VILNIUS UNIVERSITY

VALDAS BANYS

EVALUATION OF NEW PLATELETS AND INFLAMMATORY BIOMARKERS' VALUE IN THE PATHOGENESIS OF ATHEROSCLEROSIS AND METABOLIC SYNDROME

Summary of Doctoral Dissertation

Biomedical Sciences, Medicine (06 B)

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VILNIAUS UNIVERSITETAS

VALDAS BANYS

ATEROSKLEROZĖS IR METABOLINIO SINDROMO PATOGENEZĖS YPATUMAI VERTINANT NAUJUS TROMBOCITŲ IR UŽDEGIMO ŽYMENIS

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List of Abbreviations

AI – atherogenic index

AUC – area under the ROC curve

CI – confidence interval

CRP – C reactive protein (hs-CRP – high sensitivity CRP)

CVD – cardiovascular diseases

CyPA – cyclophilin A

EU – European Union

GBD – *The Global Burden of Diseases, Injuries, and Risk Factors* study

HDL-C – high density lipoprotein cholesterol

LDL-C – low density lipoprotein cholesterol

LitHir – *The Lithuanian High Cardiovascular Risk* primary prevention programme

MPV – mean platelet volume

MS – metabolic syndrome

Pct – plateletcrit

PDW – platelet distribution width

P-LCR – platelet large cell ratio

PLT – platelet count

ROC – *Receiver Operating Characteristic* curve

s/co – signal to cut-off ratio

TC – total cholesterol

TG – triglycerides

TnI – troponin I

TRAP – thrombin receptors activating peptide

vWF – von Willebrand factor

1. Introduction

Cardiovascular diseases (CVD) in Europe, as well as in Lithuania, remain the main cause of deaths. According to the GBD study (*The Global Burden of Diseases, Injuries, and Risk Factors Study*) 12.9 million people die in relation to CVD (approx. 25% of total deaths) [1], in 53 WHO European region countries – 4.35 million every year, in EU – 1.9 million [2]. Baltic states have very high rates of mortality due to CVD. In Lithuania mortality of men due to CVD is the highest in Europe [3]. According to Institute of Hygiene under the Ministry of Health of the Republic of Lithuania in 2013 47.6% of men and 65.0% of women deaths were due to CVD [4].

The development of CVD is associated with fundamental mechanisms of the pathogenesis of atherosclerosis, which is a chronic disease of arterial wall resulting in formation of atherosclerotic plaque. With the progression of the disease intima thickens, lipid streaks can be seen, and foam cells accumulate lipids. Filled with connective tissue fibroatheroma starts to ulcerate, it lacerates and manifests as atherothrombosis, one of major complications of atherosclerosis [5]. Processes are determined by endothelial lesion, chronic inflammation propagation, tethering of platelets and leucocytes on the endothelial wall, migration of macrophages to subendothelial layers, accumulation of modified (oxidized) lipids, and proliferation of vascular smooth muscle cells [5, 6]. Indirect risk factors of CVD are smoking [2, 6], arterial hypertension, hypercholesterolaemia, increased concentrations of triglycerides (TG), decreased concentration of high density lipoprotein cholesterol (HDL-C), obesity, diabetes and metabolic syndrome (MS) [2, 6, 7], inappropriate nutrition, decreased physical activity [2, 6], alcohol abuse and psychological as well as social stress [2]. One individual might be affected by a group of risk factors: the more risk factors the higher risk of CVD [7].

New biomedical studies show, that role of platelets is not restricted to thrombus formation in the place of lesion. By secreting active substances, mediators of inflammation, they function as inflammatory cells, thus in given sense are related to inflammatory mechanisms in the pathogenesis of atherosclerosis. It is evident by the following facts: increased reactivity of platelets, i.e. higher aggregability, variations of platelet volume indices (irrespectively of altered parameters' combinations: increased mean platelet volume, increased platelet large cell ratio, etc.) [8–12], intense expression of P-selectin [13] and other related molecules, active interaction of platelets and monocytes or other populations of leucocytes [14, 15].

The search of early diagnostic markers of atherosclerosis outcomes determine the introduction of new biomarkers to routine clinical practice. One of such biomarkers of CVD might be cyclophilin A (CyPA). It is an intracellular protein secreted by monocytes/macrophages, endothelial cells, vascular smooth muscle cells and platelets inducted by reactive oxygen species. The relation of CyPA to CVD has been described in several studies. It has been noted, that increased concentrations of the protein are judged as risk factor of acute coronary syndromes [16–19].

2. Aim of the study

The aim of the study was to evaluate the value of platelets as inflammatory cells in early stages of cardiovascular diseases and metabolic syndrome in the context of traditional and by applying the new biomarkers of atherosclerosis.

3. Tasks of the study

1. To evaluate the applicability of cyclophilin A in the diagnosis and prognosis of CVD and MS, to determine the value of the protein in estimating the condition of patients with diagnosed CVD.
2. To assess the tendency of platelets' increased reactivity and links of this tendency with inflammatory biomarkers in men with dyslipidaemia and other traditional CVD and MS risk factors, to determine the value of platelet hyperreactivity in estimating the risk of CVD and MS.
3. To define the value of lipid ratios, as well as lipid-C reactive protein (lipid-CRP) ratios, in estimating risk of CVD and MS.
4. To determine the value of inflammatory biomarkers (CRP, fibrinogen and von Willebrand factor (vWF)) in estimating the risk of CVD and MS in men with dyslipidaemia and other traditional CVD and MS risk factors.

4. Significance and novelty of research work

It is a great challenge for clinicians, scientists, and clinical chemists to develop the screening method or model, which could provide an opportunity to determine individuals with high risk of CVD precisely, accurately, but cost-effectively long before the onset of disease symptoms. Presumably, preventive measures would be most effective in such cases, because it takes atherosclerosis decades to manifest [20]. It is universally accepted to evaluate the risk of CVD by traditional laboratory risk factors such as total cholesterol (TC), HDL-C, low density lipoprotein cholesterol (LDL-C), TG and systolic blood preasure values [21]. New biomarkers are valuable alternatives. For example, the use of high sensitivity CRP methodology. It is known, that higher CRP values account for 60% higher risk of CVD [22].

Despite the fact, that any matter reflecting the biological process (from genetic markers to radiological visualization techniques) can be defined as a biomarker, "soluble" markers are most attractive, because testing material is relatively easy to collect, and results are usually reproducible [20]. For the first time in Lithuania we have performed the new CVD biomarker CyPA testing. We have determined, that in patients with acute CVD concentration of CyPA increases significantly, but in individuals with dyslipidaemia and/or other risk factors, on the contrary, concentration decreases. Such results are in contrast to other studies on CyPA [16, 23–25]. Furthermore, we have cleared out that CyPA in the context of CVD plays a role in inflammatory processes, but not in platelet function. Our results show, that despite the promising data in scientific literature, CyPA application is still very limited.

Furthermore, evaluation of individual biomarkers is not always enough in clinical practice. In our study an integrated complex view was applied in risk assessment of CVD and MS [26]. The selection of laboratory risk factors was unique, including traditional lipid metabolism biomarkers, also new previously not studied in Lithuania CVD and MS biomarkers/methods: fibrinogen, vWF, platelet aggregation and platelet flow cytometry. We have found an indirect relationship between platelet hyperreactivity and inflammatory processes. We have applied the new calculation method of lipid-CRP ratios and evaluated their use in estimating risk of CVD and MS. We have defined the additional value of these ratios in prognosis of CVD and the presence of three biochemical markers of MS.

5. Materials and methods

42 relatively healthy individuals, 160 patients with high risk of atherosclerosis and MS and 64 patients with CVD were included to **control**, **risk** and **event groups** respectively. Inclusion criteria of **risk group** were as follows: disturbances in lipids profile (high TC and/or high LDL-C and/or low HDL-C and/or high TG), increased weight, smoking, acute or chronic stress (evaluated by physician), no previous or current acute CVD, high risk confirmed by physician. Inclusion criteria for **event group**: acute CVD, increased troponin I.

Laboratory analysis was performed in the Center of Laboratory Medicine of Vilnius University Hospital Santariskiu Klinikos. The list of performed routine and specific tests, methods (and/or used analyzers) are summarized in the Table 1. All laboratory tests were performed the same day when blood was drawn, except for vWF and CyPA. Citrated plasma was kept frozen –20 °C and vWF analyzed in several batches. Serum was kept frozen –70 °C and CyPA was performed in several batches. Due to zero CyPA μg/mL values (76 of 160) additional units for this parameter were calculated (signal to cut-off ratio, s/co).

Table 1. Analytical methods of the study.

Variable, units	Method details
CyPA, μg/mL	ELISA (AMS Biotechnology (Europe) Ltd., UK) on Gemini analyzer (Stratec Biomedical, Germany)
CyPA, s/co	
Fibrinogen, g/L	Claus method (STA Compact, Stago, France)
TRAP, U	Whole blood aggregation with TRAP agonist on Multiplate analyzer (Roche Diagnostics, Germany)
vWF, %	Immunoturbidimetric (STA Compact, Stago, France)
TC, mmol/L	Enzymatic colorimetric (Architect ci8200, Abbott, USA)
TG, mmol/L	Enzymatic colorimetric (Architect ci8200, Abbott, USA)
HDL-C, mmol/L	Enzymatic colorimetric (Architect ci8200, Abbott, USA)
LDL-C, mmol/L	Friedewald formula, but if TG>4.5 mmol/L – direct enzymatic colorimetric (Architect ci8200, Abbott, USA)
hs-CRP, mg/L	High sensitivity immunoturbidimetric (Architect ci8200, Abbott, USA)
Glucose, mmol/L	Hexokinase (Architect ci8200, Abbott, USA)
PLT, $\times 10^9$/L	Part of full blood count by hematology analyzer SYSMEX XE-5000 (Sysmex Corporation, Japan)
MPV, fL	
Pct, %	
PDW, %	
P-LCR, %	
PAC-1/CD42a, %	FLow cytometric analysis by BD FACSCanto (BD Biosciences, USA)
CD63/CD42a, %	
CD42a/CD14, %	

Different lipid ratios (TG/HDL-C, atherogenic index (AI) – lg[TG/HDL-C], TC/HDL-C and LDL-C/HDL-C) and lipid-CRP ratios (LDL-C/CRP, CRP/LDL-C, TC/CRP, CRP/TC, HDL-C/CRP, CRP/HDL-C and lg[CRP/HDL-C]) were calculated. Lipid-CRP ratios were evaluated as absolute numbers without estimated units of measure.

In order to evaluate prognostic value of lipid ratios or lipid-CRP ratios, we have used cut-offs of each ratio. Cut-offs for lipid ratios and lipid-CRP ratios where CRP was used as denominator were calculated as means of values obtained from individuals with risk factors and individuals with diagnosed CVD. In case of lipid-CRP ratios were

CRP was used as numerator cut-offs were calculated as means and one standard deviation of values obtained from individuals with risk factors.

Biochemical metabolic syndrome basis was confirmed as present (n=23, 14.4%) if all three biochemical criteria (TG ≥1.7 mmol/L; HDL-C <1.03 mmol/L and glucose >5.6 mmol/L) were met.

Statistical analysis was performed using SPSS software (*PASW Statistics 18* version, SPSS Inc., USA). Continuous variables were expressed as means and standard deviations (SD). Data comparison was made by Student t-test, ANOVA or Mann-Whitney U test where appropriate. Categorical variables were presented as frequencies and percentages and were compared by Chi-square test. The Spearman coefficient (r) was calculated to quantify the correlation between variables. Correlation was considered weak when r value was below 0.3, moderate – between 0.3 and 0.7, strong – above 0.7. Diagnostic characteristic of markers was evaluated by ROC curves. Markers were considered as useful if area under the ROC curve (AUC) was >0.5. Linear regression models were considered applicable if coefficient of determination r^2 was ≥0.25. All reported p values were two tailed and a p value of <0.05 was considered as statistically significant.

6. Results

6.1. Control group results

In total 42 relatively healthy young individuals (mean age 22 years): 10 (23.8%) males and 32 (76.2%) females were tested. After exclusion of deviant results (high TC and/or high LDL-C and or high TG and/or low HDL-C) the final control group consisted of 31 individual (8 (25.8%) males and 23 (74.2%) females). Subsequent comparison of control group results by gender revealed statistically significant differences of TG and several lipid ratios between men and women (Table 2).

Table 2. Statistically significant differences of lipids and their ratios between males and females in control group. Values provided in mean and standard deviations.

Variable	Males	Females	p value
TG, mmol/L	0.89±0.27	0.65±0.24	0.025
TG/HDL-C	0.66±0.21	0.42±0.19	0.005
AI	–0.21±0.18	–0.41±0.19	0.012
TC/HDL-C	3.31±0.53	2.76±0.48	0.010
LDL-C/HDL-C	2.01±0.47	1.57±0.41	0.018

CyPA and glucose were moderately related while comparing CyPA with other analytes and calculated indexes in control group (CyPA in μg/mL units r=0.396, p=0.027; CyPA in s/co units r=0.405, p=0.024 respectively). Correlations of lipid values corresponded to previously in literature described. CRP values correlated with fibrinogen (r=0.562, p=0.001), glucose – with different lipids (e.c. TG r=0.541, p=0.002; HDL-C r=–0.416, p=0.020; LDL-C r=0.374, p=0.038). Among calculated ratios TG/HDL-C, AI, TC/HDL-C and LDL-C/HDL-C correlated with glucose (r between 0.434 and 0.585, p≤0.015).

6.2. Risk group results

In total 160 men (age 40–54 years; mean 48 years, SD 4 years) were included.

CyPA results were grouped into two groups: the first group consisted of zero values in μg/mL units (n=76, 47.5%), the second – remaining values, which accordingly were considered positive (n=84, 52.5%). There were no crucial differences between analysed parameters and calculated indexes except CD42a/CD14% (7.92±2.11 vs. 9.06±2.90, p=0.006) and CD42a/CD14 absolute numbers (527±201 vs. 635±264, p=0.004). In Table 3 risk group results are compared with control group results while taking into account the distribution of CyPA results.

In order to stratify risk of CVD CRP values were grouped into four categories. The first group consisted of CRP values <1.0 mg/L (n=26, 16.3%) and the risk of CVD was considered as minimal/low. The second group consisted of CRP values equal or higher than 1.0 mg/L but <3.0 mg/L (n=68, 42.5%) and the risk of CVD was considered as moderate. The third group consisted of CRP values equal or higher than 3.0 mg/L but <5.0 mg/L (n=38, 23.8%) and the risk of CVD was considered as high. The fourth group consisted of CRP values equal or higher than 5.0 mg/L (n=28, 17.5%) and the risk of CVD was considered as very high.

Table 3. Comparison of risk and control groups while taking into account distribution of CyPA values as zero and positive. Values provided in mean and standard deviations. NS – not significant.

