VILNIUS UNIVERSITY

Vytautas
ŽĖKAS

Extracellular Vesicles as a New Biomarker of Early Atherosclerosis in Healthy and Ischemic Heart Disease-Affected Male Populations

DOCTORAL DISSERTATION

Medical and health sciences,
Medicine M 001

VILNIUS 2019

This dissertation was written between 2015 and 2019 at the Department of Physiology, Biochemistry, Microbiology, and Laboratory Medicine, Insitute of Biomedical Sciences, Faculty of Medicine, Vilnius University.
The research was supported by a doctoral grant from Vilnius University.

Academic Supervisor – Prof. Habil. Dr. Zita Aušrelė Kučinskienė (Vilnius University, Medical and health science, medicine – M 001).

VILNIAUS UNIVERSITETAS

Vytautas
ŽĖKAS

Ląstelių mikrodalelės, kaip naujas ankstyvos aterosklerozės biožymuo sveikoje ir išemine širdies liga sergančių vyrų populiacijose

DAKTARO DISERTACIJA

Medicinos ir sveikatos mokslai,
medicina M 001

VILNIUS 2019

Disertacija rengta 2015 - 2019 metais Vilniaus universiteto Medicinos fakulteto Biomedicinos mokslų instituto Fiziologijos, biochemijos, mikrobiologijos ir laboratorinės medicinos katedroje.
Mokslinius tyrimus rėmė Vilniaus universiteto Medicinos fakultetas.

Mokslinė vadovė – prof. habil. dr. Zita Aušrelė Kučinskienė (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

CONTENTS

LIST OF ABBREVIATIONS

CRP – C reactive protein
CD105 – endoglin
CD144 – VE-cadherin
CD31 – platelet endothelial cell adhesion molecule (PECAM)
CD62e – e-selectin
CD9 – tetraspanin, cell surface glycoprotein
CD61 – integrin beta 3
CD42a – glycoprotein IX
DMSO – dimethyl sulfoxide
ECVs – extracellular vesicles
ESCRT – endosomal sorting complex required for transport
ELISA - enzyme-linked immunosorbent assay
EMVs – endothelial microvesicles
HDL-C – high density lipoprotein cholesterol
HPLC - high performance liquid chromatography
HSP70 – heat shock protein 70
LDL-C – low density lipoprotein cholesterol
MAPK – mitogen actvitated protein kinase
MDA – malondialdehyde
MI – myocardial infarction
MVB – multivesicular bodies
MFI – median fluorescence intensity
NTA – nanotracking analysis
PBS – phosphate buffered saline
PECAM – platelet endothelial cell adhesion molecule
PMVs – platelet microvesicles
PVDF membrane - polyvinylidene fluoride membrane
ROCK – Rho associated protein kinase
ROS – reactive oxygen species
RPMI medium – Roswel Park Memorial Institute medium
TC – total cholesterol
TSG101 – tumour susceptibility gene 101
vWF – von Willebrand factor

1. INTRODUCTION

In the past decade, extracellular vesicles (ECVs) have been understood as the potential players of intercellular communication. Now, these vehicles are intensively investigated in the range of pathological processes in hopes to use them as biomarkers for clinical diagnostics. This heightened interest is not surprising, since current biomarkers have left unmet needs in the early diagnosis of several diseases. Atherosclerosis and its clinical manifestations, such as ischemic heart disease, are one good example out of many. ECVs concentrations in peripheral blood present a previously unexploited source of biological and clinical information. Extracellular vesicles could represent the cell functions and indicate a damaged tissue better and sooner than any of the traditional marker. Besides, the cargo of these vesicles participates in the range of almost all cellular processes, both positive and negative. The investigation into the function of these vesicles is in its infancy. But this information has the potential to not only help understand the pathogenesis of atherosclerosis but also change the way we look at clinical diagnostics. The work presented here deals with the possibility of using ECVs as biomarkers. To achieve this objective, several methods were used to describe in detail the endothelial, platelet microvesicles (large extracellular vesicles) and exosomes (small extracellular vesicles), their numbers, associations with risk factors, and oxidative stress markers.

1.1 Relevance and Scientific Novelty of the Research

The data presented in this work is important for better understanding the relationship between oxidative and chronic stress from one side and the formation of endothelial microvesicles in early atherosclerosis environment from the other. Associations between atherosclerosis risk factors, oxidative and chronic stress markers (malondialdehyde, thioredoxin, and cortisol), and the total counts of microvesicles and exosomes found in this work are important for future clinical research. Extracellular vesicles participate in delivering diverse messages and influencing target cells. This could give a not only better understanding of the processes involved but also better information about the state of disease. Ultimately, microvesicle and exosome numbers could be established as biomarkers in the field of the early diagnosis of atherosclerosis and its clinical manifestations.

1.2 Research Objective

To detect and investigate endothelial and platelet-derived microvesicles and exosomes in young (25–39 year old) and healthy elder (40–60 year old) as well as post-myocardial infarction (MI) (40–60 year old) male populations and show their association with atherosclerosis risk factors and oxidative stress.

1.3 Research Tasks

1. To measure and evaluate the total counts of endothelial and platelet microvesicles in the peripheral blood of myocardial infarction patients and healthy subjects by flow cytometry.
2. To investigate endothelial microvesicles expressing CD62e (activation level) by using flow cytometry.
3. To detect and investigate exosomes and measure their count in peripheral human blood of MI patients and healthy subjects using the Western blot, a nano tracking analysis, and flow cytometry.
4. To measure the malondialdehyde concentration in the serum of myocardial infarction patients and healthy subjects using high-performance liquid chromatography and evaluate its link to the microvesicle number and activation level.
5. To measure the concentrations of lipid markers (total cholesterol, high and low density lipoprotein cholesterol), C reactive protein, and glucose in the serum of myocardial infarction patients and healthy individuals and evaluate their relation to the microvesicle counts.

1.4 Statements to be Defended

1. The total count of extracellular vesicles and their ratios from the total count of EMVs are different between healthy subjects and those suffering from disease.
2. The activation level of endothelial microvesicles is different between healthy and diseased subjects and important in understanding the pathogenetic mechanisms of atherosclerosis and endothelium injury.
3. The exosome count is different between healthy and diseased subjects and is associated to oxidative processes.
4. Extracellular vesicles (microvesicles and exosomes) are associated to the oxidative stress factor – malondialdehyde concentration in healthy subjects.

5. Atherosclerosis risk factors (such as lipid concentrations and systolic blood pressure) are associated to the total counts of microvesicles and exosomes in a diseased state.

1.5 Internship

Tartu University Technology Institute, Tartu, Estonia, May 14–25, 2018 and January 15–February 1, 2019. Host – director of the institute, Dr. Reet Kurg. During the internship, exosome counts and their association with oxidative stress were evaluated. Methods used during the internship: Western blot, flow cytometry, nano tracking analysis, determination of protein concentration by Bradford, and enzyme-linked immunosorbent assay.

2. LITERATURE REVIEW

2.1 Atherosclerosis and Extracellular Vesicles

Cardiovascular diseases remain one of the main causes of death worldwide, according to the American Heart Association [1]. In Lithuania alone, there were 168 male deaths for 1000 inhabitants in 2015, according to data from the Statistical Bureau of the European Union [2]. Avoidable death statistics show that most of these unfortunate deaths could be avoided if better health care or diagnostic protocols would be developed [2]. Atherosclerosis is still considered as one of the most important pathologies in the modern world, and the detection of early changes in the vasculature and prognosis of this disease is still lacking [3]. The definition of extracellular vesicles covers a wide range of vesicles differing in size and function. These vesicles participate in the communication processes, so they could be dully named comunicasomes. During the last decade, the overall interest in the extracellular vesicles increased, especially in various biomedical research areas including cardiology. Some studies used these markers to investigate diabetes, [4] neurodegeneration and ischemic stroke, [5] also chronic heart failure. [6] Studies have shown the association of microvesicles positive for CD144 with high systolic blood pressure [7]. Endothelial microvesicles (EMVs) carrying CD144+ and CD41a- or CD31+ and CD41- were found to be associated with ischemic stroke and significantly correlated with stroke intensity [8]. These studies identified different microvesicle populations that appeared to have different associations with disease prediction. Intensive investigations are now targeted toward explaining the role of ECVs in intercellular communication within a range of pathological processes, such as atherosclerosis and its clinical manifestation – ischemic heart disease [9].

As markers, these ECVs could undoubtedly be sensitive, but their use in the clinical field is still problematic [10]. Some of the ECVs' assays, mainly in cancer diagnostics, are already demonstrating clinical utility and gaining FDA approval [11]. Yet we still lack the standardization of isolation and characterization methods for the ECVs' populations, and these isolation procedures typically yield complex mixtures of different and distinct subgroups of ECVs, which spoil the result and make it hard to produce a sound clinical decision [12]. Also, most of today's methods of isolation for ECVs are very time-consuming and expensive for clinical laboratories. This makes it challenging to demonstrate biomarkers or drivers of diseases [12]. These inconsistent data, especially concerning the phenotypes of ECVs, muddy their importance as effective biomarkers [13].

2.2 Oxidative Stress and Extracellular Vesicles

Oxidative stress is implicated in the pathogenesis of atherosclerosis [14] and is caused by the production of excess reactive oxygen species (ROS). The ROS are formed naturally and act as regulators of various cell functions and biological processes. The oxidative stress processes are important in the pathogenesis of myocardial infarction and post-stroke survival [15]. Malondialdehyde (MDA) concentration in blood plasma is proposed as an effective oxidative stress biomarker. It has been shown that this marker correlates with tissue damage in both acute and chronic diseases [16]. This substance was found to have an ability to form epitopes on endothelial cells, which could have importance as a biomarker in cardiovascular disease [17]. Most of this substance is produced during the reaction process from the decomposition of products of lipid peroxidation, and it helps evaluate the oxidative stress level in the vascular system [18]. Lipids are the class of molecules mostly involved in the oxidative stress. Malondialdehyde is the principal and mostly studied product of peroxidation of polyunsaturated fatty acid. MDA can predict the progression of coronary artery disease three year in advance of the disease and is implicated in the pathogenesis of atherosclerosis [19]. This means that MDA is a useful biomarker and could be used as a reliable tool for the determination of oxidative stress [20]. Since an uncontrolled production of reactive oxygen species is implicated in vascular injury [21], the quantity of ECVs with it is important. Fundamental studies describe the involvement of ECVs in oxidative stress and inflammation affecting angiogenesis [22]. MDA epitopes were also found on microvesicles of the platelet [23] and monocytic origin [24]. It is possible that these MDA epitopes carrying ECVs could be pro-inflammatory, since in the presence of anti-MDA antibodies, ECVs lost their ability to stimulate the production of interleukin 8 [23]. These findings show that immunization against MDA epitopes could be valuable in the therapeutic field as well [16]. But most importantly, ECVs could serve as a possible tool for diagnostic purposes, by showing the amount of damage inflicted on the tissue. Yet we still need further evaluations of chronic inflammation and sustained stress involvement in the recurrence of coronary stenosis and cardiac failure [25].

2.3 Extracellular Vesicles in Vascular Biology

The vascular wall is an active organ constantly adapting to its environment. Usually, it does this by synthesizing biological factors, depending on signals from the outside, allowing the renewal of the organ itself. This process is better

known as vascular remodeling. It involves cell death, migration, or growth, as well as changes in the extracellular matrix [26]. ECVs are important factors in vascular remodeling and, as agents of intercellular communication, they probably control the processes in the vascular wall [27]. The participation in coagulation function is strongly implicated in the literature, especially for platelet microvesicles (PMVs). PMVs are most abundant in peripheral blood plasma and are strongly pro-thrombotic [24]. It has been shown that in the ischemic processes associated with coagulation defects, the number of ECVs increases [28]. Endothelial derived microvesicles could be a novel biological marker for endothelial injury [29]. These ECVs are important players in vascular remodeling and endothelial regeneration [30]. Their role in ischemic heart disease is probably underestimated and needs more intensive research. Exosomes are also important in atherosclerosis research [31], but it is difficult to investigate them since their functions or their cargo are still largely unknown. But in the recent years, more studies are exploring their possibilities as biomarkers [32].

2.4 Generation and Classification of Extracellular Vesicles

Extracellular vesicles are of various sizes, between 100 and 1000 nm, and they carry inside a variety of proteins, lipids, sugars, and genetic material that may influence various cellular processes and the function of the recipient cell. ECVs can be classified very broadly into 3 main classes: ectosomes (large extracellular vesicles), exosomes (small extracellular vesicles), and apoptotic bodies (Table 1), which are discussed below [32]. This classification is far from perfect and mainly deals with vesicle size and their pathway of synthesis in the cell. For clinical settings, this classification is not sufficient enough, since it does not give the site of injury. The classification of ECVs depending on size and cell origin is discussed below.

Table 1. Classification of ECVs according to their size.

Extracellular vesicles	Size	Synthesis	Molecular markers
Exosomes (small extracellular vesicles)	50–100 nm	Endosomal pathway	TSG101, CD63, CD81, CD9, Flotillin-1, HSP70.
Microvesicles (ectosomes, large extracellular vesicles)	100–1000 nm	Forms by bubbling from cell membrane	Integrins, selectins, various adhesion molecules (CD144, CD105, CD62e).
Apoptotic bodies	> 1000 nm	Forms during cell apoptosis	Cell specific markers (for instance CD3 for lymphocytes).

2.5 Microvesicles (Large Extracellular Vesicles)

The size of these membranous structures does not reach more than 1 μm (micrometer) and they carry on their membrane markers from the cell surface, which could be the remnant of their cell of origin. Biogenesis occurs via direct outward blebbing and pinching of the plasma membrane, which in turn releases ectosomes in the extracellular medium [33]. Mechanisms of their formation are generally concluded from experiments with cell cultures investigating their capacity to generate ECVs after stimulation with some triggers, like cytokines or reactive oxygen species, and this is still inconclusive [34]. Thus far, the mechanism of release *in vivo* or *in vitro* is not fully understood, but some findings suggest that this mechanism could include elements that are common with exosome formation [35]. The mechanism of release could be associated with the asymmetry of the membrane. Membrane lipid synthesis is inherently asymmetric. Phospholipids are generally synthesized on the cytosolic side of the endoplasmic reticulum [36]. However, the addition of phospholipids to one side of the membrane generates an unstable membrane and leads to changes in the shape of the membrane-like membrane bending [37]. This is rectified by a lipid transporter [38] that redistributes lipids across a membrane. But sphingolipids, which are generally on the outlet leaflet of the membrane, in addition to more sphingolipids in this leaflet, could also lead to changes in the membrane structure [39] and lead to outward budding. This outward budding mechanism does remind us of exosome formation when a vesicle forms by budding inwards

the membrane of the multivesicular body (MVB). It has been shown that the ECVs of both types are enriched in cholesterol, ceramide, and other lipids implicated in the formation of a lipid raft, leading to membrane changes discussed here [40]. For instance, ceramide production could promote membrane curvature during ectosome formation [41]. These localized changes in the plasma membrane are complemented by a vertical redistribution of the microvesicle cargo, which is distinct for every type of the membrane shedding microvesicle. But microvesicles are not enriched by random cargo; these are selectively recruited [42].

Another proposed mechanism is associated with an event when phosphatidylserine shows on the outward leaflet of the cell membrane. This event usually precedes the shedding of the ectosomes. It has been found that this happens through the activation of ROCK (Rho-associated protein kinase) and p38 MAP kinase (mitogen-activated protein kinase) by energizing the P2X7 receptor. This is similar to the formation of the apoptotic bodies, but in the case of ectosomes, the process is reversible [43]. This event could lead to scramblase, floppase, and flippase activities, which will form membrane curvature during ectosome formation [44]. The buildup of the membrane is also observed at microvillar tips, which shows another possible mechanism of release [45].

