REVIEW
from Prof. Dr. Krasimira Ilieva Ikonomova, PhD
Head of Clinical Laboratory and Immunology
National Transport Hospital – Sofia
on
Dissertation work for obtaining the educational and scientific degree "Doctor"
on
Dr. Gergana Mladenova Chausheva

LABORATORY ASSESSMENT OF CARDIOVASCULAR RISK IN PERSONS WITH LONG-TERM DIABETES TYPE I- ADIPOKINES, OSTEOPROTOGERIN, ASYMMETRIC DIMETHYL-ARGININE

Supervisor: Assoc. prof. Yana Dimitrova Bocheva, PhD

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Diabetes mellitus is a socially significant disease, mainly manifesting in two forms - type 1 diabetes (T1D) and type 2 diabetes (T2D). The frequency of T1D is approximately 10%, and T2D - 90% of patients. Assessment of SSR in patients with T1D is most often based on risk assessment inherent to type 2 diabetes. Due to the significant differences in the pathophysiological mechanisms of development of T1D and T2D, this approach is not accepted by most researchers.

CVD has been found to be more frequent and occur earlier in T1D patients compared to the general population. The incidence of CVD in T1D varies significantly depending on the duration of diabetes, age and gender. It is believed that in T1D the atherosclerotic process begins its development at a young age and in most cases is asymptomatic. The most commonly used models for T1D risk calculation are the Steno Type 1 Risk Score (ST1RE) and the 2019 ESC. These include clinical and laboratory markers and classify and predict 10-year CVD risk. To optimize the laboratory assessment of CVR in T1D, the introduction of new laboratory biomarkers is necessary. Asymmetric dimethylarginine (ADMA), osteoprotegerin (OPG), adiponectin (ADNC) and leptin (Lep) are biomarkers of high informative value for clinical practice regarding the stratification of CVR. Their follow-up will contribute to a better quality of life and survival of patients with T1D. A multidisciplinary team of laboratory specialists and

clinicians was formed to validate the laboratory indicators, which is a prerequisite for obtaining reliable results.

In the scientific literature, no comprehensive studies were found related to the analysis and capabilities of the listed biomarkers in relation to specific scales for assessing CVR in T1D. This makes the topic of the dissertation current, modern and significant.

The dissertation is written on 175 pages, including literature review - 40 pages, aim and tasks - 2 pages, material and method - 6 pages, results - 67 pages, discussion - 28 pages, conclusions - 2 pages, contributions - 2 pages. The dissertation is illustrated with 56 tables and 52 figures. The bibliography contains 307 literary sources, of which 14 are in Cyrillic and 293 are in Latin.
при Т1Д.

The literature review consists of 5 sections and a conclusion. First, epidemiological data on type 1 diabetes are presented. The disease occurs more often among Europeans and less often among Asians. The incidence of T1D is increasing by 3% every year. One third of the risk of total mortality in T1D is associated with the development of CVD.

The most common models for calculating CVR in T1D are Steno Type 1 Risk Score (ST1RE) and ESC (European Society of Cardiology) guidelines from 2019. ST1RE includes 10 risk factors (age, sex, duration of diabetes, HbA1C, systolic blood pressure, LDL-cholesterol, glomerular filtration rate, albuminuria, smoking and physical activity). The 2019 ESC CVR Calculator includes 5 risk factors - age, sex, smoking, systolic blood pressure and total cholesterol.

A thorough review of the candidate biomarkers - asymmetric dimethyl-arginine (ADMA), osteoprotegerin (OPG), leptin (Lep) and adiponectin (ADNC) for the assessment of CVR in T1D was performed. It has been noted that research interest in biomarkers has increased manifold in recent years. In 1990, 21 clinical trials of cardiovascular biomarkers were registered, while in 2010 the number increased to 2032.

It is pointed out that the scientific database provides information primarily on research into the etiology, development and treatment of CVD in T1D. Currently, diabetes mellitus is defined as the equivalent of CVD, and therefore most of the biomarkers characteristic of its accompanying macro- and microvascular complications are potential markers for the assessment of CVD. While cardio-vascular complications in T2D are in most cases already present at the time of diagnosis, in T1D they appear on average 5-10 years after its diagnosis. Therefore, the selection of appropriate biomarkers for the assessment of CVD can significantly improve the screening, diagnosis, and prognosis of CVD in T1D.

The aim of the present study is to analyze the prognostic value of: ADMA, OPG, ADNC and Lep against specific tools for the assessment of SSR - ST1RE and ESC 2019 and against hematological indicators in persons with long-standing T1D.

Six tasks are logically displayed. These included assessment of the prognostic value of ADMA, OPG, ADNC and Lep against T1D-specific tools to assess SSR. Of particular importance is the analysis of dependencies between serum levels of ADMA, OPG ADNC, Lep and hematological indicators in T1D.