Variable	Comparison of full groups			Comparison of groups with zero CyPA values excluded		
	Risk group (n=160)	Control group (n=31)	p	Risk group (n=84)	Control group (n=28)	p
CyPA, μg/mL	0.56±0.78	3.51±2.59	<0.001	1.07±0.78	3.89±2.43	<0.001
CyPA, s/co	1.04±0.12	1.91±0.66	<0.001	1.13±0.11	2.01±0.61	<0.001
Fibrinogen, g/L	3.78±0.74	2.85±0.46	<0.001	3.85±0.83	2.90±0.41	<0.001
TRAP, U	123.4±20.0	123.3±17.0	NS	125.8±20.2	124.6±17.1	NS
vWF, %	123.6±41.2	97.4±38.1	<0.001	122.9±40.8	100.0±38.3	0.010
TC, mmol/L	6.45±1.31	4.39±0.46	<0.001	6.38±1.31	4.43±0.46	<0.001
TG, mmol/L	2.52±1.86	0.71±0.27	<0.001	2.53±1.92	0.71±0.27	<0.001
HDL-C, mmol/L	1.09±0.25	1.55±0.28	<0.001	1.07±0.23	1.55±0.28	<0.001
LDL-C, mmol/L	4.20±1.12	2.51±0.42	<0.001	1.13±1.00	2.56±0.38	<0.001
CRP, mg/L	3.35±3.90	0.43±0.39	<0.001	3.52±4.83	0.45±0.40	0.001
Glucose, mmol/L	5.67±0.48	4.96±0.39	<0.001	5.68±0.50	4.97±0.39	<0.001
TG/HDL-C	2.57±2.35	0.48±0.21	<0.001	2.54±2.23	0.48±0.21	<0.001
AI	0.30±0.30	–0.36±0.21	<0.001	0.30±0.28	–0.36±0.20	<0.001
TC/HDL-C	6.17±1.66	2.90±0.54	<0.001	6.18±1.74	2.94±0.51	<0.001
LDL-C/HDL-C	3.99±1.17	1.68±0.46	<0.001	3.99±1.18	1.72±0.44	<0.001
LDL-C/CRP	2.72±2.96	8.65±4.08	<0.001	3.01±3.31	8.67±4.18	<0.001
CRP/LDL-C	0.85±0.98	0.17±0.15	<0.001	0.89±1.18	0.17±0.16	0.002
TC/CRP	4.13±4.25	15.27±6.90	<0.001	4.57±4.79	15.14±7.12	<0.001
CRP/TC	0.54±0.65	0.10±0.09	<0.001	0.57±0.81	0.10±0.09	0.003
HDL-C/CRP	0.70±0.69	5.49±2.68	<0.001	0.78±0.77	5.38±2.73	<0.001
CRP/HDL-C	3.31±4.56	0.29±0.29	<0.001	3.57±5.80	0.31±0.31	0.004
lg[CRP/HDL-C]	0.33±0.40	–0.66±0.30	<0.001	0.30±0.44	–0.65±0.31	<0.001

Using ANOVA models different means of laboratory test results were compared and test dependency on the CRP category was evaluated. There was statistically significant difference of CyPA values in both units between four CRP categories (p values 0.004 and 0.018 respectively), but arrangement of mean values was not linear (linearity p values 0.307 and 0.549 respectively). Interestingly, the lowest CyPA mean was noted in the third group of CRP results (0.30±0.56 μg/mL or 1.00±0.09 s/co). While comparing only positive CyPA values statistically significant difference was absent in s/co units (p=0.463), but existed in μg/mL units (p=0.004). Consequently, linearity of latter values was statistically significant (p=0.005) (Figure 1).

In full risk group statistically significant linear difference of fibrinogen (p<0.001, linearity p<0.001) and vWF (p=0.005, linearity p=0.007) mean values between four CRP categories existed. Fibrinogen values increment was 3.50±0.53 g/L to 4.37±0.70 g/L. vWF values increment was 117.8±34.4% to 139.5±52.3%. With several exceptions, there was no relationship of lipid markers, platelet volume indices and most flow cytometric parameters between CRP categories. Exception noted: nonlinear increase of CD42a absolute numbers (783±105 to 840±137; p=0.040, linearity p=0.253).

Lipid-CRP ratios dependency on CRP category is depicted in Figure 2. Depending on the position of CRP value in the formula (numerator or denominator) absolute values of ratios and directions of change differed. For every ratio p was <0.001 (both for the difference and the linearity).

Figure 1. Dependency of CyPA values in four CRP result groups. A1 and A2 – full risk group (n=160). B1 and B2 – risk group with zero CyPA µg/mL values excluded.

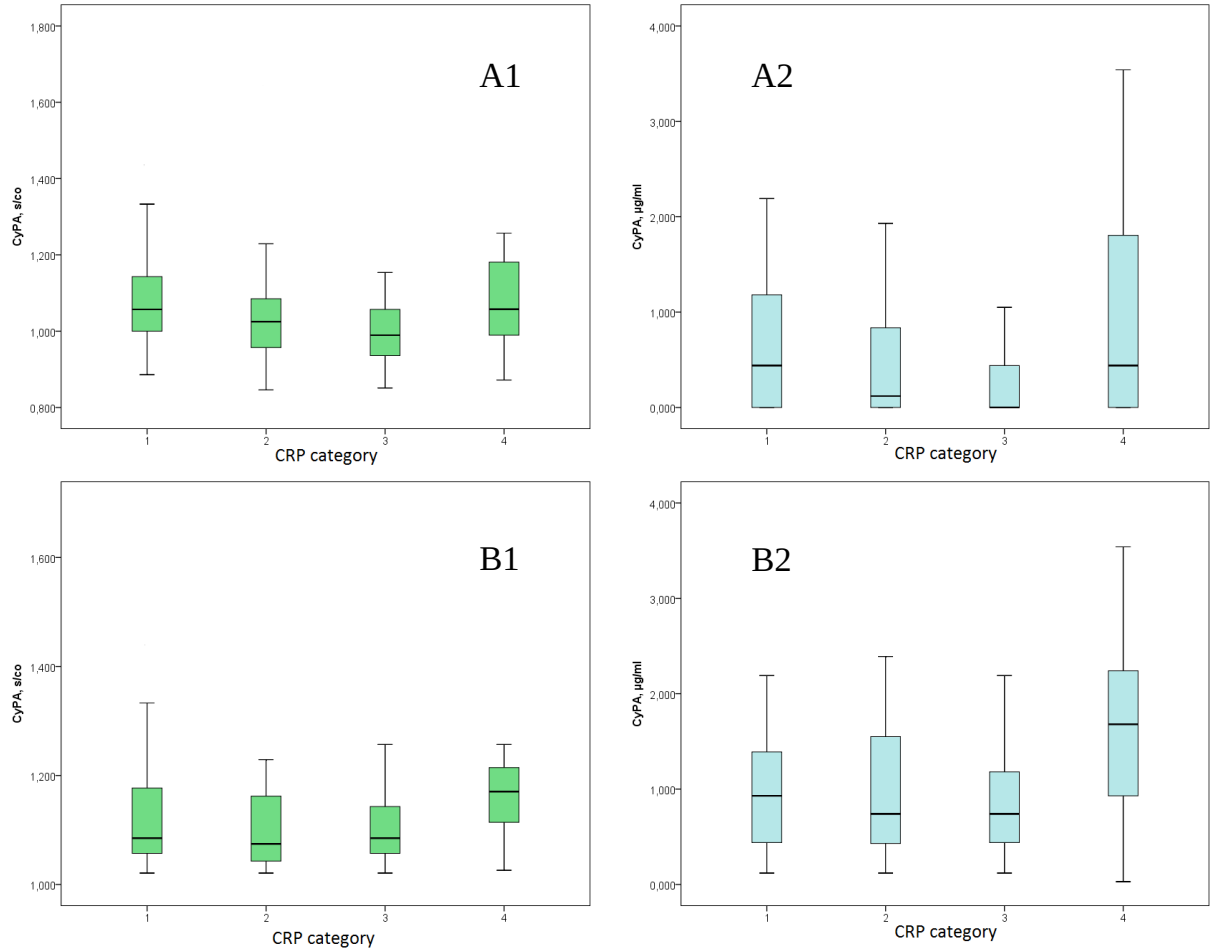
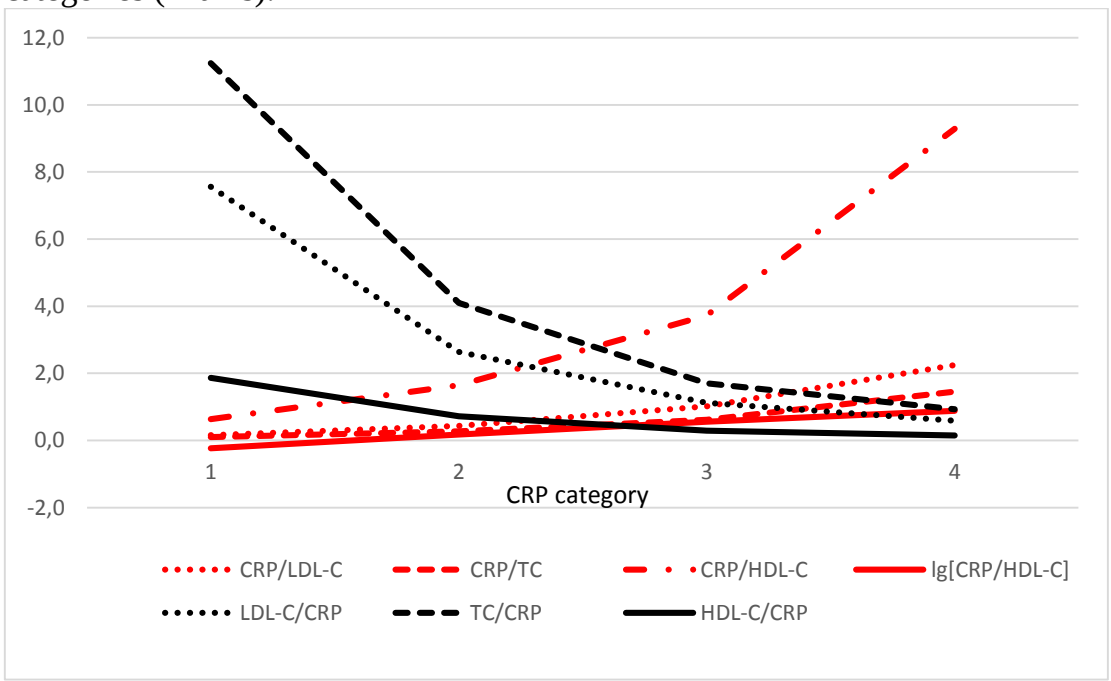


Figure 2. Dependency of lipid-CRP ratios (absolute values in Y axis) between four CRP categories (X axis).



In the whole investigated risk group population (n=160) analysis correlations of lipids (TC, TG, HDL-C and LDL-C) were as expected: TC correlation with other lipids was moderate to strong and significance level was high ($p \leq 0.009$); HDL-C significant negative correlation was noted with TG ($r=-0.333$; $p<0.001$).

Statistically significant interrelatedness of inflammatory factors was noted: vWF correlated positively with fibrinogen ($r=0.229$; $p=0.004$) and CRP ($r=0.224$; $p=0.004$), whereas CRP correlation with fibrinogen ($r=0.471$; $p<0.001$) was moderate and positive.

The following statistically significant correlations were noted for PLT: platelet numbers correlated positively with TRAP ($r=0.158$; $p=0.045$), negatively with MPV ($r=-0.379$; $p<0.001$), PDW ($r=-0.416$; $p<0.001$) and P-LCR ($r=-0.387$; $p<0.001$). PLT also correlated with flow cytometry parameters: positively with CD42a/CD14 percentage ($r=0.479$; $p<0.001$), CD14 percentage ($r=0.185$; $p=0.019$) and negatively with CD42aPE events number ($r=-0.287$; $p<0.001$) and CD63/CD42a percentage ($r=-0.177$; $p=0.025$). PLT correlations with CD42a/CD14 and CD42a are depicted in Figure 3.

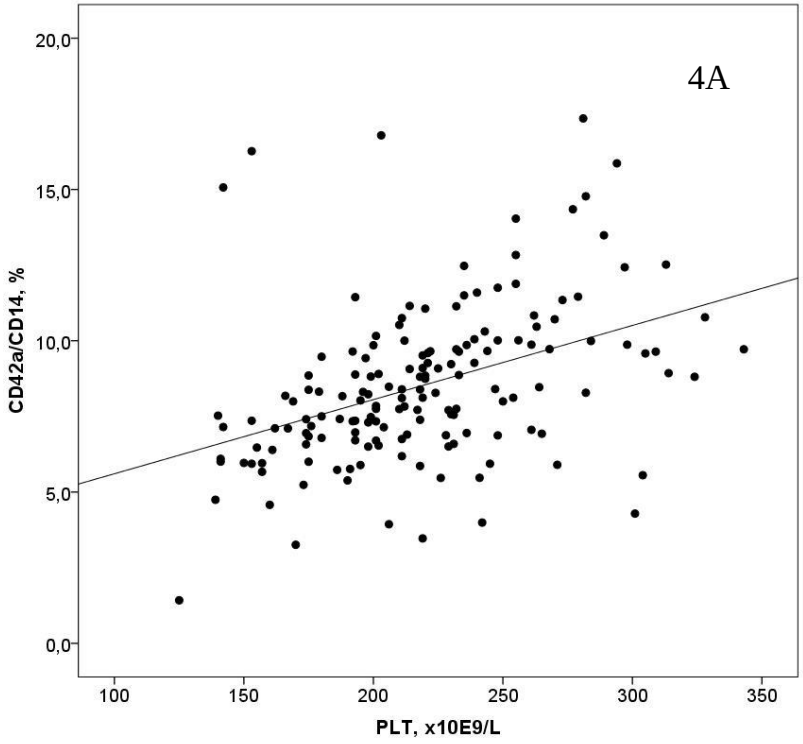
Mean platelet volume correlated positively with TRAP ($r=0.173$; $p=0.029$), PDW ($r=0.964$; $p<0.001$) and P-LCR ($r=0.994$; $p<0.001$), also with CD42aPE events number ($r=0.605$; $p<0.001$), PAC-1 events number ($r=0.200$; $p=0.011$) and CD63/CD42a percentage ($r=0.195$; $p=0.013$). Negative correlation of MPV with CD42a/CD14 percentage ($r=-0.281$; $p<0.001$), CD42a/CD14 events number ($r=-0.327$; $p<0.001$) and CD14 number ($r=-0.198$; $p<0.012$) was registered. Similar statistically significant correlations were seen for PDW and P-LCR, except their negative correlations with CD14 number (PDW $r=-0.240$, $p=0.002$ and P-LCR $r=-0.209$, $p=0.008$ respectively). MPV correlations with CD42a/CD14 and CD42a are depicted in Figure 4.

Associations of inflammatory and platelet markers: CRP weak positive correlation with TRAP ($r=0.166$; $p=0.036$) and weak negative correlation with CD42a/CD14 percentage ($r=-0.191$; $p=0.015$) and CD42a/CD14 events number ($r=-0.174$; $p=0.028$) existed; vWF correlated negatively with TRAP ($r=-0.203$; $p=0.01$), Pct ($r=-0.156$; $p=0.036$) and CD42a/CD14 percentage ($r=-0.208$; $p=0.008$); fibrinogen correlated with PLT ($r=0.192$; $p=0.015$).