2.6 Exosomes (Small Extracellular Vesicles)

Exosomes are formed within the endosomal network and released in exocytic bursts upon a fusion of multi-vesicular bodies with the plasma membrane. Generally, exosomes are smaller than microvesicles (Table 1). How they are enriched with specific cargoes and how distinct are the budding of exosomes in the lumen of MVB is still a debate. It is suggested that ceramide enrichment could be the driving force in exosome formation [46]. The release of exosomes in the extracellular medium could occur through two different mechanisms, which involves: an endosomal sorting complex required for transport (ESCRT) [47] and an ESCRT-independent tetraspanin action [48]. But the impulse that leads to the release of exosomes still needs more explanation. These vesicles are found all over the body: in blood, saliva, urine, milk, semen, bile juice, ascites, and bronchoalveolar and gastrointestinal lavage fluid [49]. Exosomes could be associated with the immune system, and the inflammation could play a part in metabolic diseases [50].

2.7 Uptake of Microvesicles and Exosomes

Extracellular vesicle uptake by target cells appears to depend on the type of recipient cells. In most instances, the uptake of ECVs seems to occur through phagocytosis [51]. Macropinocytosis may also be another way through which ECVs may transfer their content [52]. Membrane fusion requires pH 5.0, since this creates a similar fluidity between the 2 fusing membranes [53]. It has been observed that multivesicular bodies (MVB) have a pH of ~5 and that the fusion of the intraluminal vesicles to the MVB membrane has been reported [54]. The key influence of the microenvironment's pH suggests that the differences in the electrostatic charges between ECVs and the plasma membrane of the cells should be considered concerning the physiological roles of the ECVs. This could be important in understanding their functions and their targets *in vivo.*

2.8 Other Types of Extracellular Vesicles

Another type of extracellular vesicles are the apoptotic bodies – they originate from fragmented apoptotic cells with sizes up to several micrometers. They are usually formed when a cell's cytoskeleton breaks up, and this, in turn, causes the cell membrane to bulge outward [55]. These entities are now understood as a type of ECVs. It was recently understood that the fragmentation of the cell during apoptosis is controlled by several steps, known as apoptotic cell disassembly, which includes the generation of membrane blebs, apoptotic membrane protrusions, and fragmentation into apoptotic bodies [56]. Their functions within the body are largely unknown and were thought to mainly help phagocytes clear the cell debris after apoptosis [57], but it seems that inside they carry DNA and other biological molecules and could be divided inside into other subgroups by determining their origin and surface molecules [58]. This could show them as agents and biomarkers of diseases.

More exotic particles are being found in the recent years. One type of them are exomeres, which are lipoprotein-like vesicles that have a one-layer lipid membrane [59]. It looks like these new facts could change the classifications and understanding of ECVs and their importance in the body.

2.9 Classification by Cell Origin

Although ECVs can be classified according to their cellular origin, recent studies suggest a high degree of heterogeneity [60]. Specific characteristics have been proposed for different subgroups of ECVs in some instances [61], but currently, there is still a lack of widely accepted specific

phenotype markers to distinguish these populations by their origin [62]. This leads researchers to use more and more markers that could eventually be too complex and too expensive for clinical settings. That is why we need more established and specific biomarkers.

2.10 History of Extracellular Vesicles

ECVs first were observed as procoagulant platelet-derived particles in normal plasma [63], called “platelet dust” by Wolf in 1967 [64]. In 1969, Anderson found matrix vesicles during bone calcification [65]. Virus-like particles were found in human cell cultures and bovine serum [66]. Cancerous ECVs were observed in the 1980s and were shown to be procoagulants [67]. In 1983, multi-vesicular bodies (MVB) were shown fusing with the cell membrane during the differentiation of immature red blood cells [68]. In 1996, exosomes, isolated from Epstein-Barr virus-transformed B lymphocytes, were antigen-presenting and able to induce T cell responses [69]. In 2006–2007, the discovery that ECVs contain RNA, including microRNA, was made [70]. This discovery led to a renewed scientific interest. In 2010s, ECVs have been isolated from most cell types and biological fluids and shown to be important as mediators in intercellular communication [71]. Since 2011, the members of the International Society of Extracellular Vesicles (ISEV: www.isev.org/) were collaborating in the work to unify the nomenclature and the methodologies of ECVs.

3. METHODS

3.1 Subject Inclusion Criteria

Eighty-one healthy individuals were examined between December 2015 and July 2017 and included in the work. Healthy subjects had no prior history of acute cardiovascular disease and were not under any treatment for cardiovascular diseases. All subjects were 25 to 60-year-old males. Healthy subjects were grouped according to age into two groups: (1) healthy young individuals (25–39 years old) and (2) healthy elder individuals (40–60 years old) (Figure 1). No female subjects were included in this work, as males usually have an earlier onset of disease than their female counterparts [72]. This work design also allowed having a more homogenous population and more representative results, since biomarkers between sexes are slightly different and it was not in the scope of this work to compare differences between sexes. Additionally, the exosomes were investigated in the healthy elder and post-MI patients, and this was not done in the healthy young group, since more obvious differences were found between the healthy elder group and post-myocardial infarction patients.

The test group included fifteen patients. They have been treated for MI in a cardiac intensive care unit, an intensive care unit, and the first cardiology ward of Vilnius University Santaros Klinikos Hospital three months prior to the inclusion. Patients were enroled later in the work than healthy subjects – between February and May of 2018. Workflow for the inclusion of healthy subjects and patients is shown in Figure 1. All of them were aged 40 to 60 years old. Patients had myocardial infarction for the first time in their life, and coronary angiography confirmed coronary occlusion of more than 50% in at least two arteries. Coronary catheterization and coronary angioplasty were performed on all patients. Patients with acute venous thromboembolism were excluded, since this condition might increase the number of tissue factor-positive EMVs in peripheral blood plasma [73]. At the time of blood sample collection, patients were under antihypertensive treatment and received aspirin and statins. Twelve patients had primary arterial hypertension. Five patients have been diagnosed with diabetes mellitus. Various systemic inflammatory diseases (such as psoriasis, bacterial infections, autoimmune diseases, and others) affected four patients at the time of blood collection. Only one patient had systemic inflammation, diabetes mellitus, and primary arterial hypertension all together. The protocol was approved by the local Vilnius University Bioethics Committee (No.: 158200-18-990-495 and 158200-15-807-319).

Patients and healthy subjects provided written informed consent. Signed informed consent forms were archived.

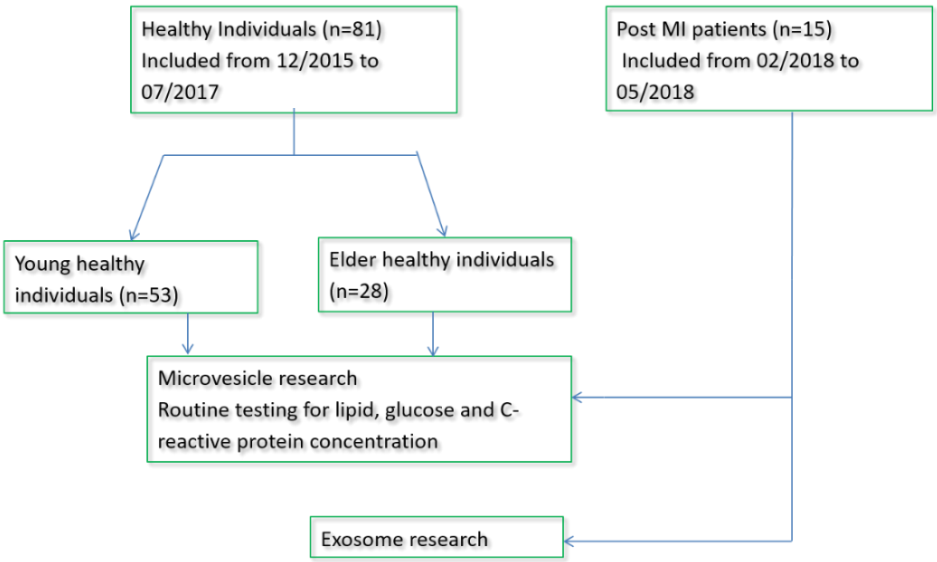


Figure 1. Summary of the workflow of the study.

3.2 Blood Sampling and Testing

For the enumeration of EMVs and PMVs, 4 ml of blood was drawn from the arm vein into the citrated vacutainer tubes. To produce platelet-free plasma, the tubes were then subjected to differential centrifugation. First, they were centrifuged at 3000g rcf for 10 minutes, and three-quarters of recovered plasma were transferred into a sterile tube and centrifuged again at 10000g rcf for 30 minutes. The resulting pellet was reconstituted with 1 ml of a serum-free RPMI (Roswell Park Memorial Institute) medium (Biological Industries, Israel) and analyzed using the LSR Fortessa (BD, California) flow cytometer.

For exosome preparation, an additional centrifugation step of 100000g for 4 hours was undertaken. The resulting pellet was constituted with 1 ml PBS and tested using flow cytometry, Western blot, or enzyme-linked immunosorbent assay (ELISA). This workflow is presented in Figure 2.

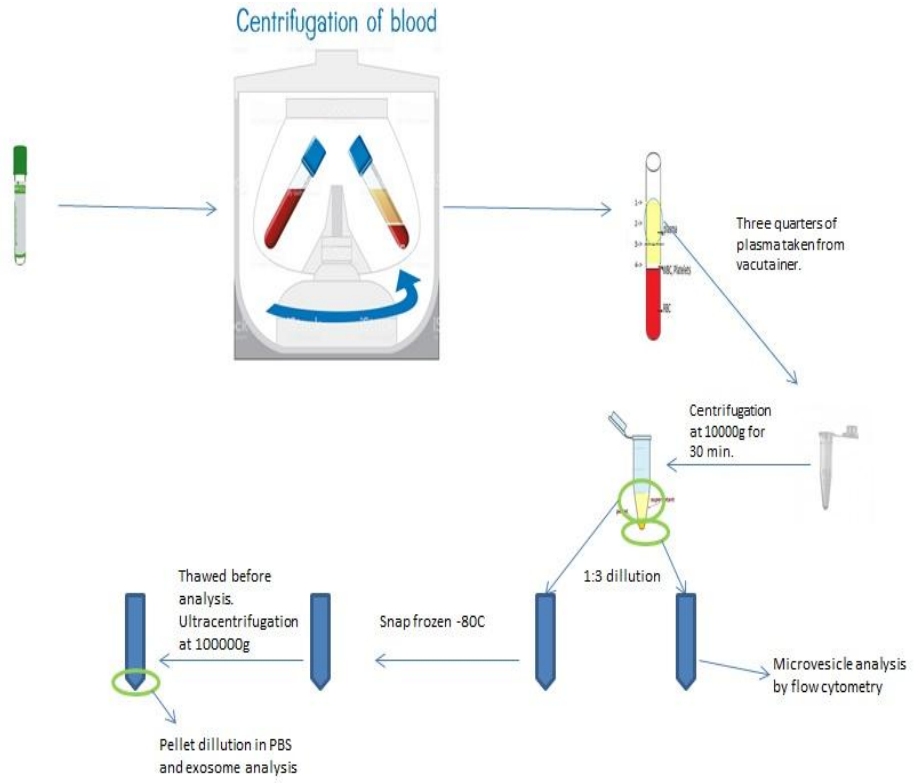


Figure 2. Workflow scheme for microvesicle and exosome sample preparation.

For C reactive protein (CRP), malondialdehyde (MDA), triglyceride, and total cholesterol (TC) concentration measurements, another 4 ml of blood were drawn from a vein into serum vacutainer tubes containing serum separation gel. Both vacutainers were taken at the same time – in the morning, after night fasting. These tubes were always taken first.

3.3 Clinical Chemistry Tests

The concentrations of C reactive protein (CRP), glucose, total cholesterol, triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were measured in the blood of patients and healthy subjects using routine techniques of a clinical chemistry laboratory (Architect ci8200, Abbott, Chicago, Illinois).

3.4 Derivatization and the High Potency Liquid Chromatography Method for the Determination of Malondialdehyde in the Serum

MDA is a marker of lipid peroxidation and is often used to represent the level of oxidative stress [74]. The serum MDA concentration was measured using a method by Khoschsorur G.A. et al. [75]. In brief, 50 µl of thawed serum was mixed with 750 µl of 0.44 M phosphoric acid solution, 250 µl of 42 mM thiobarbituric acid solution, and of 450 µl of deionized water. The prepared samples were incubated in a thermostat at 100°C for 60 minutes. After incubation, the samples were rapidly cooled in an ice bath. In the next step, the 500 µl of the sample was diluted in a methanol solution (1:1). The sample was mixed by shaking and then centrifuged for 3 min at 10000 g. After centrifugation, the 500 µl of the centrifugate was added to the chromatographic vial, and the sample was analyzed via the high-performance liquid chromatography (HPLC) method. The malondialdehyde concentration was measured by a Shimadzu Nexera X2 UHPLC system (Shimadzu). Data were collected and processed using the LabSolutions software (Shimadzu).

3.5 Flow Cytometry

3.5.1 Endothelial and Platelet-Derived Microvesicles from Human Plasma

Commercial fluorescent polystyrene beads were used: Megamix-Plus FSC (Bio-Cytex, France). This mix contained green fluorescent bead populations with sizes of 100, 300, 500, and 900 nm from the Megamix-Plus FSC bead set. Using these beads the probable location of microvesicles smaller than 1 µm was determined on a dot plot. Data on a flow cytometer were acquired with thresholds on SSC or fluorescence, and at low flow rates. Buffer controls were used to identify background signals for all instruments. A standard sheath fluid without further filtration was used in all measurements.

All samples were labeled with anti CD144-FITC (anti VE-cadherin), anti CD105-BV421 (anti endoglin), anti CD42a-PerCP (anti glycoprotein IX), anti CD62e-PE (anti e-selectin), anti CD31-APCy7 (anti PECAM (platelet endothelial cell adhesion molecule)-1), and anti CD61-APC (anti Integrin beta-3) (BD, San Jose, California). All events were gated on a forward vs. side-scattered light dot plot according to their forward scatter intensity (size). Each analysis included 1 000 000 events. Total counts of populations of EMVs and PMVs were quantified by flow cytometry and expressed as microvesicles/µl. The CD62e expression was calculated as a percentage from the total count of respective endothelial microvesicles populations. When constructing the panel

of cellular markers for this work, the antigens that were selected were highly expressed by endothelial and platelet cells or exosomes to achieve the most precise labelling of ECVs. A summary of the chosen markers is presented in Table 2.

Table 2. Summary of biomarkers for the analysis of microvesicles.

Marker	Function	Origin
CD144	VE-cadherin. Responsible for angiogenesis and intercellular communication.	Endothelial cells
CD105	Endoglin. Important for angiogenesis.	Endothelial cells, macrophages
CD31	Platelet endothelial cell adhesion molecule (PECAM-1). Important for angiogenesis and integrin activation. Associated with endothelial cell apoptosis.	Platelets, endothelial cells, monocytes, granulocytes, and B-cells
CD62e	E-selectin. Adhesion molecule. Participates in inflammation and recruiting leukocytes.	Activated endothelial cells
CD42a	Glycoprotein IX. It forms a 1-to-1 noncovalent complex with glycoprotein Ib, a platelet surface membrane glycoprotein complex that functions as a receptor for the von Willebrand factor.	Platelets
CD61	Integrin subunit beta 3. Integrins are known to participate in cell adhesion as well as cell-surface mediated signalling.	Platelets

The size and granularity of endothelial microvesicles were analyzed using a forward-scattered light set to a logarithmic scale and side-scattered light set to a logarithmic scale. As shown in Figure 3, the population of endothelial microvesicles was first identified according to their light scattering characteristics, representing their relative size.

