severe haemophilia.* ***Methods:*** *This study included 20 adults patients with severe haemophilia. Patients were divided into three groups according to treatment regimen: full-dose prophylaxis (5 pts), intermediate-dose prophylaxis (5 pts) and on demand treatment (10 pts).. The follow-up period was 3 months. In ETP, calculated values of area under the thrombin generation curve (ETP.AUC.cal) and calculated values of maximal clotting (ETP.Cmax.cal) were estimated.* ***Results:*** *The ETP.AUC.cal was significantely improved after 3 months of full-dose in comparison with intermediate-dose prophylaxis (p=0.042). ETP.Cmax was not significantely changed between these groups. In patients treated on demand, ETP values were not changed.* ***Conclusion:*** *The full-dose prophylaxis regimen improved coagulation test results better than intermediate-dose prophylaxis.*

**Keywords**: haemophilia, laboratory assays, prophylaxis regimens, efficacy monitoring.

## Introduction

Haemophilia is a genetic disorder mainly characterized by recurrent and often spontaneous bleeding into joints [1]. It is caused by deficiencies of coagulation factor (F) VIII (haemphilia A) or FIX (haemophilia B) and patients have traditionally been classified according to their plasma concentrations [1]. Thus, severe haemophiliacs with FVIII/IX levels <1% of normal, typically bleed spontaneously into joint and muscles, while patients with factor concentrations from 1 to 5% are classified as moderate hemophiliacs with infrequent spontaneous bleeding episodes or haemorrhage after mild trauma [2]. Patients with factor levels between >5 and 30% of normal are mild haemophiliacs who bleed only after major trauma or surgery [2].

Patients with severe haemophilia may be treated by replacement of the missing clotting factor during bleeding episodes only (on de-

mand), or prophylactically in order to prevent haemorrhages. Prophylaxis is defined as the administration of clotting factor at regular intervals to the bleeding and was conceived from the observation that moderate haemophilia patients with clotting factor levels > 1% of normal rarely bleeding spo-ntaneously and have much better preservation of joint function [3,4]. Therefore, the goal of prophylaxis is to keep the clotting factor level at a minimum of 1 % [5,6]. Primary prop-hylaxis is defined as continuous treatment started before the age of 2 years or after the first joint bleed, whereas secondary prop-hylaxis starts at age > 2 years or after two or more joint bleeds [3].

Recently the plasma level of FVIII/IX was observed to have a relatively poor pre-dictive value for bleeding risk [7]. Namely, it is known that, despite FVIII/IX plasma levels <1%, approximately 10% of patients with severe haemophilia exhibit a milder bleeding phenotype and do not have haemophilic arthropathy [7]. On the other hand, about 25% of patients with moderate haemophilia frequently bled and developed joint disease [8]. These findings led to expanded interest in new laboratory tools able to evaluate the overall coagulating capacity of haemophiliac patients. The endogenous thrombin potential (ETP) method measures the overall capacity of plasma to form thrombin after induction of coagulation given the central role of thrombin in blood clotting [9,10]. Recent studies have demonstrated that patients with mild haemophilia A, but exhibiting severe bleeding tendencies and spontaneous haemorrhages, had low ETP results, comparable with those obtained in severe haemophilia [11]. Likewise, it was shown that, thrombin generation capacity may be significantly different in patients with similar plasma levels of FVIII/IX [1].

Longterm prophylactic treatment represents the optimal management, but countries with low per capita income are still unable to provide it. Therefore, access to prophylaxis in developing countries should be improved by research that defines the minimum effective prophylactic regimen in comparison with on demand therapy. New haemostasis test, ETP assays, could provide useful data about the coagulation capacity of haemophiliac patients and in that way help in making decisions regarding optimal dose-regimen prophylaxis.

Our objective was to estimate the validity of laboratory assay – ETP, in monitoring and evaluating different prophylactic treatment regimens in patients with severe haemophilia. Assessment of our results may be helpful to guide decisions about the minimum prophylactic-dose regimen that would be useful for long-term prophylaxis of haemophilia patients by monitoring its efficacy with these new, more precise laboratory assay.

## Methods

This study included 20 adult males with severe haemophilia involving plasma FVIII/ IX < 1% of normal. Inclusion criteria were no FVIII/IX administration in the previous 72 hours and no evidence of active bleeding or inhibitors.

Patients were divided into three groups according to treatment regimen. All patients received a plasma-derived concentrate of FVIII/IX. The first group involved five patients with haemophilia A treated with full-dose prophylaxis: 20 U/kg three times per week. The second group included five patients given intermediate-dose prophylaxis: 10-15 U/kg three times per week. The third group consisted of 10 patients treated on demand (i.e. only in acute bleeding episodes) and seven of them had hamophilia A, whereas three had haemophilia B. The control group consisted of 10 healthy adult male donors. The follow-up period was 3 months.Blood samples were collected at the following time points: a) initially, before the start of treatment for patients on

prophylaxis or at the begining of the follow-up period for those treated on demand (marked as ETP-1); b) 20 minutes after administration of the first dose to the patients on prophylaxis (marked as ETP-2); c) after 3 months of prophylactic therapy, before administration of the next dose, or after 3 months of follow-up for patients treated on demand (marked as ETP-3).

***Endogenous thrombin potential***

The endogenous thrombin potential (ETP) measures the tendency of plasma to generate thrombin. ETP was performed using 5 ml citrated plasma prepared by centrifugation of whole blood, obtained as previously descrybed, at 1500 g for 15 minutes. Plasma aliquots were stored at -80 ˚C and thawed at 37 ˚C for 15 minutes before analysis. ETP was measured by calibrated automated thrombography (CAT). The substrate conversion kinetics was recorded by the BCS® System/BCS® XP System. Thrombin formation was activated by adding the following components to 135 µL plasma: 30 µL of tissue factor reagent (Innovin® Dade Behring), 40 µL of ETP reag-ent, provided from the BCS® System/BCS® XP System (This contains a fibrin aggregation inhibitor, chromogenic substrate, salts and stabilizers.), 40 µL of ETP buffer (Tris-HCl 50 mmol/L, pH 7.4) and 15 µL of $CaCl_2$ 250 mmol/L. The ETP parameters obtained were: ETP – area under the curve (AUC) representing the total amount of thrombin formed and ETP – maximal clotting (Cmax), which reflects the maximal achieved clot formation. A mathematical algorithm was applied to correct for the activity of $\alpha_2$-macroglobulin bound thrombin. The necessary calculations (cal) were performed automatically by the installed ETP software for the BCS® System/BCS® XP System. The measured ETP values are marked as ETP.AUC.cal and ETP. Cmax.cal and expressed as a percentage of the plasma values for the 10 healthy donors ($value_{patient}$ / $value_{normal\ plasma}$ x 100). The measurement lasted 20 minutes (1200s).

Differences in the values of ETP parameters obtained for the different therape-utic regimens were recorded within the groups as well as between the groups.

The following tests were used for statistical analysis: Student's t-test for paired samples, Fisher's exact test, Pearson chi-square test and unifactorial analysis of variance (ANOVA).

**Results**

The mean age of our 20 patients was 32 years (range 19-55 years), while that of the 10 healthy donors was 34 years (range 21-55 years). There was no significant difference between the groups regarding age (p = 0.621, standard deviation (SD) = 11.366), which is important for valid comparison between them (Table 1).

**Table 1**

**The distribution of mean age between the studied groups**

| Groups | N | mean age | SD | minimum | maximum |
|---|---|---|---|---|---|
| Full dose prophilaxis | 5 | 34 | 11.584 | 25 | 53 |
| Intermediate dose prophilaxis | 5 | 26 | 4.219 | 21 | 31 |
| On-demand | 10 | 34 | 14.635 | 19 | 55 |
| Healthy donors | 10 | 34 | 10.371 | 21 | 55 |
| Total | 30 | 33 | 11.366 | 19 | 55 |

In the group of patients receiving full-dose prophylaxis, the mean value of ETP.AUC.cal-3 after 3 months was higher than the initial value (ETP.AUC.cal-1; 96.59 vs 92,77, F = 8.742, p = 0.042; Figure 1).

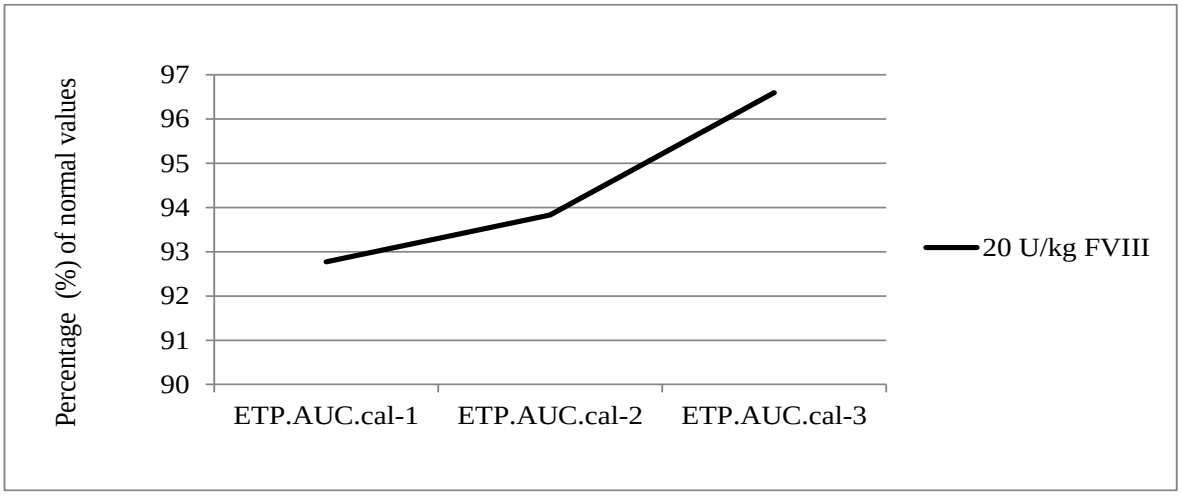



**Figure 1.** Differences of ETP.AUC.cal in the group of patients treated with full-dose rophylaxis

However, for the group treated with intermediate-dose prophylaxis, the mean value of ETP.AUC.cal-3 was similar to that for ETP.AUC.cal-1 (105.24 vs 102.36, F = 1.108, p = 0.352; Figure 2).

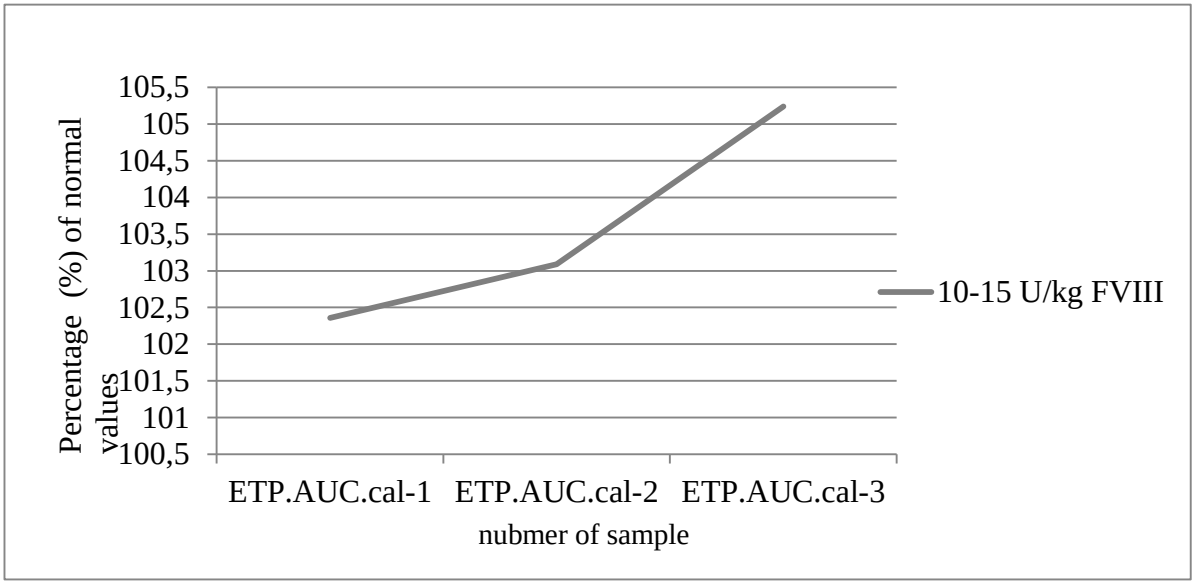



**Figure 2.** Differences of ETP.AUC.cal in the group of patients treated with intermediate-dose prophylaxis

Moreover, no significant difference between the mean values of ETP.AUC.cal-3 and ETP.AUC.cal-1 in the group treated on demand was found either (p = 0.561), nor for mean values of ETP.AUC.cal-3 between the groups (F = 0.097, p = 0.764). An interesting observation was that mean values of ETP.AUC.cal-2 (20 minutes after the first dose) were not significantly increased over the initial values in either prophylaxis group: a) full-dose group (93.83 vs 92.77; F = 0.303, p = 0.611; b) intermediate-dose group (103.09 vs 102.36; F = 0.755, p = 0.434. Moreover, the mean values of ETP.AUC.cal-3 after 3 months prophylaxis, were higher in both the full and intermediate-dose groups in comparison with mean values for ETP. AUC. cal-2.

The differences between the mean values of ETP.Cmax.cal-3 and ETP.Cmax.cal-1 were not significant in any of the studied groups: a) full-dose prophylaxis (98.75 vs 91.78; p = 0.183); b) intermediate-dose (101.63 vs 99.17; p = 0.305); c) on demand group (92.60 vs 92.14; p = 0.108). Also, there were no significant differences between the groups for the mean values of ETP.Cmax.cal-3, (F = 1.419, p = 0.268).

## Discussion

The principle goal of this study was to explore the feasibility of using ETP assay as tool for monitoring the efficacy of different-dose prophylaxis approaches in patients with severe haemophilia. Prophylactic treatment of haemophilia was introduced some 40 years ago and has clearly shown benefits for patients when compared with on demand treatment. The gold standard prophylaxis regimen (Malmö protocol) was pioneered in Sweden and involves the administration of 20-40 U/kg of FVIII three times per week for haemophilia A and twice weekly for haemophilia B [12,13]. This investigation addressed the question of whether a smaller could be useful for patients with severe haemophilia by measuring the improvement of coagulation status by laboratory assay ETP. We compared an intermediate-dose regimen, which consisted of FVIII replacement therapy at 10-15 U/kg thrice weekly with the full-dose prophylaxis regimen of FVIII 20 U/kg thrice weekly (all patients on prophylaxis were haemophiliacs A). Intermediate-dose prophylaxis has been described by a Dutch group and involved the administration of factor replacement therapy at 15-25 U/kg twice or three times per week for haemophilia A, with an overall weekly dose of 32-40 U/kg [14]. Our patients receiving intermediate-dose prophylaxis, were given a mean weekly dose of 32 U/kg, which is similar to that of the Dutch group.

The recent studies have shown that FVIII/IX level has relatively poor predictive value for bleeding risk in patients with haemophilia, which led to interest in new haemostasis test, such as ETP [1]. Therefore, we decided to record the changes of ETP parameters in response to different-dose prophylaxis regimens.

Our results showed that the parameter, ETP – ETP.AUC.cal was significantly impro-ved in patients after 3 months of full-dose prophylaxis when compared with initial values (p = 0.042), whereas significant ch-anges of ETP.AUC.cal were not recorded in the intermediate-dose prophylaxis group nor in the patients treated on-demand. The ETP parameter – ETP.Cmax.cal did not alter significantly either within or between the groups.

After 3 months receiving prophylaxis ROTEM values were longer (in seconds) than those measured 20 minutes after the first dose, when they were in the normal range in both full and intermediate-dose prophylactic groups. Opposite to this, ETP.AUC.cal values after 3 months prophylaxis were relatively higher than those measured 20 minutes after

the first dose in both treatment groups. The ETP parameter – ETP.AUC.cal, in both prophylaxis groups improved gradually during treatment and it seems that prophylaxis has a cumulative effect on thrombin generation potential. ETP.AUC.cal represents the capa-city for total thrombin formation and this was significantly improved in the full-dose prophylaxis group. ETP.Cmax.cal represents the capacity for maximal achieved clotting but neither applied prophylactic dose improved it.

The object of this study was not to investigate the relation between the results of ETP and the clinical bleeding phenotype. Several investigations have shown significant correlations between low ETP results and bleeding tendencies [12,15,16]. Our aim was to estimate the minimal prophylactic dose that could improve the coagulation status of patients with haemophilia by recording para-meters of coagulation assay, such as ETP. Data regarding changes of ETP parameters in conditions of different-dose prophylaxis regi-mens are scarce. One study comparing the effect of full and intermediate-dose prophy-laxis regimens was published by Fischer et al [6]. Bleeding frequency and its influence on joint damage (haemophilic arthropathy) after treatment by the Swedish (full-dose) and Dut-ch (intermediate-dose) prophylaxis regimens were recorded. The Swedish full-dose prop-hylaxis regimen was superior in decreasing the frequency of bleeding episodes and preserving joints, but the laboratory data for these groups were not compared.

This study has shown that ETP assay provide data about the coagulation status of haemophiliac patients receiving prophylactic therapy, more exactly – changes in capabilty for thrombin generation. The full-dose prophylaxis regimen improved coagulation test results better than intermediate-dose prophylaxis. The ETP assays is promising tool for more-dose of prophylaxis precise evaluation of the minimal efficacious prophylactic regimen.

## References


1. Dargaud Y, Negrier C. Thrombin generation testing in haemophilia comprehensive care centres. Haemophilia 2010; 16: 223-230.
2. Beltran-Miranda CP, Khan A, Jaloma-Cruz AR, Laffan MA. Thrombin generation and phenotypic correlation in haemophilia A. Haemophilia 2005; 11: 326-334.
3. Berntorp E, Astermark J, Bjorman S, Bla-chette VS, Fisher K, Giangarde PLF, et al. Concensus perspectives on prophylactic the-rapy for haemophilia: summary statement. Haemophilia 2003; 3 (suppl. 1): 1-4.
4. Berntorp F. Prophylactic therapy for haemo-philia: early expiriance. Haemophilia 2003; 9 (suppl. 1):5-9.
5. Van den Berg HM, Fisher K. Prophylaxis for severe hemophilia: experience from Europe and the United States. Sem Thromb Hemost 2003; 29 (1): 49-54.
6. Fisher K, Astermark J, van der Bom JG, Ljung R, Berntrop E, Grobees DE, van den Berg HM. Prophylactic treatment for severe haemophilia: comparison of an intermediate-dose to high-dose regimen. Haemophilia 2002; 8: 753-760.
7. Van den Berg HM, De Groot PH, Fisher K. Phenotypic heterogeneity in severe hemophilia. J Thromb Haemost 2007; 5 (Suppl. 1): 151-156.
8. Den Uijl IEM, Fisher K, van der Born JG, Grobbee DE, Rosendaal R, Plug I. Clinical outcome of moderate hemophilia compared with severe and mild hemophilia. Haemophilia 2009; 15: 83-90.
9. Varadi K, Turecek PL, Schwarz HP. Thrombin generation assay and other unive-rsal tests for monitoring haemophilia therapy. Haemophilia 2004; 20 (Suppl. 2): 17-21.
10. Marchetti M. Evaluation of the overall coagulation potential by the thrombin gene-ration assay. Hematol Meet Rep 2008;2 (1): 6.
11. Trossaert M, Regnault V, Sigaud M, Boisseau P, Fressinaud E, Lecompte T. Mild hemo- philia A with factor VIII asay discrepancy: using thrombun generation assay to assess the bleeding phenotype. J Thromb Haem 2007; 6: 486-493.
12. Nilsson IM, Hedner U, Ahlberg A. Haemo-philia prophylaxis in Sweden. Acta Paediatr Scand 1976; 65: 129-135.
13. Nilsson IM, Berntorp F, Lofqvist T, Pettersson H. Twenty five years' expiriance of prophylactic treatment in severe hemophilia A and B. J Intern Med 1992; 232: 25-32.
14. Van den Berg HM, Fisher K, Mauser-Bunschoten EP, Beek FJA, Roosendaal G, van

der Bom JG, Nieuwehnuis HK. Longterm outcome of individualized prophylactic treatment of children with severe haemophilia. Br J Haem 2001; 112; 561-565.
15. Brummel-Ziedins KE, Whelihan MF, Gissel M, Mann KG, Rivard E. Thrombin generation and beeding in haemophilia A. Haemophilia 2009; 15: 1118-1125.
16. Beltran-Miranda CP, Khan A, Jaloma-Cruz AR, Laffan MA. Thrombin generation and phenotypic correlation in haemophilia A. Haemophilia 2005; 11: 326-334.