Other correlations of biochemical parameters: glucose correlated positively with HDL-C ($r=0.164$; $p=0.038$), but negatively with PLT ($r=-0.181$; $p=0.022$), CD42a/CD14 percentage ($r=-0.199$; $p=0.012$) and CD42a/CD14 events number ($r=-0.193$; $p=0.014$); PLT correlated positively with LDL-C ($r=0.223$; $p=0.005$); TC and LDL-C correlation with CD42a/CD14 percentage was noted ($r=0.157$, $p=0.047$ and $r=0.165$, $p=0.038$ respectively).

CyPA correlations were as follows: CyPA μg/mL with CD42a/CD14 events number ($r=0.158$, $p=0.046$), CyPA s/co with CD42a/CD14 percentage ($r=0.175$, $p=0.027$) and CD42a/CD14 events number ($r=0.202$, $p=0.010$). After removing zero CyPA μg/mL values correlation analysis of remaining 84 subjects resulted in more or less the same tendencies in associations of analyzed parameters.

Figure 3. Distribution of CD42a/CD14 (4A) and CD42a (4B) values according to PLT counts: PLT and CD42a/CD14 (r^2=0.171); PLT and CD42a (in combination with PAC-1, r^2=0.064; and in combination with CD63, r^2=0.077). All measures had p≤0,001.



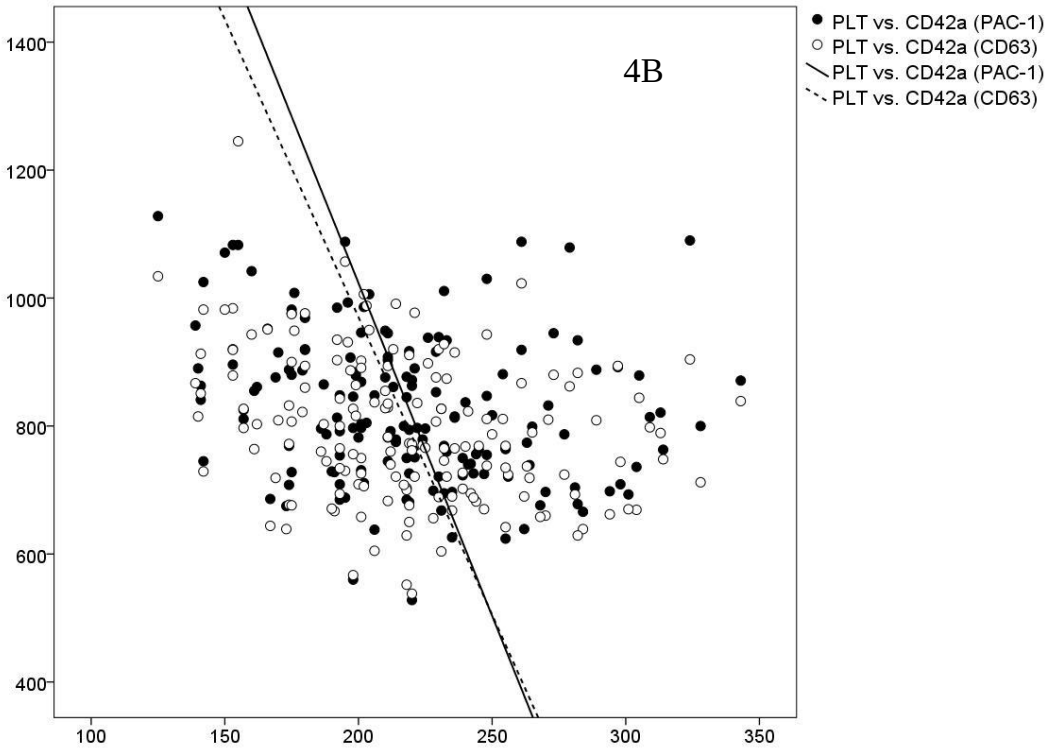
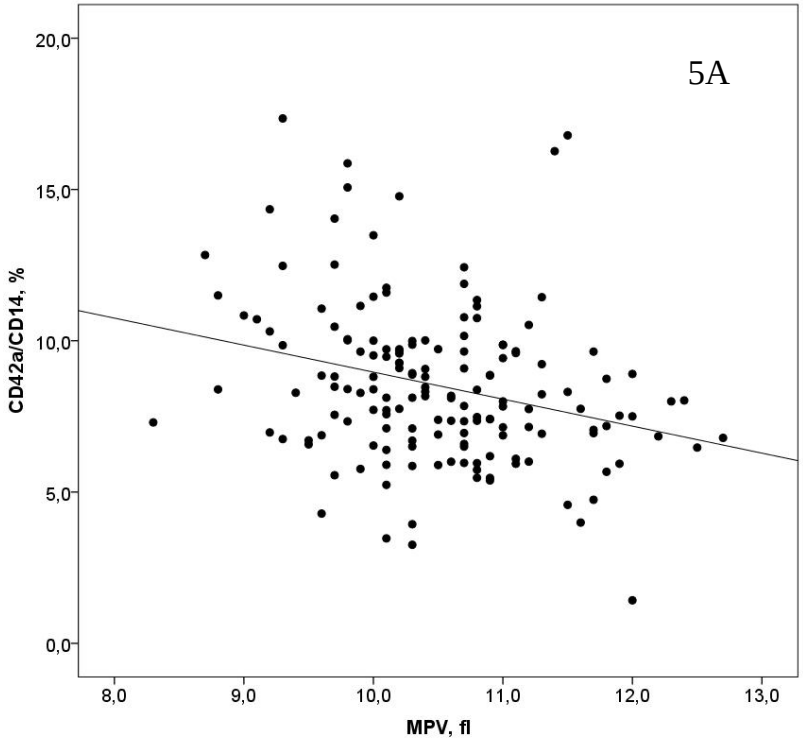
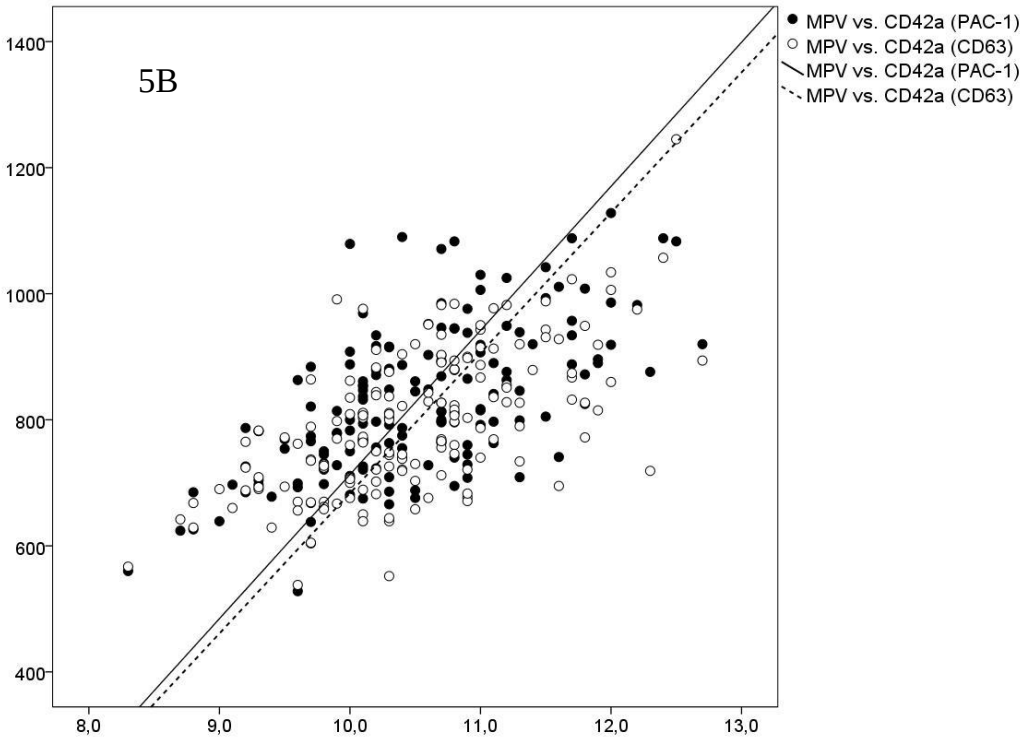


Figure 4. Distribution of CD42a/CD14 (5A) and CD42a (5B) values according to MPV: MPV and CD42a/CD14 (r^2=0.078); MPV and CD42a (in combination with PAC-1, r^2=0.388; and in combination with CD63, r^2=0.394). All measures had p<0,001.





Further on parameters were evaluated according to the approved reference values at the Center of Laboratory Medicine. Frequencies of abnormal results, reference values and estimated abnormality are summarized in Table 4. CRP values were divided into four groups: only results <1.0 mg/L were considered normal with low risk of CVD events. Each variable deviation from reference range was considered as a separate risk factor, and analyzed according to total number of risk factors (ranging from 2 to 7) registered among subjects.

Table 4. Frequencies of abnormal results according to laboratory reference ranges.

Variable, units	Reference values	Estimated abnormality	Frequency of abnormality (%)
Fibrinogen, g/L	2.0 – 4.0	>4.0	57 (35.6%)
TRAP, U	92 – 150	>150	14 (8.8%)
vWF, %	50 – 160	>160	55 (34.4%)
TC, mmol/L	<5.2	≥5.2	132 (82.5%)
TG, mmol/L	≤1.8	>1.8	100 (62.5%)
HDL-C, mmol/L	>0.91 (men only)	≤0.91	40 (25.0%)
LDL-C, mmol/L	2.6 – 3.5	>3.5	117 (73.1%)
hs-CRP, mg/L	≤5.0	1.0 – 3.0 (average risk)	68 (42.5%)
		3.0 – 5.0 (high risk)	38 (23.8%)
		>5.0 (very high risk)	28 (17.5%)
Glucose, mmol/L	4.2 – 6.1	>6.1	29 (18.1%)
MPV, fL	7.4 – 10.4	>10.4	78 (48.8%)

ANOVA analysis revealed, that increasing number of deviations gives statistically significant elevations in fibrinogen (from 3.52 to 4.32 g/L; $p<0.001$), TRAP (from 110.7 to 147.9 U; $p<0.001$), hs-CRP (from 1.17 to 3.68 mg/L; $p=0.005$), glucose (from 5.54 to 6.01 mmol/L; $p=0.026$), MPV (from 9.95 to 10.83 fL; $p=0.033$) and P-LCR (from 24.19 to 31.33%; $p=0.036$) with extremely significant linearity ($p\leq0.004$) (Figure 5). The same tendency is seen with lipids and their ratios: TG (from 1.58 to 2.50 mmo/L; $p=0.044$), TC (from 5.10 to 6.27 mmol/L; $p<0.001$), LDL-C (from 3.29 to 4.16 mmol/L; $p=0.007$), AI (from 0.14 to 0.38; $p=0.001$), TC/HDL-C (from 4.87 to 6.60; $p<0.001$), LDL-C/HDL-C (from 3.11 to 4.41; $p<0.001$), CRP/LDL-C (from 0.38 to 0.88; $p=0.030$) and lg[CRP/HDL-C] (from –0.03 to 0.50; $p<0.001$) (Figure 6). It should be noted that none of the platelet flow cytometry results depended on the number of risk factors (changes were not significant; $p\geq0.05$).

Figure 5. Statistically significant, dependent on total number of laboratory risk factors (X axis) increase of fibrinogen, glucose, CRP, TRAP, MPV and P-LCR values (absolute values in Y axis).

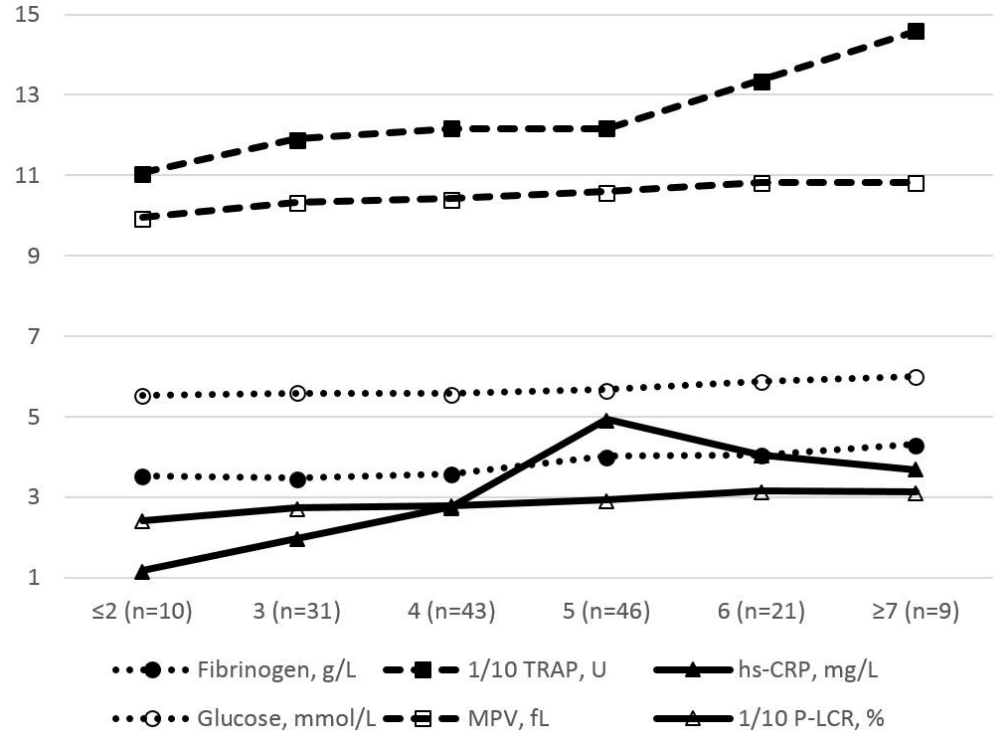
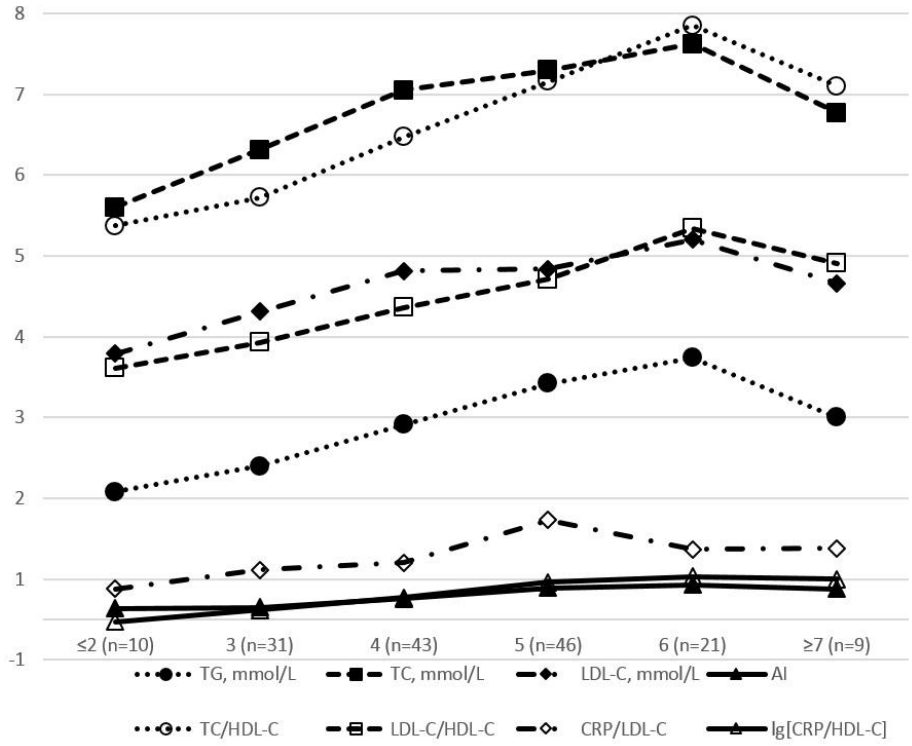


