REVIEW

of the dissertation work of full-time doctoral student Vancho Donev on the topic "Characterization of potential

immunoprophylactic and immunotherapeutic

properties of human blood plasma"

for acquiring the educational and scientific degree "Doctor"

Doctoral Program "IMMUNOLOGY"

Field of higher education: 4. Natural sciences, mathematics and

informatics

Professional field: 4.3 Biological Sciences

Scientific specialty: Immunology

Scientific supervisor: Assoc. Prof. Dr. Georgi Nikolov, MD

Biographical data about the candidate: Vancho Donev was born on 17.06.1994 in the city of Kavadarci, Republic of North Macedonia. After completing his high school education in his hometown in 2013, he studied molecular biology at the Faculty of Biology of Sofia University "Kliment Ohridski" and successively obtained a bachelor's (2017) and master's degree (2019). He also has a master's degree in "Business Administration and Business Communications" (2019) from the University of National and World Economy. This dissertation was developed and proposed for defense at the National Center for Biomedical Research "BulBIO".

The dissertation is written on 130 pages and contains the following sections:introduction and literature review – 39 pp., aim and objectives - 2 pp., materials and methods – 16 pp., results – 25 pp., discussion – 11 pp., conclusions and contributions – 6 pp., literature – 29 pp. with 317 titles in English, scientific articles and participation in scientific forums in connection with the defense – 2 pp. A list of abbreviations used is also presented at the beginning. The text includes 7 tables and 23 figures.

Relevance of the topic. Attempts at blood transfusion were made as early as the second half of the 19th century, but the discovery of blood groups in the early 20th century by Karl Landsteiner and the subsequent need for large quantities of blood during the two world wars led to the widespread use of blood transfusion as a therapeutic procedure. Gradually, procedures were

developed for the separation and storage of not only whole blood, but also blood products: plasma, serum, albumin, blood clotting factors, fibrinogen, hyperimmune serums, immunoglobulins, etc. The pandemic caused by SARS-CoV-2 and the subsequent COVID-19 crisis showed the possibilities of treating severely ill COVID-19 patients, refractory to therapy with other medications, with plasma from patients who had already recovered. The range of therapeutic applications of human blood plasma and its products, incl. immunoglobulin fractions, is very broad and includes: replacement therapy for primary and secondary immune deficiency, autoimmune diseases, transplantation, viral diseases, etc. It is no coincidence that WHO recognizes the importance of treatment with plasma proteins by adding immunoglobulins and coagulation factors to its list of essential medicines. These data show the relevance of studying the composition of blood and blood plasma in the 21st century.

Literature review. All these problems are competently considered by the author of the dissertation work in chronological order. Attention is paid to the known data on the composition of blood plasma, immunoglobulins with an emphasis on their intravenous administration, methods for their preparation and mechanism of action. The Bulgarian experience in the person of the National Center for the Study of Immunoglobulin, in the preparation of immunoglobulin preparations, dating back to the seventies of the last century, is emphasized. The data on the application of intramuscular and intravenous immunoglobulin preparations are presented chronologically. The presentation of the possibilities of subcutaneous administration of immunoglobulins and other plasma fractions would enrich the literature review. At the end of the review, the author also defines the unclear questions regarding the relationship between the composition of plasma and immunoglobulin preparations and the possibilities for their application, which stand as the goal and tasks for resolving his thesis.

The goal and objectivesof the dissertation are clearly and well formulated: "Study of the main immune components of human blood plasma used for the production of the final IVIG product Immunovenin Intact 5% and characterization of the pathogen-specific antibodies contained therein, with a view to possible immunoprophylactic and immunotherapeutic application."

Materials and methods: The study included:

1. Plasma: 90 COVID-19 convalescent donor plasma collected between January 1 and May 5, 2021; 32 from clinically healthy individuals, 10 plasma pools collected between January and December 2001.

2. IVIG (Immunovenin-intact 5%): – 90 pcs. regular production batches from 2019-2024 and batches of plasma collected during the COVID-19 pandemic – 13 pcs.

To determine the different antibodies were used:

A. Immunoenzymatic methods based on ELISA technology:

- for the determination of RBD-IgG and -IgA antibodies against SARS-CoV-2

-for the determination of specific IgG against Candida albicans

- home made immunoenzymatic method for determining anti-Hla antibodies in two versions

- method for determining antinuclear antibodies (ANA)

B. Immunoblot method for determining ANA Profile

C. Luminex technology

-quantitative method for determining a large number of cytokines in pg/ml

- kit for determining neutralizing antibodies against different variants of SARS-CoV-2. The % of antibodies to the original wild-type virus and five variants - B.1.1.529 (o), B.1.617.2 (δ), P.1 (γ), B.1.351 (β) and B.1.1.7 (α) is determined

- quantitative method for determining immunoglobulin classes and IgG isotype – in g/L