Corresponding author: Irena Djunic,
Clinic for Hematology, Clinical Center of Serbia, Belgrade
Koste Todorovica 2,
11000 Belgrade, Serbia
Tel: +381642050629; Fax: +381113065112
e-mail: **irena.djunic@gmail.com**

# DIAGNOSTIC TOOLS IN MANAGEMENT OF BLEEDING

**Zoran Stanojkovic[1,2], Ana Antic [1], Milan Lazarevic[3], Miodrag Vucic[2,4], Nebojsa Vacic [4]**

[1]Blood Transfusion Institute Nis, Serbia;
[2] Medical Faculty Niš, University of Niš, Serbia;
[3]Clinic for Cardiovascular and Transplantation Surgery, Clinical Center Nis, Serbia;
[4]Clinic for Hematology, Clinical Center Nis, Serbia

## Abstract

*Management of bleeding requires preoperative risk evaluation, the causal diagnosis of the complex pathophysiology of bleeding and rapid routine and specific coagulation testings. Coagulation screening tests include prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). Specialized tests include measuring coagulation factors (activity and antigen concentration) and vWF, euglobulin clot lysis time, D-dimer and plasminogen.*
*Treatment and transfusion algorithms, based on repeated and timely point-of-care coagulation testing and on the clinical judgment, are to be encouraged. The time lapse for reporting results and insufficient identification of the hemostatic defect are obstacles for conventional laboratory coagulation tests. The evidence is growing that rotational thrombelastometry or modified thrombelastography are superior to routine laboratory tests in guiding coagulation management.*
*Specific platelet function tests may be of value in platelet-dependent bleeding associated e.g. with extracorporeal circulation, antiplatelet therapy, inherited or acquired platelet defects. Therapeutic approaches include the use of blood products (red cell concentrates, platelets, plasma), coagulation factor concentrates (fibrinogen, prothrombin complex, von Willebrand factor), pharmacological agents (antifibrinolytic drugs, desmopressin), and local factors (fibrin glue).*

**Key words:** bleeding, coagulation, point of care, transfusion

## Normal haemostasis

Haemostasis is one of the most important systems that protects the integrity of the vascular system after tissue injury and is responsible for minimizing blood loss. There is a constant balance of factors that stimulate the activation of haemostasis and the factors activation of hemostasis can lead to thrombosis (1,2).
It is critical that formation of blood clot in response to a breach in the vascular endothelium occurs rapidly. Immediate vasoco-nstriction of the injured vessel and reflex constriction of adjacent small arteries and arterioles are responsible for an initial slowing of blood flow to the injured area (3). The endothelial cell has an active role in haemostatic response, including synthesis of tiss-ue factor, prostacyclin, von Willebrand factor (vWF), plasminogen activator, antithrombin III, and thrombomodulin (the surface protein responsible for active tion of protein C).
The reduced blood flow enables contact activation of platelets and initial adherence of platelets to exposed connective tissue follows

endothelial lining breakage. The platelet adhesion is potentiated by von vWF. Collagen and thrombin generated at the site of injury cause the adherent platelets to release their granules, including ADP, serotonin, fibrinogen, lysosomal enzymes, and heparin-neutralising factor (PF-4). Collagen and thrombin activate platelet prostaglandin synthesis, leading to the formation of thromboxane A2 (TxA2), which potentiates platelet release reactions and platelet aggregation (4,5). TxA2 is also a powerful vasoconstrictor.

The coagulation cascade involves sequential activation of a number of blood clotting factors, resulting in the formation of fibrin. It is divided into three pathways - intrinsic, extransic, and common, which are closely related and dependent, and the entire process is regulated by a number of mechanisms of the negative and positive feedback (3,6). The intrinsic pathway ensues when the negatively charged subendothelium activates factor XII which, in turn, leads to activation of factor XI that activates factor IX. In association with calcium and with factor VIII as a cofactor, activated factor IX activates factor X on the membrane surface provided by platelet phospholipid (platelet factor 3). The intrinsic pathway is mediated via the contact factor system; following limited activation, factor XII activates prekallikrein to kallikrein, which in turn, activates factor XII. In the extrinsic pathway tissue factor activates factor VII, which in turn, activates factor X both directly and indirectly via activating factor IX. The common pathway is concluded when activated factor X, in association with cofactor factor V on phospholipid surface and calcium, converts prothrombin to thrombin. Thrombin then converts fibrinogen to fibrin and after a few hours after injury there is a solid mass of cross-linked-fibrin on that place. During the same time, the plug begins to lyse due to the incorporation of plasminogen and tissue plasminogenactivator (t-PA) in the plug, resulting in plasmin generation (6-8).

The prevention of unlimited thrombin generation and, therefore, unchecked thrombosis is catalysed by the important, naturally occurring anticoagulant proteins. These include antithrombin III, heparin co-factor II, protein C, protein S and tissue factor pathway inhibitor (TFPI).

**Routine coagulation monitoring**

Defective haemostasis with abnormal bleeding may result from thrombocytopenia (quantitative platelet defect), platelet function disorder (qualitative platelet defect) or defecti-ve blood coagulation (1,3). In association with the clinical picture, including family history, the haemostatic function may be evaluable using a number of initially simple laboratory tests to assess the platelets, vessel wall and coagulation factors (coagulation screening). These tests are rapid (results are obtained for up to one hour), do not require expensive equipment and reagents, can be performed in the laboratories of all sizes and significantly contribute to the determination of the emergency level and the proper selection of therapy (9,10).

As thrombocytopenia is a common cause of abnormal bleeding, patients with suspected bleeding disorders should initially have a blood count, including ***platelet count*** (PLT) to confirm the presence of thrombocytopenia.

The cause of thrombocytopenia may be obvious, e.g., acute leukaemia or DIC. The decline of platelet count is a highly individual phenomenon, some patients are even able to recruit platelets from storage pools. Most patients approach the critical platelet count of 50.000 μ/L after losing two blood volumes (11).

Coagulation screening tests include prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT).Along with these tests is recommended also determination of fibrinogen.

The ***prothrombin time* (*PT*)** measures the extrinsic system (factor VII) and factors common to both systems (factors X, V, prothrombin, and fibrinogen). It is performed by incubating plasma with tissue thromboplastin and calcium at 37 °C at a standardized pH. The time until fibrin strand formation is determined. Reference values are 11-13 sec (70-100 %). PT is prolonged in a deficiency of factors II, V, VII, X, fibrinogen, in the presence of inhibitors of fibrinogen-fibrin reaction (heparin and FDP), in liver disease, vitamin K deficiency in oral anticoagulant therapy and in DIK. Standardization of the prothrombin time (PT) for laboratory control of oral anticoagulant treatment is based on the responsiveness of one type of thromboplastin, measured by its international sensitivity index, and conversion into the international norma-lized ratio (INR). Direct INR determi-nation is performed by local calibration using plasma of certified levels of PT. For PT = 70% INR is 1,0. A PT activity above 30-40% generally ensures normal coagulation within a safe margin (10-12). The PT is a more reliable marker of critically low coagulation factor levels than the aPTT, possibly due to the high rate of false negative aPTT results when acute phase reactant factor VIII is elevated (13).

The ***activated partial thromboplastin time (aPTT)*** measures the intrinsic system (factors VIII, IX, XI, and XII) in addition to factors common to both systems (factors X, V, prothrombin, and fibrinogen). It is performed by incubating plasma with partial thromboplastins, calcium, and kaolin powder at 37 °C at a standardized pH. The endpoint of measurement is the formation of fibrin strands.

Normal values are 25-35 sec. aPTT is prolonged in a deficiency of all coagulation factors except FVII and FXIII, particularly in hemophilia, in DIC, liver disease, the syndrome of massive transfusion, heparin therapy and in the presence of acquired inhibitors of coagulation [9,10,12]. Multiple factor deficiencies tend to show a greater prolongation for a given factor level than single factor deficiencies. Standardization, however, is difficult due to the large variation in calibration constants and methods of endpoint detection, as well as the wide range of pro- and anticoagulant factors affecting aPTT results (14). Severe aPTT prolongations >1.8 times normal are associated with bleeding. Similarly, INR elevations in trauma patients are only indicative for risk of generalized bleeding if they are >1.5- 1.8 times normal and are associated with an elevated aPTT (15). aPTT can be used for laboratory monitoring of heparin therapy (mean interval between the two doses, one hour before the next dose; therapeutic limits: 1.5 - 2.5 more than the reference witness).

The ***thrombin time* (*TT*)** is sensitive to abnormalities of fibrinogen (quantitative or qualitative) or to inhibition of thrombin. It is performed by adding thrombin to citrate plasma without platelets, which converts fibrinogen to fibrin and then measures the speed of the clot formation. TT is prolonged in the presence of hypo and afibrinogenemia (DIK, primary fibrinolysis, congenital hypofibrinogenemia), in the presence of inhibitors of converting fibrinogen to fibrin, such as heparin and FDP (4,9). TT is used in the control of thromboembolic therapy. Normal values are 16-20 sec.

***Fibrinogen*** plays a major role in routine coagulation tests, such as PT and aPTT. There are two methods used in specific fibrinogen assays: determination of the amount of fibrinogen molecules *per se*, (by immunologic, gravimetric, or heat precipitation) and determination of clottable fibrinogen. In the conventional Clauss method, where thrombin is-added to plasma, the fibrinogen concent-ration is proportional to the coagulation time measured. This test is affected by heparin and fibrinogen degradation products. Normal values are 200 - 400 mg/dL, excessive bleeding has been reported at fibrinogen levels <50 mg/dL (14).

**Specific tests**

When the result of the screening test indicates a disorder of haemostasis special tests are performed. Assays are available for measuring ***coagulation factors*** (activity and antigen concentration) and ***vWF***. Determination of factors II, V, VII and X is based on PT (normal values 0,7-1,5 IU/dl), while deterimation of factors VIII:C, IX, XI and XII is test based on aPTT (normal values 0,5-1,5 IU/dl).

Increased levels of circulating plasminogen activator may be detected by a shortened ***euglobulin clot lysis time*** (normal > 2hrs). Immunological methods are available for the detection of fibrinogen and fibrin degradation products (FDPs) as well as ***D-dimer*** in serum. In patients with enhanced fibrinolysis, low levels of circulating ***plasminogen*** may be detected (reference range 75 – 130 %). Spe-cific assays for plasminogen, t-PA, and PAI-1 are also available (10,16).

**Assessing platelet function**

When the platelet count and the blood film examination are normal, other tests are performed to detect abnormal platelet fun-ction. They help to determine the cause of or potential for excessive bleeding, to monitor and evaluate platelet function and to monitor the presence and effectiveness of antiplatelet drugs (aspirin, clopidogrel, prasugrel) (17,18).

Determination of ***bleeding time*** is the only *in vivo* test of platelet function. In principal, the closure time of a prick or incision injury of the skin by a platelet rich thrombus is measured. Several methods have developed over the decades which differ in location where the injury is performed (*Duke*: ear lobe; *Ivy*: lower arm), whether constant pressure is applied (*Ivy*: pressure cuff with 40 mmHg on upper arm), and whether the blood is swabbed or flows freely into sterile water into which the ear lobeis immersed (*Marx and Ressels*). Since this is dependent on how fast a platelet aggregate forms and how stable it is, the count and functionality of the platelets are decisive factors (19). With decreasing platelet counts from 100.000 to 10.000/µl the bleeding time prolongs (normal values are - Duke: 1-3 min, Ivy: 3-10 min).

***Platelet aggregation*** testing measures the ability of various platelet agonists (ADP, prostaglandin E1, arachidonic acid, TRAP-6, collagen, ristocetin) to induce in vitro activation and platelet-to-platelet activation. Classically *Born aggregometry* uses platelet rich plasma (PRP), but whole blood aggregometry can be also used (impedance aggregomery). In the Born aggregometer, PRP is stirred in a cuvette at 37°C and the cuvette sits between a light course and a photocell. When an agonist is added the platelets aggregate and absorb less light and so the transmission increases and this is detected by the photocell (17). The most widespread aggregometers today are *Platelet Function Analyzer* ***(PFA-100)*** (Siemens, Deerfield, IL, USA) and ***Multiplate analyzer*** (*Multiplate Platelet Function Analyzer, Roche*). *PFA-100* has a high negative predictive value and thus must help in identifying patients who are unlikely to need platelet transfusions to reduce bleeding. On the other hand, *Multiplate* is superior in monitoring of antiplatelet therapy and determination of therapy resistence, which have a significant impact on patient care (20,21). On the other hand, the use of term resistance should not be taken lightly, as it may have detrimental effects when misinterpreted. Falsely identified, it may infer an increased risk of thrombosis if treatment were discontinuted or alternatively an increased risk of haemorrhage if dosage were mistakenly increased. It is also confirmed that preoperative antiplatelet drug use in patients undergoing coronary artery bypass grafting (CABG)

and variations in patient response to antiplatelet drugs are important to assess pre-operative risk and at tempt to limit perioperative bleeding (22). The use of platelet function monitoring for bleeding patients will increase as we continue to perform more complex surguries in cardiovascular patients.

## Point-of-care coagulation monitoring – thromboelastography and rotational thromboelastometry

***Thrombelastography (TEG) / rotational thrombelastometry (ROTEM)*** measure the viscoelastic properties of non-anticoagulated or (citrate) anticoagulated blood after induction of clotting under low shear conditions, resembling the rheologic properties in venous vessels *in vivo*. The pattern of changes in viscoelasticity reflect the kinetics of all stages of thrombus formation (*reaction* [r] and *coagulation* [k] *time, clotting time* [CT] and *clot formation time* [CFT]), the stability and firmness of the clot, which is a function of platelet-fibrin interaction and fibrin polymer-rization (*maximum amplitude* [MA], *maximum clot firmness* [MCF]), as well as dissolution (*fibrinolysis*) (23,24). In contrast to routine coagulation testing in plasma, TEG/ROTEM can be performed at the bedside, relevant information can be obtained within a few minutes and, therefore, goal-directed coagu-lation therapy can be readily initiated (25).

TEG/ROTEM is a fibrinolysis-sensitive assay and allows for diagnosis of hyperfibrinolysis in bleeding patients. Addition of different coagulation-activating agents and/or platelet in hibiting agents allows the detection and quantification of specific coagulation defects, such as hypofibrinogenemia, factor deficiency, thrombocytopenia, heparin effect, and hyperfibrinolysis. All these aspects of the coagulation *scenario* come into play when an individual is injured or undergoing surgery (26). Thus, ROTEM not only provides a global picture of the injured patient's hemostatic status, but also permits differential dia-gnosis of the major underlying pathome-chanism of coagulopathy. TEG was found to be an early predictor of transfusion in blunt injury patients. Normal viscoelastic test results are unlikely to coincide with bleeding (high negative predictive value). As a consequence, another important implication of TEG/RO TEM monitoring is the immediate initiation of surgical reexploration if no hemo-staseolgical cause of bleeding is observed (27,28).

The approximate cost of test combinations of routine coagulation and ROTEM are closely equivalent. However, if ROTEM helps to shorten surgical procedures, lowers the frequency of re-openings, shortens the stay in the Intensive Care Unit, and minimizes the direct costs of blood products by also avoiding costly adverse effects of transfusion, the bility of ROTEM coagulation monitoring to save costs is significant in clinical practice.

## Literature


1. Reininger AJ. Primary haemostasis and its assessment by laboratory tests. Hämostaseologie 2006; 26: 42–7.
2. Rodak BF. Hematology, Clinical Principles and Applications. 2nd ed. Philadelphia: W.B Saunders; 2002: 609-753.
3. Shlebak A. Pathophysiological aspects of coagulation. In: Hakim N and Canelo R, editors. Haemostasis in Surgery. Imperial College Press 2007, pp. 1-15.
4. Baklaja R, Elezović I, Miljić P, Miković D, Lučić A. Laboratorijska dijagnostika poremećaja hemostaze. Interlab exim, Beograd, 2008.
5. de Groot PG, Urbanus RT, Roest M. Platelet interaction with the vessel wall. Handb Exp Pharmacol 2012; 210: 87-110.
6. Butenas S, Mann KG. Blood coagulation. Biochemistry (Mosc) 2002; 67(1): 3-12.
7. Kleinegris MC, Ten Cate-Hoek AJ, Ten Cate H. Coagulation and the vessel wall in thrombosis and atherosclerosis. Pol Arch Med Wewn 2012; 122(11): 557-66.
8. Furie B, Furie BC. Molecular and cellular biology

of blood coagulation. New Engl J Med 1992; 326: 800-6.
9. Rochon AG, Shore-Lesserson L. Coagulation monitoring. Anaesthesiol Clin North Am 2006; 24: 839-56.
10. Rydz N, James PD. Approach to the diagnosis and management of common bleeding disorders. Semin Thromb Hemost2012;38(7):711-9.
11. Hiippala ST, Myllyla GJ, Vahtera EM. Hemostatic factors and replacement of major blood loss with plasma-poor red cell concentrates. Anesth Analg1995;81:360-5.
12. Kozek-Langanecker S. Management of massive oprative blood loss. Minerva Anestesiol 2007; 73(7-8): 401-15.
13. Yuan S, Ferrell C, Chandler WL. Comparing the prothrombin time INR versus the APTT to evaluate the coagulopathy of acute trauma. Thromb Res 2007; 120(1): 29-37.
14. Ciavarella D, Reed R, Counts R, Baron L, Pavlin E, Heimbach D, *et al*. Clotting factor levels and the risk of diffuse microvascular bleeding in the massively transfused patient. Br J Haematol 1987; 67: 365-8.
15. Counts RB, Haisch C, Simon TL, Maxwell NG, Heimbach DM, Carrico CJ. Hemostasis in massively transfused trauma patients. Ann Surg 1979; 190: 91-9.
16. White RH. The epidemiology of venous thromboembolism. Circulation 2003; 107: 14-8.
17. Gori AM, Marcucci R, Paniccia R. Thrombotic events in high risk patients are predicted by evaluating different pathways of platelet function. Thromb Haemost 2008; 100: 1136-45.
18. Harrison P, Frelinger AL III, Furman MI, et al. Measuring antiplatelet drug effects in the laboratory. Thromb Res 2007; 120: 323-36.
19. Lind SE. Bleeding time. In: Michelson AD (ed). Platelets. San Diego: Academic Press 2002: 283–9.
20. Antić A, Stanojković Z, Stanojević G, Mačukanović-Golubović L. Laboratory monitoring of the aspirin and clopidogrel therapy effect. Bilt Transfuziol 2011; 57(1-2): 64-9.
21. Paniccia R, Antonucci E, Maggini N, Romano E, Gori AM, Marcucci R, Prisco D, Abbate R. Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy. Am J Clin Pathol 2009; 131(6): 834-42.
22. Ferraris VA, Ferraris SP, Suha SP. Antiplatelet drugs: Mechanisms and risks of bleeding following cardiac operations. Int J Angiol 2011; 20(1): 1-18.
23. Theusinger OM, Nürnberg J, Asmis LM, Seifert B, Spahn DR. Rotation thromboelastometry (ROTEM) stability and reproducibility over time. Eur J Cardiothorac Surg 2010; 37(3): 677-83
24. Di Benedetto P, Baciarello M, Cabetti L, Martucci M, Chiaschi A, Bertini L. Thrombe-lastography. Present and future perspectives in clinical practice. Minerva Anestesiol 2003; 69: 501-9.
25. Stanojković Z, Antić A, Stanojević G, Stanojković M. Point-of-care coagulation testings. Bilt Transfuziol 2011; 57(1-2): 41-8.
26. Luddington RJ. Thrombelastography /thromboelastometry. Clin Lab Haematol 2005; 27:81-90.
27. Lang T, Bauters A, Braun SL, Potzsch B, von Pape KW, Kolde HJ, et al. Multi-centre investigation on reference ranges for ROTEM thromboelastometry. Blood Coagul Fibrinolysis 2005; 16: 301-10.
28. Cammerer U, Dietrich W, Rampf T, Braun SL, Richter JA. The predictive value of modified computerized thromboelastography and platelet function analysis for postoperative blood loss in routine cardiac surgery. Anesth Analg 2003; 96: 51-7.