Figure 6. Statistically significant, dependent on total number of laboratory risk factors (X axis) increase of lipids and their ratios values (absolute values in Y axis).



vWf values depended on age (r=0.313; p=0.018) in a group of abnormal fibrinogen values (n=57) of risk group. Statistically significant differences of other analytes and/or calculated indices in this group are provided in Table 5. In this group of fibrinogen results correlation of CyPA in both units and LDL-C/HDL-C ratio was noted (r=0.296, p=0.026 and r=0.303, p=0.022 respectively). Other important correlations were as follows: fibrinogen with LDL-C (r=0.280, p=0.035), TC/HDL-C (r=0.442, p=0.001) and LDL-C/HDL-C (r=0.458, p<0.001), also TC with CD63 (r=0.289, p=0.030) and CD42a/CD14 % (r=0.281, p=0.034). Relation of platelet indices (MPV, PDW and P-LCR) with CD42a was relatively strong and significant (r>0.675, p<0.001). Flow cytometric analysis revealed significant correlations of PAC-1/CD42a % with AI (r=0.320, p=0.015) and CD42a/CD14 % with LDL-C/HDL-C and LDL-C/CRP ratios (r=0.293, p=0.027 and r=0.391, p=0.003 respectively).

Table 5. Statistically significant differences of variables taking into account abnormal values of fibrinogen. Values provided in mean and standard deviations.

Variable	Reference values	Abnormal values	p
Fibrinogen, g/L	**2–4 (n=103)**	**>4 (n=57, 35.6%)**	
Age, years	47.3±4.2	48.7±3.5	0.043
CRP, mg/L	2.18±1.66	5.47±5.57	<0.001
Pct, %	0.22±0.04	0.24±0.04	0.047
vWF, %	117.7±37.7	134.2±45.4	0.015
LDL-C/CRP	3.27±3.22	1.72±2.10	0.001
CRP/LDL-C	0.56±0.47	1.37±1.37	<0.001
TC/CRP	5.03±4.63	2.52±2.86	<0.001
CRP/TC	0.34±0.26	0.89±0.94	<0.001
HDL-C/CRP	0.86±0.75	0.43±0.44	<0.001
CRP/HDL-C	2.11±1.65	5.49±6.82	<0.001
lg[CRP/HDL-C]	0.20±0.34	0.55±0.40	<0.001

In a small group of abnormal TRAP aggregation results of risk group (n=14, 8.8%) only fibrinogen values difference was statistically significant (3.74±0.73 g/L in a group of normal TRAP results vs. 4.20±0.69 g/L in a group of abnormal TRAP results, p=0.028). Higher TRAP results meaning more active platelet aggregability correlated with CRP/TC (r=0.563, p=0.036), lg[CRP/HDL-C] values (r=0.576, p=0.031) and AI values (r=–0.558, p=0.038). CyPA results in both units correlated well with LDL-C/HDL-C ratio (μg/mL r=0.557, p=0.038, and s/co r=0.558, p=0.038).

Concerning abnormally high vWF values (n=55) of risk group significantly lower aggregability with TRAP and lower expression of CD42a (in combination with PAC-1 and CD63) was noted (Table 6). CD42a results in this group depended on TG and glucose concentrations (r=0.304, p=0.024 and r=0.341, p=0.011 respectively). There was also CD42a (in combination with PAC-1) correlation with AI values (r=0.313, p=0.020) and PAC-1/CD42a % relation to TC/CRP (r=0.280, p=0.039).

Among risk group individuals 24 (15%) had at least one, 52 (32.5%) had two, 67 (41.9%) had three and the rest 17 (10.6%) – four dyslipidaemia markers positive. Comparing different analytes and calculated ratios between these groups, no significant differences were noted, except PLT and Pct. Significance levels of difference were marginal, but increase linearity was significant (linearity p=0,007 and p=0,011 respectively). Statistically significant differences of variables taking into account abnormal values of lipids are provided in Table 7.

Table 6. Statistically significant differences of variables taking into account abnormal values of vWF. Values provided in mean and standard deviations.

Variable	Reference values	Abnormal values	p
vWF, %	**70–130 (n=105)**	**> 130 (n=55, 34.4%)**	
TRAP, U	125.8±18.2	118.9±22.6	0.037
Creatinine, μmol/L	75.0±9.2	78.4±12.0	0.050
HDL-C/CRP	0.78±0.76	0.55±0.49	0.047
CD42a (in combination with PAC-1)	844±121	797±99	0.015
PAC-1	43±15	50±25	0.038
CD42a (in combination with CD63)	809±121	768±94	0.028
CD63	–74±43	–55±49	0.012
CD42a/CD14, %	8.87±2.69	7.84±2.34	0.018

Table 7. Statistically significant differences of variables taking into account abnormal values of lipids. Values provided in mean and standard deviations.

Variable	Reference values	Abnormal values	p
TC, mmol/L	**<5.2 (n=28)**	**≥5.2 (n=132, 82.5%)**	
TG, mmol/L	1.82±1.30	2.67±1.94	0.029
HDL-C, mmol/L	0.99±0.25	1.11±0.25	0.028
LDL-C, mmol/L	2.93±0.50	4.47±1.03	<0.001
TC/HDL-C	5.05±1.37	6.41±1.62	<0.001
LDL-C/HDL-C	3.10±0.84	4.18±1.14	<0.001
LDL-C/CRP	1.72±1.31	2.93±3.17	0.050
LDL-C, mmol/L	**2.6–3.5 (n=43)**	**>3.5 (n=117, 73.1%)**	
TC, mmol/L	5.41±1.28	6.83±1.10	<0.001
TG, mmol/L	3.24±2.60	2.25±1.43	0.003
PLT, $\times 10^9$/L	200.3±39.1	225.6±44.0	0.001
Pct, %	0.21±0.04	0.23±0.04	0.003
TG/HDL-C	3.69±3.55	2.16±1.54	<0.001
AI	0.39±0.40	0.26±0.24	0.012
TC/HDL-C	5.63±2.09	6.37±1.43	0.012
TG, mmol/L	**≤1.8 (n=60)**	**>1.8 (n=100, 62.5%)**	
TC, mmol/L	5.87±1.16	6.79±1.28	<0.001
HDL-C, mmol/L	1.16±0.27	1.04±0.23	0.003
AI	0.04±0.17	0.45±0.24	<0.001
TC/HDL-C	5.23±1.24	6.73±1.62	<0.001
LDL-C/HDL-C	3.69±1.11	4.17±1.17	0.012
HDL-C, mmol/L	**Males >0.91 (n=120)**	**Males ≤0.91 (n=40, 25.0%)**	
TG, mmol/L	2.24±1.51	3.35±2.50	0.001
LDL-C, mmol/L	4.34±1.08	3.78±1.16	0.006
AI	0.22±0.26	0.52±0.29	<0.001

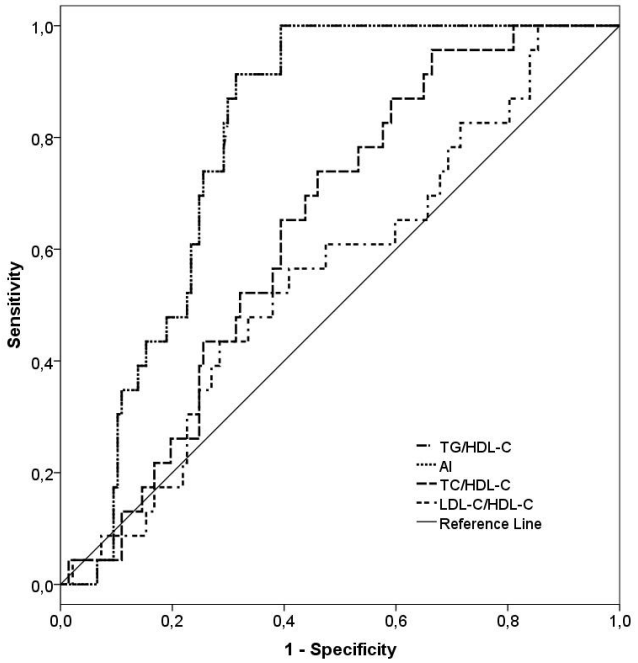
In a group of abnormal MPV values there was considerable amount of differences between the analysed variables (Table 8). Here CyPA results in both units correlated with TRAP aggregation results (r=0.262, p=0.020 and r=0.291, p=0.010 respectively). TRAP aggregation depended on platelet numbers and fibrinogen concentration (r=0.284, p=0.012 and r=0.240, p=0.034 respectively). Selected flow cytometry parameters, especially CD42a correlated with platelet volume indices (MPV, PDW and P-LCR) (r between 0.336–0.360, p≤0.004).

Table 8. Statistically significant differences of variables taking into account abnormal values of MPV. Values provided in mean and standard deviations.

Variable	Reference values	Abnormal values	p
MPV, fL	**7.4–10.4 (n=82)**	**>10.4 (n=78, 48.8%)**	
Fibrinogen, g/L	3.91±0.80	3.65±0.64	0.022
TRAP, U	119.8±17.5	127.2±21.8	0.019
PLT, $\times 10^9$/L	234.2±42.9	202.7±39.6	<0.001
PDW, %	11.04±0.84	13.62±1.63	<0.001
P-LCR, %	23.48±3.76	34.11±4.51	<0.001
CD42a (in combination with PAC-1)	776±99	882±108	<0.001
CD42a (in combination with CD63)	738±89	855±107	<0.001
CD42a/CD14, %	9.05±2.72	7.96±2.38	0.007
CD42a/CD14	650±269	514±186	<0.001
CD14	7134±1927	6487±1645	0.024

Risk group individuals were also categorised into four groups according to the number of biochemical MS markers (TG ≥1.7 mmol/L and/or HDL-C <1.03 mmol/L and/or glucose >5.6 mmol/L) present: individuals without biochemical criteria (MS[–] n=19, 11.9%), individuals having one criterion (MS[+1] n=42, 26.3%), individuals having two criteria (MS[+2] n=76, 47.5%) and individuals having all three biochemical MS criteria (MS[+3] n=23, 14.4%). Only absolute numbers of PAC-1 and CD42a/CD14 % differences were seen among these subgroups. The highest PAC-1 numbers (50±22) were seen in the MS[+2] subgroup and the lowest (37±13) – in MS[–] subgroup (p=0.019). Sequential decrease of CD42a/CD14 percentage was seen among MS subgroups (from 10.06±3.45 to 8.11±1.55, p=0.047, linearity p=0.018). Using MS[+3] as a target of prognosis, AI, TG/HDL-C ratio and TC/HDL-C ratio were found to be the better markers (Figure 7). Other analytes and lipid-CRP ratios did not meet AUC criterion of >0.5 and cannot be used as markers of MS[+3] prediction.

Figure 7. ROC curves for MS[+3] prediction. TG/HDL-C ratio AUC=0.797 (95% CI 0.728–0.865, p<0.001), AI index AUC=0.797 (95% CI 0.728–0.865, p<0.001), TC/HDL-C ratio AUC=0.640 (95% CI 0.540–0.741, p=0.031).



According to significant correlations, linear regression models were adapted for each laboratory risk factor. Successful models of different independent markers are shown in Tables 9 and 10. Fibrinogen concentration depended on combination of three variables, i.e. vWF, PLT and Pct (one of those, but not together), and CRP or one of lipid-CRP ratios (when CRP is positioned in fraction as numerator). Two examples of the latter are shown in Table 10.

Table 9. Linear regression analysis of LDL-C, TG and HDL-C.

Dependent variable	Independent variables	B	t	p	VIF	r^2
LDL-C, mmol/L	(constant)	1.139	3.636	<0.001		0.453
	TC/HDL-C	0.666	10.955	<0.001	2.302	
	AI	–3.507	–10.299	<0.001	2.302	
TG, mmol/L	(constant)	–1.416	–3.106	0.002		0.426
	TC/HDL-C	0.732	10.606	<0.001	1.021	
	LDL-C/CRP	–0.119	–2.795	0.006	1.237	
	CRP, mg/L	–0.076	–2.343	0.020	1.249	
HDL-C, mmol/L	(constant)	1.270	53.394	<0.001		0.416
	lg[CRP/HDL-C]	–0.091	–2.308	0.022	1.042	
	AI	–0.512	–9.653	<0.001	1.042	

Table 10. Linear regression analysis of fibrinogen, CRP, glucose and MPV.

Dependent variable	Independent variables	B	t	p	VIF	r^2
Fibrinogen, g/L	(constant)	2.199	6.630	<0.001		0.267
	vWF, %	0.003	2.712	0.007	1.026	
	CRP/HDL-C	0.064	5.676	<0.001	1.018	
	Pct, %	4.192	3.493	0.001	1.022	
	(constant)	2.149	6.960	<0.001		0.281
	vWF, %	0.003	2.358	0.020	1.034	
	CRP/TC	0.456	5.880	<0.001	1.024	
	PLT, $\times 10^9$/L	0.003	4.112	<0.001	1.010	
CRP, mg/L	(constant)	0.234	2.625	0.010		0.951
	CRP/TC	5.809	55.155	<0.001	1.000	
	(constant)	0.623	5.955	<0.001		0.923
	CRP/HDL-C	0.821	43.495	<0.001	1.000	
	(constant)	0.856	3.483	0.001		0.620
	lg[CRP/HDL-C]	7.677	16.070	<0.001	1.000	
Glucose, mmol/L	CRP, mg/L	0.729	11.020	<0.001	1.000	0.433
	CRP, mg/L	0.064	1.953	0.053	1.739	0.921
	TC/HDL-C	0.820	31.182	<0.001	1.739	
	CRP, mg/L	0.144	4.290	<0.001	1.586	0.908
	CD42a/CD14, %	0.544	28.581	<0.001	1.586	
	CRP, mg/L	0.087	2.382	0.018	1.720	0.899
	CD42a (in combination with CD14)	0.002	27.027	<0.001	1.720	
MPV, fL	(constant)	7.815	17.158	<0.001		0.478
	CD42a (in combination with PAC-1)	0.002	2.074	0.040	4.039	
	CD42a (in combination with CD63)	0.003	3.350	0.001	3.978	
	CD42a/CD14, %	–0.064	–3.431	0.001	1.048	
	CD14, %	–0.057	–2.161	0.032	1.020	

Despite statistically significant TRAP correlations with vWF, CRP, PLT, Pct, MPV, P-LCR, CD42a (in two different combinations) and lipid-CRP ratios, there were no successful linear regression models for TRAP. Univariate regression analysis revealed that prognostic marker for vWF is fibrinogen, but not CRP. CRP linear regression model with fibrinogen shows reliable dependency of these analytes (F 41.880, constant –5.782, $p<0,001$), although $r^2=0,210$ is lower than the established criterion. Relations of glucose with CRP, CD42a/CD14 %, CD42a (in combination with CD14) and TC/HDL-C ratio were reliable only when constant factor was not used. There were no successful models of MPV with TRAP, PDW and P-LCR, but significant link existed with flow cytometry parameters (Table 10).