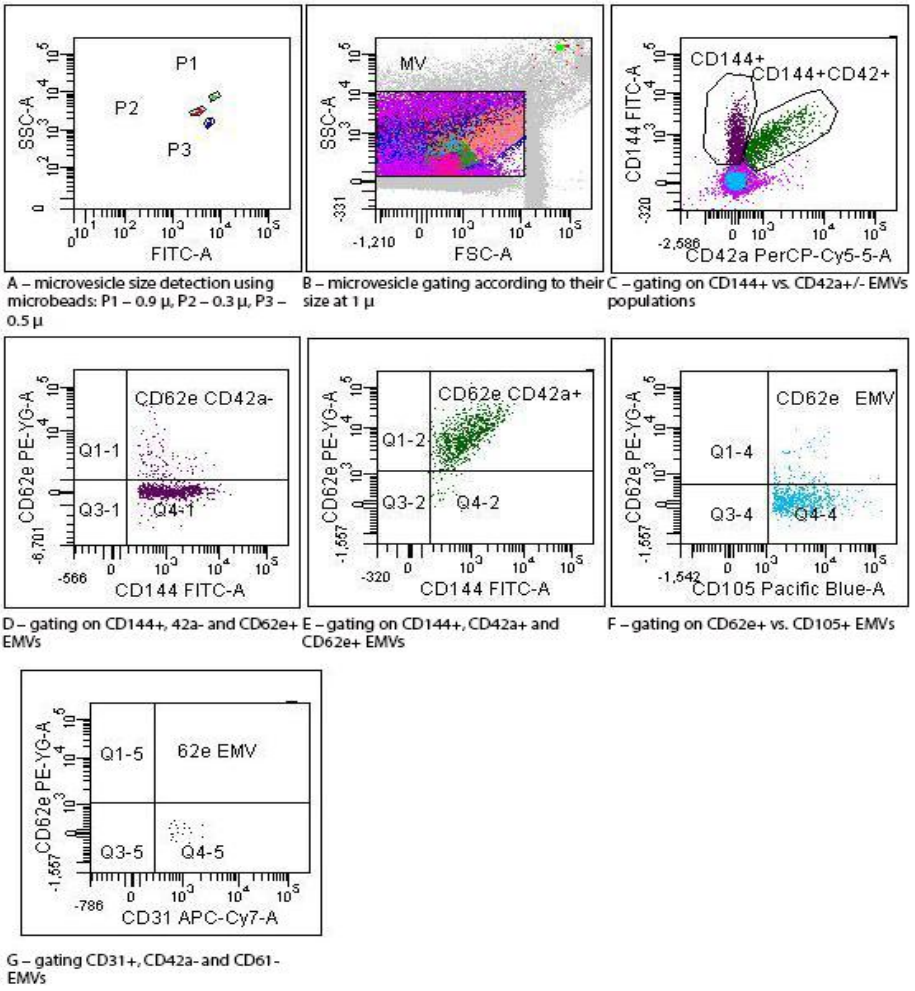


Figure 3. Flow cytometric analysis of endothelial-derived microvesicles from plasma:

A – microvesicle size detection using microbeads: P1 – 0.9 µ, P2 – 0.3 µ, P3 – 0.5 µ; B – microvesicle gating according to their size at 1 µ; C – gating on CD144+ vs. CD42a+/- EMVs populations; D – gating on CD144+, 42a- and CD62e+ EMVs; E – gating on CD144+, CD42a+ and CD62e+ EMVs; F – gating on CD62e+ vs. CD105+ EMVs; G – gating CD31+, CD42a- and CD61- EMVs

The total numbers of platelet microvesicles (PMVs) were also determined by detecting CD61 on the membrane. Two unexpected, distinct PMV populations were found: large and small. The large PMVs could be the leftovers of small platelets, and because of this fact, only small platelet microvesicles were included in the statistical analysis.

3.5.2 Exosomes from Human Plasma

A summary of the chosen markers for the analysis of exosomes is presented in Table 3.

Table 3. Summary of biomarkers for the analysis of exosomes.

Marker	Function	Origin
CD9	Tetraspanin. Important in cell communication. Enriched in exosomes.	All cells
Flotillin-1	Endosomal protein. May act as a scaffolding protein within caveolar membranes, participating in the formation of caveolae-like vesicles	All cells
Thioredoxin	Small redox protein. Plays a central role in the response to oxidative stress specifically to reactive oxygen species.	All cells
TSG101	Tumour susceptibility gene 101. Participates in MVB vesicle budding	All cells
HSP70	Heat shock protein 70. Interacts with extended peptide segments of proteins as well as partially folded proteins to prevent aggregation, remodel folding pathways, and regulate the activity of proteins.	All cells

A different method was used for CD9 detection on exosomes, described by Mellisho et al. [76]. The first sample volume was adjusted to total protein concentration, and phosphate-buffered saline (PBS) was added to an equal amount of 200 µl. The vial containing only PBS was used as a negative control. To all samples and negative control, 5 µl of Latex beads were added and left overnight at 4^{O}C. In the morning, 1 µl of 2M glycine was added to each sample and the negative control vial and kept for 45 min at room temperature. Then, two washing steps for 4000 rpm for 5 min at 4^{O}Cwere made using a PBS/BSA 0.5% block solution, and samples were left for 20 min. at room temperature; 47 µl of anti-CD9 (Abcam, Germany) were diluted with a stock solution of 94 µl

PBS by the subsequent adding of 3 µl of the said solution to each tube. Samples with antibodies were incubated for 1 hour at room temperature and centrifuged once at 4000 rpm for 5 min at 4^{O}C. The supernatant was removed until 100 µl was left in the tubes, and 400 µl of PBS was added and carefully suspended. This solution was tested on a LSRII flow cytometer (BD, San Jose, California).

3.6 Western Blotting

For the Western blot analysis, samples were loaded onto 10% or 12% polyacrylamide gels. The samples were boiled in a Laemmli buffer for 10 minutes and resolved in a polyacrylamide gel at 150V. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Biorad) for 30 min at 15V. Membranes were subsequently blocked with 5% nonfat dry milk and incubated overnight at 4^{O}C with primary antibodies (anti-tumor susceptibility gene 101 (TSG101), anti-flotillin-1, anti-heat shock protein 70 (HSP70)) at 4^{O}C. After washing, membranes were probed with secondary antibody anti-mouse or anti-rabbit (depending on the antibodies used) horseradish peroxidase (Thermo Fisher Scientific).

3.7 Nanotracking Analysis

The measurements of particle size and concentration in ECVs containing samples by nanotracking analysis (NTA) were performed on the ZetaView platform (ParticleMetrix, Germany) equipped with a 488 nm laser. All samples were analyzed at 25 ^{O}C. Samples were diluted in PBS to an appropriate concentration (according to Bradford, total protein data) before analysis. Video acquisition was performed with fixed settings for all samples (scatter mode: sensitivity 85, shutter 75; fluorescence mode: sensitivity 95, shutter 32; both: minimum brightness 20, minimum size 5, and maximum size 200). Videos of all 11 positions were recorded for each sample at each position and analyzed with the ZetaView analysis software (Version 8.03.08.02).

3.8 Statistical Analysis of Extracellular Microvesicles and Exosomes

Results were presented as a median and interquartile range if not stated otherwise. Variables were tested by the paired non-parametric Wilcoxon test and Spearman test for the correlation coefficient. The confidence level was calculated using a non-parametric Wilcoxon test, and the level for the test was set at 0.05; all P values were two-sided. To clearly demonstrate the statistically signficant difference in variable distribution between the groups, *$P<0.05$, **$P<0.01$, and ***$P<0.001$ were used in the figures. All variables were tested

for normality using the Shapiro-Wilk test. Statistical analysis was carried out using the R statistical software version 1.0.136 and SPSS version 21 (IBM, California).

4. RESULTS

4.1 Clinical Characteristics

Demographic and clinical data are displayed in Table 4.

Table 4. Demographic and clinical characteristics of the population studied. The data values are presented as means (SD) or as medians and an interquartile range (IQR) depending on the variable distribution. NS indicates not significant.

Variables	Median (IQR) or means (SD)			P
	Healthy 25–40-year olds (52)	Healthy 40–60-year olds (n=29)	Post-MI group (40–60-year olds) (n=15)	
Age, years	Means (SD): 30.31 (0.49)	Means (SD): 47.28 (0.84)	Means (SD): 51.93 (1.54)	<0.001
Systolic blood pressure	Means (SD): 131.27 (1.64)	Means (SD): 131.41 (2.51)	Median (IQR): 120 (15)	0.021
Diastolic blood pressure	Means (SD): 78.67 (1.17)	Means (SD): 82.21 (1.73)	Median (IQR): 80 (6)	NS
Heart rate	Means (SD): 71.35 (1.84)	Means (SD): 71.55 (2.28)	Means (SD): 67.07 (9.09)	NS
CRP (mg/l)	Median (IQR): 0.46 (0.70)	Median (IQR): 0.60 (1.70)	Median (IQR): 1.78 (1.90)	0.002
LDL-C (mmol/l)	Median (IQR): 2.92 (0.98)	Median (IQR): 3.14 (0.97)	Median (IQR): 2.02 (0.90)	<0.001
HDL-C (mmol/l)	Median (IQR): 1.20 (0.30)	Median (IQR): 1.18 (0.35)	Median (IQR): 1 (0.36)	NS
TG concentration (mmol/l)	Median (IQR): 1.12 (0.59)	Median (IQR): 1.29 (1)	Median (IQR): 1.44 (0.52)	NS
Total cholesterol concentration (mmol/l)	Median (IQR): 4.79 (1.31)	Median (IQR): 5.08 (1.13)	Median (IQR): 3.73 (1.31)	<0.001
Glucose concentration (mmol/l)	Median (IQR): 5.13 (0.57)	Median (IQR): 5.32 (0.66)	Median (IQR): 5.82 (3.29)	0.03
Malondialdehyde concentration (μg/L)	Median (IQR): 92.17 (30.98)	Median (IQR): 101.61 (34.85)	Median (IQR): 154.72 (8.21)	<0.001
Left ventricle ejection fraction value (EFV)	-	-	Means (SD): 45.70 (7.50)	Not evaluated

4.2 Flow Cytometry of Microvesicles from Human Blood Plasma

Characteristics of all four populations of microvesicles detected in human blood plasma and their CD62e expression are presented in Table 5 and 6.

Table 5. Characteristics of the EMVs (endothelial microvesicles) populations presented as medians and an interquartile range (IQR). Also given are P values for median differences between groups.

	Microvesicles count (microvesicles/µl) (Median±IQR)			
EMVs populations	Healthy 25–40-year olds (younger healthy group)	Healthy 40–60-year olds (elder healthy group)	Post-MI 40–60-year olds (post MI group)	P
Endoglin EMVs	22.89±75.20	10.93±53.81	33.68±205.47	NS
VE-cadherin without thrombocytic markers EMVs	322.79±232.64	173.37±426.84	215.56±133.67	NS
VE-cadherin with thrombocytic markers EMVs	254.68±185.15	304.73±210.64	73.29±49.23	<0.001
PECAM EMVs-	0.63± 2.16	1.94±2.62	1.38±2.02	0.04
PMVs	257.14±353.43	186.92±307.86	338.99±375	NS

Table 6. Characteristics of the activation of EMVs (endothelial microvesicles) (CD62e expression) presented as medians and an interquartile range (IQR). CD62e expression is calculated as a percentage derived from the total microvesicles count. Also given are P values for median differences between groups.

	CD62e expression (%) (Median±IQR)			
EMVs populations	Healthy 25–40-year olds (younger healthy group)	Healthy 40–60-year olds (elder healthy group)	Post MI 40–60-year olds (post MI group)	P
Endoglin EMVs	1.75±4.58	1.40±5.05	14.80±17.60	<0.001
VE-cadherin without thrombocytic markers EMVs	5.55±6.13	7±5.95	1.80±6	0.030
VE-cadherin with thrombocytic markers EMVs	41.25±87.80	63.70±79.45	98.90±5.80	0.002
PECAM EMVs-	-	-	-	
PMVs	-	-	-	

Four different populations of endothelial microvesicles were detected (Figures 3 and 4): 1) CD105+, CD42a-, and CD61- or endoglin EMVs; 2) CD31+, CD42a-, and CD61- or PECAM (platelet endothelial cell adhesion molecule) EMVs; 3) CD144+, CD42a-, and CD61- or VE-cadherin EMVs without thrombocytic marker; 4) CD144+, CD42a+, and CD61- or VE-cadherin EMVs with thrombocytic marker. Each subpopulation had one endothelial cell marker CD105 (endoglin), CD144 (VE-cadherin), or CD31 (PECAM) that could reflect their function according to the association with the marker. The split of the subpopulation of CD144+ EMVs into two groups, positive and negative for the thrombocytic marker CD42a, was intentional, since not only it reflected the flow cytometric results but because both subpopulations associated differently with clinical factors and the oxidative stress marker MDA.

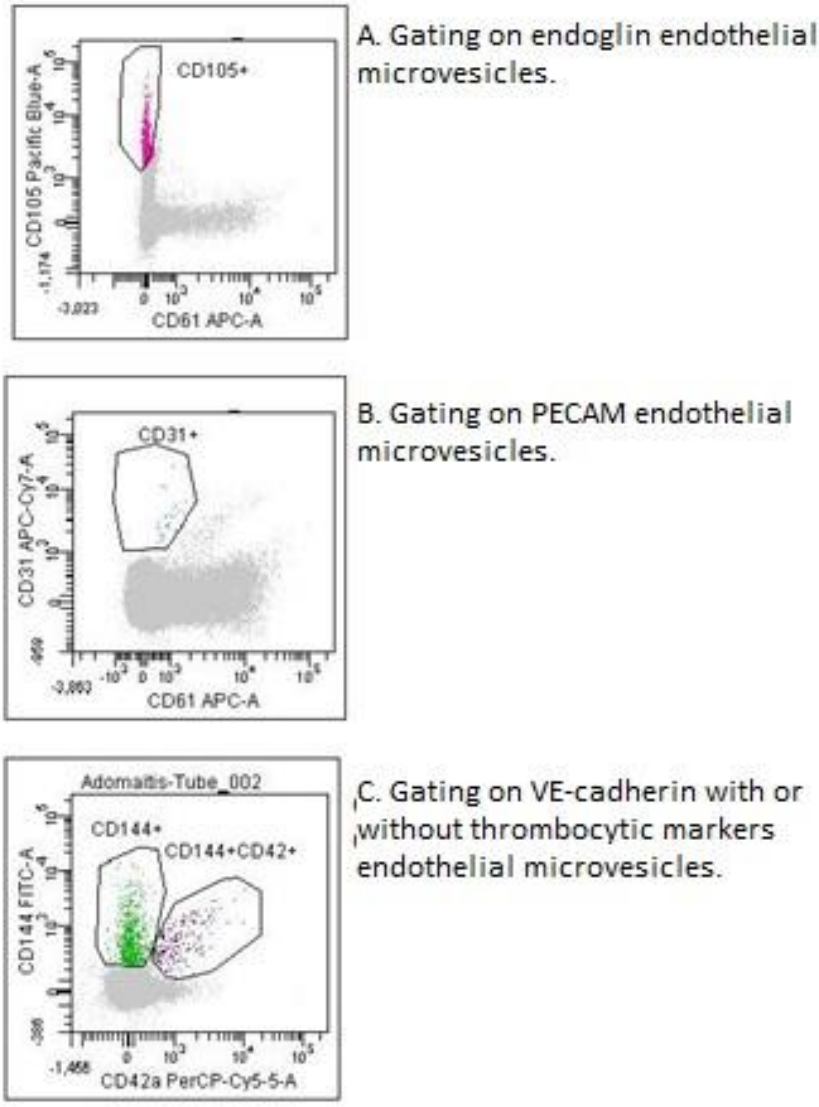


Figure 4. Gating on different populations of EMVs. A. Gating on endoglin endothelial microvesicles; B. Gating on PECAM endothelial microvesicles; C. Gating on VE-cadherin positive endothelial microvesicles (with or without a thrombocytic marker).