Corresponding author: Ass. prof. Zoran Stanojkovic, MD, PhD
Blood Transfusion Institute Nis
Boul. dr Zoran Djindjica 48, Nis
Tel.+381631039005
e-mail: **mizoz1@mts.rs**

# PRIMENA GENETSKIH TESTOVA U DETEKCIJI NEDOSTATKA ANTITROMBINA

**Mirjana Kovač[1,2], Gorana Mitić[3], Valentina Đorđević[4], Branko Tomić[4], Zsuzsanna Bereczky[5]**

[1]Medicinski fakultet Beograd,
[2]Institut za transfuziju krvi Srbije, Beograd,
[3]Institut za laboratorijsku medicinu, Kliničkog centra Vojvodine,
Medicinski fakultet Novi Sad,
[4]Institut za molekularnu genetiku i genetsko inženjerstvo, Beograd,
Division of Clinical Laboratory Research, Department of Laboratory Medicine, Faculty of
[5]Medicine, University of Debrecen, Debrecen, Hungary

## Sažetak

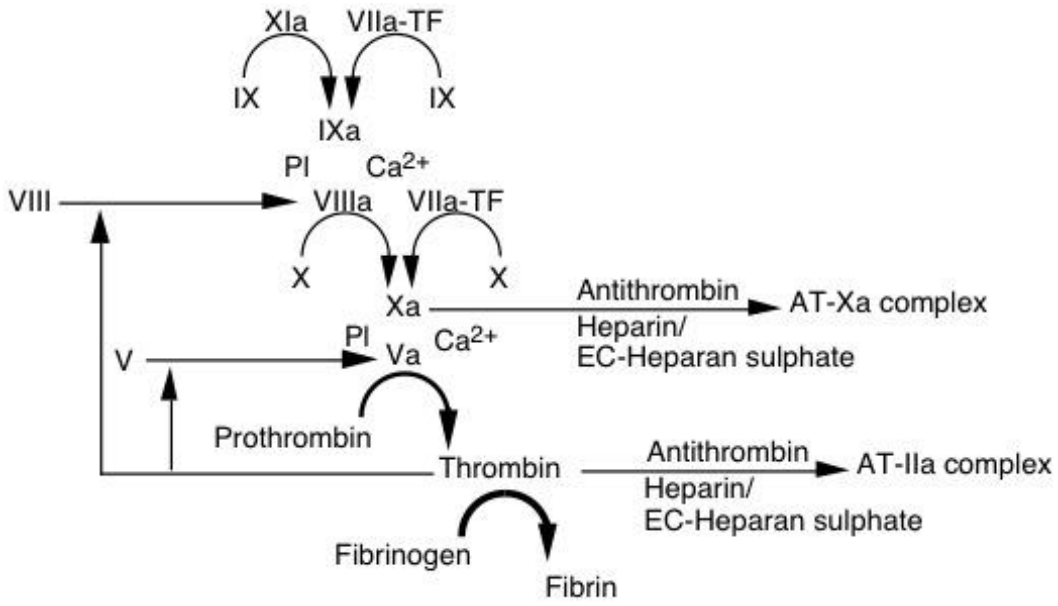
*Urođeni nedostatak AT je redak autozomni poremećaj, uzrokovan mutacijom na nivou AT gena (SERPINC1). Klasifikuje se u dva tipa: tip I je kvantitativni poremećaj, dok je tip II funkcionalni poremećaj koji se deli u tri podtipa. Klinička slika i ozbiljnost trombotičkih događanja zavise od tipa nedostatka AT i mesta mutacije.*
*Cilj naše studije je bio da se primenom genetskih testova (PCR i MLPA) utvrdi tip mutacije koja dovodi do nedostatka AT, za pacijente kod kojih je prethodno utvrđeno da imaju nedostatak AT i članove njihovih porodica. Zatim, nakon sprovedenog funkcionalnog testiranja primenom reagenasa različite osetljivosti, proceniti usaglašenost sa genskim analizama. U ispitivanje su uključene 24 porodice (ukupno 71 ispitanik) kod čijih članova je prethodno utvrđen nedostatak AT. U skladu sa genetskim nalazom kod 20 (36%) ispitanika utvrđen je tip I, kod 33 (60%) tip II HBS (15 homozigoti i 18 heterozigoti); 2 (4%) ispitanika sa tipom II PE i 16 članova porodice bez mutacije AT. Rezultati aktivnosti AT mereni primenom hc. assay anti IIa (Berichrom) pokazali su da u grupi sa tipom II HBS u homozigotnoj varijanti kod 80% ispitanika postoji dobra usaglašenost sa nalazom genetskih analiza, dok u grupi sa heterozigotnom varijantom kod svega 33% ispitanika. Aktivnost AT, merena primenom Innovance i Stachrom eseja pokazuje 100% korelaciju sa genskim analizama.*
*Imajući u vidu da postavljanje dijagnoze nedostatka AT počinje određivanjem aktivnosti AT, a završava se eventualno genskim testiranjem koje još uvek nije obavezno, te dobijene rezultate genenetike AT, primena hromogenih eseja InnovanceAT i STA-Stachrom AT bila bi najpodesnija za ispitivanje aktivnosti AT u našoj populaciji.*

**Ključne reči:** nedostatak antitrombina (AT), genske PCR analize, funkcionalni testovi

## Uvod

Antitrombin (AT) pripada porodici serinskih proteaza inhibitora (*SERPINI*) koji predstavljaju najbrojniju porodicu proteaza inhibitora (1,2). AT predstavlja veoma značajan regulator hemostaznog sistema, a njegov potpuni nedostatak je nespojiv sa životom. Inhibitorni efekat AT je polivalentan i usmeren ka inaktivaciji velikog broja faktora koagulacije, plazmina, kalikreina i KMWK. Njegova primarna uloga se ogleda u inhibiciji stvorenog trombina (IIa) koji nastaje kao krajnji produkt aktivacije hemostaznog sistema, uslovljavajući nastanak fibrina odnosno fibrinskog koaguluma. Takođe, inhibitorni efekat ispoljava u odnosu na brojne faktore koagulacije: unutrašnjeg puta (FIXa, FXIa, FXIIa) i aktivirane faktore spoljašnjeg puta (kompleks FVIIa-TF). Treba naglasiti da se njegova inhibitorna uloga u odnosu na FVIIa, odvija samo ukoliko je FVIIa vezan za tkivni faktor (TF) (Grafikon 1), (1, 3, 4).

**Grafikon 1. Inhibitorna uloga AT u hemostaznom sistemu**




Takođe, AT ima antiproliferativnu i antiinflamatornu ulogu, koja je primarno vezana za njegovu sposobnost da inhibira trombin (5). Prema nedavno publikovanom istraživanju, AT ima važnu ulogu u tumorskoj genezi. Svoj antitumor efekat ostvaruje preko inhibitornog efekta na enteropeptidazu, proteazu koja je uključena u proces širenja i angiogeneze tumorskog tkiva (6).

U toku 1965. godine Egeberg je opisao prvu porodicu sa nedostatkom AT i epizodama venskog tromboembolizma (VTE) (7), a prvi funkcionalni defekt AT (AT *Budapest)* je opisao Sas i saradnici u toku 1974. godine (8). Gen za humani AT (*SERPINC1)* je lociran na 1q23-q25 poziciji. Sadrži 7 egzona produkujući 1.4 kb mRNA i 6 introna. Vodeća sekvenca od 32 aminokiseline je kodirana preko egzona

1 i 5′ i egzona 2. Heparin vezujuće mesto AT molekule je kodirano preko egzona 2 i 3. Reaktivno mesto molekule AT je locirano na karboksi-terminalnom delu proteina i kodirano je preko egzona 6 (9-11). Deficit AT se nasleđuje autozomno dominantno. Do sada otkrivene i publikovane mutacije AT sumirane su u *Antithrombin Mutation Database* u sklopu *Human Gene Mutation Data (HGMD).* Opisano je više od 310 različitih mutacija na nivou gena AT (http://www.hgmd.cf.ac.uk;), a poslednja je prijavljena u martu 2017. god. U skladu sa preporukama *International Society on Thrombosis and Haemostasis* (ISTH) AT deficit se klasifikuje u dva tipa: AT deficit definisan kao tip I (kvantitativni) i tip II (kvalitativni), tabela 1. (1, 13).

**Tabela 1.**

**ISTH klasifikacija deficita AT**

| Tip | Interpretacija |
|---|---|
| Tip I | Kvantitativna varijanta - Tip I deficit je udružen sa redukcijom funkcionalnog i imunološkog nivoa antitrombina za oko 50% od normalnih vrednosti. |
| Tip II | Kvalitativna varijanta - Tip 2 deficit je udružen sa značajno većom redukcijom funkcionalnog u odnosu na imunološki. |
| Tip II HBS: Mutacija zahvata heparin vezujući domen molekule antitrombina. | |
| Tip II RS: Mutacija pogađa reaktivno mesto molekule antitrombina (*thrombin- binding site*). | |
| Tip II PL (Pleotropni): Mutacija pogađa oba, heparin vezujući domen molekule i reaktivno mesto molekule antitrombina. | |

Urođeni nedostatak AT prisutan je u svim rasnim i etničkim grupama. Takođe, nema razlike u zastupljenosti u odnosu na pol. Procenjuje se da je prevalenca urođenog nedostatka AT u opštoj populaciji između 1:2000 i 1:5000, a kod pacijenata sa VTE između 1:20 i 1:200 (14). Nedostatak AT je češće zastupljen u okviru porodica sa VTE (14–17) i predstavlja snažan rizik za razvoj VTE. U većini slučajeva tromboze su retke u starosnoj dobi do 20 godina, što najverovatnije proizlazi iz protektivne uloge visokog nivoa takođe prirodnog inhibitora, α-makroglobulina. Međutim, rizik raste nakon druge decenije života i do 50 godine gotovo 50% nosilaca nedostatka AT razvije trombozu. U odnosu na prevalencu u opštoj populaciji i pacijente sa VTE, procenjeni relativni rizik od VTE kod pacijenata sa AT deficitom je aproksimativno 25–50 puta veći (18 ). U odnosu na tip deficita AT, homozigotna varijanta tipa I je inkompatibilna sa životom, a nosioci heterozigotne varijante deficita se suočavaju sa ozbiljnim trombotičkim komplikacijama koje se rano razvijaju. Prema prethodno publikovanim studijama, tip II-HBS u homozigotnoj varijanti je praćen ozbiljnim trombotičkim komplikacijama, a pored rizika za venski tromboembolizam predstavlja snažan rizik za pojavu juvenilnih venskih tromboza, arterijskih tromboza (19–24), te komplikacija trudnoće (25–27). Ukoliko je nedostatak AT tip II-HBS prisutan u heterozigotnoj varijanti predstavlja manji rizik za razvoj tromboze u odnosu na druge podtipove tipa II ili nedostatak AT tip I (11, 28).

Cilj naše studije je bio da se primenom genetskih testova (PCR i MLPA) utvrdi tip mutacije koja dovodi do nedostatka AT, za pacijente kod kojih je prethodno utvrđeno da imaju nedostatak AT, kao i članove njihovih porodica. Zatim, nakon sprovedenog funkcionalnog testiranja primenom reagenasa različite osetljivosti proceniti usaglašenost sa genskim analizama.

## Materijal i metode

U ispitivanje su uključene 24 porodice (ukupno 71 ispitanik), kod čijih članova je prethodno utvrđen nedostatak AT. Nakon sprovedenih funkcionalnih i genetskih testiranja u studiju je uključeno ukupno 55 ispitanika, (22 muškarca i 33 žene), kod kojih je utvrđen nedostatak AT i 16 najbližih članova porodice, kod kojih nije utvrđen nedostatak AT. Starosna dob ispitanika u momentu uključenja u studiju izražena kao medijana je 45.5 (IQ 32 ).

Za sve ispitanike uzeti su uzorci venske krvi na natrijum citratu, 2 epruvete od 3,6 ml venske krvi, za sledeće analize: aktivnost AT, antigen AT i genetske (PCR) analize za detekciju mutacije molekule AT. Takođe, kod svih ispitanika uzeti su anamnestički podaci o pojavi prvog i ponovljenog trombotičkog događaja, faktorima rizika u vreme pojave trombotičkih događaja, pojavi tromboza u toku primene antikoagulantne terapije.

Kod svih ispitanika urađena je PCR metoda za detekciju protrombotičkih mutacija: FVLeiden i *F2* 20210G>A. DNA je izolovan primenom QIAamp DNA mini kita (QIAGEN), u skladu sa protokolom proizvođača i čuvan na +4°C. Mutacije su detektovane polimeraza lančanom reakcijom (PCR), a zatim razlaganjem sa specifičnim restrikcionim enzimima (MnlI-FV Leiden i HindIlI-FIIG20210A, NEBiolabs). Normalni i mutirani aleli se razlikuju po veličini restrikcionih fragmenata posle elektroforeze na poliakrilamidnom gelu. Za analizu AT (*SERPINC1*) gena primenjena je Sanger fluorescentna sekvenciona metoda, koja predstavlja zlatni standard. Istraživanje je obuhvatilo egzon, egzon-intron i promoter region *SERPNC1* gena. Fluorescentno direktno sekvenciranje je izvedeno u ABI Prisma 3130-Avant genetičkom analajzeru (*Applied Biosistems Foster Citi, CA*), a za procenu rezultata korišćen je *Sequencing Analysis 5.4 software.* U detekciji velikih genskih rearanžmana, korišćena je metoda *Multiplex ligation-dependent probe amplification* (MLPA), pogodna za detekciju delecije i duplikacije u sklopu *SERPINC1* gena.

Funkcionalno testiranje, određivanjem aktivnosti AT u plazmi je izvršeno primenom tri različita funkcionalna eseja koji se baziraju na inaktivaciji faktora IIa (*Berichrom*, Simens i *Stachrom*, Stago), odnosno inaktivaciji faktora Xa (*Innovance*, Simens). Analize su izvedene pod istim uslovima na koagulometru BCS-XP (Simens) odnosno na STA-Compact (Stago). Referentna vrednost za *Berichrom* navedena od strane proizvođača je 79–111%, za *Innovance* 83–113%, a za *Stachrom* je 80–120%. Dodatno je urađen imunološki esej za određivanje antigena AT primenom *LIATEST* ATIII (Stago diagnostika, Francuska). Referentne vrednosti 80–120%.

## Statistička analiza

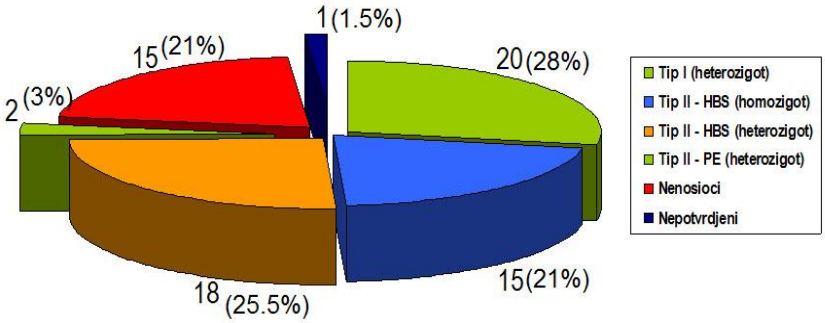
Za statističku obradu podataka korišćen je softver SPSS, verzija (SPSS, Chicago, IL, USA). U svrhu prikaza osnovnih karakteristika ispitanika korišćene su statističke

deskriptivne metode. Za ispitivanje statističke značajnosti razlika za kontinuirane varijable sa normalnom distribucijom korišćen je *Mann-Whitney U* test. Za ispitivanje razlike učestalosti pojedinih parametara između dve grupe (Tip I i Tip II deficita) korišćen je *Fisher*-ov test tačne verovatnoće i *Pearson*-ov $\chi^2$ test. Primenom *Kaplan Meier* statističkog testa utvrđeno je da li vremenska pojava prve trombotičke komplikacije, odnosno prve retromboze zavisi od tipa deficita AT.
P vrednost < 0.05 je smatrana statistički značajnom u svakom od pomenutih testova.

**Rezultati**

Genskim analizama je utvrđeno da 20 (36%) ispitanika nosi AT defict tip I u heterozigotnoj varijanti. Kod 33 (60%) ispitanika je utvrđen deficit AT tipa II-HBS, kod 15 u homozigotnoj varijanti, dok je kod 18 mutacija prisutna u heterozigotnoj varijanti. Kod 2 (4%) ispitanika je utvrđen deficit AT tip II-PE (pleotropni) (Grafikon 2).

**Grafikon 2. Distribucija učestalosti tipa nedostatka AT**




U tabeli 2. je dat prikaz starosne dobi ispitanika u momentu uključivanja u studiju, polna struktura, utvrđena vrsta mutacije u sklopu genotipizacije u odnosu na prisustvo FVLeiden, FII *G20210A* i *SERPINC 1* mutacije na nivou AT gena. Takođe je dat prikaz nivoa AT utvrđenog metodom merenja biološke aktivnosti, primenom hromogene metode preko inaktivacije F II odnosno F X. Na nivou *SERPINC1* gena kod ispitanika sa utvrđenim nedostatkom AT tip I, otkriveno je 6 različitih mutacija, od toga 3 su novootkrivene, a preostale mutacije su već poznate i kao takve se nalaze u bazi podataka za *AT (Human Gene Mutation Data)*. U grupi sa nedostatkom AT tip-II, utvrđene su dve vrste mutacije: tip II-HBS (*Heparin binding site)* sa utvrđenom mutacijom c.391 C>T, p.Leu99(131)P (*Budapest 3*) koja je detektovana u homozigotnoj i heterozigotnoj varijanti i tip II-PE (pleotropni) sa utvrđenom mutacijom c.1301T >C, p.Phe402(434)Ser (*Torino)* u heterozigotnoj varijanti. Obe mutacije koje su uzrok nedostatka AT tip II su nastale kao posledica zamene redosleda aminokiselina i kao već poznate mutacije se nalaze u bazi podataka za *AT (Human Gene Mutation Data)*.
Analiza rezultata merene aktivnosti AT, primenom hromogenog eseja, u odnosu na

utvrđeni genotip, pokazuje da postoji neslaganje između nivoa aktivnosti AT utvrđene primenom *Berichrom* testa, kod onih ispitanika kod kojih je utvrđen nedostatak AT tip II-HBS. Naime, kod 20% homozigota i 67% heterozigota iz grupe tip II-HBS merena aktivnost AT pokazuje potpuno normalne rezultate, dok se kod deficita AT tip I i tip II-PE u potputnosti poklapaju genetski i nalaz funkcionalne aktivnosti. Aktivnost AT merena primenom *Innovance* i *Stachrom* pokazuje potpunu usaglašenost sa rezultatima genetike, bez obzira na tip mutacije.