In univariate regression analysis we have found that flow cytometry parameters of platelets can be predicted by established lipid ratios and lipid-CRP ratios cut-offs. Among other analytes high values of vWF and MPV were predictors of platelet activity (absolute numbers of CD42a, PAC-1 and CD63) and formation of platelet monocyte complexes (absolute numbers of CD42a/CD14). Results of platelet function univariate analysis are presented in Tables 11 and 12. The former Table depicts platelet function parameters prediction capabilities from increased CVD risk point of view (individuals having 5–7 laboratory risk factors), while the latter one – from increased MS risk point of view (individuals with having 3 MS biochemical risk factors). Only absolute numbers of PAC-1/CD42a and CD42a (in combination with CD14) were statistically significant predictors of increased CVD risk ($p\leq0.045$) and none of flow cytometry parameters could reach the level of significance for MS[+3] prediction.

Table 11. Univariate regression analysis of flow cytometry parameters of platelet function when presence of 5–7 laboratory CVD risk factors is set as a fixed factor.

Dependent variable	Independent variables	B	95% CI		t	p
			Min	Max		
PAC-1/CD42a, %	AI >0.26	0.054	0.018	0.090	2.9	0.004
	LDL-C/CRP >2.32	0.192	0.065	0.318	3.0	0.003
	TC/CRP >3.53	–0.180	–0.305	–0.055	–2.8	0.005
PAC-1/CD42a *	AI >0.26	15.6	5.1	26.1	2.9	0.004
	LDL-C/CRP >2.32	55.5	18.6	92.4	3.0	0.003
	TC/CRP >3.53	–52.4	–89.0	–15.9	–2.8	0.005
CD42a (in combination with PAC-1)	vWF >130%	–43.7	–76.7	–10.7	–2.6	0.010
	MPV >10.4 fL	99.0	66.7	131.3	6.0	<0.001
	CRP/LDL-C >1.83	60.0	7.4	112.6	2.3	0.026
PAC-1	LDL-C/CRP >2.32	15.4	1.8	29.0	2.2	0.026
	HDL-C/CRP >0.63	–17.2	–31.1	–3.3	–2.4	0.016
CD63/CD42a, %	MPV >10.4 fL	0.058	0.005	0.111	2.1	0.034
	lg[CRP/HDL-C] >0.73	0.080	0.004	0.155	2.1	0.039
CD63/CD42a	MPV >10.4 fL	16.6	1.2	32.0	2.1	0.034
	lg[CRP/HDL-C] >0.73	22.0	0.06	44.0	2.0	0.049
CD42a (in combination with CD63)	MPV >10.4 fL	113.7	82.1	145.4	7.1	<0.001
CD63	vWF >130%	18.1	3.7	32.4	2.5	0.014
	CRP/LDL-C >1.83	31.7	8.9	54.7	2.7	0.007
CD42a/CD14, %	vWF >130%	–0.99	–1.8	–0.2	–2.3	0.021
CD42a/CD14	MPV >10.4 fL	–128.2	–203.2	–53.1	–3.4	0.001
CD42a (in combination with CD14) *	TC/HDL-C >6.0	–439.1	–730.9	–147.2	–3.0	0.003

Table 12. Univariate regression analysis of flow cytometry parameters of platelet function when presence of three MS biomarkers is set as a fixed factor.

Dependent variable	Independent variables	B	95% CI		t	p
			Min	Max		
PAC-1/CD42a, %	AI >0.26	0.047	0.010	0.084	2.5	0.013
	LDL-C/CRP >2.32	0.214	0.087	0.340	3.3	0.001
	TC/CRP >3.53	–0.195	–0.321	–0.069	–3.1	0.003
PAC-1/CD42a	AI >0.26	13.5	2.6	49.4	2.5	0.015
	LDL-C/CRP >2.32	62.1	25.3	98.9	3.3	0.001
	TC/CRP >3.53	–57.0	–93.8	–20.2	–3.1	0.003
CD42a (in combination with PAC-1)	vWF >130%	–41.5	–75.1	–7.8	–2.4	0.016
	MPV >10.4 fL	104.7	72.7	136.6	6.5	<0.001
PAC-1	vWF >130%	6.2	0.02	12.5	2.0	0.049
	LDL-C/CRP >2.32	16.0	2.7	29.4	2.4	0.019
	HDL-C/CRP >0.63	–17.3	–30.7	–3.8	–2.5	0.012
CD42a (in combination with CD63)	vWF >130%	–36.5	–67.9	–5.1	–2.3	0.023
	MPV >10.4 fL	115.4	85.5	145.2	7.6	<0.001
	CRP/LDL-C >1.83	62.4	12.6	112.3	2.5	0.014
CD63	vWF >130%	19.0	4.2	33.7	2.5	0.012
CD42a/CD14, %	vWF >130%	–1.1	–1.9	–0.3	–2.6	0.011
	MPV >10.4 fL	–1.2	–2.0	–0.4	–2.9	0.004
CD42a/CD14	MPV >10.4 fL	–135.6	–208.5	–62.7	–3.7	<0.001
CD42a (in combination with CD14)	TC >5.2 mmol/l	395.4	5.5	785.2	2.0	0.047
	TC/HDL-C >6.0	–416.4	–718.0	–114.9	–2.7	0.007
CD14	MPV >10.4 fL	–626.6	–1185	–68.6	–2.2	0.028
CD14, %	MPV >10.4 fL	–0.6	–1.2	–0.05	–2.1	0.033

According to the established cut-offs the presence of MS[+3] could be prognosticated by decreased HDL-C (cut-off <0.91 mmol/L, OR 3.41, 95% CI 1.37–8.53, Chi square 7.464, p=0.009) and increased glucose concentrations (cut-off >6.1 mmol/L, OR 2.95, 95% CI 1.11–7.82, Chi square 5.023, p=0.030), but not by deviations of fibrinogen, vWF, TRAP, TG, TC, LDL-C, CRP or MPV. The presence of 5–7 laboratory CVD risk factors could be prognosticated by most analysed factors (Table 13).

Table 13. Likelihood of having 5-7 laboratory CVD risk factors according to established cut-offs of separate markers. NS – not significant.

Variable	Cut-off	OR	95% CI	Chi square	p
Fibrinogen, g/L	4.0	4.979	2.455–10.097	21.191	<0.001
TRAP, U	151	4.569	1.223–17.065	5.940	0.024
vWF, %	130	1.231	0.641–2.367	0.391	NS
TC, mmol/L	5.2	7.200	2.367–21.904	15.014	0.001
TG, mmol/L	1.8	6.156	2.948–12.854	25.691	<0.001
HDL-C, mmol/L	0.91*	3.009	1.414–6.404	8.555	0.004
LDL-C, mmol/l	3.5	4.271	1.926–9.469	13.860	<0.001
CRP, mg/L	3.0**	2.360	1.223–4.515	6.486	0.009
Glucose, mmol/L	6.1	4.481	1.788–11.233	11.425	0.001
MPV, fL	10.4	2.771	1.460–5.259	9.931	0.002
Acute stress	–	1.138	0.542–2.391	0.116	NS
3 MS markers	–	1.527	0.627–3.719	0.877	NS
CRP and MPV	–***	2.778	1.170–6.595	5.640	0.021

* – cut-off for men only. ** – cut-off for differentiation of high CVD risk individuals.
*** – the same cut-offs as above.

Considering that CRP values below 1.0 mg/L are associated with low CVD risk, CRP values corresponding to high CVD risk may influence increment of MPV values (>10.4 fL) with odds ratio of 2.46 (95% CI 1.00–6.05, Chi square 4.017, p=0.050) and vice versa – high MPV values are associated with increased CRP values. Higher concentrations of fibrinogen (>4 g/L) may be determined by increased TRAP aggregability (>150 U) (odds ratio 5.27, 95% CI 1.57–17.67, Chi square 8.576, p=0.007). Likewise higher TG concentrations (>1.8 mmol/l) may be detected 5.12 times more often when acute stress fact is in concern (95% CI 1.37–19.14, Chi square 6.731, p=0,015).

6.3. Event group results

In total 64 individuals (mean age 65 years): 47 males (73.4%) and 17 females (26.6%) were included in the study. Age related variations were noted for HDL-C (r=0.267, p=0.033), TG (r=–0.440, p<0.001), AI (r=–0.445, p<0.001), TC/HDL-C (r=–0.399, p=0.001) and LDL-C/HDL-C (r=–0.335, p=0.007). Comparison of laboratory tests results by gender revealed differences in TG and HDL-C values, TG being lower (1.12±0.60 mmol/L vs. 1.95±1.30 mmol/L, p=0.014) and HDL-C being higher (1.27±0.26 mmol/L vs. 1.00±0.33 mmol/L, p=0.003) in women. Consequently, AI was significantly higher in men (0.24±0.34 vs. –0.10±0.23, p<0.001). Comparison of event and control groups results is provided in Table 14.

Table 14. Comparison of event and control groups while taking into account distribution of CyPA values to zero and positive. Values provided in mean and standard deviations. NS – not significant.

Variable	Comparison of full groups			Comparison of groups with zero CyPA values excluded		
	Event group (n=64)	Control group (n=31)	p value	Event group (n=12)	Control group (n=28)	p value
CyPA, µg/mL	5.79±24.69	3.51±2.59	0.611	30.9±51.5	3.89±2.43	0.008
CyPA, s/co	1.04±0.49	1.91±0.66	<0.001	1.54±1.03	2.01±0.61	0.074
TC, mmol/L	5.68±1.57	4.39±0.46	<0.001	6.25±1.91	4.43±0.46	<0.001
TG, mmol/L	1.73±1.21	0.71±0.27	<0.001	2.02±1.06	0.71±0.27	<0.001
HDL-C, mmol/L	1.07±0.33	1.55±0.28	<0.001	1.01±0.29	1.55±0.28	<0.001
LDL-C, mmol/L	3.82±1.25	2.51±0.42	<0.001	4.31±1.53	2.56±0.38	<0.001
CRP, mg/L	26.6±45.5	0.43±0.39	0.002	32.7±56.8	0.45±0.40	0.004
Glucose, mmol/L	7.93±5.03	4.96±0.39	0.001	8.15±3.72	4.97±0.39	<0.001
TG/HDL-C	1.92±1.62	0.48±0.21	<0.001	2.27±1.74	0.48±0.21	<0.001
AI	0.15±0.35	–0.36±0.21	<0.001	0.27±0.26	–0.36±0.20	<0.001
TC/HDL-C	5.69±2.11	2.90±0.54	<0.001	6.60±2.94	2.94±0.51	<0.001
LDL-C/HDL-C	3.81±1.55	1.68±0.46	<0.001	4.55±2.29	1.72±0.44	<0.001
LDL-C/CRP	1.32±1.78	8.65±4.08	<0.001	1.05±1.73	8.67±4.18	<0.001
CRP/LDL-C	10.2±21.3	0.17±0.15	0.010	13.1±31.2	0.17±0.16	0.032
TC/CRP	2.02±2.91	15.27±6.90	<0.001	1.50±2.39	15.14±7.12	<0.001
CRP/TC	6.16±12.20	0.10±0.09	0.007	7.76±17.12	0.10±0.09	0.021
HDL-C/CRP	0.44±0.87	5.49±2.68	<0.001	0.24±0.36	5.38±2.73	<0.001
CRP/HDL-C	34.3±71.6	0.29±0.29	0.010	53.2±120.5	0.31±0.31	0.023
lg[CRP/HDL-C]	0.90±0.77	–0.66±0.30	<0.001	1.10±0.77	–0.65±0.31	<0.001

As in the risk group, event group individuals were categorised into four CRP subgroups: <1.0 mg/L CRP n=5 (7.8%), 1.0–3.0 mg/L n=13 (20.3%), 3.0–5.0 mg/L n=9 (14.1%), and >5.0 mg/L n=37 (57.8%). Partial relation of HDL-C was noted between four subgroups and difference of values was significant (p=0.010) HDL-C values being significantly lower in the highest CRP category. Although difference of CyPA values in both units was not statistically significant, in subgroup of 1.0–5.0 mg/L CRP these were slightly higher than in subgroups with lower CRP concentrations. After exclusion of zero CyPA μg/mL values, difference between CyPA means was still not significant, but the tendency of CyPA concentrations decrease towards the higher CRP concentrations was seen.

Correlations of lipids was also as expected. TC correlated with TG (r=0.379, p=0.002), LDL-C (r=0.952, p<0.001). HDL-C correlated with TG (r=–0.459; p<0.001). Other significant correlations in event group were as follows: CRP was in relation with glucose (r=0.273; p=0.029) and creatinine (r=0.387; p=0.002), also with lipids such as TC (r=–0.289, p=0.021), HDL-C (r=–0.346, p=0.005) and LDL-C (r=–0.259; p=0.039). Creatinine correlated with glucose (r=0.517; p<0.001) and TG (r=0.288; p=0.021). Consequently, creatinine correlation with AI (r=0.304; p=0.015) and all lipid-CRP ratios (r varied between 0.362–0.373 and was with or without negation sign, p ≤0.003) was noted. CyPA values in both units correlated with creatinine (r=0.251, p=0.046 and r=0.308, p=0.013 respectively).

Event group individuals also were categorized according to deviations from reference ranges of analytes. Differences are summarized in Tables 15 and 16. Here essential remarks could be made on lipids and their ratios, as well as lipid-CRP ratios. CyPA differences would be of note, these were close to the established significance criterion.

Table 15. Statistically significant differences of variables in event group taking into account abnormal values of TC and TG. Values provided in means and standard deviations.