4.3 Characterization of Groups

Post-myocardial infarction patients had a significantly elevated concentration of MDA (154.72) vs. the healthy elder group (101.61) and vs. the healthy younger group (92.17) ($P<0.001$). In comparison to both healthy

groups, post-MI patients had lower LDL-C (2.02) vs. the healthy young group (2.92) and vs. the healthy elder group (3.14), and lower total cholesterol concentrations (3.73) vs. the healthy young group (4.79) and vs. the healthy elder group (5.08) ($P<0.001$) (Table 4, Figures 5, 6). There was a mean age difference (Table 4) between the post-MI group and the healthy elder group, since there were more post-MI individuals between 50–60 years of age, while the individuals were more between 40 to 50 years of age in the healthy elder group ($d= -4.83\pm1.62$, $P=0.005$). When comparing the healthy young group with the post-MI group, statistically significant differences in MDA concentration (92.17 vs. 154.72, $P<0.001$), LDL-C concentration (2.92 vs. 2.02, $P<0.001$), HDL-C concentration (1.2 vs. 1, $P=0.023$), CRP concentration (0.46 vs. 1.78, $P=0.001$), total cholesterol concentration (4.79 vs. 3.73, $P<0.001$), glucose concentration (5.13 vs. 5.82, $P=0.029$), and systolic blood pressure (131.27 vs. 120, $P=0.005$) were found (Figures 5, 6). While comparing the healthy elder group with the post-MI group, statistically significant differences were found in MDA concentration (101.61 vs. 154.72, $P<0.001$), total cholesterol concentration (5.08 vs. 3.73, $P<0.001$), LDL-C concentration (3.14 vs. 2.02, $P<0.001$), and systolic blood pressure (131.41 vs. 120, $P=0.029$) (Figures 5, 6). It would seem that only MDA concentration, total cholesterol concentration, LDL-C concentration, and systolic blood pressure were significantly different between both healthy groups and the post-MI group (Figure 5, 6).

Comparing both healthy groups between each other, only total cholesterol (4.79 vs. 5.08, $P=0.021$) and glucose (5.13 vs. 5.32, $P=0.05$) concentrations' medians were statistically reliably different.

Blood pressure in healthy individuals is higher in comparison to the post-MI patients because of the antihypertensive treatment the patients received at the time. The same could be said about concentrations of the cholesterols, since lipids were lower in the post-MI group in comparison to both healthy groups due to statin treatment, which the patients had received at the time of sample collection. The MDA concentration reflects the oxidative stress environment, and data shows that the highest levels of oxidative stress were found in the post-MI patients (154.72).

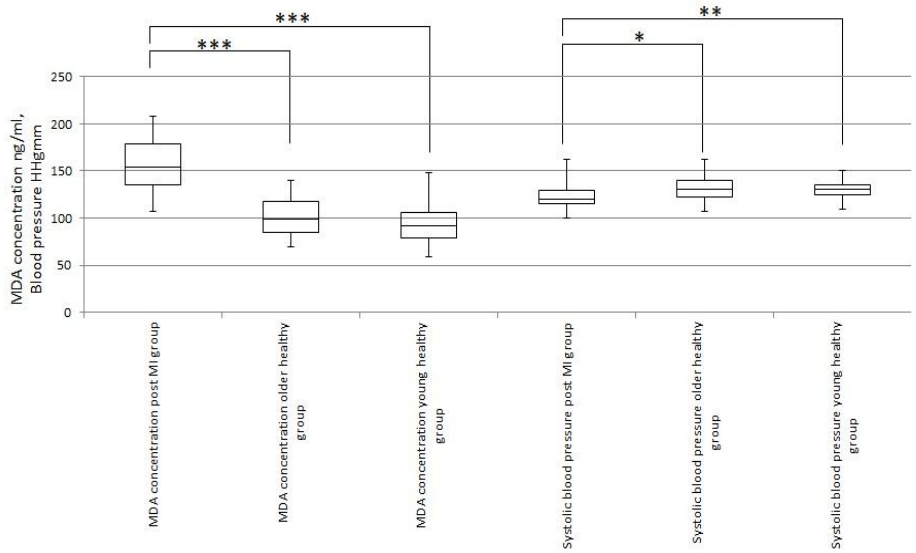


*Figure 5. Differences in MDA concentration and systolic blood pressure between individuals of the post-MI group versus healthy individuals. The bars represent median (interquartile range) numbers of the respective variable *P<0.05, **P<0.01, ***P<0.001 for comparisons between the post-MI group and healthy subjects.*

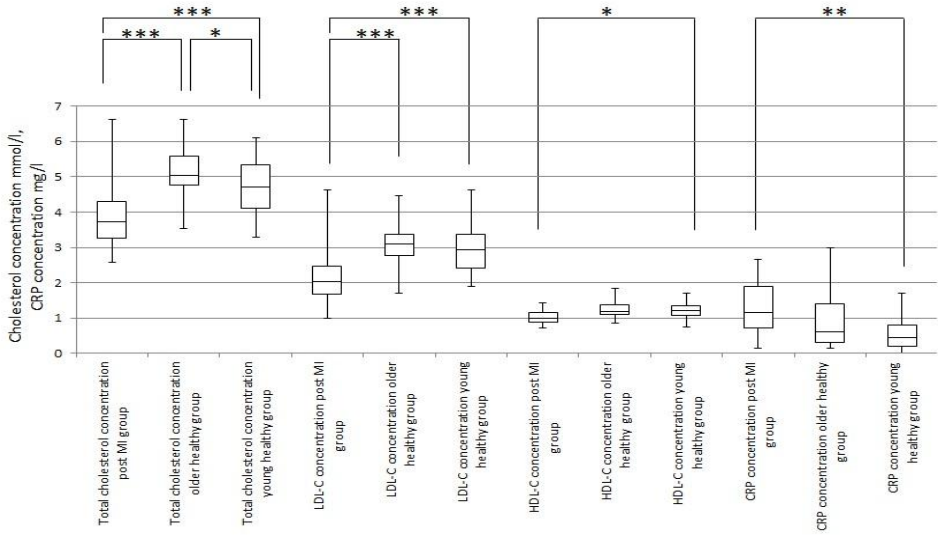


*Figure 6. Differences in lipid and CRP concentrations between individuals of the post-MI group versus healthy. The bars represent median (interquartile range) numbers of the respective variable *P<0.05, **P<0.01, ***P<0.001 for comparisons between the post-MI group and healthy subjects.*

4.4 Numbers of Circulating Endothelial and Platelet Microvesicles

The healthy younger group had a significantly elevated amount of VE-cadherin-EMVs with the thrombocytic marker (322.79) vs. the healthy elder group (173.37) and vs. the post-MI group (215.56), (P<0.001), (Table 5, Figure 8). The amount of PECAM EMVs also differed significantly between groups, and the healthy elder group had the highest amount (1.94) vs. the healthy younger group (0.63) and vs. the post-MI group (1.38), P=0.04) (Table 5, Figure 7).

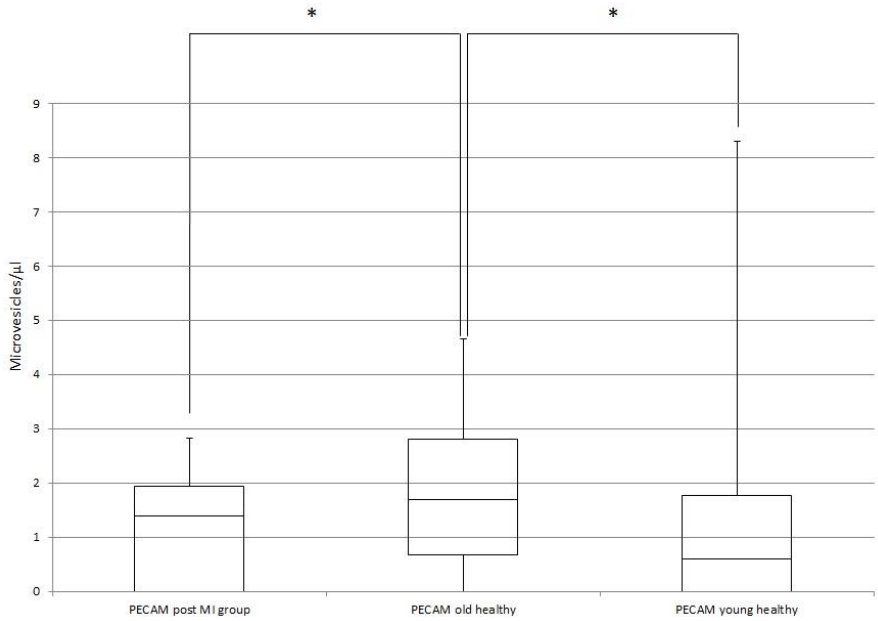


*Figure 7. Differences in the total count of PECAM EMVs (microvesicles/µl) between individuals of the post MI group versus healthy subjects. The bars represent median (interquartile range) numbers of the respective subset of EMVs. *P<0.05, **P<0.01, ***P<0.001 for comparisons between the post MI-group and healthy subjects.*

The only median difference of the total count of VE-cadherin EMVs with thrombocytic marker was found when healthy young and post-MI groups were compared (254.68 vs. 73.29, P<0.001). The healthy elder group and post-MI group had median differences in all EMV populations except for VE-cadherin without thrombocytic marker: 1) for VE-cadherin, EMVs with thrombocytic marker 304.73 vs. 73.29, P<0.001 (Figure 8); 2) for endoglin EMVs, 10.93 vs. 33.68, P=0.009 (Figure 9); 3) for PECAM EMVs, 1.94 vs. 1.38, P=0.032

(Figure 7). When comparing both healthy groups between each other, a median difference was found between PECAM EMVs (0.63 vs. 1.94, P=0.025) (Figure 7).

The highest amounts of PECAM EMVs were detected in the healthy elder group, a lower amount in the post-MI group, and the lowest in the healthy younger group. This could show a higher degree of endothelial damage in the healthy elder individuals with comparison to the younger ones. On the other hand, bearing in mind their disease history, the patients should have a definitely higher level of endothelial damage than healthy individuals, but this damage could be managed with treatment and could have influenced these results. Looking to endoglin-EMVs, the highest amount was detected in the post-MI group and the lowest in the healthy elder group. This shows a definitive link to the PECAM-EMVs, since the results are reversed in the case of endoglin-EMVs.

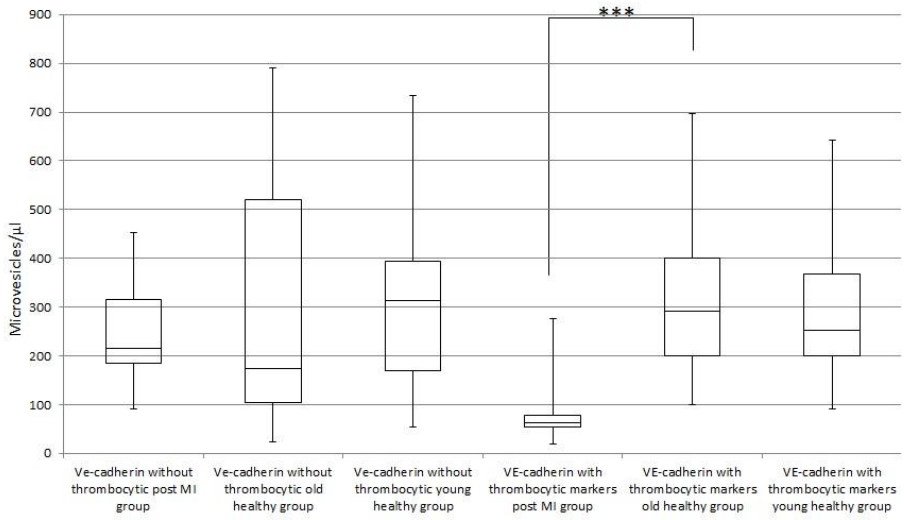


*Figure 8. Differences in the total count of VE-cadherin with or without thrombocytic marker EMVs (microvesicles/µl) between individuals of the post MI group versus healthy subjects. Bars represent median (interquartile range) numbers of respective EMVs subset. *P<0.05, **P<0.01, ***P<0.001 for comparisons between the post-MI group and healthy subjects.*

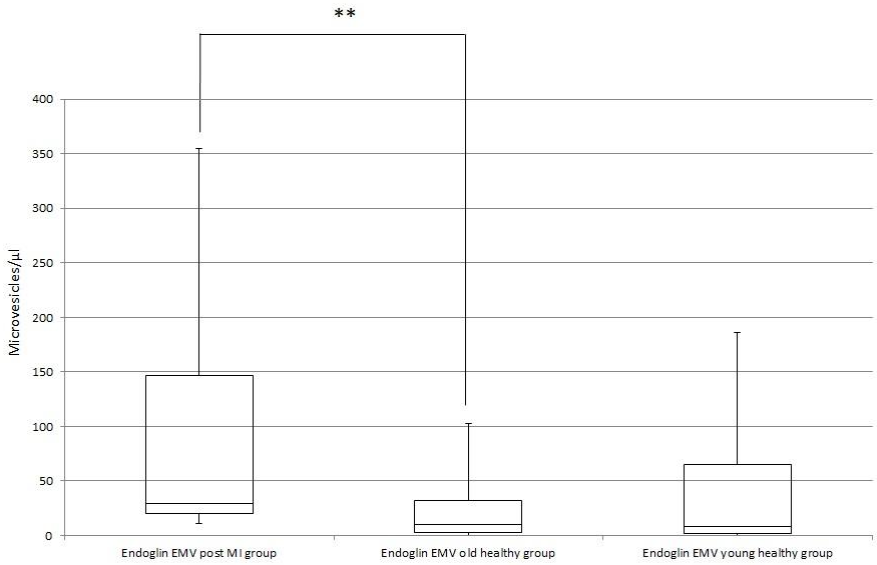


*Figure 9. Differences in the total count of endoglin EMVs (microvesicles/µl) between individuals of the post-MI group versus healthy subjects. The bars represent the median (interquartile range) numbers of their respective EMV subset. *P<0.05, **P<0.01, ***P<0.001 for comparisons between the post-MI group and healthy subjects.*

When comparing CD62e expression (activation level) on the EMV membrane between healthy individuals and MI patients, all EMV populations differently expressed CD62e on their membranes (Table 6, Figure 10). There was also a median difference between healthy groups when comparing the activation levels of EMVs: endoglin EMVs (1.75 vs 1.4, P<0.001) and VE-cadherin without thrombocytic marker (5.55 vs. 7, P=0.002) differently expressed CD62e. When the healthy elder group were compared with the post-MI group, the CD62e expression was found to be statistically significantly different in all populations except for PECAM EMVs – in endoglin EMVs (1.4 vs. 14.8, P<0.001), in VE-cadherin without thrombocytic marker (7 vs. 1.8, P=0.013), and in VE-cadherin with thrombocytic marker (63.7 vs. 98.9, P=0.001). Between the healthy young group and the post-MI group, there was also a statistically significant difference in the activation level of EMV populations, except for VE-Cadherin EMVs without thrombocytic marker and PECAM EMVs – in endoglin EMVs (1.75 vs. 14.8, P<0.001) and in VE-cadherin with thrombocytic marker (41.25 vs. 98.9, P=0.002) (Table 6, Figure 10). Median differences were not found in terms of EMV activation levels

between the healthy groups. The PECAM microvesicle population had no CD62e expression in all three groups.