**Tabela 2.**

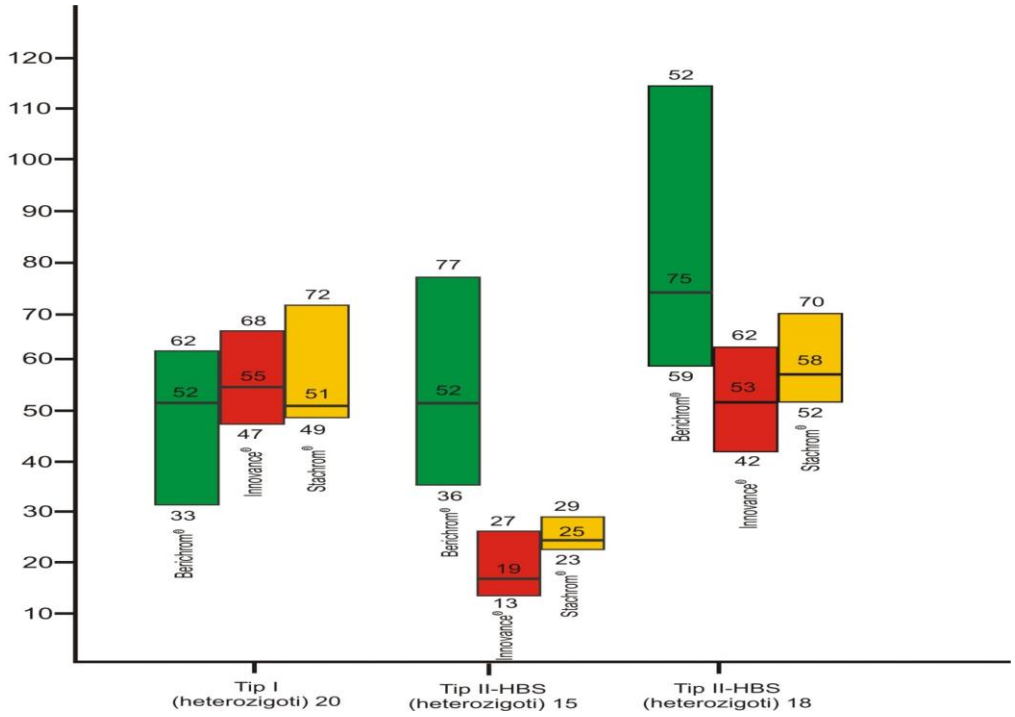
**Rezultati genotipizacije i nivo aktivnosti AT u grupi ispitanika**

| **AT deficit** | **Tip I (heterozigot)** | **Tip II- HBS (homozigot)** | **Tip II- HBS (heterozigot)** | **Tip II- PE (heterozigot)** |
|---|---|---|---|---|
| Broj porodica | 9 | 11/13 | 8/13 | 1 |
| Broj ispitanika | 20 | 15 | 18 | 2 |
| Starosna dob, medijan (IQ) | 40.5 (36) | 31.5 (14) | 52(55) | 50 (20) |
| Pol M/Ž | 7/13 | 6/9 | 9/9 | 0/2 |
| FVLeiden / FII G20210A | 1/1 | | 2 | |
| *SERPINC 1* mutacije | IVS5 +27 G>C *heterozygous*, IVS1 -18 C>G *heterozygous;* c.652-654 ATC del, p.Ile218(186)del *heterozygous;* c.1171 C>T, p.Arg391(359) STOP *heterozygous;* (IVS5 +27 G>C *heterozygous;* c.1019-1022TGGAdel, p.Leu308(340)LeufsX5 *heterozygous* (nova mutacija); c.548Cdel, p.Ser151(183)SerfsX100 *heterozygous* (nova mutacija); c.415-416AAdel, p.Lys139(171)ValfsX16 *heterozygous* (nova mutacija) | c.391 C>T, p.Leu99(131)P (AT *Budapest 3) homozygous* | c.391 C>T, p.Leu99(131)P (AT *Budapest 3) heterozygous* | c.1301T>C, p.Phe402(434)Ser (*AT Torino*) heterozygous |

| | | | | |
|---|---|---|---|---|
| AT hc. esej (IU/mL)<br>*Berichrom*®<br>Saglasnost sa gen. anal. n(%) | 0.52 (0.33-0.62)<br>20 (100) | 0.52 (0.36-0.77)<br>12 (80) | 0.75(0.59-1.14)<br>6 (33) | 0.53(0.52-0.52)<br>2 (100) |
| *Innovance*<br>Saglasnost sa gen. anal. n(%) | 0.55 (0.47-0.68)<br>20 (100) | 0.19 (0.13-0.27)<br>15(100) | 0.53(0.42-0.62)<br>18 (100) | / |
| *Stachrom*®<br>Saglasnost sa gen. anal. n(%) | 0.51 (0.49-0.72)<br>20 (100) | 0.25 (0.23-0.29)<br>15(100) | 0.58(0.52-0.70)<br>18 (100) | / |
| *LIATEST* ATIII antigen | 56 (49-75) | 125 (93-143) | 94 (93-119) | / |

*Berichrom*® (metoda IIa), *Innovance*®(metoda Xa), *Stachrom*® (metoda IIa)

**Grafikon 3. Rezultati aktivnosti AT mereni različitim reagensima (Berichrom®, Innovance ®, Stachrom ®)**




U tabeli 3. date su kliničke karakteristike ispitanika u odnosu na utvrđeni tip nedostatka AT. U grupi sa nedostatkom tip I, 12/20 (60%) ispitanika je imalo trombozu, u grupi sa tip II-HBS (homozigoti) 12/15 (80%), u grupi tip II-HBS (heterozigoti) 4/18 (22%), a u grupi tip II-PE svi nosioci su imali trombozu. U odnosu na vreme ispoljavanja simptomatologije, najranije trombozu razvijaju nosioci nedostatka AT tip II-HBS (homozigoti) sa prosečnom starosnom dobi od 17 godina,

dok heterozigoti za isti tip najkasnije ispoljavaju simptomatologiju, sa prosečnom starosnom dobi od 36 godina, P=0.002. Kod nedostatka AT tip II-HBS u homozigotnoj varijanti je češća spontana pojava tromboza koja se beleži kod 67% ispitanika. Kod tip I i tip II-HBS u heterozigotnoj varijanti polovina ispitanika ima spontane tromboze, dok je kod pleotropnog tipa prva tromboza udružena sa određenim faktorom rizika (trudnoća). U odnosu na lokalizaciju prve tromboze, distribucija učestalosti je podjednaka osim za nastanak abdominalnih tromboza (v. cava inferior, v. lienalis, v. porte i v. renalis), koje se predominantno javljaju kod homozigotne varijante tip II-HBS. U odnosu na broj pacijenata sa ponavljanim trombozama, distribucija učestalosti je podjednaka i kreće se 35–50%, osim kod heterozigota tip II-HBS, kod kojih je ponavljana tromboza značajno manje učestala i registruje se kod 16% ispitanika. U odnosu na vreme nastanka retromboze, ispitanici iz grupe homozigota tip II-HBS, najmlađi razvijaju retrombozu, P=0.002. U toj grupi prosečna starosna dob kod nastanka retromboze je 21.5 godina, dok je u ostalim grupama starosna dob podjednaka i kreće se od 35 godina kod tipa I, 36 godina kod tipa II-HBS (heterozigoti) do 42 godine kod tipa-PE. Razliku beležimo i u odnosu na pojavu retromboze u toku primene antiko-agu-lantne terapije, gde se retromboza u toku antikoagulantne terapije beleži kod 28% ispitanika sa nedostatkom AT tip I, dok iz grupe tip II-HBS (homozigoti) 83% razvija retrombozu, P=0.002.

**Tabela 3.**

**Kliničke karakteristike ispitanika sa utvrđenim nedostatkom AT**

| | **Tip I (heterozigoti)** | **Tip II-HBS (homozigoti)** | **Tip II-HBS (heterozigoti)** | **Tip II-PE (heterozigoti)** |
|---|---|---|---|---|
| Broj porodica | 9 | 11/13 | 8/13 | 1 |
| Broj ispitanika | 20 | 15 | 18 | 2 |
| Pol M/Ž | 7/13 | 6/9 | 9/9 | 0/2 |
| Nosioci sa najmanje jednom VTE; n/N (%) | 12/20 (60) | 12/15 (80) | 4/18 (22) | 2/2 (100) |
| Starosna dob kod prvog trombotičkog događaja; medijana (IQ) | 24.5 (14) | 17(7) | 36 (55) | 35 (5) |
| Spontane tromboze n/N, (%) | 5/12 (42) | 8/12 (67) | 2/4 (50) | 0/2 (0) |
| Lokalizacija tromboze:<br>Distalna DVT<br>Proksimalna DVT<br>PE<br>Abdominalna<br>Cer. venski sinus Arterijske tromboze | <br>3<br>6*<br>1<br>0<br>1*<br>0 | <br>1<br>5<br>1<br>4&<br>0<br>1 | <br>2*<br>0<br>2<br>0<br>0<br>0 | <br>1<br>1 |
| Broj sa rekurentnom trombozom (RT) n/N, (%) | 7/12 (58) | 6 /12 (50) | 3/4 (75) | 1/2 (50) |
| Starosna dob kod RT; | 35 (31) | 21.5 (11) | 36 (/) | 42(/) |
| Retromboza u toku AK n (%) | 2/7 (28) | 5/6 (83) | 0 | 0 |

Dva pacijenta iz grupe tip I i jedan iz grupe tip II-HBS (heterozigoti) sa PE kao komplikacijom DVT, & (tromboza v. cave, v. lienalis, v. porte i v. renalis)

## Diskusija

Dobijeni genski rezultati pokazali su da od 55 ispitanika sa utvrđenim nedostatkom AT, 36% ima nedostatak AT tip-I, dok 64% ima tip-II, predominantno tip II-HBS koji je utvrđen kod 60% ispitanika. Veoma mali broj od 4% se odnosi na tip II-PE, koji je utvrđen u sklopu jedne porodice. Geno-tipizacija je pokazala da kod naših ispitanika kod kojih je utvrđen nedostatak AT tip I, pored već poznatih mutacija postoje i potpuno tri nove do sada nepoznate mutacije na nivou molekule AT. Sve ove genske mutacije, u grupi sa tipom I su posledica delecije ili zamene redosleda aminokiselina i ispoljene su u heterozigotnoj varijanti. Nasuprot tome, kod nedostatka AT tipa II, prisutna je samo po jedna genska mutacija i to c.391 C>T, p.Leu99(131)P (AT *Budapest 3)* u slučaju tip II-HBS i ispoljena u homozigotnoj i heterozigotnoj varijanti. U slučaju tipa II-PE radi se o mutaciji c.1301T>C, p.Phe402(434)Ser (*AT Torino*) u heterozigotnoj varijanti, koja je takođe nastala kao posledica zamene redosleda aminokiselina. Druge do sada poznate mutacije, kao što su *Cambridge RS* (p.Ala-416Ser); zatim *Basel* (Phro73Leu), *Padua* (p.Arg79His) i *Truro* (p.Glu269Lys) koje uzrokuju tip II-HBS nisu detektovane u našoj populaciji. Analiza rezultata do sada publikovanih studija genetskih mutacija koje su rađene u različitim populacijama poka-zuju da slične rezultate daju istraživači za mađarsku populaciju. U njihovoj studiji predominantni tip deficita je tip II-HBS sa izraženim *founder* efektom gde se uglavnom detektuje (AT *Budapest 3* p.Leu99(131)P) mutacija, utvrđena kod 79% ispitanika, a mali broj se odnosi na ostale mutacije koje su odgovorne za nastanak tipa II ili tipa I (29). U finskoj populaciji *Puurunen* i saradnici takođe naglašavaju *fonder* efekat, potenciran prisustvom predominantno *Basel* (Phro73Leu) mutacije, koja izaziva nedo-statak AT tip II-HBS, koja je utvrđena kod 88% ispitanika sa nedostatkom AT (22). U ostalim evropskim populacijama najčešće je zastupljena mutacija AT *Cambridge* II (p.Ala 416Ser), AT *Basel* (p.Pro73Leu) i AT *Padua* (p.Arg79His) (10,22,24, 31- 33), te nisko zastupljeni polimorfizam (AT *Dublin*; p.Val 30Glu) (34). AT *Cambridge* II je zastupljen u britanskoj populaciji; a ova mutacija ima prevalencu od oko 0.5–2.0% u francuskoj, španskoj i germanskoj populaciji (34).

U sklopu prethodnog postupka utvrđivanja prisustva trombofilije, kod naših ispitanika, isključeno je postojanje drugih trombofilija osim protrombotičkih mutacija. Genotipizacija dva markera FVLeiden i protrombin G20210A, pokazala je da 4 (7%) ispitanika imaju kombinovanu urođenu trombofiliju. Mađarski istraživači takođe navode slične podatke gde kod svojih ispitanika nalaze isključivo ove dve trombofilije kao dodatni faktor rizika za tromboze (29).

Poređenje rezultata funkcionalne aktivnosti AT, dobijenih primenom eseja koji se bazira na anti-IIa aktivnosti (*Berichrom*), sa rezultatima genetike, pokazuje da kod detekcije nedostatka AT tip I i tip II-PE ovaj test u svim slučajevima ukazuje na nedostatak AT. S druge strane kod 80% pacijenata sa nedostatkom AT tip II-HBS u homozigotnoj varijanti, rezultati funkcionalne aktivnosti ukazuju na nedostatak AT, dok je kod preostalih 20% nalaz funkcionalne aktivnosti AT potpuno normalan. Kod heterozigotne varijante snižena aktivnost AT utvrđena je kod svega 33% pacijenata, što znači da 67% njih ne bi bilo dijagnostikovano bez genetskih analiza. S druge strane, primena *Innovance* i *Stachrom* eseja pokazuje odličnu korelaciju sa genetskim analizama bez obzira na tip nedostatka. Ovi rezultati ukazuju koliko je značajan odabir adekvatnog funkcionalnog eseja koji se koristi u rutinskom skriningu trombofilija u našoj populaciji, imajući u vidu učestalost nedostatka AT tip II-HBS u našoj populaciji. Prethodno publikovane studije su takođe

pokazale da postoji jasna razlika u sposobnosti različitih AT esej metoda da otkriju sve varijante deficita AT (22, 24, 35, 36). *Kovacs* i saradnici daju prednost eseju baziranom na anti-Xa aktivnosti (*Innovance AT)* u odnosu na anti-IIa (*Berichrom AT)* u detekciji pacijenta sa deficitom AT tip II-HBS. Autori navode da je esej baziran na anti-Xa aktivnosti mnogo potentniji u detekciji deficita AT tip II-HBS u heterozigotnoj varijanti uslovljen mutacijom *Budapest3*, koji predstavljaju najveći problem za dijagnostiku. Naime, uobičajeni komercijalni eseji ne pokazuju dovoljnu senzitivnost kod ovog tipa deficita, a koji je visoko zastupljen u mađarskoj populaciji, tako da su rezultati ili normalni ili na donjoj granici referentnih vrednosti. S druge strane, u detekciji AT deficita tip I, oba različito bazirana eseja daju gotovo identične rezultate (36), što je potvrđeno i u našoj studiji. *Puurunen* i saradnici ističu da primena komercijalnih dostupnih funkcionalnih eseja, koji se najčešće primenjuju, ne može da otkrije tip II-HBS uzrokovan *Basel* mutacijom, koja je visoko prisutna u finskoj populaciji. Ispitujući pet različitih testova za detekciju funkcionalne aktivnosti AT u odnosu na rezultate genetskih analiza, oni preporučuju primenu *STA-Stachrom* AT ili *Innovance AT* (Siemens), funkcionalnih testova, koji su pokazali najbolje karakteristike u detekciji nedostatka AT tipa II, dok su preostala tri testa nesenzitivna i daju normalan nalaz AT kod pacijenta sa tipom II-HBS, koji je uzrokovan *Basel* mutacijom (22). Analiza kliničke slike u odnosu na utvrđeni genotip pokazuje da je tip II-HBS najraznovrsnija grupa sa nedostatkom AT, gde je klinički i laboratorijski fenotip veoma različit i zavisi od specifične mutacije odnosno da li se ona ispoljava u homo ili heterozigotnoj varijanti. Analiza pokazuje da je broj ispitanika koji su razvili bar jedan trombotički događaj najveći u grupi sa nedostatkom AT tip II. Naime, u grupi sa tipom II-HBS u homozigotnoj varijanti, 80% nosilaca ima bar jednu manifestnu VTE, koje se ispoljavaju veoma rano, gde je prosek starosne dobi za ispoljavanje prve tromboze 17, a kod retromboze 21,5 godina. U ovoj grupi se takođe beleži najveći broj spontanih tromboza, utvrđenih kod 67%, kao i tromboza neuobičajene lokalizacije kao što su abdominalne tromboze, koje uključuju trombozu: v. cava inferior, v. lienalis, v. renalis i v porte. Takođe, u ovoj grupi beležimo visoku stopu rekurentnih tromboza od 50%, pri čemu broj rekurentnih tromboza u toku primene antikoagulantne terapije predstavlja predominantnu karakteristiku ovog tipa deficita AT.

S druge strane, u grupi sa tipom II-PE svi nosioci imaju trombotičke manifestacije, ali se one ispoljavaju kasnije, sa prosečnom starosnom dobi od 35 godina i u svim slučajevima su provocirane. Najmanji broj trombotičkih manifestacija beležimo kod ispitanika sa tipom II-HBS u heterozigotnoj varijanti (22%), pri čemu je to grupa u kojoj se najkasnije javljaju tromboze, prosečno u 36 godini života. Kada su u pitanju nosioci deficita AT tip I, trombotičke događaje beležimo kod 60% nosilaca deficita AT, u 58% provocirane, sa prosečnom starosnom dobi od 24,5 kod prve tromboze, odnosno 35 kod rekurentne tromboze, sa stopom rekurentnosti od 58%. Nekoliko studija je procenjivalo rizik za nastanak tromboze kod osoba sa nedostatkom AT (14–18), međutim veoma malo studija se bavi ispitivanjem efekta tipa mutacije na nivou SERPINC gena, odnosno korelacijom genotipa i fenotipa. Ove malobrojne studije pokazale su da tip urođenog deficita ima direktan uticaj na stepen rizika za razvoj VTE, ali takođe i za lokalizaciju tromboze. *Finazzi* i sar. pokazuju da je prevalenca VTE značajno niža kod pacijenata sa tip II-deficitom u poređenju sa tipom I (37). U studiji *Luxemburg*-a i sar. prikazan je efekat mutacije na nivou SERPINC gena u odnosu na ispoljavanje defekta, odnosno kliničku sliku. Prema njihovom istraživanju nosioci deficita AT tip II, koji nastaje kao posledica *missense* mutacija, imaju manji rizik za nastanak VTE u odnosu na mutacije koje dovode do nedostatka AT tip I, pri čemu je najmanji rizik povezan sa nedostatkom AT tip II-HBS. Takođe rizik za razvoj PE kao komplikacije tromboze, je niži kod tipa II u odnosu na tip I. Nasuprot tome, rizik za razvoj arterijske tromboze je visok kod tipa II, sa 5,9 puta većim rizikom kod nosilaca nedostatka tipa II-HBS (28). Treba napomenuti da u studiji *Luxemburg*-a i saradnika nije rađena analiza podtipova u grupi sa tipom II-

HBS u odnosu na homo ili heterozigotni status. Nedavno su *Gindele* i sar. publikovali studiju u kojoj se bave procenom kliničke slike kod tipa II-HBS, gde ukazuju na ozbiljne kliničke manifestacije ovog tipa u homozigotnoj varijanti. *Purumnen* i sar. daju slične rezultate u studiji koja se odnosi na finsku populaciju, gde dominira tip II-HBS uzrokovan *Basel* mutacijom. Oni beleže učestalost arterijskih tromboza od 11,3% sa učestalošću od 5,7% do 45. godine života (29).

Posebno je važan aspekt kliničkog praćenja asimptomatskih nosilaca AT mutacije, s obzirom da deficit AT predstavlja najveći apsolutni rizik za pojavu VTE među svim urođenim trombofilijama (18). Takođe veoma značajan korak u kliničkom praćenju predstavlja skrining porodice, kod čijeg se člana utvrdi uzročna mutacija. Međutim, ono što je polazna osnova u adekvatnom postavljanju dijagnoze nedostatka AT, je odabir relevantnih testova kojim se meri aktivnost AT. Iako je ova metoda deo skrininga na trombofilije (38), treba naglasiti da dostupni komercijalni eseji, koji se rutinski primenjuju u laboratorijama, nisu podjednako osetljivi na određene mutacije AT, što je pokazalo i naše istraživanje. Ta činjenica ima veliki značaj u postupku ispitivanja trombofilije i direktno utiče na ishod lečenja bolesnika jer neutvrđeni funkcionalni deficit dovodi do nepostavljanja dijagnoze ove najteže urođene trombofilije. *Kovacs* i sar. predlažu da vrednost AT <80% zahteva ponavljanje analize iz novog uzorka, a ako dobijena vrednost bude u intervalu 70–80%, preporučuju izvođenje genskih analiza (39), dok *Zeng* i sar. preporučuju gensku analizu na nivou *SERPINC* gena, kod svih osoba sa neprovociranom trombozom čak i kad je aktivnost AT normalna (40). Koliki je značaj testiranja i otkrivanja ne samo jasno izraženog već i blagih odnosno graničnih slučajeva nedostatka AT, pokazuju rezultati nedavno publikovane studije *Di Minno* i sar. Naime, oni su utvrdili da je rizik za rekurentnu VTE kod pacijenata sa nivoom AT manjim od 70% od HR 3.48, dok je u grupi sa nivoom AT od 70 do 80% HR 2.4, u odnosu na pacijente sa normalnim nivoom AT (41).

Premda naša studija daje prve podatke o genetskoj pozadini nedostatka AT u našoj populaciji, ona ima ograničenja s obzirom da u studiju nisu uključeni svi članovi ispitivanih porodica. Takozvani *selection bias* proizlazi iz činjenice da su u studiju uključeni svi oni simptomatski bolesnici sa ozbiljnim tromboembolijskim komplikacijama, koji su mnogo više bili motivisani da učestvuju u studiji, nego asimptomatski članovi porodice. Ta činjenica je važna u pretpostavci da bi ispitivanjem svih članova porodice došli do većeg broja nosilaca već utvrđene mutacije u porodici. Međutim, dobijeni rezultati koji pokazuju zastupljenost pojedinih vrsta mutacija AT u našoj populaciji, predstavljaju veoma dragocen podatak, i na njega ne utiče koliko je asimptomatskih članova uključeno. S druge strane, dobijeni podatak o vrsti mutacija koje su zastupljene u našoj populaciji je veoma važan i treba da utiče na odabir adekvatnog hromogenog eseja, dovoljno osetljivog za određeni tip.

**Zaključak:** Postavljanje dijagnoze nedostatka AT počinje određivanjem aktivnosti AT, a završava se eventualno genskim testiranjem, koje još uvek nije obavezno. Imajući u vidu genske karakteristike naše populacije, primena hromogenih eseja *Innovance* AT i *STA-Stachrom* AT bila bi najpodesnija za ispitivanje aktivnosti AT u našoj populaciji.