Variable	Reference values	Abnormal values	p
TC, mmol/L	**<5.2 (n=27)**	**≥5.2 (n=37, 57.8%)**	
TG, mmol/L	1.22±1.30	2.11±1.41	0.003
HDL-C, mmol/L	0.96±0.34	1.15±0.31	0.027
LDL-C, mmol/L	2.71±0.72	4.62±0.88	<0.001
CRP, mg/L	43.6±60.4	14.2±24.6	0.010
LDL-C/HDL-C	3.08±1.13	4.34±1.61	0.001
CRP/LDL-C	19.6±29.7	3.3±6.4	0.002
CRP/HDL-C	61.6±100.4	14.3±26.8	0.008
lg[CRP/HDL-C]	1.13±0.91	0.73±0.60	0.036
TG, mmol/L	**≤1.8 (n=42)**	**>1.8 (n=22, 34.4%)**	
CyPA, μg/mL	1.44±6.04	14.08±40.60	0.051*
CyPA, s/co	0.96±0.11	1.20±0.82	0.065*
TC, mmol/L	5.22±1.27	6.56±1.74	0.001
HDL-C, mmol/L	1.16±0.32	0.90±0.29	0.003
LDL-C, mmol/L	3.56±1.15	4.30±1.32	0.025
TC/HDL-C	4.69±1.22	7.60±2.14	<0.001
LDL-C/HDL-C	3.22±1.12	4.95±1.64	<0.001

* – level of significance does not meet <0.05 criterion.

Table 16. Statistically significant differences of variables in event group taking into account abnormal values of HDL-C, LDL-C and glucose. Values provided in means and standard deviations.

Variable	Reference values	Abnormal values	p
HDL-C, mmol/L	**Male >0.91; Female >1.20 (both genders n=33)**	**Male ≤0.91; Female ≤1.20 (both genders n=31, 48.4%)**	
CRP, mg/L	11.97±20.93	42.23±58.18	0.007
LDL-C/CRP	1.76±1.98	0.85±1.42	0.040
CRP/LDL-C	3.71±7.87	17.08±28.08	0.011
TC/CRP	2.74±3.39	1.25±2.07	0.039
CRP/TC	2.41±5.07	10.16±15.91	0.010
LDL-C, mmol/L	**2.6–3.5 (n=25)**	**>3.5 (n=39, 60.9%)**	
TC, mmol/L	4.19±0.72	6.63±1.18	<0.001
TG, mmol/L	1.33±0.63	1.99±1.41	0.033
HDL-C, mmol/L	0.96±0.35	1.14±031	0.030
CRP, mg/L	44.29±62.30	15.31±25.27	0.012
TC/HDL-C	4.83±1.61	6.24±2.22	0.008
CRP/TC	12.01±17.53	2.42±3.97	0.002
CRP/HDL-C	64.1±103.8	15.2±26.9	0.007
Glucose, mmol/L	**4.2–6.1 (n=30)**	**>6.1 (n=34, 53.1%)**	
CRP, mg/L	13.59±21.96	38.13±56.84	0.030
CRP/LDL-C	4.22±8.23	15.45±27.26	0.034
CRP/TC	2.86±5.35	9.08±15.50	0.041
CRP/HDL-C	14.4±22.4	51.9±93.1	0.035

In event group results regression analysis there were no successful models for CyPA or TnI. There was a significant relation of creatinine and glucose values, but only when constant is not used in regression analysis (Table 17). CRP and HDL-C as well as LDL-C model suitability criterion $r^2 \geq 0.25$ was not reached, but was close to it.

Table 17. Linear regression analysis of event group results.

Variable	Model	B	t	p	VIF	r^2
Creatinine, µmol/L	(constant)	60.452	2.474	0.016		0.063
	Glucose, mmol/L	5.336	2.074	0.045	1.000	
Creatinine, µmol/L	CyPA, s/co	46.490	2.699	0.009	2.365	0.526
	Glucose, mmol/L	6.455	3.049	0.003	2.365	
CRP, mg/L	(constant)	113.862	5.399	<0.001		0.230
	HDL-C, mmol/L	–41.936	–2.662	0.010	1.053	
	LDL-C, mmol/L	–11.092	–2.650	0.010	1.053	

6.4. Comparison of three output groups

Creatinine values difference was statistically significant between risk and event groups (76±10 µmol/L vs. 103±107 µmol/L, p=0.002). Atherosclerosis risk factors such as lipids, CRP, glucose, lipid ratios as well as lipid-CRP ratios differed significantly between all three output groups (in most cases p values were <0.001, but always met <0.05 criterion).

CyPA was evaluated in two aspects: first, results were compared in full collection and consequently after exclusion of zero CyPA µg/mL values, secondly, CyPA values were compared using two different measurement units. Control and risk group CyPA values in both units differed significantly (p<0.001), values being higher in controls. In full collection of event and control groups CyPA results there was only CyPA s/co

significant difference (p<0.001). After exclusion of zero values, CyPA values became significantly different in both units (p values 0.008 and 0.007 respectively). Comparing CyPA values between risk and event groups, difference is evident only after exclusion of zero values.

In evaluation of CyPA value in prognosis of CVD we have found only the chance of 0.21 to have CVD if values are higher than 1.04 s/co (mean value of event group) (odds ratio 0.210, 95% CI 0.097–0.455, Chi square 17.648, p<0.001). CyPA prognostic capabilities for MS[3+] were not approved.

For lipids and their ratios as well as lipid-CRP ratios likelihoods to have CVD were calculated and summarized in Table 18. Likewise likelihoods to have MS[3+] were calculated and summarized in Table 19. Odds ratios are highest when CRP is used in numerator of lipid-CRP ratio formula. The same ratios demonstrate statistically sound results in differentiating CVD affected individuals in ROC curve analysis (Figure 8). Among other analytes creatinine, CRP and glucose could meet the criteria of CVD markers' AUC values (AUC 0.616, 0.739 and 0.668 respectively, p≤0.007). Merely lipids ratios ROC curve analysis results were statistically insignificant or did not meet AUC criterion >0.5.

Table 18. Likelihood of CVD according to the established cut-offs of separate markers. OR – odds ratio. CI – confidence interval. NS – not significant.

Variable	Cut-off	OR	95% CI	Chi square	p
TG/HDL-C	2.39	0.569	0.293-1.104	2.823	NS
AI	0.26	0.529	0.292-0.959	4.467	0.036
TC/HDL-C	6.00	0.547	0.301-0.994	3.965	0.048
LDL-C/HDL-C	3.94	0.767	0.427-1.377	0.791	NS
LDL-C/CRP	2.32	0.320	0.159-0.646	10.708	0.001
CRP/LDL-C	1.83	8.455	4.148-17.233	40.737	<0.001
TC/CRP	3.53	0.281	0.137-0.578	12.830	0.001
CRP/TC	1.19	12.333	5.736-26.521	53.312	<0.001
HDL-C/CRP	0.63	0.337	0.167-0.681	9.697	0.002
CRP/HDL-C	7.87	9.977	4.712-21.124	44.253	<0.001
lg[CRP/HDL-C]	0.73	8.163	4.201-15.859	43.983	<0.001

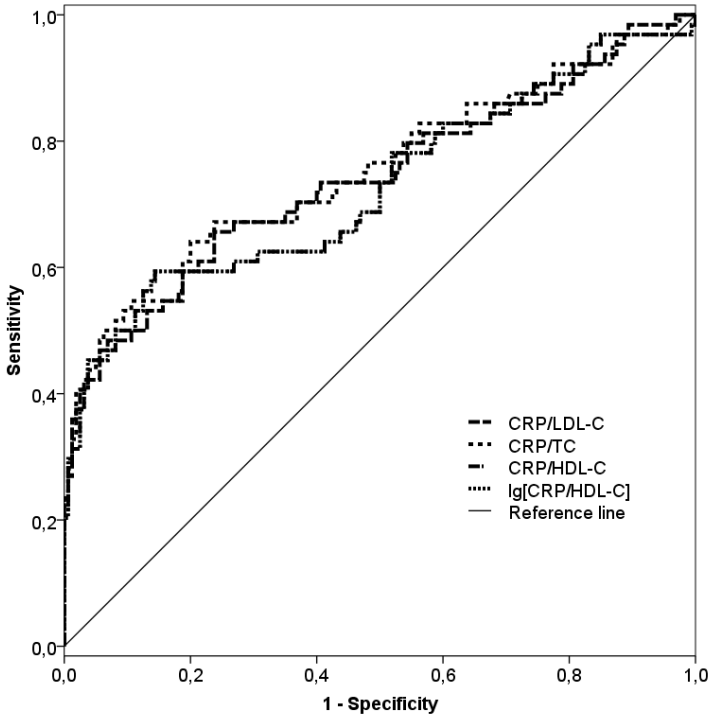
Table 19. Likelihood of MS[3+] according to the established cut-offs of separate markers. OR – odds ratio. CI – confidence interval.

Variable	Cut-off	OR	95% CI	Chi square	p
TC/HDL-C	6.00	3.265*	1.797-4.734*	24.555	<0.001
LDL-C/HDL-C	3.94	4.628	1.454-14.729	7.133	0.009
CRP/HDL-C	7.87	2.240*	0.096-4.383*	5.647	0.041
lg[CRP/HDL-C]	0.73	2.667*	0.543-4.792*	8.947	0.014

* - logarithmic expression of OR and CI.

It was found that single traditional CVD risk factors such as HDL-C or LDL-C increase risk of having MS[3+] despite occurrence of CVD: HDL-C – 3.7 times higher (logarithmic odds ratio value 3.689, 95% CI 1.564–5.813, Chi square 20.642, p=0.001), and LDL-C – around 30 % (OR 0.316, 95% CI 0.100–0.997, Chi square 4.003, p=0.049). Among individuals who are not affected by CVD increased vWF values raise risk of having MS[3+] by 2.7 (OR 2.724, 95% CI 0.991–7.487, Chi square 4.013, p=0.052).

Figure 8. ROC curves for CVD prediction. CRP/LDL-C ratio AUC=0.741 (95% CI 0.661–0.821, p<0.001), CRP/TC ratio AUC=0.752 (95% CI 0.673–0.831, p<0.001), CRP/HDL-C ratio AUC=0.727 (95% CI 0.645–0.809, p<0.001) and lg[CRP/HDL-C] AUC=0.727 (95% CI 0.645–0.809, p<0.001).



7. Conclusions

1. According to the study results cyclophilin A (CyPA) test is not suitable for early detection and prognosis of cardiovascular diseases (CVD) and metabolic syndrome (MS). The determined link between CyPA and platelets-monocytes complexes gives evidence on protein's liaison with inflammatory processes, but not the platelet function. CyPA reflects severity of acute CVD and presumably is an outcome of the event, but not the cause.
2. Platelet function in men with dyslipidaemia and other traditional risk factors of CVD and MS are prone to increased reactivity. According to the study results increased platelet reactivity can be considered as independent risk factor of CVD, but not the MS. Links between platelet functions and inflammatory processes are week and indirect. Presumably platelets are involved in the platelet driven thrombosis, but not inflammatory processes, which determine development of CVD and MS.
3. Ratios of lipid metabolism markers are independent prognostic markers of CVD and MS, and allow individuals to be differentiated between CVD cases and those who are only affected by the risk factors. Lipid-CRP ratios can be used as additional valuable tool for the estimation of risk of CVD, but not MS. CRP position in numerator of lipid-CRP ratios demonstrates better value for prediction of CVD.
4. Increased values of inflammatory markers (CRP, fibrinogen and vWF) in men with dyslipidaemia and other traditional risk factors of CVD and MS are independent risk factors of CVD, but not MS.

8. List of publications, theses and oral presentations

8.1. Publications

1. Banys V. **Role of platelet hyperfunction in pathogenesis of atherosclerosis and metabolic syndrome.** Laboratorinė medicina 2012; 3(55):136–146.
2. Banys V, Kučinskienė ZA, Andrejevaitė V, Kaminskas A, Jablonskienė V, Vitkus D. **Significance of cyclophilin A and traditional atherosclerosis biomarkers in cardiovascular diseases.** Laboratorinė medicina 2014; 16(1): 3–13.
3. Banys V, Andrejevaitė V, Šapoka V, Kučinskienė ZA. **Platelet function and indices in Lithuanian men with dyslipidemia: associations with inflammatory biomarkers.** Acta medica Lituanica 2014; 21(4): 161–7.

8.2. Theses and posters

1. Pikta M, Banys V, Olovjankina N, Viigimaa M, Tonne J, Titova T. **Samples storage time influence on whole blood impedance aggregometry results.** Poster at 45th Nordic Coagulation Meeting 2012, Stockholm, Sweden.
2. Banys V, Cimoš R. **Design and application of lupus anticoagulant diagnostic algorithm.** Biochemia Medica 2012; 22(3): A122. Poster at 2nd European Joint Congress of EFLM and UEMS "Laboratory Medicine at the Clinical Interface".
3. Banys V. **A case report of EDTA contamination and high potassium result in non-hemolyzed serum.** Biochemia Medica 2013; 23(1): A24–A25. Poster at 2nd EFLM-BD European Conference on Preanalytical Phase.
4. Bagdonaitė L, Banys V. **Relation between oxidized low density lipoproteins and chronic liver inflammation process.** Atherosclerosis 2014; 235(2): e163. Poster at 82nd European Atherosclerosis Society Congress.
5. Bagdonaitė L, Banys V, Povilonytė D. **Association of oxidative system components with chronic liver inflammation.** Clin Chem Lab Med 2014; 52: S1404. Poster at IFCC-WorldLab 2014 Congress.
6. Banys V, Kaminskas A, Kučinskienė ZA. **Cyclophilin A as a potential platelet hyperreactivity marker in patients with high risk of atherosclerosis and metabolic sindrome.** Clin Chem Lab Med 2014; 52: S253. Poster at IFCC-WorldLab 2014 Congress.

8.3. Oral presentations

1. 2012-12-06 "**Metabolic syndrome and atherosclerosis issues: the search of platelet and inflammation markers**" presented at conference of Lithuanian Society of Laboratory Medicine, Vilnius.
2. 2013-04-18 "**Pre-analytical issues of coagulation testing**" presented at joint international conference of Lithuanian Society of Laboratory Medicine and BD® "Importance of quality management in laboratory medicine: relevant pre-analytical and analytical questions", Vilnius.
3. 2013-10-03 "**Platelet function testing: what choice is best?**" presented at conference of Lithuanian Society of Laboratory Medicine, Klaipėda.
4. 2013-11-07 "**Aggregometry: plasma or whole blood?**" presented at conference of Lithuanian Society of Laboratory Medicine, Vilnius.

5. 2013-12-12 "**Review of Clinical Chemistry and Journal of Thrombosis and Haemostasis through the year 2013**" presented at conference of Lithuanian Society of Laboratory Medicine, Vilnius.
6. 2014-09-19 "**Coherence of platelet function with biochemical atherosclerosis and metabolic syndrome risk factors**" presented at XIIth Baltic Congress of Laboratory Medicine 2014, Riga.