Endoglin-EMVs had a much higher activation level in the post-MI group when compared with other populations of EMVs. This result could show that these endoglin- and CD62e-bearing EMVs could bear a more important role in the damage of endothelium, but at this point without hard proof this notion is only a speculation.

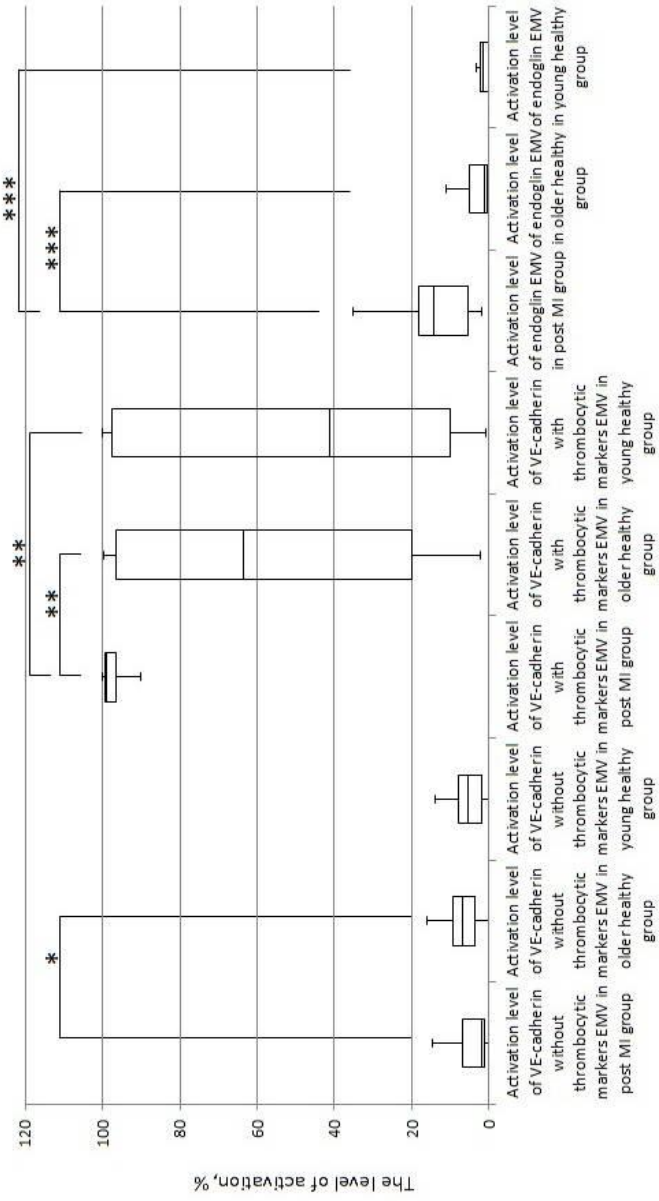


*Figure 10. Differences in CD62e expression (%) in the subsets of endothelial microvesicles between the post-MI group and healthy subjects. The bars represent median (interquartile range) numbers of their respective EMV subset. *P<0.05, **P<0.01, ***P<0.001 for comparisons between post the-MI group and healthy groups.*

4.5 Association between Traditional Markers and the Total Count of Endothelial and Platelet Microvesicles Populations and Their CD62e Expression

The data are presented in Table 7.

Table 7. Associations between the populations of EMVs, their CD62e expression, and cholesterol and glucose concentrations in human serum.

	Healthy elder group			Post-MI group			
	Activated endoglin EMVs	Endoglin EMVs	VE-cadherin EMVs without CD42a	VE-cadherin EMVs without CD42a	Activated VE-cadherin EMVs without CD42a	Activated VE-cadherin EMVs with CD42a	Endoglin EMVs
Total cholesterol concentration	NS	NS	NS	R=-0.63, P=0.011	NS	NS	R=-0.64, P=0.01
LDL-C concentration	NS	NS	NS	R=-0.68, P=0.005	NS	NS	R=0.69, P=0.004
HDL-C concentration	R=0.64, P<0.001	R=-0.47, P=0.011	R=0.37, P=0.046		R=0.78, P=0.001	NS	NS
Glucose concentration	NS	NS	NS	NS	NS	R=0.65, P=0.009	NS

In the younger healthy group, the CD62e+ level in endoglin EMVs negatively correlated with age (R=-0.54, P<0.001).

In the healthy elder group: 1) the total count of VE-cadherin EMVs without thrombocytic marker negatively correlated with the HDL-C concentration (R=-0.37, P=0.046) 2) the total count of PMVs positively correlated with age (R=-0.39, P=0.036). 3) The total count of endoglin EMVs negatively correlated with the HDL-C concentration (R=-0.47, P=0.011); 4) The CD62e+ level in endoglin EMVs negatively correlated with the HDL-C concentration (R=-0.64, P<0.001).

In healthy groups, the CD62e+ level in VE-cadherin without thrombocytic marker had no associations.

In the post-MI group: 1) The total count of VE-cadherin EMVs without thrombocytic marker negatively correlated with the total cholesterol concentration (R=-0.63, P=0.011) and LDL-C concentration (R=-0.68, P=0.005); 2) The total count of endoglin EMVs negatively correlated with the total cholesterol concentration (R=-0.64, P=0.01) and LDL-C concentration (R=-0.69, P=0.004) and positively with systolic blood pressure (R=0.54,

P=0.038). 3) The CD62e+ level in VE-cadherin without thrombocytic marker positively correlated with the HDL-C concentration (R=0.78, P=0.001) and negatively with systolic blood pressure (R=-0.54, P=0.037); 4) The CD62e+ level in VE-cadherin with thrombocytic marker negatively correlated with the glucose concentration (R=-0.65, P=0.009), systolic blood pressure (R=-0.53, P=0.04), and cortisol concentration in blood serum (R=-0.56, P=0.029). No correlations with the CD62e+ level in endoglin EMVs were observed. This shows a possible difference between VE-cadherin expressing and endoglin expressing EMVs. Since more associations were found in the post-MI group and the healthy elder group, the activation level could possibly be understood as a negative factor in disease progression.

Heart rate and diastolic blood pressure had no associations with endothelial microvesicles in all three groups.

4.6 Association of Total Counts of Microvesicles and Malondialdehyde Concentration in Human Blood Serum

The data is presented in Table 8.

Table 8. Associations between atherosclerosis risk factors and MDA concentration and the total count of EMV populations and their CD62e expression.

Groups	Healthy younger group			Healthy elder group			
EMVs and PMVs populations	VE-cadherin EMVs without CD42a	Activated VE-cadherin EMVs without CD42a	Activated Endoglin EMVs	PMVs	VE-cadherin EMVs without CD42a	Activated Endoglin EMVs	Endoglin EMVs
MDA concentration in serum	P=0.04 R=0.28	NS	NS	R=0.64 P<0.001	R=0.49, P=0.007	R=0.49, P=0.008	R=0.38 P=0.04
Age	NS	NS	R=-0.54, P<0.001	NS	NS	NS	NS

In the healthy younger group, only the total count of VE-cadherin EMVs without thrombocytic marker positively correlated with malondialdehyde concentration (R=0.28, P=0.04). In the healthy elder group, MDA concentration positively correlated with the total count of PMVs (R=0.64, P<0.001), the total count of VE-cadherin EMVs without thrombocytic marker (R=0.49, P=0.007), the total count of endoglin EMVs (R=0.38, P=0.04), and with the CD62e expression in the population of endoglin EMVs (R=0.49, P=0.008).

In the post-MI group, there was no association between MDA concentration and the total counts of EMVs or their activation levels.

4.7 Associations between Populations of Human Blood Endothelial Microvesicles

The data is presented in Tables 9 and 10.

Table 9. Associations between different EMV populations in healthy groups.

	Healthy younger group			Healthy elder group			
Total counts of EMVs	VE-cadherin EMVs without CD42a	Activated VE-cadherin EMVs with CD42a	Activated VE-cadherin EMVs without CD42a	PMVs	Activated VE-cadherin EMVs with CD42a	VE-cadherin EMVs with CD42a	VE-cadherin EMVs without CD42a-
Endoglin EMVs	R=0.41, P=0.003	R=0.66, P<0.001	R=0.42, P=0.002	NS	R=0.53, P=0.003	R=0.47, P=0.001	R=0.69, P<0.001
Activated endoglin EMVs	R=0.37, P=0.007	NS	NS	R=0.47, P=0.018	NS	NS	R=0.48, P=0.009

Table 10. Associations between different EMV populations in the post-MI group.

Total counts of EMVs	PMVs	VE-cadherin EMVs with CD42a	Activated VE-cadherin EMVs with CD42a	VE-cadherin EMVs without CD42a	PECAM EMVs
Endoglin EMVs	NS	R=-0.76, P=0.001	R=-0.76, P=0.001	R=0.64, P=0.001	NS
Activated endoglin EMVs	NS	NS	NS	NS	R=-0.57, P=0.027
Activated VE-cadherin EMVs with CD42a	R=-0.56, P=0.031	NS	-	R=-0.76, P=0.001	NS

In younger healthy group total count of VE-cadherin without thrombocytic marker EMVs positively correlated with the total count of endoglin EMVs (R=0.41, P=0.003) and activation level in endoglin EMVs (R=0.37, P=0.007). Total count of endoglin EMVs positively correlated with activation level in VE-cadherin without thrombocytic marker EMVs (R=0.42, P=0.002) and activation level in VE-cadherin with thrombocytic marker EMVs (R=0.66, P<0.001).

In elder healthy group total count of endoglin EMVs positively correlated with VE-cadherin without thrombocytic marker EMVs (R=0.69, P<0.001) and VE-cadherin with thrombocytic marker EMVs (R=0.47, P=0.01). Activation level in endoglin EMVs had negative associations with PMVs concentration (R= 0.47, P=0.018) and positively correlated with the total count of VE-cadherin without thrombocytic marker EMVs (R=0.48, P=0.009). Activation level in VE-cadherin with thrombocytic marker EMVs positively correlated with the total count of endoglin EMVs (R=0.53, P=0.003).

In the post MI group, VE-cadherin without thrombocytic marker EMVs positively correlated with the total count of endoglin EMVs (R=0.64, P=0.01) and negatively with CD62e expression in VE - cadherin with thrombocytic marker EMVs (R=-0.763, P=0.001). CD62e expression in endoglin EMVs negatively correlated with total counts of PECAM EMVs (P=0.027, R=-0.57). Activation level in VE-cadherin with thrombocytic marker EMVs negatively correlated with total amounts of PMVs (R=-0.56, P=0.031) and endoglin EMVs (R=-0.76, P=0.001).

The correlations between different populations of EMVs presented here shows that both endoglin and VE-cadherin EMVs activated are linked together and could participate in the same processes in the pathogenesis of atherosclerosis. These associations complete the picture and shows these EMVs acting together in the endothelium tissue. Comparing correlations between groups it would seem that post MI group had more negative correlations than both healthy groups. Also it would seem that correlations in both healthy groups were comparably alike. This could show a definitive difference between the disease and health and ratios between amounts or activation levels of EMVs should be counted.

4.8 Ratios between Different Populations of Extracellular Vesicles and Their Level of Activation

Since the associations between EMVs of different populations were observed, the different ratios of their total numbers and activation levels were also evaluated. The total number of EMVs and their activation levels were analyzed separately, since no relation between the total number of EMVs and their activation level was observed. Rejecting the ratios where the division by zero occurred, the four ratios showed a statistically significant difference between all three groups (the healthy young and elder as well as the post-MI groups) (Table 11). The ratio of the total number of Endoglin-EMVs with the total number of VE-cadherin-EMVs containing thrombocytic marker resulted in P<0.001, the ratio of the total number of Endoglin-EMVs with a total number of

VE-cadherin-EMVs without thrombocytic marker resulted in P=0.011, and the ratio of activated Endoglin-EMVs with activated VE-cadherin EMVs containing thrombocytic marker resulted in P=0.002. The total number of PMVs were also included in ratio counting and the ratio between the total number of PMVs and the total number of VE-cadherin-EMVs containig thrombocytic marker were shown to be significaly different between groups (P=0.001). All the ratios were higher in the post-MI group, highlighting the difference between healthy individuals and patients. It means that the investigated ratios could be used in the differentiation between ischemic heart disease and healthy individuals. The data describing these variables are presented in Table 11.

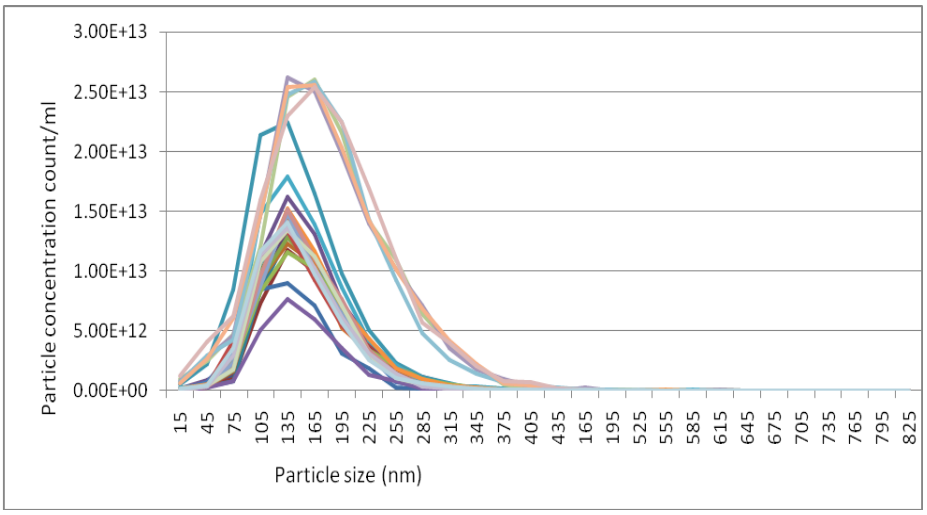
The possible correlation between ratios and atherosclerosis risk factors was also tested. Correlation data were observed only in the healthy elder group and the post-MI group. In the healthy elder group, the ratio of PMVs with VE-cadherin-EMVs containing thrombocytic marker negatively correlated with the MDA concentration (R=-0.65, P<0.001). The HDL-C concentration negatively correlated with the ratio of activated Endoglin-EMVs with activated VE-cadherin-EMVs containing thrombocytic marker (R=-0.56, P=0.002) and with the ratio of Endoglin-EMVs with VE-cadherin-EMVs containing thrombocytic marker (R=-0.4, P=0.033). In the post-MI group, the ratio of Endoglin-EMVs with VE-cadherin-EMVs without thrombocytic marker negatively correlated with the LDL-C concentration (R=-0.64, P=0.011), and the ratio of Endoglin-EMVs with VE-cadherin-EMVs with thrombocytic marker negatively correlated with the TC concentration (R=-0.55, P=0.033) and LDL-C concentration (R=-0.74, P=0.002). The ratio of activated Endoglin-EMVs with activated VE-cadherin-EMVs with thrombocytic marker positively correlated with TG (R=0.54, P=0.039) and glucose concentrations (R=0.6, P=0.018).