## Literatura:


1. Lane DA, Caso R. Antithrombin: Structure, genomic organization, function and
2. inherited deficiency. Baillieres Clin Haematol 1989; 2:961-998.
3. Lane DA, Olds RJ, Boisclair V, Chowdhury V, Thein SL, Cooper DN, Blajchman M, Perry DJ, Emmerich J, Aiach M. Antithrombin III Mutation Database: First Update. Thrombosis And Haemostasis 1993;70:361-369.
4. Perry DJ. Antithrombin and its Inherited Deficiencies. Blood Reviews 1994; 8: 37-55.
5. Olds RJ, Lane DA, Mille BM, et al. Antithrombin: principal inhibitor of thrombin. Semin Thromb Hemost 1994; 20: 353-372.
6. Hoffmann J. N. et al. Benefit/risk profile of high-dose antithrombin in patients with severe sepsis treated with and without concomitant heparin. Thromb. Haemost.2006; 95, 850–56.
7. Luengo-Gil G, Calvo MI, Martín-Villar E, Águila S, Bohdan N, Antón AI, Espín S, Ayala de la Peña F, Vicente V, Corral J, Quintanilla M, Martínez-Martínez I.Antithrombin controls tumor migration,

invasion and angiogenesis by inhibition of enteropeptidase. Sci Rep. 2016; 6:27544.
8. Egeberg O. Inherited antithrombin deficiency causing thrombophilia. Thromb Diath Haemorrh. 1965; 13:516-30.
9. Sas G, Blasko G, Banhegyi D, Jako J, Palos LA. Abnormal antithrombin III (antithrombin III "Budapest") as a cause of a familial thrombophilia. Thromb Diath Haemorrh 1974; 32:105-115.
10. Olds RJ, Lane DA, Thein SL. The Molecular Genetics of Antithrombin Deficiency. Br J Haem 1994; 87: 221-226.
11. Olds RJ, Lane DA, Chowdhury V, Sas G, Pabinger I, Auberger K, Thein SL. (ATT) Trinucleotide repeats in the antithrombin gene and their use in determining the origin of repeated mutations. Hum Mutat 1994; 4:31–41.
12. Luxembourg B, Delev D, Geisen C, et al. Molecular basis of antithrombin deficiency. Thromb Haemost 2011; 105: 635-646.
13. http://www.hgmd.cf.ac.uk
14. Lane DA, Olds RJ, Conard J, Boisclair M, Bock SC, Hultin M, Abildgaard A, Ireland, H, Thompson E, Sas G, Horellou MH, Tamponi G, Thein SL. Pleiotropic Effects of Antithrombin Strand 1C Substitution Mutations. Journal Of Clinical Investigation 1993;90:2422-2433.
15. Tait, R.C., Walker, I.D., Perry, D.J., Islam, S.I.A.M., Daly, M.E., McCall, F., Conkie, J.A. & Carrell, R.W. Prevalence of antithrombin III deficiency in the healthy population. B J Haematol 1994;87: 106-112.
16. Fischer R, Sachs UJ, Heidinger KS, Eisenburger D, Kemkes-Matthes B. Prevalence of hereditary antithrombin mutations is higher than estimated in patients with thrombotic events. Blood Coagul Fibrinolysis 2013; 24:444-448.
17. De Stefano V, Leone G, Mastrangelo S, Tripodi A, Rodeghiero F, Castaman G, Barbui T, Finazzi G, Bizzi B, Mannucci PM.Clinical manifestations and management of inherited thrombophilia: retrospective analysis and follow-up after diagnosis of 238 patients with congenital deficiency of antithrombin III, protein C, protein S.Thromb Haemost 1994;72(3):352-8.
18. Sanson BJ, Simioni P, Tormene D, Moia M, Friederich PW, Huisman MV, Prandoni P, Bura A, Rejto L, Wells P, Mannucci PM, Girolami A, Büller HR, Prins MH.The incidence of venous thromboembolism in asymptomatic carriers of a deficiency of antithrombin, protein C, or protein S: a prospective cohort study. Blood 1999;94(11):3702-3706.
19. Vossen CY, Conard J, Fontcuberta J, Makris M, VAN DER Meer FJ, Pabinger I, Palareti G, Preston FE, Scharrer I, Souto JC, Svensson P, Walker ID, Rosendaal FR.Risk of a first venous thrombotic event in carriers of a familial thrombophilic defect. The European Prospective Cohort on Thrombophilia (EPCOT). J Thromb Haemost 2005;(3):459-64.
20. Tu CM, Hsueg CH, Chu KM, Cheng SM, Tsao TP. Simultaneous thromboses of double coronary arteries in a young male with antithrombin III deficiency. Am J Emerg Med 2009; 27:1169 e3–6.
21. Peovska I, Maksimovic J, Kalpak O, Pejkov H, Bosevski M. Recurrent myocardial infarction in a young football player with antithrombin III deficiency. Cardiol J 2008; 15: 463–466.
22. Roldán V, Ordoñez A, Marín F, Zorio E, Soria JM, Miñano A, España F, González-Conejero R, Pineda J, Estellés A, Fontcuberta J, Vicente V, Corral J. Antithrombin Cambridge II (A384S) supports a role for antithrombin deficiency in arterial thrombosis. Thromb Haemost 2009;101:483-486.
23. Puurunen M, Salo P, Engelbarth S, Javela K, Perola M. Type II Antithrombin deficiency caused by a founder mutation Pro73Leu in the Finnish population - clinical picture. J Thromb Haemost 2013;11:1844-1849.
24. Szilágyi S, Péter A, Magyar MT, Balogh S, Bereczky Z. Recurrent arterial thrombosis associated with the antithrombin basel variant and elevated lipoprotein(a) plasma level in an adolescent patient. J Pediatr Hematol Oncol 2012; 34:276-279.
25. Orlando C, Heylen O, Lissens W, Jochmans K. Antithrombin heparin binding site deficiency: A challenging diagnosis of a not so benign thrombophilia. Thromb Res. 2015;135:1179-1185.
26. Robertson I, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GDO, Walker ID, Greaves M, Brenkel I, Regan I, Greer IA. Thrombophilia in pregnancy: a systemic review. B J Haematol 2005;132:171-196.
27. Kovac M, Mitic G, Miljic P, Mikovic Z, Mandic V, Djordjevic V, Radojkovic D, Bereczky Z, Muszbek L. Poor pregnancy outcome in women with homozygous type-II HBS antithrombin deficiency. Thromb Res 2014;133(6):1158-1160.
28. Kovac M, Mitic G, Mikovic Z, Mandic V, Djordjevic V, Muszbek L, Bereczky Z. Pregnancy related stroke in the setting of homozygous type-II HBS antithrombin deficiency. Thromb Res 2016; 139:111-113.
29. Luxembourg B, Pavlova A, Geisen C, Spannagl M, Bergmann F, Krause M. et al. Impact of the type of *SERPINC1* mutation and subtype of antithrombin deficiency on the thrombotic phenotype in hereditary antithrombin deficiency. Thromb Haemost 2014;111:249-257.

30. Gindele R, Oláh Z, Ilonczai P, Speker M, Udvari Á, Selmeczi A, Pfliegler G, Marján E, Kovács B, Boda Z, Muszbek L, Bereczky Z.Founder effect is responsible for the p.Leu131Phe heparin-binding-site antithrombin mutation common in Hungary: phenotype analysis in a large cohort.J Thromb Haemost. 2016;14(4):704-15.
31. Caspers M, Pavlova A, Driesen J, Harbrecht U, Klamroth R, Kadar J, Fisher R, Kemkes-Matthes B, Oldenburg J. Deficiencies of antithrombin, protein C and protein S - practical experience in genetic analysis of a large patient cohort. Thromb Haemost 2012; 108: 247-257.
32. Corral J, Hernandez-Espinosa D, Soria JM, Gonzalez-Conejero R, Ordonez A, Gonzalez-Porras JR, et al. Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis. Blood 2007;109:4258–4263.
33. Perry DJ, Daly ME, Tait RC, Walker ID, Brown K, Beauchamp NJ, et al. Antithrombin Cambridge II (Ala384Ser): clinical, functional and haplotype analysis of 18 families. Thromb Haemost 1998; 79: 249–253.
34. Sanchez C, Alessi MC, Saut N, Aillaud MF, Morange PE. Relation between the antithrombin Cambridge II mutation, the risk of venous thrombosis, and the endogenous thrombin generation. J Thromb Haemost 2008;11:1975-1977.
35. Navarro-Fernández, M. E. de la Morena-Barrio, J. Padilla, A. Miñano, N. Bohdan, S. Águila, I. Martínez-Martínez, T. S. Sevivas, C. de Cos, N. Fernán dez-Mosteirín, P. Llamas, S. Asenjo, P. Medina, J. C. Souto, K. Overvad, S. R. Kristensen, J. Corral, V. Vicente. Antithrombin Dublin (p.Val30Glu): a relatively common variant with moderate thrombosis risk of causing transient antithrombin deficiency. Thromb Haemost 2016; 116:146-154.
36. Javela K, Engelbarth S, Hiltunen L, Mustonen P, Puurunen M.Great discrepancy in antithrombin activity measured using five commercially available functional assays. Thromb Res. 2013;132(1):132-137.
**37.** Kovacs B, Bereczky Z, Olah Z, Gindele R, KereneyiA, Selmeczi A. et al. The superiority of anti-Xa assay over anti-IIa assay in detecting heparin-binding site antithrombin deficiency. Am J Clin Pathol 2013;140:675-679.
38. Finazzi G, Caccia R, Barbui T. Different prevalence of thromboembolism in the
39. subtypes of congenital antithrombin III deficiency: review of 404 cases. Thromb
40. Haemost 1987; 58: 1094.
41. Baglin T, Gray E, Greaves M, Beverley J. Hunt BJ, Keeling D. et al Clinical guidelines for testing for heritable thrombophilia. B J Haematol 2010;149:209-220.
42. Kovács B, Bereczky Z, Selmeczi A, Gindele R, Oláh Z, Kerényi A, Boda Z, Muszbek L. Progressive chromogenic anti-factor Xa assay and its use in the classification of antithrombin deficiencies. Clin Chem Lab Med. 2013; 52(12):1797-806.
43. Zeng W, Tang L, Jian XR, Li YQ, Guo T, Wang QW. et al. Genetic analysis should be included in clinical practice when screening for antithrombin deficiency. Thromb Haemost 2015;113:262-271.
44. Di Minno MN, Dentali F, Lupoli R, Ageno W. Mild Antithrombin Deficiency and the Risk of Recurrent Venous Thromboembolism: A Prospective Cohort Study. Circulation. 2014;129(4):497-503.

**Adresa autora:**
Mirjana Kovač
Institut za transfuziju krvi Srbije, Beograd
Svetog Save 39.
11 000 Beograd
Tel: +381 11 3812 804
Fax: +381 11 2458 328
E-mail: mkovac008@gmail.com

# FUNCTIONAL ROLE OF WUSCHEL IN STEM APICAL MERISTEMS

**Molly Rubin[1], Bela Balint[2,3], Milena Todorovic[4], Ksenija Radotic[5], Mirjana Pavlovic[1]**

[1]Department of Computer and Electrical Engineering and Computer Science, FAU, FL, USA;
[2]Institute for Transfusiology and Hemobiology, MMA, Belgrade, Serbia;
[3]Serbian Academy of Sciences and Arts;
[4] Clinic for Hematology, Clinical Center of Serbia, Belgrade, Serbia;
[5]Department of Life SciencesInstitute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia.

## Abstract

*In this paper we were focused mainly on the role of WUSCHEL within Arabidopsis and how it functions within the meristem. We used the sources listed in the references page with the main goal to write a very concise paper precisely outlining the function, location, and purpose of the WUS gene and corresponding protein. We have concluded that WUSCHEL is of essential importance in maintaining stemness in plants, and more importantly, stimulating expression of CLV3 to maintain shoot apical meristem homeostasis.*

**Key words:** WUSCHEL, WUS gene, meristem, plant stem cells

## Introduction

Plants maintain stemness, the ability for cell differentiation from the stem cell state, at specialized growth regions known as stem apical meristems (SAM) (Fig.1.). Cell differentiation is highly regulated and the same number of cells, along with cell size are maintained at these points of growth. The undifferentiated SAM cells are regulated by the home box transcription factor WUSCHEL (WUS), allowing SAM cells to maintain their stem cell state. Cells containing the WUS gene are located within the central zone (CZ) [1,2,3,4,5,7] of the meristem. WUS is regulated in a feedback loop by CLAVATA (CLV) genes which prevent uncontrolled differentiation of cells. These discoveries regarding WUS has largely been done through research in Arabidopsis. We will discuss the current and past research methods and results that has been done to identify the function of the WUSCHEL transcription factor in SAM growth and development.

## WUSCHEL (WUS) location within the SAM

WUS expression begins at the 16 cell embryo stage, where the 4 inner apical cells show homeobox expression [1,3]. And even earlier WUS is expressed in the nucellus domain of *Arabidopsis* ovules [6]. When the plant embryo is at the heart stage, the WUS expressing cells are restricted a centrally located area and beneath epidermal layers, showing early indication of its role in stem cell differentiation [1,3]. Cells containing the WUS homeobox are located in the meristem which is divided in to various zones based on the potency of the cells. The SAM is grossly divided

into various zones, the CZ, where pluripotent stem cells remain in a relatively fixed and controlled number [4], the peripheral zone (PZ) which contains multipotent cells that contribute to lateral organ formation, and the rib zone (RZ) which contains multipotent cells that give rise stem support within the meristem [1,4,7]. There are approximately 500 cells total within the SAM of the mature *Arabidopsis*, and approximately 35 located within the central zone [4]. At Maturity WUS cells are located in the CZ within the SAM underneath three layers of cells [1,3]. Within the CZ the first 3 layers of cells, L1-L3, are stem cells that move in an anticlinal (perpendicular to the surface of the SAM) manner, forming the epidermal, mesophilic, and central tissues of the plant respectively [3,4,7].

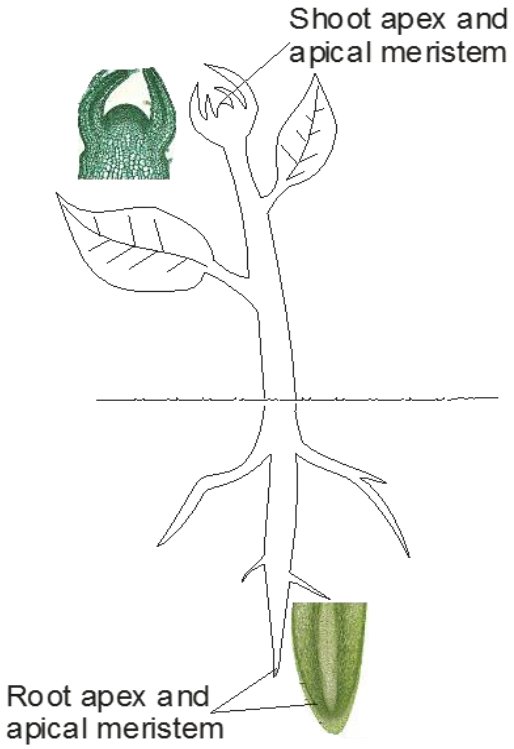



**Figure 1.** Schematic representation of the position of plant apical meristems

## WUSCHEL Function

WUS function was first hypothesized by the pattern showing WUS mutants lacked the ability to maintain normal meristems [1]. For example, in WUS mutants, down regulation or absent WUS genes, the SAM shows diminished growth and a flat apex [2].

This shows that the CZ stem cells are not proliferating, but rather are differentiating into the adult state, accounting for the small, flat meristem. In conjunction with this, WUS has been determined that it does not contribute to the organ content of cells, but rather is responsible for stem cell proliferation and maintenance of overlying pluripotent stem cells lo-

cated within the CZ [1,3,5]. Expressed WUS proteins are not found within the actual stem cells but beneath them [1] directly controlling the stem state of the overlying cells in the CZ. Since WUS is responsible for CZ cells with a stem cell identity, cases of over expression of WUS genes the shoot meristems show large, miss-hapen meristems (having a taller apex than in a wild-type SAM) showing over growth of stem cells and a lack of differentiation [2].

**Regulation of WUS by CLAVATA (CLV) genes**

As stem cells divide and move towards the periphery, leaving the range of WUS transcription, organ initiation begins to occur and the cells begin to lose their stem cell identity [2]. This occurs because, WUS works in conjunction with the CLAVATA (CLV) genes to mediate cell growth and differ-rentiation, more specifically it works in a negative feedback loop with CLV3 [2,3,4,5,7], by stimulating CLV3 expression. CLV3 is a short glycopeptide chain [4] whose expression maintains cell size and number by stimulating organ initiation within stem cells allowing for differentiation into the mature cell state [2,3]. An example being, in decreasing the expression the CLV3 in SAM, expansion of the CZ is seen as well as an increase in the rate of stem cell division, this occurs by the cells in the PZ de-differentiating and returning to their stem state due to increased expression of WUS [2]. Where in over expression of CLV3 would show a decrease in the size and number of stem cells in the CZ and an increase in differentiated cells in the PZ. This shows that CLV3 plays a role regulating in the cell growth seen within the SAM. The relationship between CLV3 and WUS, WUS maintain stem cell expression and activation CLV3, which is regulating and cell division, size, and organ initiation [2,3].

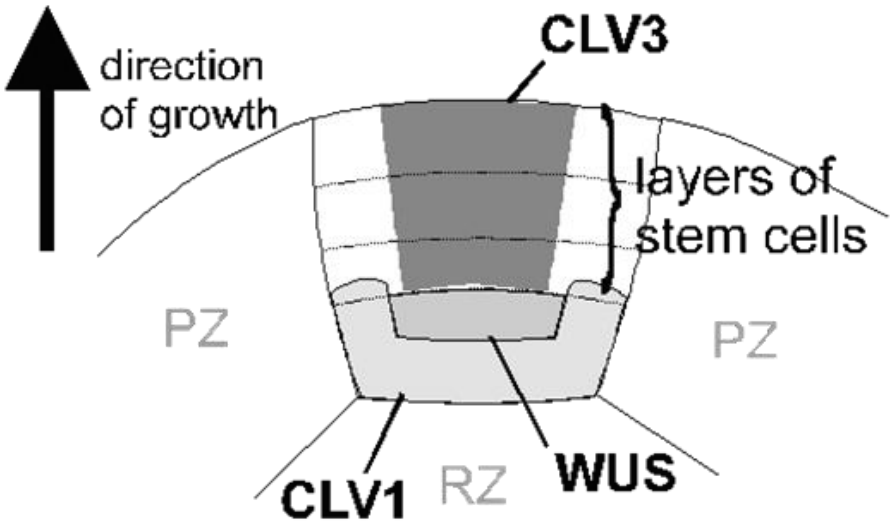



**Figure 2.** Scheme showing WUS-CLAVATA signaling cascade

The relationship between WUS and CLV3 in a wild-type plant works towards self-regulation and keeping the SAM in a state of homeostasis. Expressed protein movement with

in the cells regarding the WUS-CLV3 relationship is essential to this homeostasis. The protein movement allows not only for the communication between cells but also the triggering of CLV3 in response to WUS expression. The rates of expression within the various zones of the meristem vary. With higher levels of WUS and lower CLV3 expression within the CZ, maintaining stemness, to the opposite of higher levels of expression of CLV3 in the PZ and lower WUS expression creating a gradient. As cell movement occurs from the CZ to the PZ, through proliferation, the gradient of expressed proteins maintains the balance in the size and cell count of both stem and differentiated cells in the SAM [5], differentiated cells in the periphery and stem cells centrally. This is unique to plant meristem niches [5] and is vital in maintaining SAM homeostasis.

## Conclusions

WUSCHEL is of essential importance in maintaining stemness in plants and more importantly stimulating expression of CLV3 to maintain shoot apical meristem homeostasis. WUS is determined early with expression in the ovule state and early embryonic structures, allowing for regulation and proliferation of stem cells that allow for infinite growth within plants. By regulating SAM growth and proliferation along with CLV3, *Arabidopsis,* is able to maintain steady growth within its life cycle. Further research into WUS and CLV3 and their molecular interactions along with their homologs in other plant species will allow us to understand more completely the process of stem cell regulation and growth.

## References


1. K. Mayer, H. Schoof, A. Haecker, M. Lenhard, G. Jürgens and T. Laux, "Role of WUSCHEL in Regulating Stem Cell Fate in the Arabidopsis Shoot Meristem", Cell, vol. 95, no. 6, pp. 805-815, 1998.
2. R. Yadav, M. Tavakkoli and G. Reddy*, "WUSCHEL mediates stem cell homeostasis by regulating stem cell number and patterns of cell division and differentiation of stem cell progenitors", Development, vol. 137, pp. 3581-3589, 2010.
3. H. Schoof, M. Lenhard, A. Haecker, K. Mayer, G. Jürgens and T. Laux, "The Stem Cell Population of Arabidopsis Shoot Meristems Is Maintained by a Regulatory Loop between the CLAVATA and WUSCHEL Genes", Cell, vol. 100, no. 6, pp. 635-644, 2000.
4. S. Dodsworth, “A diverse and intricate signalling network regulates stem cell fate in the shoot apical meristem,” Developmental Biology, vol. 336, no. 1, pp. 1–9, 2009.
5. R. Yadav, M. Perales, J. Gruel, T. Girke, H. Jönsson and G.Venugopala Reddy “WUSCHEL
6. protein movement mediates stem cell homeostasis in the Arabidopsis shoot apex”, Genes and Development, 2011.
7. T. Yamada, Y. Sasaki, K. Hashimoto, K. Nakajima, C. S. Gasser, “CORONA, PHABULOSA and PHAVOLUTA collaborate with BELL1 to confine WUSCHEL expression to thenucellus in Arabidopsis ovule”, Development, vol.143, pp. 422-426, 2016.
8. M. Somssich, B. Je, R. Simon, D. Jackson, “CLAVATA-WUSCHEL signaling in the shoot meristem”, Development, vol. 143, pp. 3238-3248, 2016.