9. Summary in Lithuanian

Įvadas. Širdies ir kraujagyslių ligos (ŠKL) Lietuvoje, kaip ir visoje Europoje, buvo ir tebėra pagrindinė mirties priežastis. Mirtingumas nuo širdies ir kraujagyslių sistemos ligų užima pirmąją vietą tarp visų mirties atvejų. Nuo šių ligų, GBD studijos (angl. *The Global Burden of Diseases, Injuries, and Risk Factors Study*) duomenimis, miršta 12,9 milijono žmonių (apie 25 % visų mirčių priežasčių). Pasaulio sveikatos organizacijos Europos regiono 53 šalyse nuo jų kasmet miršta 4,35 milijono, Europos Sąjungos (ES) šalyse – 1,9 milijono žmonių. Baltijos šalyse mirtingumas dėl šios priežasties yra didžiausias, o blogiausi vyrų ir moterų rodikliai užfiksuoti Lietuvoje. Vyrų standartizuotas mirtingumo nuo ŠKL rodiklis yra vidutiniškai 53 procentais didesnis nei moterų. LR SAM Higienos instituto sveikatos informacijos centro duomenimis, 2013 m. Lietuvoje kraujotakos sistemos ligos sudarė 47,6 % vyrų ir 65,0 % moterų mirties priežasčių.

ŠKL vystymasis yra neatsiejamas nuo esminių aterosklerozės patogenezės mechanizmų. Aterosklerozė – lėtinė arterijų sienelės liga, kuri skatina aterosklerozinių plokštelių (ateromų) susidarymą joje. Ligai progresuojant storėja intima, susidaro lipidiniai ruoželiai, putotosiose ląstelėse kaupiasi riebalai ir neląsteliniai lipidai. Susidariusi ateroma dėl jungiamojo audinio gausėjimo tampa fibroateroma, ji išopėja, įplyšta ir komplikuojasi aterotromboze. Ją lemia endotelio pažeidimas, nuolatinis uždegimo reiškinių skatinimas, trombocitų ir leukocitų prikibimas prie arterijų sienelių, monocitų, transformuotų į makrofagus, migracija į poendotelinį sluoksnį, modifikuotų (oksiduotų) lipidų darinių kaupimasis, kraujagyslių lygiųjų raumenų proliferacija. Netiesioginiai savarankiški aterosklerozės komplikavimosi ŠKL rizikos veiksniai yra rūkymas, arterinė hipertenzija, hipercholesterolemija, padidėjusi triacilglicerolių koncentracija, sumažėjusi didelio tankio lipoproteinų (DTL) cholesterolio koncentracija, nutukimas, cukrinis diabetas ar metabolinis sindromas (MS), netaisyklinga mityba, mažas fizinis aktyvumas, piktnaudžiavimas alkoholiu ir psichinis bei socialinis stresas. Vienas asmuo tuo pačiu metu gali turėti visą grupę rizikos veiksnių. Didėjant šių veiksnių skaičiui, ŠKL rizika didėja.

Naujausių tyrimų duomenimis, trombocitų vaidmuo neapsiriboja trombų suformavimu pažeidimo vietoje. Išskirdami aktyvias medžiagas, uždegimo tarpininkus, jie dalyvauja kaip uždegimo ląstelės. Vadinasi, tam tikra prasme yra susiję su aterosklerozės vystymosi uždegimo patogenezės teorija. Tai pasireiškia padidėjusiu trombocitų reaktyvumu, t. y. didesniu agregaciniu aktyvumu, trombocitų tūrio indeksų pakitimais (nepriklausomai nuo pakitusių parametrų derinio – padidėjusiu vidutiniu trombocitų tūriu, padidėjusiu trombocitų pasiskirstymo pagal dydį parametru, padidėjusiu didelių trombocitų skaičiaus santykiu su bendru trombocitų skaičiumi), intensyvesne P selektino ar kitų molekulių ekspresija, aktyvesne sąveika su monocitais ar kitomis leukocitų populiacijomis.

Aterosklerozės padarinių ankstyvosios diagnostikos žymenų paieškos lemia naujų biožymenų įtraukimą į kasdienę klinikinę praktiką. Vienas tokių ŠKL žymenų yra ciklofilinas A (CyPA). Tai viduląstelinis baltymas, kurį gali išskirti aktyvių deguonies formų veikiami monocitai/makrofagai, endotelio ląstelės, kraujagyslių lygiųjų raumenų ląstelės ir trombocitai. Keliose studijose aprašytas CyPA ir ŠKL ryšys. Nurodoma, jog padidėjusios šio baltymo koncentracijos yra vertinamos kaip ūminių koronarinių sindromų rizikos veiksnys.

Darbo tikslas – tradicinių aterosklerozės biocheminių žymenų kontekste ir pasitelkiant naujus biožymenis įvertinti trombocitų, kaip uždegimo ląstelių, svarbą esant ankstyvoms širdies ir kraujagyslių ligų bei metabolinio sindromo stadijoms.

Uždaviniai:

1. Atsižvelgiant į galimą ciklofilino A ir padidėjusio trombocitų reaktyvumo žymenų ryšį, įvertinti ciklofilino A tinkamumą ankstyvai širdies ir kraujagyslių ligų bei metabolinio sindromo diagnostikai ir prognozei, nustatyti ciklofilino A reikšmę vertinant asmenų, kuriems diagnozuota ūminė širdies ir kraujagyslių liga, būklę.
2. Įvertinti vyrų, kuriems nustatyta dislipidemija bei kiti tradiciniai ŠKL ir MS rizikos veiksniai, trombocitų polinkį į padidėjusį reaktyvumą ir ryšius su uždegimo žymenimis, nustatyti padidėjusio trombocitų reaktyvumo reikšmę vertinant ŠKL ir MS riziką.
3. Nustatyti lipidų apykaitos žymenų santykių, taip pat lipidų apykaitos žymenų ir C reaktyviojo baltymo (CRB) santykių reikšmę vertinant ŠKL ir MS riziką.
4. Įvertinti vyrų, kuriems rasta dislipidemija bei kiti tradiciniai ŠKL ir MS rizikos veiksniai, uždegimą atspindinčių rodiklių (CRB, fibrinogeno ir Vilebrando faktoriaus) vertę numatant ŠKL ir MS riziką.

Praktinė darbo reikšmė ir naujumas

Gydytojams, mokslininkams ir klinikiniams chemikams sukurti atrankinių tyrimų metodą ar modelį, kuris leistų užtikrintai, tiksliai, tačiau taupiai nustatyti padidėjusios ŠKL rizikos asmenis gerokai anksčiau, nei pasireiškia simptomai, yra didžiulis iššūkis. Prevencinės priemonės, tikėtina, bus efektyviausios šiuo laikotarpiu, nes aterosklerozei pasireikšti gali prireikti dešimtmečių. Visuotinai priimta širdies ir kraujagyslių ligų riziką vertinti tradiciniais laboratoriniais rizikos veiksniais: tai bendrasis cholesterolis, DTL cholesterolis, MTL cholesterolis ir triacilgliceroliai bei sistolinio kraujospūdžio vertės. Naujų biožymenų paieška ir tyrimai – tai galima alternatyva nusistovėjusioms prevencinėms priemonėms, pavyzdžiui, didelio jautrumo C reaktyviojo baltymo (CRB) tyrimas, kurio verčių padidėjimas lemia 60 % didesnę riziką susirgti širdies ir kraujagyslių ligomis.

Nors biožymeniu gali būti bet kuris biologinį procesą atspindintis veiksnys (nuo genetinių žymenų iki radiologinių vaizdinių tyrimo metodų), „tirpūs“ žymenys yra patraukliausi, nes tiriamąją medžiagą išgauti yra palyginti nesudėtinga, o rezultatai lengvai atkuriami (pakartojami). Šiame tyrime nagrinėjome galimybes panaudoti naują žymenį (ciklofiliną A) prognozuojant ir (ar) diagnozuojant ŠKL. Rezultatais parodėme, kad, nepaisant daug žadančių literatūroje pateikiamų duomenų, CyPA panaudojimas, vis dėlto yra ribotas. Nustatėme, kad, sergant ūminėmis širdies ir kraujagyslių ligomis, ciklofilino A vertės gali reikšmingai padidėti, tačiau asmenų, turinčių dislipidemiją ir (arba) kitų rizikos veiksnių, atvirkščiai, nei nustatyta daugelio užsienio mokslininkų, jos yra mažesnės. Be to, išsiaiškinome, kad širdies ir kraujagyslių ligų rizikos veiksnių kontekste ciklofilinas A yra labiau uždegimo procesus, o ne trombocitų funkcijas atspindintis žymuo.

Paskirų tyrimų vertinimo ne visada pakanka. Išeitis tokiais atvejais yra kompleksinis daugelio rizikos veiksnių vertinimo modelis, kurį taikėme savo tyrime. Naudotas laboratorinių rizikos veiksnių rinkinys yra unikalus, apimantis ne tik tradicinius lipidų apykaitos žymenų tyrimus, bet ir naujus, iki šiol Lietuvoje širdies ir kraujagyslių

ligų, taip pat metabolinio sindromo požiūriu nenagrinėtus žymenis: fibrinogeną, Vilebrando faktorių, trombocitų aktyvumą agregacijos ir tėkmės citometrijos metodais. Nustatėme, kad trombocitų reaktyvumo ir uždegimo procesų sąsajos yra netiesioginės. Be to, ŠKL ir MS rizikai vertinti taikėme apskaičiuojamus indeksus, tokiu būdu atskleisdami galimybes panaudoti lipidų apykaitos žymenų indeksų ir lipidų apykaitos žymenų santykių su CRB rezultatus.

Tyrimo metodai. Tyrimui buvo sudarytos trys grupės: kontrolinė ir dvi tiriamųjų grupės, kurios patogumo dėlei pavadintos rizikos ir įvykio grupėmis. Kontrolinei grupei buvo pasirinkti santykinai sveiki 20–24 metų amžiaus (n=42, 10 vyrų ir 32 moterys) asmenys. Tiriamųjų rizikos grupei atrinkti asmenys, turintys aiškių aterosklerozės ir metabolinio sindromo rizikos veiksnių (n=160, amžiaus vidurkis 48 metai). Atrankos kriterijais laikyti šie veiksniai: rūkymas, ūminis ar lėtinis stresas (su nerimo komponentu ar be jo), antsvoris, lipidų apykaitos žymenų rezultatų pakitimai, ūminės ŠKL nebuvimas (taip pat ir praeityje), vyriškoji lytis. Grupę sudarė vien vyrai, nes vyriškoji lytis yra vertinama kaip nepriklausomas ŠKL rizikos veiksnys. Trečiajai, įvykio, grupei buvo pasirinkti asmenys, kurie buvo paguldyti į Vilniaus universiteto ligoninės Santariškių klinikas dėl ūminio miokardo infarkto, pablogėjusios nestabilios krūtinės anginos ar kitokio koronarinės širdies ligos varianto ir kuriems buvo nustatyta padidėjusi troponino I koncentracija (amžiaus vidurkis 65 metai, n=64, 47 vyrai ir 17 moterų).

Kontrolinės ir rizikos grupių asmenims buvo atlikti šie laboratoriniai tyrimai: lipidų apykaitos žymenų (bendrojo cholesterolio, MTL cholesterolio, DTL cholesterolio, triacilglicerolių), didelio jautrumo C reaktyviojo baltymo (hs-CRB), gliukozės, fibrinogeno, Vilebrando faktoriaus (vWF), taip pat trombocitų agregacijos kraujyje su trombino receptorius aktyvinančiu peptidu (TRAP) ir ciklofilino A (CyPA). Rizikos grupės asmenims papildomai atlikti trombocitų tėkmės citometrijos tyrimai su CD42a, PAC-1, CD63 ir CD14 antigenais ir veninio kraujo tyrimas automatizuotu būdu (analizei panaudoti šie rodikliai: trombocitų skaičius (PLT), vidutinis trombocitų tūris (MPV), trombocitų pasiskirstymas pagal dydį (PDW), trombokritas (Pct) ir didelių trombocitų skaičiaus santykis su bendru trombocitų skaičiumi (P-LCR). Tyrimą papildžius įvykio grupe, šios grupės asmenims buvo atlikti šie laboratoriniai tyrimai: lipidų apykaitos žymenų, hs-CRB, gliukozės, kreatinino, troponino I (TnI) ir CyPA.

Visi laboratoriniai tyrimai, išskyrus vWF ir CyPA, buvo atliekami kraujo paėmimo dieną bendra tvarka kartu su kitais VUL Santariškių klinikų Laboratorinės medicinos centro tyrimais. Citratinės plazmos likučiai buvo šaldomi –20 °C laipsnių temperatūroje, o vWF tyrimai atliekami vieną kartą per mėnesį. Serumo likučiai buvo šaldomi –70 °C laipsnių temperatūroje, o CyPA tyrimai atlikti keliomis serijomis. Naują CyPA metodą įdiegė ir jo tyrimus, kaip ir fibrinogeno, TRAP ir vWF, atliko disertantas.

Atliktų laboratorinių tyrimų metodikos. hs-CRB – naujos kartos didelio jautrumo CRB tyrimas, atliktas imunoturbidimetrijos metodu (Architect ci8200, Abbott, JAV). Fibrinogeno koncentracija kraujo plazmoje nustatyta Klauso metodu (STA Compact, Stago, Prancūzija). Vilebrando faktoriaus antigeno (vWF:Ag) koncentracija kraujo plazmoje tirta imunoturbidimetrijos metodu (STA Compact, Stago, Prancūzija). Bendrojo cholesterolio koncentracija serume nustatyta fermentiniu (cholesterolesterazės / cholesteroloksidazės) kolorimetriniu metodu (Architect ci8200, Abbott, JAV), triacilglicerolių (TG) koncentracija serume – fermentiniu (glicerolfosfatoksidazės) kolorimetriniu metodu (Architect ci8200, Abbott, JAV). DTL-cholesterolio koncentracija

serume nustatyta dviejų pakopų fermentiniu (cholesterolesterazės / cholesteroloksidazės) kolorimetriniu metodu (Architect ci8200, Abbott, JAV). MTL-cholesterolio koncentracija apskaičiuota taikant Friedevaldo formulę, o jeigu TG>4,5 mmol/l, – tiesioginiu dviejų pakopų fermentiniu (cholesterolesterazės / cholesteroloksidazės) klirenso metodu (Architect ci8200, Abbott, JAV). Lipidų apykaitos laboratoriniai rodikliai (TC, MTL-ch, DTL-ch ir TG) buvo vertinami apskaičiuojant atskirų lipidų verčių santykius: TG/DTL-ch, lg[TG/DTL-ch], TC/DTL-ch ir MTL-ch/DTL/ch. Papildomai buvo skaičiuojami lipidų apykaitos žymenų ir CRB verčių santykiai: MTL-ch/CRB, CRB/MTL-ch, TC/CRB, CRB/TC, DTL-ch/CRB, CRB/DTL-ch ir lg[CRB/DTL-ch]. CyPA koncentracija serume nustatyta ELISA metodu (AMS Biotechnology (Europe) Ltd., Jungtinė Karalystė) „Gemini“ (Stratec Biomedical, Vokietija) analizatoriumi. Veninio kraujo automatizuotas tyrimas atliktas hematologiniu analizatoriumi „SYSMEX XE-5000“ (Sysmex Corporation, Japonija). Trombocitų agregacija tirta veniniame (visame) kraujyje *Cardinal* ir *Flower* (elektrinės varžos pokyčio matavimo) metodu „Multiplate“ analizatoriumi su trombino receptorius aktyvinančiu peptidu-6 (TRAP-6, toliau vartojamas trumpinys TRAP) (Roche Diagnostics, Vokietija). Trombocitų funkcinis aktyvumas agonistais nestimuliuotame kraujyje tirtas tėkmės citometrijos metodu (BD FACS Canto, BD Biosciences, JAV). Tyrimams naudoti žymenys: antikūnai prieš žmogaus CD42a trombocitams identifikuoti, PAC-1 ir CD63 trombocitų aktyvumui vertinti ir antikūnai prieš žmogaus CD14 (monocitų žymuo) (BD Biosciences, JAV). Gliukozės koncentracija kraujo serume nustatyta heksokinazės metodu (Architect ci8200, Abbott, JAV), kreatinino koncentracija – modifikuotu *Jaffe* metodu (Architect ci8200, Abbott, JAV). Troponino I (TnI) koncentracija nustatyta dviejų etapų chemiliuminescencinės mikrodalelių imunoanalizės (angl. *CMIA*) metodu (Architect ci8200, Abbott, JAV).