Material and methods. The study was conducted at the UMBAL "St. Marina" - Varna for the period 2018-2020. The dissertation was prepared as part of the scientific project "Cardiovascular and metabolic risk associated with visceral adipose tissue in patients with type 1 diabetes mellitus", supported by the "Scientific Research" Fund to the Ministry of Education and Science.The project is interdisciplinary with the participation of endocrinologists, internists, pediatricians, specialists in laboratory medicine, imaging diagnostics, social medicine.

The subjects of the study were 59 healthy volunteers and 124 patients with T1D. Patients and healthy controls were selected according to established inclusion and exclusion criteria. All participants completed an informed consent protocol. Anthropometric, clinical, laboratory and imaging studies were performed on the patients. Each participant completed a questionnaire including demographic data, diabetes control, insulin regimen, and socio- economic status.

Laboratory methods include the monitoring of hematomorphological indicators, studied on a 5-diff hematology analyzer Sysmex XN 1000. Biochemical indicators CRP, HbA1C, AlbU were studied on the latest generation of biochemical analyzers (ADVIA chemistry 1800, Olimpus AU600). ADMA, OPG, ADNC and Lep were determined by the ELISA method with kits from approved European manufacturers.

The STENO Type 1 Risk Engine (ST1RE) CVD estimator calculates 10-year risk of non-fatal and fatal CVD (CHD, stroke, peripheral vascular disease). The following categories of risk are perceived: low (<10%); moderately elevated (10-20%) and high SSR (>20%).

The 2019 ESC calculator classifies patients with T1D as moderate risk (between 1% and 5%), high risk (between 5% and 10%), very high risk (above 10%).

Calculator for estimated CVD Risk Factor 3 is a combination of risk factors (RF) established in clinical practice for the development of CVD in DM: HbA1C, CRP and AlbU. The patients were defined in the following groups: group 0 – without the presence of RF. group 1 - with the presence of 1 RF; group 2 - with the presence of 2 RF and group 3 - with the presence of 3 RF.

The statistical package SPSS 19 was used for statistical processing of the data, and the following methods were applied - descriptive statistics, correlation analysis. linear regression analysis, factor analysis, non-parametric statistical methods for nominal data, binary logistic regression, ROC analysis, tabular and graphical method of data presentation. The statistical methods are perfectly suitable for the fulfillment of the set tasks.

Results. According to the ST1RE calculator, 38.7% of the studied patients fell into the low CVR category; 28.2% - with moderate risk and 33.1% with high CVR. According to the 2019 ESC criteria, 30.6% of patients had high CVR and 69.4% very high CVR. Comparing the clinical and laboratory indicators of the healthy control group and patients with long-standing T1D, it was found that T1D patients had significantly higher values for HbA1C%, AlbU and ADNC.

The following algorithm was used to establish the prognostic value of the SSR evaluation indicators (ADMA, OPG, ADNC, Lep). First, the influence of gender, age and duration of diabetes on the relevant parameter is evaluated. This is followed by an assessment of the relationship of the indicator with the ST1RE, ESC calculators from 2019 and with Risk Factor 3. Finally, ROC analyzes are performed to derive threshold values of the indicator in T1D. **ADMA** In patients with T1D, a significant positive correlation was found between AlbU value and serum ADMA levels. In combination with HbA1C determination, AlbU predicted 15% of ADMA concentration in subjects with T1D. ADMA as a stand-alone biomarker does not have a

sufficiently good diagnostic performance in differentiating patients with very high CVR according to the criteria of established models such as ST1RE and ESC from 2019. **OPG** has a good predictive value (approximately 70%) for men and women compared to ST1RE in differentiating individuals with high CVR. In the 2019 ESC, OPG has a good prognostic value in differentiating women with very high SSR - approximately 65%. OPG threshold values >5.075 pmol/l for men and >5.355 pmol/l for women vs. ST1RE and >5.025 pmol/l for women vs. ESC from 2019 suggest very high CVR and warrant urgent preventive measures. Patient age, duration of diabetes, and presence of micro/macroalbuminuria were significant positive determinants of serum OPG levels in individuals with long-standing T1D.
ADNC as a standalone biomarker does not have a sufficiently good diagnostic performance in differentiating patients with very high CVR according to the criteria of T1D-specific CVR evaluation tools - ST1RE and ESC from 2019. In women with T1D, a negative correlation between ADNC and CRP was established. and in healthy women, 58.6% of the variance in measured AlbU concentrations was associated with variance in ADNC scores.
Lep In men from both studied groups, there was a tendency to increase the concentration of Lep with advancing age. In men with T1D, the value of AlbU - 11.9% - had an independent positive influence on the serum levels of Lep. In women, significant positive determinants of serum Lep levels were AlbU and CRP. These variables predicted 23.8% of the changes in serum Lep concentration in women with long-standing T1D and 29.4% in healthy women. Lep has very good diagnostic performance in differentiating men with very high SSR to ST1RE (approx. 80%) and ESC as of 2019 (approx. 70%). The derived threshold values were >2.28 ng/ml and >1.38 ng/ml, respectively. Each increase in Lep concentration by 1 ng/ml leads to an increase in the chance in men to fall into the category of very high SSR by 1.7 times according to ST1RE and by 1.404 times according to 2019 ESC. In women, Lep has sufficient diagnostic performance compared to the 2019 ESC (approximately 60%) at a threshold value >5.475 ng/ml.
In individuals with long-standing T1D, a positive association was observed between serum levels of OPG, ADNC, Lep and a tendency for **microcytic, hypochromic anemia**. In men with long-standing T1D and esmated very high CVR, according to validated ST1RE and ESC 2019 models, the risk of developing anemia is greater than in women with T1D.
With regard **to leukocyte variables**, adipokines have opposite effects: ADNC is a negative regulator and Lep a positive one.
A significant finding in the present study was **platelet hyperreactivity** in subjects with long-standing T1D, which corresponded with serum levels of Lep and ADMA.