Corresponding author: Prof. Mirjana Pavlovic, MD, PhD
CEECS, EE-96, #515
Florida Atlantic University, Bioengineering, 33431 Boca Raton, FL, USA
Tel:561-297-234
e-mail: **mpavlovi@fau.edu**

# TERAPIJSKA IZMENA PLAZME U LEČENJU BOLESNIKA SA POREMEĆAJEM NEUROMIJELITIS OPTICA – PRIKAZ DVA SLUČAJA

**Gordna Ostojić[1,2], Dragana Obradović[2,3], Dušan Vučetić[1,2], Bela Balint[1,2,4,5]**

[1]Institut za transfuziologiju i hemobiologiju, Vojnomedicinska akademija, Beograd;
[2]Medicinski fakultet VMA, Univerzitet odbrane, Beograd;
[3]Klinika za neurologiju, Vojnomedicinska akademija, Beograd;
[4]Institut za medicinska istraživanja, Univerzitet u Beogradu;
[5]Srpska akademija nauka i umetnosti.

## Sažetak

*Poremećaj neuromijelitis optica (PNMO) je zajednički sindrom za različite vrste autoimunskih inflamacijskih demijalizirajućih bolesti prvenstveno okrenutih ka optičkom nervu ali i drugim delovima centralnog nervnog sistema.*
*Cilj prikaza je bila analiza postignutih terapijskih efekata TIP u lečenju PNMO, u odnosu na neželjene efekte ove procedure, uz osvrt na terapijsku primenu imunomodulacijskih i drugih medikamenata, što može bitno uticati na odluku o preduzimanju TIP kod neuroloških bolesnika i dovesti do zaključaka o opravdanosti njene primene, kao i mesta i uloge TIP u lečenju.*
*Izvršena je retrospektivna analiza uspešnosti primene TIP kod dva bolesnika (muškarac i žena) u dobi od 40 i 53 godine sa akutnim PNMO, lečenih od 2013. do 2017. godine, u Institutu za transfuziologiju i hemobiologiju VMA. TIP su izvedene pomoću separatora krvnih ćelija kontinuiranog toka, Cobe Spectra i Spectra Optia (Terumo BCT, USA), a prema važećim standardima i preporukama vezanim za proceduru. Analizirano je devet izvedenih procedura, procesirano je prosečno 5460 (4900-6000) kod bolesnika i 4558 mL (4300-5050) kod bolesnice, što odgovara 1,3 telesna krvna volumena oba bolesnika. Volumen izdvojene plazme iznosio je prosečno 2533 mL (2300-2800) kod bolesnika i 2092 mL (1900-2300) kod bolesnice, po jednoj proceduri izmene plazme. Titar antitela (AQP4-IgG) nakon TIP je smanjen za 75% početne vrednosti.*
*Zaključeno je da adekvatna i blagovremena primena dovoljnog broja TIP, kao i optimalnog izdvojenog volumena plazme dovela je do normalizacije kliničkog statusa bolesnika, uz primenu medikamenata, uz minimalne neželjene efekte.*

**Ključne reči:** Neuromijelitis optica, terapijska izmena plazme, anklidor aqaporin - 4 antitela.

## Uvod

Poremećaj neuromijelitis optica (PNMO), nazvan i kao Neuromyelitis Optica Spectrum Disorder ili Devicova bolest je zajednički naziv za sindrom različitih vrsta autoimunskih inflamacijskih demijalizirajućih patoloških stanja, prvenstveno sa ispadima optičkog nerva, ali i drugih delova centralnog nervnog sistema (CNS). Multipla skleroza (MS) je poremećaj CNS-a koji se odlikuje i inflamacijskim i neurodegenerativnim mehanizmi-

ma povreda mozga i kičmene moždine. Neuromijelitis optica (PNMO) je i inflama-cijska demijelinizirajuća bolest sa generalno lošom prognozom koja je selektivno usmerena prema optičkim nervima i kičmenoj moždini. Veza između MS i optičkog neuritisa je zapa-ljenje i gubitak mijelinskog pokrivača opti-čkog nerva i mrežnjače. Kod većine bolesnika sa MS bolest progredira u više epizodičnih relapsa i progresivno neurološko oštećenje tokom bolesti (1, 2). PNMO se smatra autoimunskom bolešću, što je nedavno pokazano prisustvom antitela (PNMO-IgG) usmerenih protiv akvaporina 4 - anklidor aqaporin 4 imunoglobulina G (AQP4-IgG) i antitela koja su odgovorna za lezije sa prisustvom perivaskularnih naslaga IgM i C9 (AQP4-IgM). Kod nekih pedijatrijskih bolesnika sa ponovljenim optičkim neuritisom opisano je prisustvo antitela protiv glikoproteina mijelin-oligodendrocita (anti-MOG antitela) u cirkulaciji (3).

Dijagnoza MS se može uspostaviti na osn-ovu kliničkih i radioloških kriterijuma kod bolesnika koji imaju dve ili više neurološke manifestacije u skladu sa multifokalnim zapaljenjem CNS-a koji se diseminišu u prostoru i u vremenu. Kod većine (85%) bolesnika sa MS klinički tok bolesti započinje i sa epizodama neurološke disfunkcije, nakon čega sledi potpun ili nekompletan oporavak (1, 2). Tokom vremena, većina bolesnika sa relapsirajućim oblikom - relapsing-reminding MS (RRMS) prelazi u sekundarnu progresivnu multiplu sklerozu (SPMS) tj. sa pogoršanjima neurološke nesposobnosti sa ili bez klinički otvorenih relapsa (1, 2). Približno 15% bolesnika sa MS doživljava primarni progresivni tok od početka, bez prethodnih recidiva, poznata kao primarna progresivna multipla skleroza (PPMS) ili sa nadograđenim neurološkim događajima, poznatim kao progresivna relapsirajuća MS (1, 2).

Terapija ovih poremećeja usmerena je u više pravaca. Većina studija prednost daje prevenciji napada, što podrazumeva primenu imunosupresora (azatioprin i mitoksantron) ili intravenskog γ-globulina, što smanjuje učestalost i ozbiljnost napada (1). Nasuprot tome, dostupno je manje podataka o kratkoročnom tretmanu optičkog neuritisa i mijelitisa. Visoko dozni intravenski kortikosteroidi su korišćeni ali sa manje efikasnosti. Smanjenje koncentracije antitela, primenom terapijske izmene plazme (TIP) pokazalo je efikasnost u nekoliko neuroloških bolesti koje dele slične patofiziološke mehanizme.

Pored uticaja na tok bolesti, upotreba imunomodulatornih terapija kod adolescenata sa RRMS može imati uticaj na kvalitet života. Italijanska studija je bila dizajnirana da proceni promene u kvalitetu života adolescenata sa RRMS-om koji primaju terapiju sa interferonom (IFN-β1a-Rebif) i pokazala je dugoročno poboljšanje kvaliteta života zbog subkutane primene interferona samoinjektorom (4). Intravenska primena visokih doza kortikosteroida pokazala je prednost u odnosu na oralnu primenu ali nije obezbedila dugoročnu korist. Naknadne studije su pokazale da je efekat primene oralnog prednizona uporediv sa primenom intravenskog metilprednizolona.

Terapijska izmena plazme se koristi za lečenje ozbiljnih epizoda demijelinacije CNS-a koje ne reaguju povoljno na primenjenu terapiju kortikosteroidima. TIP je aferezna procedura koja se koristi sa ciljem smanjenja

koncentracije ili otklanjanja specifičnog patogena (autoantitela, imunski kompleksi, citokini) iz plazme, uz nadoknadu otklonjenog volumena nekom pogodnom tečnošću. Tačna uloga TIP nije uvek sa sigurnošću definisana, a postignuti efekti su različiti i individualni, te su kriterijumi i sama primena TIP stalno predmet razmatranja kao terapijski modalitet (5–8). PNMO spada u drugu kategoriju indikacija za primenom TIP, što znači da služi ili kao glavna autonomna terapija ili u kombinaciji sa drugim vrstama lečenja (5).

TIP predstavlja skup afereznih postupaka vezanih za odstranjivanje dela bolesnikove krvi, njeno razdvajanje na komponente, otklanjanje određenog volumena plazme – koji je u fizikohemijskom pogledu patološki izmenjen i/ili u kome se nalazi specifični "patogeni supstrat" – i reinfuziju preostalog (modifikova-nog) dela krvi, uz upotrebu najpogodnije nadoknadne tečnosti (8). Najčešće se izvodi u što kraćem vremenu od postavljene dijagnoze, tri puta nedeljno, obično pet u tretmanu, a kod takozvanih održavajućih izmena plazme, jedan do dva puta mesečno, iako se njihova dinamika može menjati zavisno od dijagnoze i kliničkog stanja samog bolesnika. Volumen otklonjene plazme jednom procedurom TIP treba da iznosi jedan ili dva volumena bolesnikove plazme (45 – 50 mL/kg telesne mase bolesnika) tokom terapijske kure oko 3 – 5 volumena cirkulišuće plazme (5–8). Procena stvarnog terapijskog učinka TIP u bolesnika sa autoimunskim oboljenjima je često teška zbog istovremene primene medikamentozne imunosupresivne terapije (9). Povoljni efekat TIP se zasniva na adekvatnoj brzini primene, na volumenu otklonjene plazme tokom cele terapijske kure i istovremenoj medikamentoznoj (antiinflamacijskoj i imunosupresivnoj) podršci (10). Nadoknada otklonjene plazme se postiže adekvatnom količinom rastvora humanog albumina u kombinaciji sa kristaloidnim rastvorima (najčešće 5% i 20%), a ređe sa plazmom (5–10). Količina primenjenog albumina zavisi od volumena odklonjene plazme, od vrednosti albumna pre i posle tretmana i funkcionalne očuvanosti jetre bolesnika i uvek je individualna (10).

Tokom prethodnih decenija bilo je evi-dentirano više stotina bolesti i poremećaja u čijem su lečenju primenjivane TIP. Postignuti terapijski efekti nisu bili jednaki, što je dovelo do analize opravdanosti primene TIP. Cilj TIP je otklanjanje "patogenog supstrata" iz cirkulacije bolesnika, dok se imunosupresivnim sredstvima želi postići inhibicija njihovog stvaranja ili modulacija imunskog odgovora, što bi rezultovalo boljim terapijskim odgovorom (5).

Najbolji terapijski učinak se konstatuje kod bolesnika sa akutnim poliradikulo-neuritisom i mijastenijom gravis sa cirkuli-šućim antitelima protiv acetilholinskih rece-ptora (postoje u 85–90% bolesnika), ali i drugim neurološkim oboljenjima (MS), koja nemaju adekvatan terapijski odgovor na pri-menjenu terapiju. Povoljan terapijski efekat kod bolesnika sa ovom vrstom oboljenja se manifestuje povratkom oslabljenih refleksa, poboljšanjem oštrine vida, govora i gutanja, normalizovanjem motornih funkcija – uz pozi-tivan nalaz određenih laboratorijskih testova (5, 10). Procena terapijskog učinka TIP ima određena ograničenja zbog: nepotpunog poz-navanja etiopatogeneze većine imunoneuroloških poremećaja; nepostojanja odgovara-jućih kontrolnih grupa bolesnika; istovremene primene imunosupresivnih lekova i nedostatka laboratorijskih testova za kvantifikaciju "pato-genog supstrata" u bolesnikovoj krvi i u otklo-njenoj plazmi (5–10). O povoljnim efektima primene TIP ima brojnih saopštenja. Tako, Schilling i saradnici u jednoj randomiziranoj studiji (16 slučajeva) izveštavaju o neurolo-škom poboljšanju kod oko 70% lečenih bolesnika (11). U jednoj drugoj studiji (66 slučajeva) demijelinizirajućih bolesti, od kojih je 10 bolesnika imalo PNMO, postignuto je poboljšanje kod oko 60% njih nakon šestome-sečnog tretmana TIP (12).

Cilj ovog rada je kritička analiza posti-gnutih terapijskih efekta TIP u terapiji PNMO, uz primenu imunomodulacijskih medikamenata (kortikosteroida, imunoglobulina, interferona), radi procene opravdanosti ovog modaliteta lečenja - na osnovu postignutih povoljnih kliničkih efekata, naspram potencijalnih neželjenih efektata TIP (procedura sa vantelesnim krvotokom).

**Prikazi slučajeva**

Izvršena je analiza uspešnosti primene TIP kod dva bolesnika (muškarac i žena) u dobi od 40 i 53 godine sa akutnim PNMO, lečenih od 2013. do 2017. godine, u Institutu za transfuziologiju i hemobiologiju VMA. TIP su kod oba bolesnika izvedene pomoću separatora krv-nih ćelija kontinuiranog toka, Cobe Spectra i Spectra Optia (Terumo BCT, USA), a prema važećim standardima i preporukama vezanim za proceduru.

Bolesnik (40) je u početku bolesti retrobulbarnog neuritisa i bez demijenizacije CNS (2013. godine), pozitivno reagovao na pulsnu dozu imunosupresivne terapije (Azatioprin, Pronizon), sa oporavkom vida posle prvog ataka. Titar antitela (AQP4-IgG) na početku bolesti bio je 1:80, a nakon terapije 1:20. Drugi atak je išao sa teškom ambliopijom levog oka. Preduzeta medikamentozna terapija nije dala rezultate te se sprovode tri TIP. Postiže se dobar terapijski odgovor, nema novih lezija na CNS praćeno magnetnom rezonancom (MR). Prvi relaps (retobulbarni bol desno, slabost desne ruke sa diskretnom levostranom hemiparezom) usledio je 2017.god kada se ponovo sprovodi ciklus od tri TIP. Bolesnik vraća mororne funcije i dolazi do poboljšanja oštrine vida desnog oka. Trenutno je bolesnik pored stalne medi-kamentozne terapije i na održavajućim TIP jednom mesečno te se bolest uslovno rečeno drži pod kontrolom.

Sličan slučaj je i sa bolesnicom (53) koja se 2017. godine javlja zbog retrobulbarnog neuritisa i oslabljenja motorike ruku, nogu i glave, sa nemogućnošću održanja položaja glave, otežanim kretanjem, gutanjem i govorom. Bolesnica je u početku primala ade-kvatne doze kortikosteroida i Imurana, ali nije bilo dobrog terapijskog odgovora, te se pristupilo primeni izmena plazme. Titar anti-tela (AQP4-IgG) na početku 1:160, sprovodi se ciklus od šest TIP, na drugi dan do povlačenja simptoma i skoro potpunog oporavka bolesnice. Nalaz antitela nakon sprovedene terapije ispod 10. Dalja terapija se nastavlja održavajućim TIP na dve nedelje, a kasnije jednom mesečno.

Analizirano je devet izvedenih procedura, procesirano je prosečno 5460 (4900-6000) kod bolesnika i 4558 mL (4300-5050) kod bolesnice, što odgovara 1,3 telesna krvna volumena bolesnika. Volumen izdvojene plazme iznosio je prosečno 2533 mL (2300-2800) kod bolesnika i 2092 mL (1900-2300) kod bolesnice, po jednoj proceduri izmene plazme. Titar antitela (AQP4-IgG) je smanjen 75% početne vrednosti. TIP su rađene na drugi dan, a nadoknada je vršena adekvatnom količinom rastvora humanog albumina u kombinaciji sa kristaloidnim rastvorima. Kod bolesnice je zbog nezadovoljavajućeg venskog pristupa, nakon prve izmene, ugrađen centralno venski kateter. Osim blažih hipotenzivnih reakcija drugih značajnih neželjenih reakcija i komplikacija nije bilo. Bolesnici su dobro podneli terapijsku kuru, što se takođe manifestovalo smanjenjem titra antitela u cirkulaciji i njihovim značajnim kliničkim poboljšanjem, tačnije poboljšanjem oštrine vida i motornih funkcija (držanje glave, gutanja, hvatanje predmeta rukama, stabilizacija hoda).

Zaključeno je da ukoliko primena imunomodulacijskih i imunosupresorskih lekova ne daje adekvatan terapijski odgovor, kao što je bio slučaj sa našim bolesnicima sa PNMO, opravdano je primeniti TIP. Blagovremena primena i dovoljno intenzivne (broj, učestalost procedura i količina izdvojene plazme) TIP - uz upotrebu odgovarajućih medikamenata - neosporno je dovela do smanjenja titra antitela u cirkulaciji, a time i poboljšanja/normalizacije kliničkog statusa bolesnika. Međutim, za donošenje definitivnih zaključaka potrebne su veće, prospektivne i randominizirane studije sa kritičkim sagledavanjem povoljnih i sporednih efekata TIP u lečenju ove grupe bolesnika.

**Napomena:** Ovaj rad je deo projekata Ministarstva odbrane RS (MF/VMA–9/17–19) i

Ministarstva prosvete, nauke i tehnološkog razvoja RS (175061).

## Literatura


1. Fiona Costello, Optical Coherence Tomografy in Neuro-ophthalmology. Neurologic Clinics. 2017: 35(1); 153-63. DOI: http://dx.doi.org/10.1016/j.ncl.2016.08.012
2. Petzold A, Wattjes MP, Costello F, Flores-Rivera J, Fraser CL, Fujihara K et al. The investigation of acute optic neuritis: a review and proposed protocol. Nature Reviews Neurology 10: 2014; 447–58. doi:10.1038/nrneurol.2014.108
3. Jarius S, Franciotta D, Bergamaschi R, Wildemann B, Wandinger KP. Immunoglobulin M antibodies to aquaporin-4 in neuromyelitis optica and related disorders. Clin Chem Lab Med. 2010; 48 (5): 659-63. doi: 10.1515/CCLM.2010.127.
4. Ghezzi A, Bianchi A, Baroncini D, Bertolotto A, Malucchi S, Bresciamorra V t al. A multicenter, observational, prospective study of self- and parent-reported quality of life in adolescent multiple sclerosis patients self-administering interferon-β1a using RebiSmart™-the FUTURE study. Neurol Sci. 2017; 38(11): 1999-2005. doi: 10.1007/s10072-017-3091-6. Epub 2017 Aug 22.
5. Roback JD, Grossman BJ, Harris T, Hillyer CD. Technical Manual, 18th ed. AABB (American Association of Blood Banks); 2014.
6. Balint B, Radovic M, Malesevic M, Jovicic A, Stamatovic D, Ostojic G, et al. The rationale for therapeutic apheresis in the treatment of hematologic and immune–mediated neurologic disorders. Blood Banking Transf Med 2003; 1: 73–80.
7. van Der Meche FG, van Doorn PA. Guillain–Barre Syndrome. Curr Treat Options Neurol 2000; 2: 507–516.
8. Balint B, Jovičić A, Apostolski S, Magdić B, Taseski J. Plasma exchange in myasthenia gravis and multiple sclerosis. Vojnosanit Pregl 2000; 57 (5 Suppl): 11–5.
9. Principi i klinički aspekti afereznog lečenja.U: Balint B, Trkuljić M, Todorović M (eds). Osnovini principi hemoterapije. Beograd: Čigoja štampa; 2010. p. 209–56.
10. Ostojić G. Ljubenov M, Todorović- Balint M, Obradović D, Jovičić-Gojkov D, Balint B. Terapijska izmena plazme u lečenju imunoneuroloških bolesnika. Anest Reanim Transfuziol 2015; 42: 57-61.
11. Schilling S, Linker RA, König FB, Koziolek M, König FB, Müller GA et al. Plasmaexchenge therapz for steroid-unresponsive multiple sclerosis relapses: clinical experience with 16 patients. Journal Article, English Abstract 01 2006; 77(4):430-8. DOI: 10.1007/s00115-005-2019-1
12. Llufriu S ,J. Castillo, Y. Blanco, L. Ramió-Torrentà, J. Río, M. Vallès et all. Plasma exchange for acute attacks of CNS demyelination. Neurology; 2009, 73:12949-53.