Duomenų statistinė analizė atlikta statistinės analizės programa SPSS (versija *PASW Statistics 18*). Buvo vertinamas duomenų išsidėstymo simetriškumas, grupių rezultatai išreikšti vidurkiu ir standartiniu nuokrypiu. Duomenims palyginti buvo atliekami parametriniai (neporinių modelių Stjudento t testas, vidurkių palyginimas ANOVA metodu) ir neparametriniai (Mano ir Vitnio U) statistiniai testai. Linijinės priklausomybės tarp kintamųjų laipsniui nustatyti buvo skaičiuojamas Spirmeno koreliacijos koeficientas (r). Koreliacija vertinta kaip silpna (kai $r<0,3$), vidutinė (kai $0,3\leq r\leq 0,7$) arba stipri (kai $r>0,7$). Diagnostinės kintamųjų ypatybės vertintos ROC kreivių metodu. Kintamieji buvo laikomi tinkamais diagnostiniais žymenimis, kai plotas po ROC kreive (AUC) buvo >0,5. Tiesinės regresinės analizės modeliai buvo laikomi tinkamais, jei jų determinacijos koeficientas r^2 buvo $\geq 0,25$. Visi statistinės analizės rezultatai buvo statistiškai reikšmingi, jei p vertė <0,05.

Rezultatai. Atlikti tyrimai nepatvirtino literatūroje skelbtų duomenų – baltymo CyPA koncentracija buvo statistiškai patikimai ($p<0,001$) didesnė kontrolinėje grupėje nei tiriamųjų grupėse (rizikos ar įvykio). Vis dėlto regresinės analizės duomenys parodė, kad CyPA koncentracijos didėjimas lemia kreatinino koncentracijos augimą (CyPA s/co B=46,5 t=2,7, p=0,009, VIF=2,4, $r^2=0,53$). Taip pat nustatėme, kad didesnių CyPA verčių atvejais trombocitų ir monocitų kompleksų absoliutūs ir (ar) santykiniai skaičiai yra statistiškai patikimai didesni (atitinkamai p=0,004 ir p=0,006). CyPA koncentracijos kitimų tiesinės priklausomybės nuo nukrypusių rezultatų skaičiaus nebuvo (p=0,280). Daugialypės regresinės analizės modeliai CyPA reikšmės nepatvirtino. Rizikos grupėje CyPA vertės koreliavo su trombocitų ir monocitų kompleksus atspindinčio

parametro CD42a/CD14 rezultatais: CyPA µg/ml su CD42a/CD14 įvykių skaičiumi (r=0,158, p=0,046); CyPA s/co su CD42a/CD14 % (r=0,175, p=0,027) ir CD42a/CD14 įvykių skaičiumi (r=0,202, p=0,010). Esant padidėjusioms MPV vertėms, rizikos grupėje CyPA koreliavo: su trombocitų TRAP agregacijos rezultatais (µg/ml r=0,262, p=0,020; s/co r=0,291, p=0,010) ir fibrinogeno rezultatais (s/co r=0,226, p=0,046).

Palyginus įvairių nagrinėtų kintamųjų skirtumus tarp keturių grupių, sudarytų pagal lipidų apykaitos žymenų nukrypimų skaičių, statistiškai patikimai besiskiriančių analičių nerasta. Trombocitų skaičiaus statistinio patikimumo lygmuo buvo artimas nusistatytai ribai (p=0,055), o trombokrito – lygus jai (p=0,050). Šių dviejų bendrojo kraujo tyrimo parametrų padidėjimas buvo patikimai tiesiškas (tiesiškumo p vertės buvo atitinkamai 0,007 ir 0,011). Vertinant trombocitų skaičių ir tėkmės citometrijos parametrus, nustatyta statistiškai patikima teigiama PLT koreliacija su CD42a/CD14 % (r=0,479; p<0,001), tačiau neigiama su CD42a registruojamų įvykių skaičiumi (r=–0,290; p<0,001) (PLT ir CD42a/CD14 r^2=0,171; PLT ir CD42a (PAC-1) r^2=0,064; PLT ir CD42a (CD63) r^2=0,077; visų p≤0,001). MPV atveju rezultatai priešingi: neigiama MPV koreliacija su CD42a/CD14 % (r=–0,281; p<0,001), tačiau teigiama su CD42a registruojamų įvykių skaičiumi (MPV ir CD42a/CD14 r^2=0,078; MPV ir CD42a (PAC-1) r^2=0,388; MPV ir CD42a (CD63) r^2=0,394; visų p<0,001). Rizikos grupės MPV verčių tiesinės regresinės analizės rezultatai atskleidė teigiamą MPV ryšį su CD42a, tačiau neigiamą su CD14 (p≤0,040). Tai patvirtina ir tėkmės citometrijos parametrų daugialypės regresinės analizės dviem požiūriais rezultatai: padidėjusios ŠKL rizikos požiūriu, kai asmenys turi 5–7 laboratorinius ŠKL rizikos veiksnius (p≤0,034), ir padidėjusios MS rizikos požiūriu, kai asmenys turi tris biocheminius MS rizikos veiksnius (p≤0,033). Trombocitų žymenų įtaka metabolinio sindromo diagnostikai nenustatyta. Tačiau ŠKL rizikos veiksnių požiūriu TRAP (>151 U) padidina riziką turėti 5–7 laboratorinius ŠKL rizikos veiksnius 4,6 karto (šansų santykis 4,6, 95 % PI 1,2–17,1, chi kvadratas 5,9, p=0,024), MPV (>10,4 fl) – 2,8 karto (šansų santykis 2,8, 95 % PI 1,5–5,3, chi kvadratas 9,9, p=0,002), o CRB (>3,0 mg/l) ir MPV (>10,4 fl) derinys – 2,8 karto (šansų santykis 2,8, 95% PI 1,2–6,6, chi kvadratas 5,6, p=0,021).

Iš kontrolinės grupės dėl nukrypusių lipidų apykaitos žymenų buvo pašalinta 11 narių. Nustatyti statistiškai reikšmingi lipidų, taip pat lipidų ir CRB santykių skirtumai (p≤0,017) šioje grupėje bei atitinkami statistiškai reikšmingi skirtumai tarp rizikos ir kontrolinės grupių (p<0,001). Lipidų, jų santykių bei lipidų ir CRB santykių vertės rizikos grupėje priklausomai nuo bendro laboratorinių ŠKL ir MS rizikos veiksnių skaičiaus (nuo 2 iki 7) nuosekliai didėjo (tiesiškumo p≤0,020), o vidurkiai skyrėsi statistiškai patikimai (p≤0,044). Vertinant tikimybę sirgti ŠKL, išsiskyrė CRB/MTL-ch, CRB/TC, CRB/DTL-ch santykiai ir lg[CRB/DTL-ch] vertės. Atitinkamai didesnės CRB/MTL-ch vertės (>1,83) padidina riziką sirgti ŠKL 8,5 karto (šansų santykis 8,5, 95% PI 4,1–17,2, chi kvadratas 40,7, p<0,001), CRB/TC (>1,19) – 12 kartų (šansų santykis 12,3, 95 % PI 5,7–26,5, chi kvadratas 53,3, p<0,001), CRB/DTL-ch (>7,87) – apie 10 kartų (šansų santykis 9,98, 95 % PI 4,7–21,1, chi kvadratas 44,3, p<0,001), o lg[CRB/DTL-ch] (>0,73) – 8 kartus (šansų santykis 8,2, 95 % PI 4,2–15,9, chi kvadratas 44,0, p<0,001). Pastarųjų tinkamumą ŠKL diagnozuoti įrodo ROC kreivių metodu gauti rezultatai: CRB/MTL-ch santykio AUC=0,741 (95 % PI 0,661–0,821, p<0,001), CRB/TC santykio AUC=0,752 (95 % PI 0,673–0,831, p<0,001), CRB/DTL-ch santykio AUC=0,727 (95 % PI 0,645–0,809, p<0,001) ir lg[CRB/DTL-ch] AUC=0,727 (95 % PI 0,645–0,809, p<0,001). Vertinant tikimybę turėti tris biocheminius MS žymenis, išsiskyrė TC/DTL-ch, MTL-ch/DTL-ch,

CRB/DTL-ch santykiai ir lg[CRB/DTL-ch] vertės. Atitinkamai didesnės TC/DTL-ch vertės (>6,0) padidina riziką turėti tris biocheminius MS žymenis 3,3 karto (šansų santykis 3,3, 95 % PI 1,8–4,7, chi kvadratas 24,5, p<0,001), MTL-ch/DTL-ch (>3,94) – 4,6 karto (šansų santykis 4,6, 95 % PI 1,5–14,7, chi kvadratas 7,1, p=0,009), CRB/DTL-ch (>7,87) – 2,2 karto (šansų santykio logaritminė išraiška 2,2, 95 % PI 0,1–4,4, chi kvadratas 5,6, p=0,041), o lg[CRB/DTL-ch] (>0,73) – 2,7 karto (šansų santykio logaritminė išraiška 2,7, 95 % PI 0,5–4,8, Chi kvadratas 8,9, p=0,014). ROC kreivių metodu MS prognoziniais žymenimis nustatyti: TG/DTL-ch (AUC=0,797, 95 % PI 0,728–0,865, p<0,001), aterogeniškumo indeksas (AUC=0,797, 95 % PI 0,728–0,865, p<0,001) ir TC/DTL-ch (AUC=0,640, 95 % PI 0,540–0,741, p=0,031). Trombocitų tėkmės citometrijos parametrų daugialypės regresinės analizės rezultatai patvirtina šių santykių vertę vertinant ŠKL ir MS riziką.

Iš nagrinėtų uždegimo žymenų fibrinogenas ir CRB leido patikimai prognozuoti didelius ŠKL laboratorinių žymenų skaičius (5–7): didesnės fibrinogeno vertės (>4 g/l) didino riziką apytiksliai 5 kartus (šansų santykis 4,98, 95 % PI 2,5–10,1, chi kvadratas 21,2, p<0,001), o CRB (>3,0 mg/l) – 2,4 karto (šansų santykis 2,4, 95 % PI 1,2–4,5, chi kvadratas 6,5, p=0,009). MS prognozuoti uždegimo žymenimis nepavyko. Nors didėjant laboratorinių ŠKL ir (ar) MS rizikos veiksnių skaičiui, uždegimo žymenys nuosekliai ir statistiškai patikimai didėjo (p≤0,001).

Išvados:

1. Remiantis atlikto tyrimo rezultatais, ciklofilino A (CyPA) tyrimas ankstyvai širdies ir kraujagyslių ligų (ŠKL) bei metabolinio sindromo (MS) diagnostikai ar jų išsivystymo prognozei nėra tinkamas. Nustatyta CyPA bei trombocitų ir monocitų kompleksų sąsaja rodo baltymo ryšį ne su trombocitų funkcijomis, o su uždegimo mechanizmais. CyPA atspindi ūminės ŠKL būklės sunkumą ir, tikėtina, yra įvykio padarinys, o ne priežastis.
2. Dislipidemiją ir tradicinių ŠKL ir MS rizikos veiksnių turinčių vyrų trombocitų funkcijos pasižymi padidėjusio reaktyvumo polinkiu. Remiantis tyrimo rezultatais, padidėjęs trombocitų reaktyvumas gali būti vertinamas kaip atskiras ŠKL, bet ne MS rizikos veiksnys. Trombocitų funkcijų ir uždegimo procesų ryšiai yra silpni. Tikėtina, kad trombocitų funkcijos lemia trombocitų kilmės trombozių, o ne uždegimo procesų sąlygotą ŠKL bei MS vystymąsi.
3. Lipidų apykaitos žymenų santykiai yra nepriklausomi ŠKL ir MS prognoziniai žymenys, kurie leidžia diferencijuoti rizikos veiksnių paveiktus asmenis nuo tų, kurie serga. Lipidų apykaitos žymenų ir C reaktyviojo baltymo (CRB) santykiai gali būti naudojami kaip papildoma ŠKL, tačiau ne MS rizikos vertinimo priemonė. Skaičiuojant santykius, CRB pozicija skaitiklyje geriau atspindi galimą sveikų asmenų ŠKL riziką.
4. Padidėjusios vyrų, turinčių dislipidemiją ir tradicinius ŠKL ir MS rizikos veiksnius, uždegimo rodiklių (CRB, fibrinogeno ir Vilebrando faktoriaus) vertės yra nepriklausomi ŠKL, bet ne MS rizikos veiksniai.

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12. Curriculum vitae

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- 1998–2004 – Vilnius University, Faculty of Medicine (Study program in medicine, MD);
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Postgraduate training courses:

1. Advanced course in thrombosis and hemostasis, European School of Hematology (ESH) and International Society on Thrombosis and Hemostasis (ISTH), 2011, Cascais, Portugal.
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Membership in professional societies and organisations:

- Member of American Association for Clinical Chemistry (AACC) (since 2006);
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- Board member and treasurer of Lithuanian Society of Laboratory Medicine (LLMD) (since 2012);
- Member of International Society on Thrombosis and Hemostasis (ISTH) (since 2012);
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Approbation:

Oral presentations:

1. Banys V. Platelet function testing: opportunities and experience. Xth Baltic Congress of Laboratory Medicine, 2010, Tallin, Estonia.
2. Banys V. Metabolic syndrome and atherosclerosis issues: the search of platelet and inflammation markers. Conference of Lithuanian Society of Laboratory Medicine, 2012, Vilnius, Lithuania.
3. Banys V. Pre-analytical issues of coagulation testing. Joint international conference of Lithuanian Society of Laboratory Medicine and BD® „Importance of quality management in laboratory medicine: relevant pre-analytical and analytical questions“, 2013, Vilnius, Lithuania.
4. Banys V. Platelet function testing: what choice is best? Conference of Lithuanian Society of Laboratory Medicine, 2013, Klaipėda, Lithuania.

5. Banys V. Aggregometry: plasma or whole blood? Conference of Lithuanian Society of Laboratory Medicine, 2013, Vilnius, Lithuania.
6. Banys V. Review of Clinical Chemistry and Journal of Thrombosis and Haemostasis through the year 2013. Conference of Lithuanian Society of Laboratory Medicine, 2013, Vilnius, Lithuania.
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