Table 11. Characteristics of ratios between the EMVs and their activation levels and PMVs presented as medians and an interquartile range (IQR).

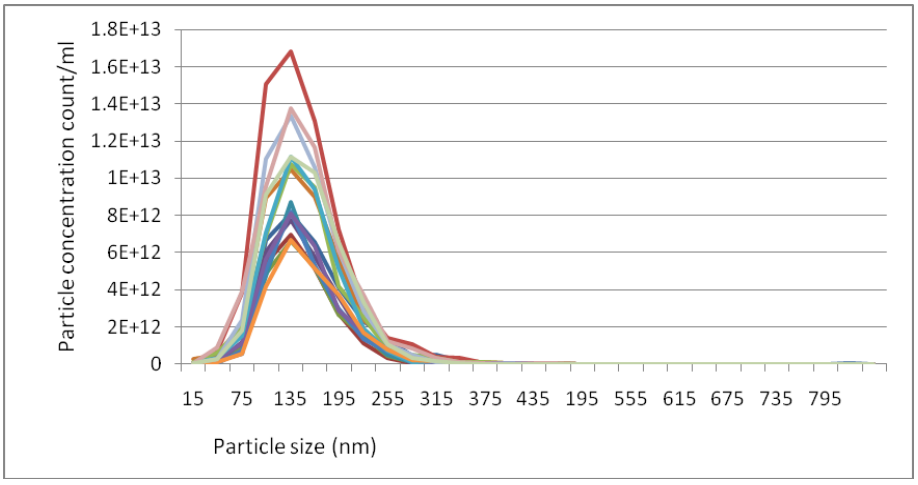
Ratios	Median ±IQR healthy younger group	Median ±IQR healthy elder group	Median ±IQR post-MI group	P
Endoglin EMVs with VE-cadherin EMVs with thrombocytic marker	0.05±0.31	0.05±0.16	0.37±3.38	P<0.001
Endoglin EMVs with VE-cadherin EMVs without thrombocytic marker	0.07±0.23	0.07±0.17	0.19±0.48	P=0.011
Activated endoglin EMVs with activated VE-cadherin EMVs with thrombocytic marker	0.02±0.07	0.05±0.1	0.16±0.18	P=0.002
PMVs with VE-cadherin EMVs with thrombocytic marker	0.95±1.35	0.67±1.47	4.4±7.95	P=0.001

4.9 Numbers of Circulating Exosomes in Human Blood

According to nanoparticle tracking analysis (NTA) (Zeta View, Particle Metrix, Germany), a median difference between the post-MI and healthy elder groups ($3.1*10^{10}$/ml±1.9 vs. $7.07*10^{10}$/ml ±3.1, P<0.001) was found (Figure 11). Healthy subjects had a higher concentration of particles than subjects from the post-MI group. There was no significant difference between the mean volume or diameter of exosomes in groups (Figure 11).



Healthy elder group



Post-MI group

Figure 11. NTA analysis of exosomes isolated from the healthy elder group and post-MI group. The concentrations of particles according to their size are shown. Each line in the graph represents a different sample.

The tetraspanin CD9 expression on the exosome membrane was tested using flow cytometry (Figure 12). A flow cytometric analysis of median fluorescence intensity (MFI) and the percentage from total events of exosomes expressing CD9 revealed the healthy elder group to have a higher amount of CD9+ exosomes than individuals from the post-MI group (MFI - 275±39.5 vs. 252±13, $P<0.001$, percentage of events – 23.01%±17.4 vs. 1.3%±0.6, $P<0.001$). The total protein content was higher in samples from the post-MI group (1.01 µg/ml±0.49 vs. 1.95 µg/ml ± 1.07, $P=0.001$). The sample impurity could be the

reason for this phenomenon, since the numbers of NTA correlated with the Bradford results in the healthy elder group but not in the post-MI group.

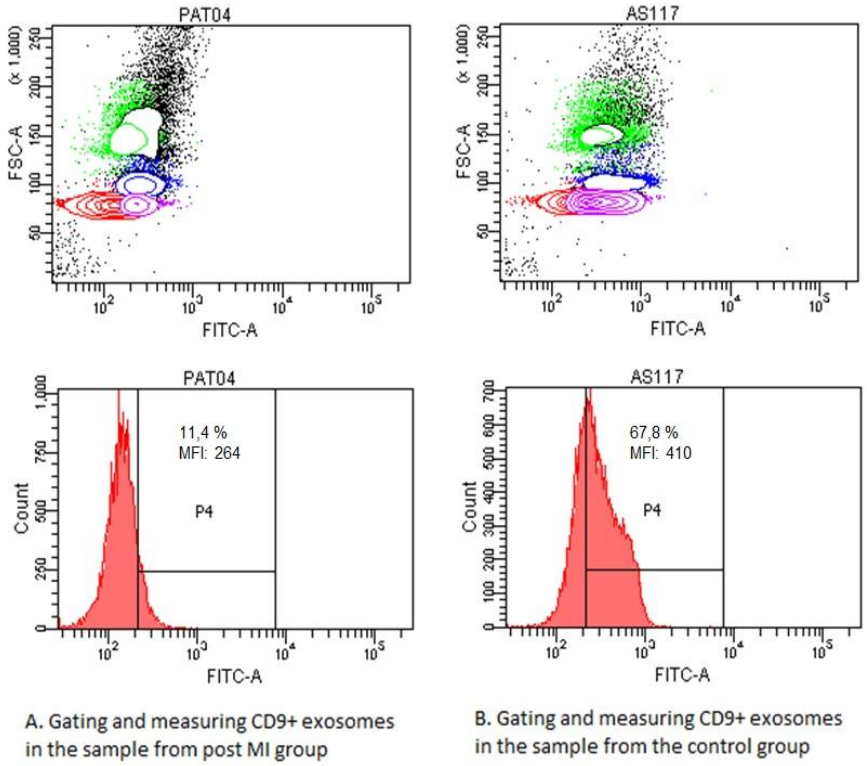


Figure 12. Measuring CD9+ expressing exosomes by flow cytometry A. Gating and measuring CD9+ exosomes in the sample from the post-MI group; B. Gating and measuring CD9+ exosomes in the sample from the healthy elder group. The result shows a higher amount of CD9+ exosomes in the healthy elder group.

4.10 Phenotyping of Human Blood Isolated Exosomes

The presence of TSG101, flotillin-1, HSP70, and tetraspanin CD9 were tested in the exosome samples (Figures 13, 14). The TSG101, flotillin-1, and HSP70 were tested using Western blotting, but only TSG101 were visible (SDS page, Bio-Rad, California) (Figure 13). The Protein level in each sample was equalized using data from the Bradford method.

Figure 13. Western blot of TSG101.

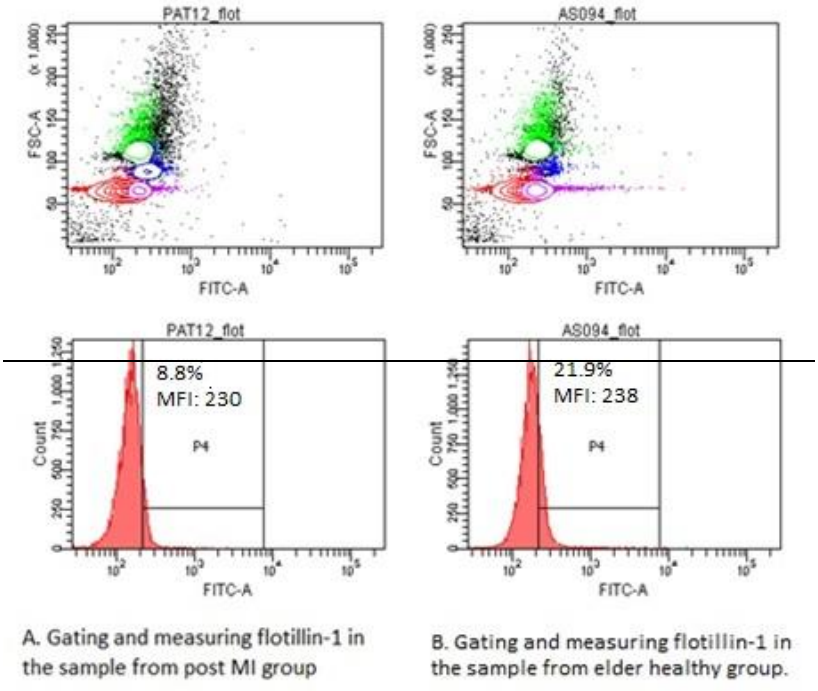


Figure 14. Detection of flotillin-1 by flow cytometry. A. Gating and measuring flotillin-1 in the sample from post MI group. B. Gating and measuring flotillin-1 in the sample from elder healthy group.

Since flotillin-1 was not found using the Western blot, flow cytometry was employed. The presence of Flotillin-1 on the exosome membrane was confirmed by flow cytometry, using 4 samples: two from each group (Figure 14). The Western blot test for flotillin-1 was negative, probably due to the higher sensitivity of flow cytometry and too low amounts of flotillin 1 on the membranes of exosomes. In conclusion, flotillin-1 was found on the membrane of exosomes.

4.11 Associations between Traditional Atherosclerosis Markers, MDA and Exosome Concentration, and the Number of CD9-Containing Exosomes

In the healthy elder group, the concentration of malondialdehyde correlated with the total exosome concentration determined by NTA (R=0.4, P=0.003) the median of fluorescence intensity of CD9-expressing exosomes (R=0.49, P=0.008), and the percentage of the total events of CD9-expressing exosomes (R=0.55, P=0.002). In the post-MI group, there was no association with MDA concentration.

In the healthy elder group, the HDL-C concentration positively correlated with exosome concentration (R=0.46, P=0.01).

It shows that as with the larger microvesicles, exosome amounts correlated with the MDA concentration only in the healthy elder group but not in the post-MI group. Also, a correlation with lipids, in this case the HDL-C concentration, was also found in the healthy elder group but not in the post-MI group.

4.12 Testing Thioredoxin in Human Blood Isolated Exosomes Using Western Blotting

An expression of thioredoxin in samples of the healthy elder and post-MI groups was detected (Figure 15). However, no thioredoxin activity was detected while performing ELISA. This leads to the conclusion that thioredoxin is, probably, located inside the exosomes and is not expressed on the membranes. In the Western blot images, different expression levels of thioredoxin can be seen, but it seems that healthy subjects have a lower amount of thioredoxin than MI patients. This could mean that exosomes participate in the antioxidative function, and exosomes could have a more prominent role in the post-MI patients, since endothelium damage should be more pronounced in these patients.

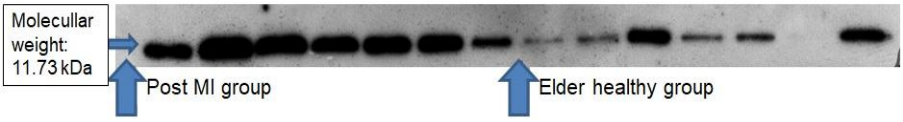


Figure 15. Thioredoxin detection using the Western blot. On the left of the picture, the samples from the post MI group are shown. On the right, starting from the second arrow, the samples from the healthy elder group are shown. The thioredoxin amount in each sample was not calculated.

DISCUSSION

The objective of this work was to determine the differences between the numbers of microvesicles and exosomes in healthy individuals (at different age intervals – 25–39 and 40–60-years old) and in patients that had suffered from myocardial infarction three months before. Three main markers were used to describe EMVs in this work: CD105 (endoglin), CD144 (VE-cadherin), and CD31 (PECAM). CD62e was used as an activation marker of human blood EMVs. VE-cadherin is required to maintain an endothelial barrier, since it serves as a protein of cell junction [77]. VE-cadherin could be associated with endothelial injury during inflammation [78], as its phosphorylation and degradation allows the migration of lymphocytes and neutrophils through the endothelium [79]. Endoglin serves as an auxiliary receptor for transforming growth factor-β (TGFβ) and is required during and after the activation of endothelium [80]. Endoglin is usually associated with a healthy endothelium and inversely correlates with the severity of coronary atherosclerosis [81]. CD62e or E-selectin is an endothelial activation marker associated with inflammation and is well-studied in atherosclerosis [82]. This marker plays a significant role in the pathogenesis of endothelium dysfunction [82]. Essentially only two markers, CD144 and CD62e, could be considered as truly endothelial [83], whereas CD62e is mainly attributed as a negative factor in endothelial injury [84]. The presence of the CD62e on the EMVs membrane could be related to the involvement of EMVs in endothelial injury. Different studies show that increased CD144+ (VE-cadherin) and CD62e+ (E-selectin) microvesicles are associated with dyslipidaemia [85]. Higher numbers of CD105+ (endoglin) and CD144+ endothelial microvesicles were associated with acute ischemic stroke [86, 87]. In chronic heart disease, the levels of CD105+ and CD144+ EMVs were found to be undistinguished from healthy individuals [88]. A percentage of CD62e-positive EMVs was counted in each subpopulation to show their level of activation. The presence of CD62e on the membranes of EMVs can be associated with oxidative stress or atherosclerosis risk factors.

The concentration of MDA was also associated with an increase in the total number of EMVs and exosomes in the healthy group. Moreover, only the total number of VE-cadherin-containing EMVs without thrombocytic marker and the activation level of endoglin-EMVs were associated with MDA concentration. It seems that VE-cadherin-EMVs were differently associated with MDA concentration or atherosclerosis risk factors depending on the presence of CD42a on their membranes. VE-cadherin-EMVs containing thrombocytic marker had no associations with MDA or clinical markers in healthy

individuals, yet in the patients, its associations followed VE-cadherin-EMVs without thrombocytic marker. It is possible that VE-cadherin microvesicles carrying thrombocytic marker are probably not important in oxidative stress, unlike EMVs with VE-cadherin and without thrombocytic marker. It is hard to explain this phenomenon, but it could be due to the different functions of these EMVs in the body. Interestingly, the association of the total number of EMVs with MDA concentration was not observed in these patients. It could be partly explained by the associations that EMVs had with lipids. Activated (i.e., carrying CD62e) endoglin-EMVs had a positive association with MDA concentration and a strong negative association with HDL-C concentration in healthy subjects. In the post-MI group, these activated EMVs were negatively associated with total cholesterol or LDL-C concentrations. There was a clear difference between endoglin and VE-cadherin containing EMVs comparing correlation data between their numbers and MDA or cholesterol concentrations. It could be influenced by the statin treatment that the patients had received at the time of sample collection. Statins, it seems, decrease the release of endothelial microvesicles [89], but this is still debatable [90]. The increased number of EMVs detected in the healthy elder group in comparison to the healthy younger group could mean an association between EMV numbers and pathological processes in the endothelium could be possible. Healthy individuals did not received any treatment when the samples were collected and, looking at the amounts of EMVs detected, data from the healthy younger group were comparable with data from the post-MI group. This indicates a possible influence of the treatment. Drugs could help reduce the damaging factors to the endothelium and, in turn, change the total number of EMVs, leading to healthier endothelial tissue. An association between numbers and the activation levels of the EMVs and the concentration of HDL-C in the blood was observed in both the post-MI and healthy elder groups. HDL-C could have an antioxidative function at least in early atherosclerosis and could serve as a protective factor [91]. But for the post-MI patients, the activated VE-Cadherin-EMVs correlated with the HDL-C concentration but not with the activated endoglin-EMVs, while for the healthy elder group, the opposite could be said. On the other hand, the activation level of endoglin-EMVs was increased in the post-MI group, whereas the healthy elder group had a higher expression of CD62e in VE-Cadherin-EMVs without a thrombocytic marker population. The same was observed when the total numbers of EMVs were compared. The calculated ratios mainly involved, again, these two population of EMVs: VE-cadherin – EMVs and endoglin - EMVs. Both types of EMVs containing VE-cadherin or endoglin participate in endothelial damage and repair processes (as mentioned above) but from the data, it seems, their involvement is different. It is possible that

endoglin carrying EMVs are participating in the regulation of healthy endothelial function and VE-cadherin carrying EMVs, probably, are more associated with inflammation. These VE-cadherin and endoglin containing EMVs could be related to the degeneration processes of the endothelial tissue.