Correspondence to: Ass. prof. Gordana Ostojic, MD, PhD
Institute of Transfusiology and Hemobiology, Military Medical Academy,
Crnotravska 17,
11 000 Belgrade, Serbia.
e-mail: **gocao@yahoo.com**

# ENDOTRAHEA INTUBACIJA KOD PACIJENATA SA POVREDOM MAKSILOFACIJALNE REGIJE

**Krasimirka Grujić, Snježana Zeba**

Klinika za anesteziologiju i intenzivnu terapiju, Vojnomedicinska akademija, Beograd

## Sažetak

*Zbog svoje lokalizacije povrede maksilofacijalne regije nose sa sobom visok stepen kako fizičke tako i psihičke treaume po pacijenta.Mogućnosti i način povreda mogu biti saobraćajne nesreće, sportske povrede, slučajni padovi, tuče,povrede na radu itd.*
*Povrede ove regije mogu se klasifikivati na povrede mekih tkiva, kosti i izolovane povrede zuba. Povrede mogu biti izolovane(60%) ili udružene sa kraniocerebralnim povredama(8%), povredama vratne kičme (10%), lokomotornog sistema (17%), te sa povredama abdomena i toraksa.*
*Kod pacijenata sa povredom mekih i koštanih struktura lica može nastupiti sekundarno, delimično ili potpuno zatvaranje gornjeg disajnog puta zbog : prisutnosti stranih tela u usnoj šupljini i orofarinksu (izbijeni zubi, zapadanje zubnih proteza, komadi otrgnutih mekih tkiva i kosti, povraćeni komadi hrane idr.), zapadanje jezika ili mekog nepca kod preloma mandibule ili maksile, obilnog krvarenja u usnoj šupljini naročito kod preloma nosnih kostiju i maksile, opsežnih edema poda usne šupljine , orofarinksa ili hipofarinksa, te povrede larinksa i traheje.*
*Najveći problem sa kojim se anesteziolog susreće jeste osiguravanje zadovoljavajuće oksigenacije i ventilacije tokom uvoda u anesteziju. Endotrahealna intubacija može biti oralna, nazalna ili kroz traheostomu.Ako hirurški zahvat ili postoperativni tok ne zahtevaju traheotomiju, kod otežane intubacije pacijenta je bolje intubirati uz pomoć fiberoptočkog bronhoskopa.Indikacije, kontraindikacije, oprema i priprema samog pacijenta biće diskutovani u ovom radu.*
*Uspešnost anestezije u zbrinjavanju povreda maksilofacijalne regije zavisit će u velikoj meri od znanja i spretnosti anesteziologa , kao i mogućnosti monitoriranja svih sistema organizma tokom reanimacije i hirurškog zahvata.*

**Ključne reči**: endotrahealna intubacija, politrauma, maksilofacijalna regija, monitoring

## Uvod

Trauma je nasilno oštećenje organizma izazvano dejstvom spoljašnjih faktora.
Trauma se definiše i kao,,telesno oštećenjena oorganskom nivou koje nastaje kao posledica akutnog izlaganja energiji ( mehaničkoj, hemijskoj, toplotnoj, električnoj ili zračenju )".

Zbog svoje lokalizacije povrede maksilofacijalne regije nose sa sobom visok stepenkako fizičke tako i psihičke traume po pacijenta. Mogućnosti i način povreda mogu biti saobraćajne nesreće, sportske povrede, slučajni padovi, tuče, povrede na radu itd.

Povrede ove regije mogu se klasifikovati na povrede mekih tkiva, kosti i izolovane povrede zuba. Povrede mogu biti izolovane (60%) ili udružene sa kraniocerebralnim povredama (8%), povredama vratne kičme (10%), lokomotornog sistema(17%), te sa povredama abdomena i toraksa. Kod pacijenata sa povredom mekih i koštanih struktura lica može nastupiti sekundarno , delimično ili potpuno zatvaranje gornjeg disajnog puta zbog: prisutnosti stranih tela u usnoj šupljini i orofarinksu ( izbijeni zubi, zapadanje zubnih proteza, komadi otrgnutih mekih tkiva i kosti, povraćeni komadi hrane itd.), zapadanje jezika ili mekog nepca kod preloma mandibule ili maksile, obilnog krvarenja u usnoj šupljini naročito kod preloma nosnih kostiju i maksile , opsežnih edema poda usne šupljine , orofarinksa ili hipofarinksa, te povrede larinksa i traheje. Zbinjavanje pacijenata sa povredom maksilofacijalne regije zahteva adekvatnu, sigurnu , brzu i strucnu intervenciju tima sa adekvatnom saradnjom svih aktera koji učestvuju u zbrinjavanju , primenom aktuelnih protokola i smernica , korišćenjem savremene opreme, povećava se stopa preživljavanja sa jedne strane a sa druge smanjuje procenat komplikacija i trajnog invaliditeta traumatizovanih pacijenata.

Specifičnosti anesteziološkog rada u maksilofacijalnoj hirurgiji baziran je na činjenici da vćina pacijenata zbog specifčne patologije ulazi u kategoriju onih kod kojih se očekuje otežana ili onemogućena ventilacija i teška intubacija. Protokoli za zbrinjavanje teškog disajnog puta su slični u većini država koje ih imaju , osvežavani i dopunjavani u različitim vremenskim intervalima.

Danas su nam na raspolaganju brojni uređaji i sredstva kojima se olakšava uspostavljanje problematičnog disajnog puta.Supraglotična sredstva (LMA) različitog dizajna ,laringealne tube, rigidni fiberoptički vodič, fleksibilni bronhoskop,GlideScope i setovi za perkutanu traheostomiju su neki od njih, koje imamo na raspolaganju u našoj ustanovi.

Svaki od ovih uređaja i pomoćnih sredstava su neizmerna pomoć u situaciji očekivano ili neočekivano problematičnog disajnog puta , ali samo u rukama osoba koje su prošle obuku i imaju dovoljno iskustva.Naravno, evaluacija pacijenata je najvažniji faktor koji će uticati na izbor strategije u proceni obezbeđivanja disajnog puta naročito tamo gde je neophodno omogućiti operatoru nesmetan rad.

Klinička stanja koja su najčešće povezana sa otežanom intubacijom su mikrognatija, fraktura mandibule, laringealne lezije koje parcijalno opstruiraju disajni put, nestabilni prelomi vratne kičme , potreba za intubacijom u budnom stanju, reumatoidni artritis, trismus i kraniofacijalne malformacije. Fiberoptička intubacija je kontraindikovana kod pacijenata kojima je neophodno uspostaviti disajni put hirurškim putem , kao i kod laringealne traume , posebno u slučajevima kada se sumnja na krikotrahealnu separaciju.

## Preoperativna priprema

U preoperativnoj pripremi ovih pacijenata mora se uzeti u obzir mogućnost udružene frakture kostiju lica sa potresom mozga i razvojem postkomocionog sindroma ( gubitak svesti,amnezija, mučnina , povraćanje...), kao i udružene povrede grudnog koša – okolnosti povređivanja tuča, saobraćajni traumatizam, te je neophodno isključiti frakture rebara , kontuziju pluća , pneumotoraks..Procena prohodnosti disajnog puta prvi je u nizu postupaka kod svih trauma – tizovanih pacijenata pa i ovih.Pacijent sa izolovanom traumom lica sam će zauzeti položaj koji mu olakšava disanje (sedeći plożaj sa pognutom glavom, bočni položaj.). Pacijent bez svesti ili pacijent sa pridruženom traumom (naročito vratne kičme) koja ne dopušta postavljanje pacijenta u nbočni položaj , strana tela iz disajnog puta treba ukloniti manuelno ili sukcijom, te pritiskom na temporomandibularni zglob pomaknuti bradu napred i gore bez pome – ranja glave i vrata.Krvarenje je redovno

prisutno kod pacijenata sa povredom kostiju lica ,naročito nosnih kostiju i maksile što može uzrokovati hipovolemiju.Kad su prisutni znaci hipovolemije treba isključiti postojanje krvarenja iz drugih organa ( jetra, slezina idr..)

Postavljanje pacijenta u Trendelenburgov položaj (za oko 20%) sprečit će slivanje krvi i sluzi u orofarinks i hipofarinks. Endotrahealna intubacija indikovana je kod vitalno ugroženog pacijenta sa oslabljenom ventilacijom i oksigenacijom (SaO2<90%) i jakim krvarenjem.

Nakon osiguravanja disajnog puta i zaustavljanja krvarenja potrebno je pacijenta detaljno pregledati i isključiti povrede ostalih organa (mozga, vratne kičme, oka, larinksaitd..). Sigurna anestezija se temelji na detaljnoj preoperativnoj obradi, tačnoj dijagnozi i odgovarajućem monitoringu tokom operacije.Tokom pripreme potrebno je monitorirati sve organe u organizmu (RR, EKG, TA,GCS,SaO2., diurezu), te uspostaviti bar dva široka venska puta( 14G ili bar 16G), za nadoknadu volumena.Postupak mora biti kod svakog povređenog kao u pacijenta sa ,,punim želucem".Premedikaciju je najbolje dati na operacionom stolu. Svrha premedikacije je da sedira pacijenta , smanji salivaciju a, a ne kompromituje spontano disanje.Odabir i doze lekova su individualne i prilagođavaju se stanju svakog pacijenta pojedinačno.Položaj pacijenta na operacionom stolu zavisi od stanja pacijenta i krvarenja.Pacijent sa obilnim krvarenjem u usnu šupljinu , koje otežava disanje , dobro je stavite u Trendelenburgov položaj.

Pre uvoda u anesteziju potrebna je dobra oksigenacija pacijenta u položaju koji mu najviše odgovara.

## Endotrahealna intubacija

Procena prohodnosti disajnog puta prvi je u nizu postupaka kod svih traumatizovanih pacijenata .Pri izvođenju endotrahealne intubacije kod pacijenata sa povredom maksilofacijalne regije potrebno je misliti na mogućnost anatomskih promena gornjih disajnih puteva uzrokovanih edemom ili hematomom.Naravno, potrebno je pripremiti ,,set za otežanu intubaciju koji pored bronhoskopa treba da sadrži i laringoskop, kao i različite vrste tubusa i laringealnih maski , Kilijanov spekulum,, Magilove hva-taljke" i dr potreban materijal.

Po pravilu , frakture sa dislokacijom većeg stepena zahtevaju uz repoziciju i osteosintezu koštanih fragmenata te se izvode u opštoj endotrahealnoj anesteziji, dok se frakture koje se tretiraju instrumentalnom repozicijom koristi tehnika analgosedacije.

Intubacija budnog pacijenta može se učiniti ,,na slepo" ili fiberoptičkim bronhoskopom. Obe metode smanjuju ali ne isključuju, verovatnoću aspiracije sadržaja usne šupljine.

Lekovi izbora moraju biti brzog delovanja i doziranje je individualno.Savremeni pristup zbrinjavanju povreda kostiju i mekih tkiva lica zahteva neposrednu ili ranu potpunu rekonstrukciju prelomljenih kostiju i struktura mekih tkiva.Endotrahealni tubus potre-bno je skloniti iz usne šupljine , pa u ovim stanjima prednost ima nazalna intubacija.Još uvek se provlači pitanje da je nazalna intubacija apsolutno kontraindi-kovana kod pacijenata sa prelomom LeFortII i LeFort III zbog mogućeg pridruženog preloma baze lobanje i rinoreje.

Strah od širenja infekcije u intrakranijalni prostor nije opravdana (prema mišljenju nekih autora)jer je tubus sterilan a pacijenti su pre i perioperativno zaštićeni antibiotici-ma.

Koma nije apsolutna kontraindikacija za anesteziju pacijenata sa povredom lica , ali je rizik anestezije povećan kod pacijenata starijih od 40 godina.Tokom operacije komatoznog pacijenta potrebno je monitorirati i intrakranijalni pritisak (ako je to moguće ).Traheotomiju treba učiniti ako se očekuje produžena intubacija, zbog panfacijalnog preloma, postoperativnog opsežnog edema mekih tkiva, baze jezika i orofarinksa, povrede larinksa, traheje, opekotine lica II i III stepena i udružene povrede lica i grudnog koša.

## Zaključak

Specifičnost anesteziološkog rada kod pacijenata sa povredom maksilofacijalne regije bazirana je na činjenici da većina povređenih zbog lakalizacije povreda ulazi u kategoriju onih kod kojih se očekuje otežana ili onemogućena ventilacija i teška intubacija.
Danas su nam na rapolaganju brojni urežaji i sredstva kojima se olakšava uspostavljanje problematičnog disajnog puta.Supraglotična sredstva različitog dizajna, laringealne tube, rigidni fiberoptički vodič, fleksibilni bronhoskop, GlideScope i setovi za perkuta-nu traheostomiju su neki od njih koje imamo na raspolaganju u našoj ustanovi.
Svaki od ovih urežaja i pomoćnih sredstava su neizmerna pomoć u situaciji očekivano ili neočekivano problematičnog disajnog puta, ali samo u rukama osoba koje su prošle obuku i imaju dovoljno iskustva.

## Literatura


1. Skerman JH.AnestheticManagment of Craniofacial Trauma. The Middle East J Emmerg Med;2002;2
2. Hollier L, Grantcharova EP, Kattash M.Fatial Gunshut Wounds:A 4-Year Expe-rience. J Oral Maxillofac Surg; 2001;59; 277-282
3. Tor-GoranH,Valdemar T Skoog. Identifi-cation of childrenat high anesthetic riskat time of primary palatoplasty. Scand J Plast Reconstr Hand Serg 2001;35: 177-182
4. Sossis MB (ed.) Anesthesia for otolaryngologic and head neck surgery.Vol.11,No. 3.of Anesth clin NAm.WB Saunders and Company, 1993.
5. Ovasapian A, Yelich SI, Dukes MF & Brunner EE. Blood pressure and heart rate changes during awake fiberoptic nasotracheal intubation. Anesthesia and Analgesia 1983;62: 951-954.
6. Carollo DS, Nossaman BD, Ramadhyani U. Dexmedetomidine: a review of clinical applications. Curr Opin Anesthesiol. 2008; 21(4): 457-461
7. Pinsky MR, Payen D:Functional hemodynamic monitoring. Crit Care 2005;9566-572

# POVREDE VRATA – ANESTEZIOLOŠKI PRISTUP

**Ivana Krstić Lečić**

Klinika za anesteziologiju i intenzivnu terapiju, Vojnomedicinska akademija, Beograd

**Sažetak**

*Značaj povreda vrata u mirnodopskom traumatizmu određuju učestalost, neposredna smrtnost i mogućnost da se danas pomogne većini preživelih na neposrednom mestu povrede u okviru „Advanced trauma life support" programa. Zbrinjavanje ovog tipa povreda zahteva dobro uvežbano osoblje i adekvatnu opremu. Specifičnost povreda vrata ogleda se u karakterističnoj anatomiji vrata i prisutnosti vitalnih anatomskih struktura (arterijski i venski krvni sudovi karotidnog i jugularnog sliva, karotodni glomus,cervikalna kičma, traheja, blizina kupole pleure i dr.) čije zbrinjavanje veoma često zahteva multidisciplinarno lečenje i timski rad.*

## Uvod

Povrede vrata spadaju u relativno retke (1,5% ukupnog traumatizma) ali su po svom karakteru izuzetno ozbiljne zbog mogućnosti ozleđivanja većeg broja organa, vaskularnih i nervnih struktura sadržanih u prostoru ispred vratnog dela kičme. Zbog toga njihovo zbrinjavanje podleže prioritetu povreda prvog reda hitnosti i dobro obučeno osoblje.
Prognostički su ove povrede izuzetno ozbiljne – veliki broj povreda vrata završava smrtno na mestu ranjavanja zbog iskrvarenja, ugušenja ili traumatskog šoka. Kod preživelih postoje česte komplikacije u vidu infekcije na mestu ozlede i šire (celulitis, medijastinitis) razvoj hematoma, razvoj posttraumatskih arterijskih i arteriovenskih malformacija, ostećenje nerava (pareza/paraliza rekurensa) i dr.
Povrede vrata se dele na povrede zatvorenog i otvorenog tipa.

## Zatvorene povrede vrata

Mehanizam nastanka: Ove povrede nastaju direktnim delovanjem tupe sile na predeo vrata pri čemu mogu uzrokovati akutnu tau mu neurovaskularnih struktura vrata praćenih direktnim nadražajem cerviko-vagalnih struktura (sinkopa/srčani zastoj), kao I kontuziju, luksaciju ili frakturu grkljana, traheje ili pod jezične kosti.
Dijagnoza zatvorenih povreda vrata na terenu je bazirana na osnovu postojanja kliničkih simptoma: bol u predelu grkljana, otežan /nemoguć govor, gutanje usled pritiska na larinks, postojanje potkožnog emfizema vrata, lica, gornjeg dela grudnog koša, iskasljavanje krvi, postojanje stridoroznog disanja, abnormalna pokretljivost grkljana, postojanje hematoma i krepitacija.
U bolničkim uslovima, ukoliko stanje unesrećenog to dozvoljava, može se uraditi dodatna dijagnostika – direktna laringoskopija, RTG i MSCT snimak vrata, kojima se dodatno sagledava obimnost povrede.
Osnovni princip zbrinjavanja baziran je na evaluaciji stepena težine povrede i mogućnosti naknadnog razvoja komplikacija koje mogu životno ugroziti unesrećenog (rastući hematom sa potencijalnom kompresijom disajnog puta i sl.), što kao imperativ nameće potrebu za dugoročnom medicinskom opservacijom povređenog. Pri postojanju evidentnih znakova respiratorne insuficijencije, neophodno je izvršiti obezbeđenje disajnog puta tehnikom endo trahealne intubacije ili hitne traheotomije.
Endotrahealna intubacija može biti otežana zbog povredom izmenjenih anatomskih stru-

ktura, prisutnog hematoma, udružene ozlede cervikalne kičme, pa se nameće tehnika „Crash“ intubacije kao metod izbora.

Tehnika „CRASH“ intubacije:

1. Sav pribor koji je neophodan za intubaciju i eventualnu aspiraciju mora se pripremiti unapred za brzu upotrebu (laringoskop sa dve špatule različitih veličina), armirani tubus, aspiracioni kateter. Ako su dostupna – spremiti sredstva za video asistiranu intubaciju (Video-scop, bronhoskop - fleksibilni, rigidni...).
2. Tubus treba pripremiti tako da se u njega unapred stavi mandren. Lakoj intubaciji mandren neće smetati, a teškoj će biti od neprocenjivog značaja.
3. Preoksigenirati pacijenta sa 100% kiseonikom tako da spontano diše bez upotrebe pritiska spolja i forsirane ventilacije.
4. Pacijent se uspavljuje brzo delujućim indukcionim sredstvom i brzo delujućim mišićnim relaksantom uz pravilo da se ovi pacijenti ne ventiliraju asistirano zbog potencijalnor razvoja emfizema, već se nakon 1-2 minuta po prolasku fascikulacija najpre na glavi, a potom i na dijafragmi i trbuhu pristupi brzoj intubaciji.
5. Kada fascikulacije prođu glavu jedan pomagač pritisne lako na krikoidnu hrsavicu i tako zatvori ezofagus da, eventualno regurgitovani sadrzaj ne krene na usta. To je tzv „Selikov manevar“.
6. Tubus se ubaci u traheju i kaf se naduva brzo, a pritisak na krikod prestane. Dubina tubusa se odmah proveri, a pacijent dodatno sedira i analgezira.
7. Kada je intubacija zavrsena, postavlja se želudačna sonda i prazni želudac koliko je to moguće.

Poželjno je da najiskusnji član ekipe obavi tehniku intubacije zbog svih otežavajucih okolnosti.

U slučaju nemogućeg obezbeđenja disajnog puta tehnikom endotrahealne intubacije, neophodno je obezbediti hirurško zbrinjavanje disajnog puta metodom urgentne traheotomije.

**Otvorene povrede vrata**

Izuzetno retko zahvataju samo „omotače“ vratnih organa (kožu, potkožno tkivo, fasciju mišića), češće i same vratne organe (dušnik, grkljan, jednjak, štitnu žlezdu, krvne sudove, nervne strukture, duktus toracikus, kupolu pleure..). Povrde pojedinih organa najčešće su kombinovane, veoma retko izolovane.

**Otvorene povrede grkljana i dušnika:** Izolovane povrede dušnika su retke. Najčešće su praćene povredama grudog koša, jednjaka, velikih krvnih sudova. Klinička slika je najčešće veoma dramatična i izrazena hemoragičnotraumatskim šokom, krvarenjem, smetnjama u disanju, gutanju. Pojava penušave krvi u spoljašnjoj rani, dokaz je perforacije traheje. Najčešći simptomi su svakako znaci respiratorne isuficijencije koji nastupaju bilo zbog pomeranja zdrobljenog tkiva traheje, bilo zbog kompresije na traheju usled emfizema ili hematoma, bilo zbog suženja lumena traheje usled perihondralnih i submukoznih krvarenja.