The obtained results cover the set goal and tasks of the work and show the ability of the dissertation student to build a scientific hypothesis and critical analysis of the obtained data.

The discussion shows the author's ability to objectively compare and contrast her data with world results. The pathogenetic mechanisms by which each of the investigated indicators increase the cardiovascular risk have been examined very precisely. The statistical methods used to confirm or reject certain hypotheses have also been carefully selected. In this way, recommendations are derived for the inclusion of a certain biomarker for the follow-up of patients with T1D.

The involvement of OPG in the development of endothelial dysfunction and the formation of atherosclerotic plaque has been highlighted. The role of visceral adipose tissue as a source of adipokines and inflammatory cytokines is reviewed. The role of exogenously introduced insulin,

statins, antihypertensive medications and their relation to the studied parameters is emphasized. The role of autoimmune processes in the stomach for the development of anemia in patients with T1D has been assessed. The tendency for leukocytosis in individuals with long-standing T1D is associated with the chronic low-grade inflammatory process that contributes to atherosclerotic progression and CVD. Higher platelet counts and platelet indices in individuals with T1D are due to platelet dysfunction and platelet hyperactivity. The higher the MPV, the more likely the formation of thrombus and damage to the vascular endothelium. The hyperreactive phenotype of platelets may account for the inadequate response to antiplatelet agents in patients with T1D. The ability to categorize SCC based on serum levels of certain biomarkers could provide enormous benefit in risk stratification, centralizing resources, and eliminating the need for additional testing in a large segment of patients.

The conclusions of the dissertation are 12 in number. They are clearly and precisely defined. They derive from the results and correspond to the set goals and objectives.

Nine contributions are highlighted - 4 original and 5 confirmatory.
-For the first time in Bulgaria, the prognostic value of ADMA, OPG, ADNC and Lep was evaluated against specific tools for the assessment of SSR: ST1RE and ESC from 2019 in persons with long-term T1D.
-For the first time in Bulgaria, the prognostic value of ADMA, OPG, ADNC, Lep and the indicators from the blood count was evaluated against a constructed model - a combination of established in clinical practice RF for the development of CVD in DM (RiskFactor3: HbA1C>7%, CRP > 3 mg/l and AlbU >30 mg/l).
-For the first time in Bulgaria, the influence of haematomorphological indicators on the serum levels of ADMA, OPG, ADNC and Lep and their relationship with ST1RE and ESC from 2019 was assessed in persons with long-term T1D.
-For the first time in Bulgaria, hematological changes were analyzed in persons with long-standing T1D and unsatisfactory control.
-The importance of OPG as a prognostic factor affecting the risk of CVD in individuals with long-standing T1D has been confirmed.
-The importance of Lep as a prognostic factor influencing the risk of CVD in individuals with long-standing T1D has been confirmed.
-The significance of AlbU as an independent variable on serum levels of ADMA, OPG, ADNC and Lep was confirmed. -The importance of BMI and CRP on serum levels of adipokines has been confirmed.
-The regulatory role of adipokines on hematopoiesis in individuals with long-term T1D and healthy controls has been confirmed.
- The need to derive sex-dependent reference values for platelet indices (MPV, PLC-R, PDW, PCT) and their implementation in routine clinical-laboratory practice has been confirmed.
I share the contributions of the work, which has both a scientific-theoretical and a scientific-applied nature. They are of valuable scientific and practical value.

There are 2 publications related to the dissertation work in refereed medical journals. Four reports from scientific forums are also presented.

The abstract meets the requirements. It reflects in a synthesized form the most essential moments of the dissertation work in all its sections.

In conclusion based on the material presented to me, I believe that the dissertation is completed planned tasks accurately and systematically. I believe that the volume, content and relevance of the dissertation meet the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and I recommend the respected Scientific Jury to award Dr. Gergana Mladenova Chausheva the educational and scientific degree "Doctor" in professional direction 7.1 Medicine, scientific specialty 03.01.12 "Clinical laboratory".

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Prof. Krasimira Ikonomova, MD, PhD