Another microvesicle group investigated in this work was platelet microvesicles. The difference in total number of PMVs between groups was not detected. Associations between them and risk factors or associations with total number of EMVs were little to non-existent. But, interestingly, the ratios between EMVs and PMVs proved to be completely different. Statistically significant differences were observed especially when ratio with VE-cadherin-EMVs containing the thrombocytic marker was investigated. This was the only ratio (between total numbers of VE-cadherin-EMVs containing the thrombocytic marker and PMVs) that had a negative association with MDA concentration. This association with MDA concentration not only serves to prove the possible negative effect of VE-cadherin - EMVs but also introduces PMVs as a possible effector in oxidative stress.

The data of microvesicle research was comparable to exosomal data. Mainly the integrin molecules distinguish the exosomes: CD63, CD81 and CD9 [91]. In this work, the CD9 was used to investigate the number of exosomes. Not all exosomes carried CD9 molecules on their membrane. For this reason, NTA analysis was employed to found true quantities of exosomes in the blood of the subjects. Interestingly CD9 data coincided with NTA data (the amount of exosomes detected by both measurements was lower in patients and healthy individuals). The same result was detected when investigating microvesicles. Thioredoxin was also detected in exosomal samples. It is a classical antioxidative agent used to prove exosome associations with oxidative stress [92]. Detection of thioredoxin and its association between MDA concentration and the number of exosomes could show possible participation of thioredoxin in oxidative stress and subsequent endothelium damage. Undeniably, there is a link between microvesicles and exosomes and the link with oxidative stress was found in both classes of ECVs. Data of this work show the involvement of ECVs both small and large (exosomes and microvesicles) in oxidative stress and ultimately in the pathogenesis of atherosclerosis.

Summarizing the results, the significance of CD62e or e-selectin in endothelial damage is clearly visible. The differences between the microvesicles carrying this marker between groups, the relationship between the risk factors of atherosclerosis or the concentration of malondialdehyde clearly indicate that it is important to determine not only the amounts of microvesicles themselves but also the proportion of microvesicles carrying CD62e on their membranes. This may be a significant marker of early endothelial damage.

CONCLUSIONS

1. Four distinct populations of endothelial microvesicles were detected in the human blood of healthy subjects and patients in a post-myocardial infarction period:
   a) VE-cadherin-EMVs positive and negative for a thrombocytic marker:
      1) The amount of VE-cadherin-EMVs containing thrombocytic marker was the highest (4 times) in healthy elder group (304.73±210.64 microvesicles/μl) and the lowest in the post-MI group (73.29±49.23 microvesicles/μl).
      2) The amount of VE-cadherin-EMVs without thrombocytic marker was the highest (2 times) in the healthy younger group (322.79±232.64 microvesicles/μl) and the lowest in the healthy elder group (173.37±426.84 microvesicles/μl).
   b) The number of PECAM-EMVs was the highest (3 times) in the healthy elder group (1.94±2.62 microvesicles/μl) and the lowest in the healthy younger group (0.63±2.16 microvesicles/μl).
   c) The number of Endoglin-EMVs was comparably the lowest (3 times) in the healthy elder group (10.93±53.81 microvesicles/μl) and the highest in the post-MI group (33.68±205.47 microvesicles/μl).
   d) The level of PMVs did not show any significant difference between healthy and diseased patients.
2. The activation level of CD62e-expressing EMVs in all populations of endothelial microvesicles was higher in the post-MI group (for endoglin EMVs: 14.8±17.6 %; $P<0.001$, for VE-cadherin with thrombocytic marker EMVs: 98.9±5.8 %, $P=0.002$), except for VE-cadherin-EMVs without a thrombocytic marker (1.8±6 %, $P=0.03$) in comparison to both healthy groups (for endoglin EMVs: 1.75±4.58% in the healthy younger group vs. 1.4±5.05 % in the healthy older group; for VE-cadherin without thrombocytic marker EMVs: 5.55±6.13 % in the healthy younger group vs. 7±5.55 % in the healthy older group; for VE-cadherin with thrombocytic marker EMVs: 41.25±87.8 % in the healthy younger group vs. 63.7±79.45 % in the healthy older group). This reveals that both parameters – the total number and activation levels of endothelial microvesicles in human blood – are important.
3. The total concentration of exosomes determined by a nanotracking analysis in blood samples from the healthy elder group was higher ($7.07*10^{10}$/ml ±3) if compared to the post-MI group ($3.1*10^{10}$/ml±1.9). This shows that small ECVs (or exosomes) participate in the process of endothelial injury.

4. The concentration of malondialdehyde in human blood significantly correlated: with the total amount of VE-cadherin-EMVs without a thrombocytic marker, endoglin-EMVs and their activation level, the total count of platelet microvesicles in the healthy elder group, and the exosome concentration in the blood samples gathered from the healthy elder group. Thioredoxin was also found in the exosome samples. It proves the link between microvesicles, exosomes, and oxidative stress.
5. The concentrations of total cholesterol and low- and high-density lipoproteins in the MI patients and healthy subjects significantly correlated with the total amount of endothelial microvesicles expressing VE-cadherin without thrombocytic marker and endoglin-containing microvesicles with thrombocytic marker. The CD62e expression in endoglin-containing endothelial microvesicles was associated with a high density cholesterol concentration in healthy subjects. Finally, the expression of CD62e in VE-cadherin-containing endothelial microvesicles with a thrombocytic marker significantly correlated with systolic blood pressure. The correlation data reveal that EMVs, but not PMVs, participate in human myocardial infarction.

APPLICABILITY

This work presents the possibility of using microvesicles and exosomes in the clinical field. The method is reproducible and could be adapted to clinical settings. However, it then has to be validated. The validation procedure looks simple, but there are a lot of hurdles and traps in the way [93]. The definition of normal versus abnormal ECVs sample presents a problem that could be seen from this work as well. To have clearly defined normal ranges, we need larger studies. In 2017, it was shown that clinically validated assays in this field are achievable, and this should be the goal in the future [93]. The large variability of ECVs' levels also presents a problem in need of addressing [94]. Contaminating factors, such as proteins, platelets, and lipids, are hard to remove from a sample, and the recover efficiency of ECVs is low [95]. There are new methods under development, such as microfluidics, which give some interesting results [96]. But in general, we lack a reliable isolation method. And as highlighted in the recent Clinical Wrap-Up session at ISEV 2018, the strict requirements for the samples could be hard to achieve in clinical settings, and we have to keep that in mind when developing assays [97].

ACKNOWLEDGEMENTS

Throughout the writing of this dissertation, I have received a great deal of support and assistance. I would first like to thank my supervisor, Prof. Habil. Dr. Zita Aušrelė Kučinskienė, whose expertise was invaluable in formulating the research topic and producing this work as a whole. I would like to acknowledge Assoc. Prof. Dr. Rėda Matuzevičienė and my colleagues Aušra Janulionienė, Mantas Radzevičius, and Dr. Aušra Linkevičiūtė for their important methodological help in particular.

I would like to acknowledge my colleagues from my internship at the Technology Institute of Tartu University for their wonderful collaboration. You supported me greatly and were always willing to help me. I would particularly like to single out my supervisor at the Technology Institute of Tartu University, Dr. Reet Kurg – I wish to express my gratitude to you for your excellent cooperation and for all of the opportunities I was given to conduct my research.

I would also like to thank my tutor, Prof. Dr. Audronė Jakaitienė, for her valuable guidance. You provided me with the tools that I needed to choose the right direction and complete my dissertation.

REFERENCES

1. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP et al. on behalf of the American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics - 2019 update: a report from the American Heart Association [published online ahead of print January 31, 2019]. *Circulation.*
2. https://ec.europa.eu/eurostat/statistics-explained/index.php/Cardiovascular_diseases_statistics#Deaths_from_cardiovascular_diseases
3. Wang J, Tan GJ, Han LN, Bai YY, He M, Liu HB. Novel biomarkers for cardiovascular risk prediction. *Journal of geriatric cardiology*. 2017;14(2):135–150.
4. Gustafson D, Veitch S, Fish JE. Extracellular Vesicles as Protagonists of Diabetic Cardiovascular Pathology. *Frontiers in Cardiovascular Medicine*. 2017;4:71.
5. Liu W, Bai X, Zhang A, Huang J, Xu S, Zhang J. Role of Exosomes in Central Nervous System Diseases. *Frontiers in Molecular Neuroscience*. 2019;12(240).
6. Nozaki T, Sugiyama S, Koga H, Sugamura K, Ohba K, Matsuzawa Y et al. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *Journal of American College of Cardiology*. 2009;54(7):601-8.
7. Chen Y, Li G, Liu M-L. Microvesicles as Emerging Biomarkers and Therapeutic Targets in Cardiometabolic Diseases. *Genomics Proteomics Bioinformatics.* 2018;16:50–62.
8. Li P, Qin C. Elevated Circulating VE-Cadherin + CD144 + Endothelial Microparticles in Ischemic Cerebrovascular Disease. Thromb Res. 2015;135(2):375-381.
9. van der Vorst EPC, de Jong RJ, Donners MMPC. Message in a Microbottle: Modulation of Vascular Inflammation and Atherosclerosis by Extracellular Vesicles. *Frontiers in Cardiovascular Medicine.* 2018;5:2.
10. Sluijter JPG, Davidson SM, Boulanger CM, Buzás EI, de Kleijn DPV, Engel FB, Giricz Z, Hausenloy DJ, Kishore R, Lecour S, Leor J, Madonna R, Perrino C, Prunier F, Sahoo S, Schiffelers RM, Schulz R, Van Laake LW, Ytrehus K, Ferdinandy P. Extracellular

vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovascular Research.* 2018;114(1):19-34.

11. Castellanos-Rizaldos E, Grimm DG, Tadigotla V, et al.Exosome-based detection of EGFR T790M in plasma from non-small cell lung cancer patients. *Clinical Cancer Research*. 2018;24:2944–2950.
12. Hartjes TA, Mytnyk S, Jenster GW, van Steijn V, van Royen ME. Extracellular Vesicle Quantification and Characterization: Common Methods and Emerging Approaches. *Bioengineering (Basel).* 2019;6(1):7.
13. Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles*. 2015;4:27066.
14. Stocker R, Keaney JF. Role of oxidative modifications in atherosclerosis. *Physiology Reviews*. 2004;84:1381-478.
15. Moris D, Spartalis M, Spartalis E, et al. The role of reactive oxygen species in the pathophysiology of cardiovascular diseases and the clinical significance of myocardial redox. *Annals of Translation Medicine*. 2017;5(16):326.
16. Papac-Milicevic N, Busch CJ, Binder CJ. Malondialdehyde Epitopes as Targets of Immunity and the Implications for Atherosclerosis. *Advances in Immunology*. 2016;131:1–59.
17. Amir S, Hartvigsen K, Gonen A, et al. Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease. *Journal of Lipid Research*. 2012;53(7):1316–1326.
18. Lee R, Margaritis M, Channon K, Antoniades C. Evaluating Oxidative Stress in Human Cardiovascular Disease: Methodological Aspects and Considerations. *Current Medicinal Chemistry*. 2012;19(16):2504-2520.
19. Solati Z, Ravandi A. Lipidomics of Bioactive Lipids in Acute Coronary Syndromes. *International Journal of Molecular Sciences*. 2019;20(5):1051.
20. Singh S et al. Aldehyde dehydrogenases in cellular responses to oxidative/electrophilic stress. *Free Radical Biology & Medicine*; 2013;56:89-101.

21. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling*. 2014;20(7):1126-1167.
22. Bodega G, Alique M, Bohórquez L, Morán M, Magro L, Puebla L, Ciordia S, Mena MC, Arza E, Ramírez MR. Young and Especially Senescent Endothelial Microvesicles Produce NADPH: The Fuel for Their Antioxidant Machinery. *Oxidative Medicine and Cellular Longevity*. 2018:3183794.
23. Tsiantoulas D, Perkmann T, Afonyushkin T, et al. Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. *Journal of Lipid Research*. 2015;56(2):440–448.
24. Liu ML, Scalia R, Mehta JL, Williams KJ. Cholesterol-induced membrane microvesicles as novel carriers of damage-associated molecular patterns: mechanisms of formation, action, and detoxification. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2012;32(9):2113–2121.
25. Wang J, Liu J, Zhu X, Zhang M, Wang S, Zheng Z. Nonculprit lesion progression in patients with ST elevation myocardial infarction after primary percutaneous coronary intervention. *International Heart Journal* 2014;55:48-52.
26. Van Varik B, Rennenberg R, Reutelingsperger C, Kroon A, de Leeuw P, Schurgers L. Mechanisms of arterial remodeling: lessons from genetic diseases. *Frontiers in Genetics.* 2012;3(290).
27. Tadokoro H, Umezu T, Ohyashiki K, Hirano T, Ohyashiki JHJ. Exosomes derived from hypoxic leukemia cells enhance tube formation in endothelial cells. *Biology Chemistry*. 2013; 288(48):34343-51.
28. Maas SLN, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends in Cell Biology*. 2017:172-188.
29. Agouni A, Parray AS, Akhtar N, Mir FA, Bourke PJ, Joseph S, et al. There Is Selective Increase in Pro-thrombotic Circulating Extracellular Vesicles in Acute Ischemic Stroke and Transient Ischemic Attack: A Study of Patients From the Middle East and Southeast Asia. *Frontiers in Neurology*. 2019;10(251).
30. Jansen F, Li Q, Pfeifer A, Werner N. Endothelial- and Immune Cell-Derived Extracellular Vesicles in the Regulation of Cardiovascular Health and Disease. *JACC Basic to Translational Science.* 2017;2(6):790-807.

31. Wang, Yanan, Yingyu Xie, Ao Zhang, Mingyang Wang, Zihan Fang, and Junping Zhang. Exosomes: An Emerging Factor In Atherosclerosis. *Biomedicine & Pharmacotherapy.* 2019;115: 108951.
32. Chistiakov, Dimitry A., Alexander N. Orekhov, and Yuri V. Bobryshev. Extracellular Vesicles And Atherosclerotic Disease. *Cellular And Molecular Life Sciences.* 2015:72 (14): 2697-2708.
33. Su SA, Xie Y, Fu Z, Wang Y, Wang JA, Xiang M. Emerging role of exosome-mediated intercellular communication in vascular remodeling. *Oncotarget.* 2017;8(15):25700–25712.
34. Beer KB, Wehman AM. Mechanisms and functions of extracellular vesicle release in vivo-What we can learn from flies and worms. *Cell Adhesion & Migration*. 2017;11(2):135-150.
35. Shifrin DA Jr, DemoryBeckler M, Coffey RJ, Tyska MJ. Extracellular vesicles: communication, coercion, and conditioning. *Molecular Biology of the cell.* 2013;24:1253-9.
36. Lagace, T., & Ridgway, N. The role of phospholipids in the biological activity and structure of the endoplasmic reticulum. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research.* 2013;1833(11): 2499-2510.
37. Pollet H, Conrard L, Cloos AS, Tyteca D. Plasma Membrane Lipid Domains as Platforms for Vesicle Biogenesis and Shedding? *Biomolecules.* 2018;8(3):94.
38. Chauhan N, Farine L, Pandey K, Menon AK, Bütikofer P. Lipid topogenesis--35years on. *Biochimica et Biophysica Acta*. 2016;1861(8 Pt B):757–766.
39. Hall K, Lee T-H, Mechler AI, Swann MJ, Aguilar M-I. Real-time Measurement of Membrane Conformational States Induced by Antimicrobial Peptides: Balance Between Recovery and Lysis. *Scientific Reports.* 2014;4:5479.
40. W. Robert Bishop, Robert M. Bell, Assembly of the endoplasmic reticulum phospholipid bilayer: the phosphatidylcholine transporter.*Cell.* 1985;42(1):51-60.
41. Alicia Alonso and Félix M. Goñi. The Physical Properties Of Ceramides In Membranes. *Annual Review of Biophysics*. 2018;47(1):633-654.
42. Li SP, Lin ZX, Jiang XY, Yu XY. Exosomal cargo-loading and synthetic exosome-mimics as potential therapeutic tools. *Acta Pharmacologica Sinica.* 2018 Apr;39(4):542-551.