The author has mainly used ready-made test kits. The exception is the method for determiningstaphylococcal antibodies, which was developed in accordance with the needs of the dissertation work. In essence, all methods are based on the principle of using enzyme- or fluorochrome-labeled antibodies. In all developments, mainly IgG class antibodies are determined, with the exception of antibodies against SARS-CoV-2, where IgA antibodies are also determined, and cytokines, where the molecules themselves are determined. One part of the results obtained give an idea of the concentration of the substances under study, another part - are semi-quantitative in nature. I would like to clarify the concept of the substrate in the methods used - in fact, it is

correct to use the expression "chromogen/substrate complex", where the true substrate of the peroxidase enzyme is hydrogen peroxide, and TMB is the substance (chromogen), which is an indicator of the degree of positive reaction by changing its color, which is read spectrophotometrically. I find the detailed description of the preparation of Immunovenin intact 5% unnecessary in the "Results" section, since this is already a well-known commercial product produced at BulBio-NCZPB EAD.

Results

A. Serum immunoglobulins in IVIG and in convalescent plasma: The characterization of 11 batches of IVIG produced in one year showed that the variation in various parameters between batches was less than 2%, and the distribution of IgG1,2,3,4, IgA, IgM in 90 batches reproduced the typical values for human plasma. The average concentration of IgG2 in convalescent plasma was the highest - 7.01 g/L, followed by IgG1 (5.15 g/L), IgG3 (1.12 g/L) and IgG4 (0.21 g/L). The average concentration of IgA was 0.5 g/L, of IgM - 3.34 g/L and of Ig E - 0.0006 g/L.

B. Studies on antibodies and cytokines in convalescent plasma and IVIG:SARS-CoV-2 RBD-specific IgG was detected in 63% (57/90) of the tested plasmas of COVID-19 survivors, with a mean concentration of 3.11 ± 2.9. Anti SARS-CoV-2 IgA was detected in 59% (53/90) of the samples with a mean value of 3.50 ± 2.27. In 27.7% of the survivors, neither IgG nor IgA antibodies were detected. Batches of IVIG produced in the pre-pandemic period did not contain IgG and IgA antibodies against SARS-CoV-2, but during the pandemic there was a significant increase in specific IgG and less in IgA antibodies. The mean level of IgG antibodies in Immunovenin intact was higher than that in the plasmas of survivors.

The average neutralizing activity of antibodies against SARS-CoV-2 in the plasma of COVID-19 recoveries ranged from 54.2% for the Delta variant to 85.4% for Omicron. Neutralizing antibodies were found for all subtypes of SARS-CoV-2 variants in 39/57 (68.42%) of the convalescent plasmas, and almost all (94.7%) could neutralize at least one variant. No correlation was found between the level of RBD-IgG or RBD-IgA and the total neutralizing activity of RBD-positive samples.

The neutralizing activity of anti-SARS-CoV-2 antibodies against different variants of the virus (wild type, alpha, beta, gamma, delta, o-micron) were studied in 13 IVIG samples and it was

found that it was highest for the alpha variant and lowest for the gamma variant, but in all samples there was activity against some of the virus variants. No correlation was found between the titer of specific antibodies and their neutralizing effect.

The concentration of 90 convalescent plasmas was studied.25 cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-18, TNF-α; IL-9, IL-10, IL-17A (CTLA-8), IL-21, IL-22, IL-23, IL-27, IFN-α, IL-1-α, IL-1RA, IL-7, IL -15, IL-31 and TNF-β). Significantly increased concentrations of IL-18 were observed in all analyzed samples with an average level of 54.05 pg/ml; IL-27 was elevated in 92.2% (83/90) of the samples (average 18.23 pg/ml), and IL-1RA – in 76/90 (84.4%) (average 222.96 pg/ml). The remaining molecules were elevated in different concentrations and in different percentages of the samples. IL-22 and IL-10 in RBD-double positive for IgG and IgA patients were many times higher than those in double-negative patients.

The comparative analysis betweenPooled plasmas for the production of Immunovenin – intact 5%, collected during the Covid-19 pandemic and hyperimmune plasmas from individual donors who recovered from Covid-19 showed increased expression of IL-1RA in three and IL-9 in one hyperimmune donor plasma, while no elevated levels of cytokines were detected in the pooled plasmas.

C.Antibodies against Candida albicans: In all 90 batches of IVIG 5%, antibodies against Candida albicans were detected in a concentration higher than randomly selected sera.

D. Antibodies against Staphylococcus aureus in IVIG 5%: The author has developed 2 immunoenzymatic tests for the detection of IgG antibodies against alpha-hemolysin. The optimal conditions for their performance have been established. It has been found that in 89/90 IVIG batches the concentration is higher than the concentration of IgG alpha-hemolysin antibodies in the serum of healthy donors.

E. Detection and characterization of ANA in Immunovenin-intact 5% and convalescent plasma: All COVID-19 convalescent plasmas were negative for ANA, while all IVIG batches were positive for these antibodies. 5 of these batches (with the highest ANA levels) were characterized and 2 of them were found to have particularly high levels of anti-Ro-52 and anti-AMA-M2 antibodies.

Discussion: According to the data in this dissertation, the concentration and distribution of immunoglobulin classes and subclasses in Bulgarian IVIG have the same distribution as the level in healthy adults.