Anesteziološki pristup i ovde podrazumeva tehnike obezbeđenja disajnog puta endotrahealnom intubacijom koja je od samog starta u kategoriji otežane. Ako je defekt na prednjem zidu traheje moguće je direktno kroz njega plasirati trahealnu kanilu. U slučaju moguće i uspele endotrhealne intubacije pozeljno je privući trahealne patrljke hirurškim šavovima čime se održava prolaznost i anatomski polozaj. U svim ostalim slučajevima pristupa se hirurškoj tehnici niske traheotomije.

U toku zbrinjavanja neophodno je obezbediti venski put i što pre krenuti sa nadoknadom cirkulatornog volumena u cilju sprečavanja post traumatskog / hemoragičnog šoka, uključiti antibiotike širokog spekta dejstva, pacijenta sedirati i analgezirati i po potrebi ostaviti na kontrolisanoj, prolongiranoj me haničkoj ventilaciji. Neophodno je praćenje svih vitalnih parametara, uz svakodnevnu laboratorijsku i kliničku evaluaciju što podrazumeva neophodnost lečenja ovih pacijenata u jedinicama intenzivne nege.

U slučaju udruženih povreda traheje i jednjaka, neohodno je planirati dugotrajnu ishranu pacijenta tehnikom totalne parenteralne ishrane, eventualno gastrostomije.

**Komplikacije** povreda vrata mogu da budu rane komplikacije i kasne komplikacije.
Rane komplikacije su sekundarna infekcija (celulitis, medijastinitis), emfizem, opšte teško, septično stanje i drugo zavisno od stepena povrede.Kasne komplikacije: stenoze dušnika i jednjaka, stalna promuklost, nemogućnost fonacije, traheoezofagealne i ezofagokutane fistule, kao i arterijske i arterivenske aneurizme.

## Zaključak

Povrede vrata zahtevaju multidisciplinarni pristup lečenju i uključivanje timskog rada anesteziologa, maksilofacijalnog hirurga, torakalnog hirurga, neurohirurga, infektologa, a po svojoj složenosti i mogućim kom - plikacijama predstavljaju izuzetno kom plesne povrede koje kao imperative nameću potrebu observacije i lečenja traumatizovanog u jedinicama intenzivne nege nezavisno od toga da li se pristupa konzervativnom ili hirurškom načinu lečenja.

## Literatura:


1. Dr John Mills, Dr Mary T. Ho, Dr Donald D. Trunkey, Urgentna medicina, savremena dijagnostika i lečenje, Kalifornija 1983.
2. Janković, R., Inter i intra-hospitalni transport pacijenata, Katedra urgentne medicine, Medicinski fakultet Niš, 2011.
3. Ovassapian A, Yelich J, Dykes MHM, et al. Fiberoptic nasotracheal intubation - incidence and causes of failure. Anesth Analg 1983; 62:692-5.
4. Boysen PG. Fiberoptic instrumantation for airway management. Annual Refresher Course Lectures,1992.
5. Woodall NM, Harwood RJ, Barker GL. Complications of awake fiberoptic intubation without sedation in 200 healthy anaesthetists attending a training course. Br J Anaesth. 2008; 100: 850-5.
6. www.esicm.org European Society of Intensive Care Medicine
7. www.aagbi.org Association of Anaesthstists of Great Britain and Ireland


ANESTEZIJA • REANIMACIJA • TRANSFUZIJA
art

# USTVO AUTORIMA ZA PRIPREMANJE RADA

Časopis **"ANESTEZIJA, REANIMACIJA, TRANSFUZIJA"** je časopis Udruženja anestezi-sta, reanimatora i tranfuzista Srbije registrovan kao glasilo javnog informisanja 1982. godine, u kojem se objavljuju radovi članova Udruženja, i članova drugih društava medicinskih i srodnih struka. Časopis objavljuje: originnalne radove, sa-opštenja, prikaze bolesnika, preglede iz literature, radove iz istorije medicine, radove za praksu, vodiče kilničke prakse.
Prispeli rukopis Uređivački odbor šalje recenzentima radi stručne procene. Ukoliko recenzenti predlože izmene ili dopune, kopija recenzije se dostavlja autoru s molbom da unese tražene izmene u tekst rada ili da argumentovano obrazloži svoje neslaganje s primedbama recenzenta. Ko-načnu odluku o prihvatanju rada za štampu donosi glavni i odgovorni urednik.
Časopis se štampa na srpskom jeziku, sa kratkim sadržajem prevedenim na engleski jezik. Radovi stranih autora se štampaju na engleskom jeziku sa kratkim sadržajem na srpskom i engleskom jeziku.

## OPŠTA UPUTSTVA

Tekst rada kucati u programu za obradu teksta Word, latinicom, sa dvostrukim proredom, isključivo fontom Times New Roman i veli-činom slova 12 tačaka (12 pt). Sve margine podesiti na 25 mm, veličinu stranice na A4, a tekst kucati sa levim poravnanjem i uvlačenjem sva-kog pasusa za10 mm, bez deljenja reči (hifenacije). Izbegavati upotrebu tabulatora i uzastopnih prazni h karaktera (spejsova) radi poravnanja teksta, već za to koristiti alatke za kontrolu poravnanja na lenjiru i Toolbars. Posle svakog znaka interpunkcije staviti samo jedan prazan karakter. Ako se u tekstu koriste specijalni znaci (simboli), koristiti font Symbol.
Rukopis rada dostaviti e-mail-om, odštampan jednostrano na beloj hartiji formata A4 u tri primerka, zajedno sa disketom ili diskom (CD) na koje je snimljen identičan tekst koji je i na papiru. Stranice numerisati redom u okviru donje margine, počev od naslovne strane. Podaci o korišćenoj literaturi u tekstu označavaju se arapskim brojevima u uglastim zagradama – npr. [1, 2], i to onim redosledom kojim se pojavljuju u tekstu.
**Naslovna strana:** Na posebnoj, prvoj stranici rukopisa treba navesti sledeće:

- **naslov rada bez skraćenica;**
- **puna imena i prezimena autora i koautora (bez titula) sa tačnom godinom rođenja svakog od njih;**
- zvaničan naziv ustanova u kojima autori rade i mesto, i to redosledom koji odgovara indeksiranim brojevima autora;
- ukoliko je rad prethodno saopšten na nekom stručnom sastanku, navesti zvaničan naziv sastanka, mesto i vreme održavanja;
- na dnu stranice navesti ime i prezime, kontakt - adresu, broj telefona, faksa i e-mail adresu jednog od autora radi korespondencije.

**Autorstvo:** Sve osobe koje su navedene kao autori rada treba da se kvalifikuju za autorstvo. Svaki autor treba da je dovoljno učestvovao u radu na rukopisu kako bi mogao da preuzme odgovornost za celokupan tekst i rezultate iznesene u radu. Autorstvo se zasniva samo na:

- bitnom doprinosu koncepciji rada, dobijanju rezultata ili analizi i tumačenju rezultata, planiranju rukopisa ili njegovoj kritičkoj reviziji od znatnog intelektualnog značaja,
- završnom doterivanju verzije rukopisa koji se priprema za štampanje.

Autori treba da prilože opis doprinosa u ruko-pisu za svakog koautora pjedinačno. Fiansiranje, sakupljanje podataka ili generalno nadgledanje istraživačke grupe sami po sebi ne mogu opravdati autorstvo. Svi drugi koji su doprineli izradi rada, a koji nisu autori rukopisa,
trebalo bi da budu navedeni u zahvalnici s opisom njihovog rada, naravno, uz pisani pristanak.

**Kratak sadržaj – SAŽETAK:** Uz originalni rad, saopštenje, prikaz bolesnika, pregled iz literature, rad iz istorije medicine i rad za praksu, na posebnoj stranici treba priložiti kratak sadržaj rada obima 200-300 reči. Za originale radove kratak sadržaj treba da ima sledeću strukturu: uvod, cilj rada, metod rada, rezultati i zaključak. Svaki od navedenih segmenata pisati kao poseban pasus, koji počinje boldovanom reči "Uvod","Cilj rada","Metod rada","Rezultati"i "Zaključak".Navesti najvažnije rezultate(numeričke vrednosti) statističke analize i nivo značajnosti.

**Ključne reči:** Ispod kratkog sadržaja navesti ključne reči (od tri do šest). U izboru ključnih reči koristiti Medical Subject Headings – MeSH (http://gateway.nlm.nih.gov).

**Prevod na engleski jezik:** Na posebnoj stranici otkucati naslov rada na engleskom jeziku, puna imena i prezimena autora, nazive ustanova na engleskom jeziku i mesto.
Na sledećoj stranici priložiti kratak sadržaj na engleskom jeziku (Abstract) sa ključnim rečima (Key words), i to za radove u kojima je obavezan kratak sadržaj na srpskom jeziku, koji treba da ima 200-300

reči. Apstrakt na engleskom treba da ima istu strukturu kao i kratak sadržaj na srpskom.Prevesti nazive tabela, grafikona, slika, shema, celokupni srpski tekst u njima i legendu.

**Struktura rada:** Svi podnaslovi se pišu velikim slovima i boldovano.Originalni rad treba da ima sledeće podnaslove: uvod, cilj rada, metod rada, rezultati, diskusi-ja, zaključak, literatura.
Prikaz bolesnika čine: uvod, prikaz bolesnika, diskusija, zaključak, literatura. Ne treba koristiti imena bolesnika, inicijale ili brojeve istorija bolesti, naročito u ilustracijama.
Pregled iz literature čine: uvod, odgovarajući podnaslovi, zaključak, literatura. Pregledne rado-ve iz literature mogu objavljivati samo autori koji navedu najmanje pet autocitata (reference u kojima su ili autori ili koautori rada).

**Tekst rukopisa:** Koristiti kratke i jasne rečenice. Prevod pojmova iz strane literature treba da bude u duhu srpskog jezika. Sve strane reči ili sintagme za koje postoji odgovarajuće ime u našem jeziku zameniti tim nazivom.
Za nazive lekova koristiti isključivo generička imena. Uređaji (aparati) se označavaju trgovačkim nazivima, a ime i mesto proizvođača treba navesti u oblim zagradama. Ukoliko se u tekstu koriste oznake koje su spoj slova i brojeva, precizno napisati broj koji se javlja kao ekspo-nent ili kao indeks (npr. $^{99}Tc$, IL-6, $O_2$, $B_{12}$, CD8).

**Skraćenice:** Koristiti samo kada je neophodno, i to za veoma dugačke nazive hemijskih jedinjenja, odnosno nazive koji su kao skraćenice već prepoznatljivi (standardne skraćenice, kao npr. DNK). Za svaku skraćenicu pun termin treba navesti pri prvom navođenju u tekstu, sem ako nije standardna jedinica mere. Ne koristiti skraćenice u naslovu. Izbegavati korišćenje skraćenica u kratkom sadržaju, ali ako su neophodne, svaku skraćenicu ponovo objasniti pri prvom navođenju u tekstu.

**Decimalni brojevi:** U tekstu rada decimalne brojeve pisati sa zarezom, a u tabelama, na grafikonima i drugim prilozima, budući da se u njima navodi i prevod na engleskom jezi-ku, decimalne brojeve pisati sa tačkom (npr. u tekstu će biti 12,5±3,8, a u tabeli 12.5±3.8). Kad god je moguće broj zaokružiti na jednu deci-malu.

**Jedinice mere:** Dužinu, visinu, težinu i zapreminu (volumen) izražavati u metričkim jedinicama (metar–m, kilogram–kg, litar–l) ili njihovim delovima. Temperaturu izražavati u stepenima Celzijusa (°C), količinu supstance u molima (mol), a pritisak krvi u milimetrima živinog stuba (mm Hg). Sve rezultate hematoloških, kliničkih i biohemijskih merenja nvoditi u metričkom sistemu prema Internacioalnom sistemu jedinica (SI).

**Obim rukopisa:** Celokupni rukopis rada – koji čine naslovna strana, kratak sadržaj, tekst rada, spisak literature, svi prilozi, odnosno potpisi za njih *i legenda (tabele, fotografije, grafikoni, sheme, crteži), naslovna strana i kratak sadržaj na engleskom jeziku – mora iznositi za originalni rad, saopštenje, pregled iz literature i vodič kliničke prakse do 5.000 reči, za prikaz bolesnika do 2.000 reči, za rad iz istorije medicine do 3.000 reči, za rad za praksu do 1.500 reči; radovi za ostale rubrike moraju imati do 1.000 reči.*
Provera broja reči u dokumentu može se izvršiti u programu Word kroz podmeni Tools –Word Count ili File–Properties–Statistics.

**Tabele:** Tabele se označavaju arapskim brojevima po redosledu navođenja u tekstu, sa nazivom na srpskom i engleskom jeziku. Tabele raditi isključivo u programu Word, kroz meni Table– Insert–Table, uz definisanje tačnog broja kolona i redova koji će činiti mrežu tabele. Desnim klikom na mišu – pomoću opcija Merge Cells i Split Cells – spajati, odnosno deliti ćelije. U jednu tabelu, u okviru iste ćelije, uneti i tekst na srpskom i tekst na engleskom jeziku – nikako ne praviti dve tabele sa dva jezika!
Koristiti font Times New Roman, veličina slova 12 pt, sa jednostrukim proredom i bez uvlačenja teksta.
Korišćene skraćenice u tabeli treba objasniti u legendi ispod tabele na srpskom i engleskom jeziku.
Svaku tabelu odštampati na posebnom listu papira i dostaviti po jedan primerak uz svaku kopiju rada (ukupno tri primerka tabele za rad koji se predaje).

**Fotografije:** Fotografije se označavaju arapskim brojevima po redosledu navođenja u tekstu, sa nazivom na srpskom i engleskom jeziku. Za svaku fotografiju dostaviti tri primerka ili tri seta u odvojenim kovertama. Primaju se isključivo originalne fotografije (crno-bele ili u boji), na sjajnom (glatkom, a ne mat) papiru,po mogućstv u formata 9×13 cm ili 10×15 cm.
Na poleđini svake fotografije staviti nalepnicu. Na njoj napisati redni broj fotografije i strelicom označiti gornji deo slike. Voditi računa da se fotografije ne oštete na bilo koji način.
Fotografije snimljene digitalnim fotoaparatom dostaviti e-mail-om, na CD-u i odštampane na papiru, vodeći računa o kvalitetu (oštrini) i veličini digitalnog zapsa. Poželjno je da rezolucija bude najmanje 150 dpi, format fotografije 10×15 cm, a format zapisa*.JPG.
Ukoliko autori nisu u mogućnosti da dostave originalne fotografije, treba ih skenirati kao Grayscale sa rezolucijom 300 dpi, u originalnoj veličini i snimiti na CD-u.Fotografije se mogu objaviti u boji, ali dodatne troškove štampe snosi autor.

**Grafikoni:** Grafikoni treba da budu urađeni i dostavljeni u programu Excel, da bi se videle

prateće vrednosti raspoređene po ćelijama. Iste grafikone linkovati i u Word -ov dokument, gde se grafikoni označavaju arapskim brojevima po redosledu navođenja u tekstu, sa nazivom na srpskom i engleskom jeziku. Svi podaci na grafikonu kucaju se u fontu Times New Roman, na srpskom i engleskom jeziku.Korišćene skraćenice na grafikonu treba objasniti u legendi ispod grafikona na srpskom i engleskom jeziku.Svaki grafikon odštampati na posebnom listu papira i dostaviti po jedan primerak uz svaku kopiju rada (ukupno tri primerka za rad koji se prdaje).

**Sheme (crteži):** Sheme raditi u programu Corel Draw ili Adobe Ilustrator (programi za rad sa vektorima, krivama). Svi podaci na shemi kucaju se u fontu Times New Roman, na srpskom i engleskom jeziku, veličina slova 10 pt.
Korišćene skraćenice na shemi treba objasniti u legendi ispod sheme na srpskom i enleskom jeziku.
Svaku shemu odštampati na posebnom listu papira i dostaviti po jedan primerak uz svaku kopiju rada (ukupno tri primerka za rad koji se predaje).

**Zahvalnica:** Navesti sve one koji su doprineli stvaranju rada a ne ispunjavaju merila za autorstvo, kao što su osobe koje obezbeđuju tehničku pomoć, pomoć u pisanju rada ili rukovode odeljenjem koje obezbeđuje opštu po dršku. Finansijska i materijalna pomoć, u obliku sponzorstva, stipendija, poklona, opreme, lekova i drugo, treba takođe da bude navedena.

**Literatura:** Reference numerisati rednim arapskim brojevima prema redosledu navođenja u tekstu. Broj referenci ne bi trebalo da bude veći od 30, osim u pregledu iz literature, u kojem je dozvoljeno da ih bude do 50, dok za vodiče kliničke prakse broj referenci nije ograničen. Broj citiranih originalnih radova mora biti najmanje 80% od ukupnog broja referenci, odnosno broj citiranih knjiga, poglavlja u knjigama i preglednih članaka manji od 20%.
Izbegavati korišćenje apstrakta kao reference, a apstrakte starije od dve godine ne citirati. Reference članaka koji su prihvaćeni za štampu treba označiti kao "u štampi" (in press) i priložiti dokaz o prihvatanju rada.
Reference se citiraju prema tzv. vankuverskim pravilima (Vankuverski stil), koja su zasnovana na formatima koja koriste National Library of Medicine i Index Medicus. Naslove časopisa skraćivati takođe prema načinu koji koristi Index Medicus (ne stavljati tačke posle skraćenica!). Za radove koji imaju do šest autora navesti sve autore. Za radove koji imaju više od šest autora navesti prva tri i et al. Stranice se citiraju tako što se navede početna stranica, a krajnja bez cifre ili cifara koje se ponavljaju (npr. od 152. do 157. stranice navodi se: 152-7).

**Prilikom navođenja literature treba se pridržavati pomenutog standarda, jer je to vrlo bitan faktor za indeksiranje prilikom klasifikacije naučnih** časopisa. Pravilnim navođenjem literature Srpski arhiv bi dobio na kvalitetu i bolje bi se kotirao na listi svetskih naučnih časopisa.

**Primeri:**

**1. Članak u časopisu:**
Maisch B, Seferovic PM, Ristic AD, et al. Guide lines on the diagnosis and management of pericardial diseases. Executivesummary.Eur Heart J 2004; 25(7): 587 -610.

**2. Poglavlje u knjizi:**
Maisch B, Ristic AD, Funck R, et al. Dilated cardiomyopathies and congestive heart failure. In: Singal PK, Dixon IMC, Kirshenbaum LA, Dhalla NS, editors. Cardiac Remodeling and Fai lure. Bos- ton-Dordrecht-London:Kluwer Academic Publis he- rs; 2003. p.35-65.

**3. Knjiga:**
Seferovic PM, Spodick DH, Maisch B, editors, Maksimovic R, Ristic AD, assoc. editors. Perica r- diology: Contemporary Answers to Continuing Challenges. Beograd: Nauka; 2000.

**Propratno pismo:**

**Uz rukopis obavezno priložiti pismo koje su potpisali svi autori, a koje treba da sadrži:**

- **puna imena i prezimena autora (bez titula) sa tačnom godinom rođenja sva kog od njih;**
- **izjavu da rad prethodno nije publikovan i da nije istovremeno podnet za objavljivanje u nekom drugom časopisu i**
- **izjavu da su rukopis pročitali i odobrili svi autori koji ispunjavaju merila au torstva.**

**Propratno pismo mora biti potpisano, skenirano i u PDF ili JPG formatu poslato elektronski uz rad.**
**Takođe je potrebno dostaviti kopije svih dozvola za:reprodukovanje prethodno objavljen-og materijala, upotrebu ilustracija i ojavljivanje informacija o poznatim ličnostima ili imeno-vanje osoba koje su doprinele izradi rada.**

**Slanje rukopisa:** Rukopis rada i svi prilozi uz rad dostavljaju se u elektronskoj formi putem e-mail-a glavnom uredniku Časopisa ili u pisanoj formi, zajedno sa CD - om na kome je snimljen identičan rad koji je u papirnoj formi. Rad se šalje preporučenom pošiljkom na adresu:

**Udrženje anestezista, reanimatora i transfuzista Srbije,**
**ul.Svetog Save 39, 11000 Beograd,**
**sa naznakom za časopis ANESTEZIJA, REANIMACIJA, TRANSFUZIJA**

**E-mail: udruzenjeart@gmail.com**

Telefon glavnog urednika: **064 2400-652**

***Napomena:***

**Rad koji ne ispunjava uslove ovog uputstva ne može biti upućen na recenziju i biće vraćen autorima da ga dopune i isprave. Pridržavanjem uputstva za pisanje rada znatno će se skratiti vreme celokupnog procesa do objavljivanja rada u časopisu, što će pozitivno uticati na kvalitet i redovnost izlaženja časopisa.**