43. Bianco F, Perrotta C, Novellino L. et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. *The EMBO Journal.* 2009;28(8): 1043-1054.
44. D'Souza-Schorey C, Clancy JW. Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes & Development* 2012; 26(12):1287-99.
45. Verhoef, P. A., M. Estacion, et al. P2X7 Receptor-Dependent Blebbing and the Activation of Rho-Effector Kinases, Caspases, and IL-1Ī² Release. *The Journal of Immunology.* 2003;170(11): 5728-5738.
46. Isabella Panfoli, Laura Santucci, Maurizio Bruschi, Andrea Petretto, Daniela Calzia, Luca A. Ramenghi, Gianmarco Ghiggeri, Giovanni Candiano. Microvesicles as promising biological tools for diagnosis and therapy. *Expert Review of Proteomics.* 2018;15(10):801-808.
47. Juan, T., & Fürthauer, M. Biogenesis and function of ESCRT-dependent extracellular vesicles. *Seminars In Cell & Developmental Biology*. 2018;74:66-77.
48. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brugger B, Simons M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science.* 2008;319(5867): 1244-1247.
49. Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell & Bioscience*. 2019;9:19.
50. Console L., Scalise M, Indiveri, C. Exosomes in inflammation and role as biomarkers. *Clinica Chimica Acta.* 2019;488:165-171.
51. Admyre C, Johansson SM, Qazi KR, Filen J-J, Lahesmaa R, Norman M, et al. Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. *The Journal of Immunology.* 2007;179(3):1969-78.
52. Console L, Scalise M, Indiveri C. Exosomes in inflammation and role as biomarkers. *Clinica Chimica Acta.* 2019;488:165–71.
53. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience.* 2015;65(8):783-797.
54. Record M, Silvente-Poirot S, Poirot M, Wakelam MJO. Extracellular vesicles: lipids as key components of their biogenesis and functions. *Journal of Lipid Research*. 2018;59(8):1316-1324.
55. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495–516.

56. Atkin-Smith, G. K., Poon, I. K. Disassembly of the Dying: Mechanisms and Functions. *Trends in Cell Biology*. 2017;27: 151–62
57. Orlando KA, Stone NL, Pittman RN. Rho kinase regulates fragmentation and phagocytosis of apoptotic cells. *Experimental Cell Research.* 2006;312(1):5-15.
58. Jiang L, Paone S, Caruso S, Atkin-Smith GK, Phan TK, Hulett MD, et al. Determining the contents and cell origins of apoptotic bodies by flow cytometry. *Scientific Reports.* 2017;7(1):14444.
59. Zhang, H., D. Freitas, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature Cell Biology*. 2018;20(3): 332-343.
60. Willms E, Cabañas C, Mäger I, Wood MJA, Vader P. Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. *Frontiers in Immunology*. 2018;9(738).
61. Lv J-X, Kong Q, Ma X. Current advances in circulating inflammatory biomarkers in atherosclerosis and related cardio-cerebrovascular diseases. *Chronic Diseases and Translational Medicine*. 2017;3(4):207-12.
62. Lawson C, Vicencio JM, Yellon DM, Davidson SM. Microvesicles and exosomes: new players in metabolic and cardiovascular disease. *Journal of Endocrinology*. 2016;228(2):R57–R71.
63. Chargaff E, West R. The biological significance of the thromboplastic protein of blood. *Journal of Biological Chemistry*. 1946; 166: 189–97.
64. Wolf P. The nature and significance of platelet products in human plasma. *British Journal of Haematology*. 1967; 13: 269–88.
65. Anderson HC. Vesicles associated with calcification in the matrix of epiphyseal cartilage. *Journal of Cell Biology*. 1969; 41: 59–72.
66. Benz EW Jr., Moses HL. Small, virus-like particles detected in bovine sera by electron microscopy. *Journal of the National Cancer Institute*. 1974; 52: 1931–4.
67. Dvorak HF, Quay SC, Orenstein NS, Dvorak AM, Hahn P, Bitzer A Met al. Tumor shedding and coagulation. *Science*. 1981; 212: 923–4.
68. Yang Y, Hong Y, Cho E, Kim GB, Kim IS. Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery. *Journal of Extracellular Vesicles*. 2018;7(1):1440131.

69. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV , Melief CJet al. B lymphocytes secrete antigen-presenting vesicles. *Journal of Experimental Medicine*. 1996; 183: 1161–72.
70. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. E xosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology*. 2007; 9: 654–9.
71. Lasser C, O'Neil SE, Ekerljung L, Ekstrom K, Sjostrand M, Lotvall J. RNA-containing exosomes in human nasal secretions. *American Journal of Rhinology & Allergy*. 2011; 25: 89–93.
72. Fairweather DL. Sex Differences in Inflammation During Atherosclerosis. *Clinical Medicine Insights: Cardiology*. 2014; 8(3):49-59.
73. Bucciarelli P, Martinelli I, Artoni A et al. Circulating microparticles and risk of venous thromboembolism. *Thrombosis Research*. 2012; 129:591-597.
74. Torun AN, Kulaksizoglu S, Kulaksizoglu M, Pamuk BO, Isbilen, E, Tutuncu NB. Serum total antioxidant status and lipid peroxidation marker malondialdehyde levels in overt and subclinical hypothyroidism. *Clinical Endocrinol (Oxf).* 2009;70:469-474.
75. Khoschsorur GA., Winklhofer-Roob BM, Rabl H. et al. Evaluation of a Sensitive HPLC Method for the Determination of Malondialdehyde, and Application of the Method to Different Biological Materials. *Chromatographia* 2000;52:181.
76. Mellisho EA, Velásquez AE, Nuñez MJ, Cabezas JG, Cueto JA, Fader C, et al. Identification and characteristics of extracellular vesicles from bovine blastocysts produced in vitro. *PLoS ONE.* 2017;12(5): e0178306.
77. Giannotta JM, Trani M, Dejana E. VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Development. Cell*. 2013;26:441–454.
78. Adam AP. Regulation of Endothelial Adherens Junctions by Tyrosine Phosphorylation. *Mediators of Inflammation*. 2015;2015:24.
79. Turowski P, Martinelli R, Crawford R, Wateridge D, Papageorgiou A-P, Lampugnani MG et al. Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. *Journal of Cell Science*.2008;121: 29-37.
80. Park SY, DiMaio TA, Liu W, Wang S, Sorenson CM, Sheibani N. Endoglin regulates the activation and quiescence of endothelium by

participating in canonical and non-canonical TGF-β signaling pathways. *Journal of Cell Science*. 2013;126: 1392-1405.

81. Saita E, Miura K, Suzuki-Sugihara N, Miyata K, Ikemura N, Ohmori R et al. Plasma Soluble Endoglin Levels Are Inversely Associated With the Severity of Coronary Atherosclerosis—Brief Report. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2016;37:49-52.
82. Galkina E, Ley K. Vascular Adhesion Molecules in Atherosclerosis. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2007;27:2292-2301.
83. Dignat-George F1, Boulanger CM. The many faces of endothelial microparticles. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2011;31(1):27-33.
84. Deng F, Wang S, Zhang L. Endothelial microparticles act as novel diagnostic and therapeutic biomarkers of circulatory hypoxia-related diseases: a literature review. *Journal of Cellular and Molecular Medicine*. 2017;21(9):1698–1710.
85. Hu SS, Zhang HG, Zhang QJ, Xiu RJ. CD144 + EMPs/CD62E + EMPs: A couple of new biomarkers to monitor endothelial function in hypertension with hyperlipidemia involved. *International Journal of Cardiology*. 2014;175(1):203.
86. Simak J, Gelderman MP, Yu H, Wright V, Baird AE. Circulating endothelial microparticles in acute ischemic stroke: a link to severity, lesion volume and outcome. *Journal of Thrombosis and Haemostasis.* 2006;4:1296-1302.
87. Wang Z, Cai W, Hu S, Xia Y, Wang Y, Zhang Q, et al. A Meta-Analysis of Circulating Microvesicles in Patients with Myocardial Infarction. *Arquivos Brasileiros de Cardiologia*. 2017;109:156-64.
88. Berezin AE, Kremzer AA, Berezina TA, Martovitskaya YV. Pattern of circulating microparticles in chronic heart failure patients with metabolic syndrome: Relevance to neurohumoral and inflammatory activation. *BBA Clinical.* 2015;4:69-75.
89. Tramontano AF, O'Leary J, Black AD, Muniyappa R, Cutaia MV, El-Sherif N. Statin decreases endothelial microparticle release from human coronary artery endothelial cells: implication for the Rho-kinase pathway. *Biochemical and Biophysical Research Communications*. 2004;320-1:34-38.
90. Badimon L, Suades R, Arderiu G, Peña E, Chiva-Blanch G, Padró T. Microvesicles in Atherosclerosis and Angiogenesis: From Bench

to Bedside and Reverse. *Frontiers in Cardiovascular Medicine*. 2017;4:77.

91. Brites F, Martin M, Guillas I, Kontush A. Antioxidative activity of high-density lipoprotein (HDL): Mechanistic insights into potential clinical benefit. *BBA Clinical*. 2017;8:66-77.
92. Lu J, Holmgren A. The thioredoxin antioxidant system. *Free Radical Biology and Medicine*. 2014;66:75-87.
93. Ayers L, Pink R, Carter DRF, Nieuwland R. Clinical requirements for extracellular vesicle assays. *Journal of Extracellular Vesicles*. 2019;8(1):1593755.
94. Szatanek R, Baj-Krzyworzeka M, Zimoch J, et al. The methods of choice for Extracellular Vesicles (EVs) characterization. *International Journal of Molecular Sciences*. 2017;18:1153.
95. Dickhout A, Koenen RR. Extracellular Vesicles as Biomarkers in Cardiovascular Disease; Chances and Risks. *Frontiers in Cardiovascular Medicine*. 2018;5(113).
96. Gholizadeh S, Shehata Draz M, Zarghooni M, Sanati-Nezhad A, Ghavami S, Shafiee H, et al. Microfluidic approaches for isolation, detection, and characterization of extracellular vesicles: Current status and future directions. *Biosensors and Bioelectronics*. 2017;91:588-605.
97. Rayyan M, Zheutlin A, Byrd J. Clinical research using extracellular vesicles: insights from the international society for extracellular vesicles 2018 annual meeting. *Journal of Extracellular Vesicles*. 2018;7(1):1535744.

WORK APPROBATION

List of Original Publications

1. Žėkas V, Kučinskienė ZA. Endotelinės mikrodalelės: naujas ankstyvosios aterosklerozės žymuo. *Laboratorinė medicina*. 2016;18(2):25–29.
2. Thery C, Kenneth WW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R. Žėkas V et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*. 2018;23;7(1):1535750.
3. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius M, Janilionienė A, Linkevičiūtė A, Kučinskienė ZA. Chronic and oxidative stress association with total count of endothelial microvesicles in healthy young male plasma. *Advances in Clinical and Experimental Medicine*. 2019;28(5):683–692.
4. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Vitkus D, Radzevičius M, Janiulionienė A, Linkevičiūtė A, Kutkienė S, Kučinskienė ZA. Circulating microvesicles association with oxidative and chronic stress in healthy and coronary artery disease affected male populations. *Laboratorinė medicina*. 2019;21:2(82):51–55.

Poster Presentations

1. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius M, Janiulionienė A, Kučinskienė ZA. Damaging factors on endothelial cells affect circulating endothelial microvesicle count in peripheral blood. *Atherosclerosis*. 2017;263:132–133 (The 85th EAS Congress, Prague, Czech Republic, April 23–26, 2017).
2. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius M, Janilionienė A, Linkevičiūtė A, Kučinskienė ZA.Chronic and oxidative stress association with total count of endothelial microvesicles in healthy young male plasma. *EestiArst*. 2018;97(Lisa1):46–47 (The Seventh Baltic Atherosclerosis Congress. Tallin, Estonian Republic. April 6–7, 2018).
3. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Vitkus D, Radzevičius M, Janilionienė A, Linkevičiūtė A, Kučinskienė ZA. Endothelial microvesicles as biomarkers of oxidative stress. *Medicina*; 2019;55

(supplement 1):207. (The 77th International Scientific Conference of the University of Latvia, February 22, 2019, Riga, Latvia).

Oral and Poster Presentations

1. Žėkas V (oral presentation), Matuzevičienė R, Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius M, Janulionienė A, Kučinskienė ZA. Atherosclerosis risk factors influence endothelial microvesicle count in peripheral blood. Open Readings 2017: The 60th International Conference for Students of Physics and Natural Sciences, March 14–17, 2017, Vilnius, Lithuania: programme and abstracts. Vilnius: Vilniaus Universitetas. ISSN 2029-4425. 2017, p. 105.
2. Žėkas V (oral presentation), Matuzevičienė R; Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius Mantas, Janiulionienė A, Linkevičiūtė A, Kučinskienė ZA. Endothelial microvesicles in subclinical atherosclerosis: inflammation, chronic and oxidative stress associations // *Laboratorinė medicina.* Vilnius : Lietuvos laboratorinės medicines draugija. ISSN 1392-6470. 2018, T. 20, special suppl. p. S42 (Baltic Laboratory Medicine Congress, Vilnius, Republic of Lithuania, May 10–12, 2018).

Oral Presentations

1. Žėkas V (oral presentation). "Endotelinių mikrodalelių diagnostinė reikšmė". The IX Congress of Lithuanian Laboratory Medicine: "Procesai iki tyrimų ir kiti aktualūs šiandieninės laboratorinės medicines klausimai," Vilnius, Republic of Lithuania, April 20, 2017.
2. Žėkas V (oral presentation), Matuzevičienė R, Karčiauskaitė D, Vitkus D, Radzevičius M, Janilionienė A, Linkevičiūtė A, Kučinskienė ZA. "Endothelial microvesicles and oxidative stress." The Eleventh Young Scientists Conference: "Bioateitis: gamtos ir gyvybės mokslų perspektyvos." Vilnius, Republic of Lithuania, December 14, 2018.
3. Žėkas V (oral presentation)."Ląstelinių mikrodalelių kiekio pokyčiai laikotarpyje po miokardo infarkto." The XI Congress of Lithuanian Laboratory Medicine: "Procesai iki tyrimų ir kiti aktualūs šiandieninės laboratorinės medicines klausimai," Vilnius, Republic of Lithuania, April 9, 2019.

NOTES

Vilniaus universiteto leidykla
Saulėtekio al. 9, LT-10222 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 15 egz.