One of the main focuses of Vancho Dobrev's research is the study of the composition of convalescent plasma after recovering fromCOVID 19. According to him, the highest average concentration is IgG2, followed by IgG1, IgG3 and IgG4, which he explains by very high concentrations in 4 of the tested samples. RBD-specific IgG and IgA are simultaneously elevated in 50% of the plasmas, in 27.7% these antibodies are simultaneously negative, in 13% only IgG antibodies are positive, and in 8.8% - only IgA. These data are similar to those obtained by other authors. Important for science and practice is the characterization of the formed neutralizing antibodies against SARS-CoV-2 and their concentration in convalescent plasma. This issue has been discussed throughout the COVID 19 pandemic, and even after, and here the author intervenes competently by proving that 39/57 (68.42%) of the convalescent plasmas contain neutralizing antibodies for all subtypes of SARS-CoV-2 variants and almost all (94.7%) can neutralize more than one variant. All COVID-19 convalescent plasmas were negative for ANA. Observations of this type are important in view of the still ongoing need to treat certain cases of COVID-19 with convalescent plasma.

Of course, an important conclusion for practice is thatThe neutralizing activity of IgG antibodies in different batches of the Bulgarian IVIG Immunovenin-intact 5% against several variants of SARS-CoV-2 ranges from 75.5% to 91.5%, and the comparison with convalescent plasma shows no significant difference in neutralizing activity. This observation is in line with the data of other authors.

Based on the data on the importance of the so-called "cytokine storm" as one of the factors for the deterioration of the condition of patients withCOVID 19, the author investigated the content of 25 cytokines in convalescent plasma and found a significant increase in some of them, such as IL 18, IL-27, IL 1RA. IL 22 and IL 10 were increased mainly in the convalescent plasma of patients with double positive IgG and IgA RBD antibodies. The literature data on the cytokine composition of convalescent donor plasma are contradictory and therefore the observation of Vancho Donchev contributes to this issue.

Contribution to the study of the composition of the Bulgarian preparationImmunovenin Intact 5% is the demonstration of a high level of specific anti-Candida albicans IgG in all 90 batches tested, as well as specific IgG to Hla in 89/90 batches. An immunoenzymatic method has been developed and validated for the study of specific IgG to Hla. These data present Immunovenin Intact 5% as a product with pronounced antibacterial and antifungal properties that can be used in the treatment of diseases caused by Candida albicans and Staphylococus aureus. The immunotherapeutic effect of Bulgarian IVIG in chronic inflammatory diseases has been well studied, and the data in the present work represent a new field for research both in terms of antibodies against Candida albicans and those against Staph. aureus.

The presence of antibodies against nuclear antigens in immunoglobulin preparations for intravenous administration is debatable.. The author of the present work intervenes in this discussion as according to his data these antibodies are present in 90 of the 5% Immunovenin intact series he studied. The attempt to assess the type of these antibodies in 5 series shows that anti-Ro-52 and anti-AMA-M2 antibodies are particularly common. The question of the significance of the presence of ANA in immunoglobulin preparations has not yet found its solution. The author of the dissertation correctly connects this finding with the fact that ANA are formed in the course of some autoimmune diseases, but very often after viral and bacterial infections and persist for a long time after that, although the healthy blood donor has no symptoms of the disease. Another known fact is that ANA in low concentrations are also found in 5% of healthy people, as well as in patients who have suffered from tuberculosis or oncological disease. At the same time, IVIG is an alternative to immunosuppressive therapy in systemic lupus and other autoimmune diseases. These facts are discussed very carefully by the author of the dissertation.

Conclusions and contributions:I agree with the conclusions drawn and the contributions indicated, but I believe that the formulation of conclusion N 4 (cytokine levels in the studied convalescent plasma and IVIG) and N 9 (ANA levels in IVIG) should be approached with some caution, given the powerful effect of these biologically active molecules.

Literature: Well-selected authors, including from a historical perspective.

The autobiographyreflects the author's main achievements on the researched issue.

Conclusion: The topic of the dissertation is interesting, the author has conducted a number of studies on Bulgarian IVIG, which broaden our view of important and somewhat little-known qualities of this product. In addition, a large-scale study is being conducted on the composition of convalescent plasma after recovering from COVID 19 and immunoglobulin preparations, during SARS-CoV-2 infection and afterwards. The author's participation in publications in IF journals makes a very good impression. The remarks I made are benevolent and propaedeutic in nature. They do not affect my positive opinion of this dissertation.

I believe that the presented materials fully comply with the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for its implementation, as well as with the Regulations of the National Center for the Development of the Academic Staff. I confidently vote in favor of awarding the educational and scientific degree "DOCTOR" to VANCHO DONEV in the field of higher education: 4. Natural Sciences, Mathematics and Informatics; Professional field: 4.3 Biological Sciences and Scientific specialty: Immunology.

12.8.2025

Reviewer: 

Prof. D Marta Petrova Baleva-Nikolova, MD, Dsc