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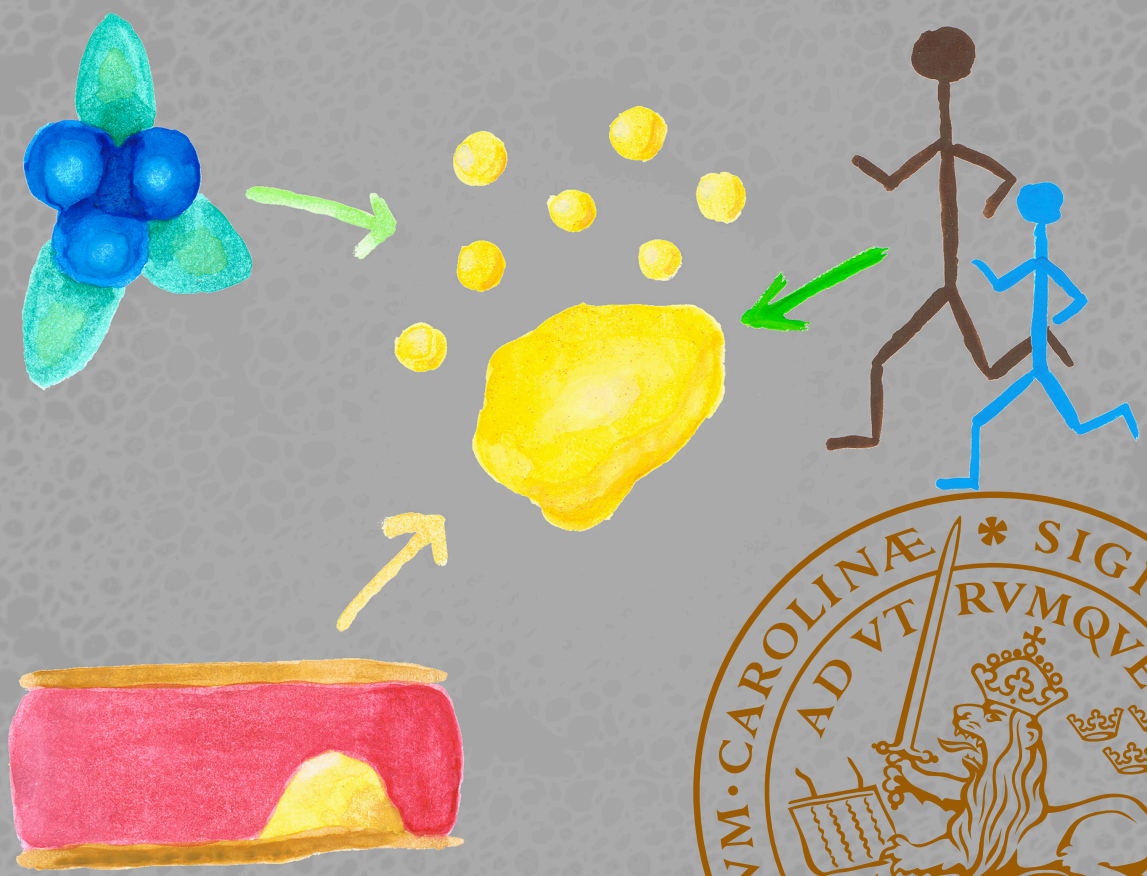

Microvesicle signaling in cardiovascular biology under normal and pathobiological conditions

Paulina Bryl-Górecka

LUND UNIVERSITY

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Abstract

Cardiovascular disease (CVD) is one of the leading causes of death worldwide. Atherosclerosis, a chronic pathology directly related to the circulatory system, develops due to an interplay between several molecular events. Atherosclerotic plaque build-up can lead to a reduction in arterial blood flow and finally, myocardial infarction (MI). To prevent or slow down of the CVD progression, it is important to primarily address modifiable risk factors connected with lifestyle and nutrition.

Microvesicles (MVs) belong to a larger group, called extracellular vesicles that also includes exosomes and apoptotic bodies. These small (<1 μm) vesicles are released from the cell by blebbing of plasma membrane, a complex process involving numerous signaling pathways. Circulating MVs originate mainly from blood cells, as well as vascular endothelial cells. The release is activated by pro-inflammatory and pro-coagulant conditions. MVs transport bioactive molecules both on their surface and inside the lumen that makes them an interesting target for biomarker discovery and potential diagnostic application. The overall aim of this thesis was to explore the role of MVs in cardiovascular-related pathologies.

To analyse plasma MVs, we optimized an acoustic trapping machine based method, employing ultrasonic standing wave, that isolates vesicles in a non-contact manner. We demonstrated that the new method is comparable to standard protocols of MV enrichment and could potentially be used for vesicle-based diagnostics . Furthermore, the results of a clinical study revealed that exercise has a protective effect on the vasculature through decreasing a release of MVs from activated endothelium. The proteomic data showed that exercise changes the pattern of the vesicular cargo.

Bilberries (*Vaccinium myrtillus*) are considered to be a beneficial dietary component for patients with CVD and in the BEARSMART randomized clinical study we explored if there was an effect of bilberry powder supplementation on circulating MVs in MI patients. After eight weeks of dietary intervention, there was a significant reduction in platelet and endothelial MV concentration. Moreover, the *in vitro* part of the study demonstrated that bilberry extract decreased endothelial vesiculation, which was related to the $P2X_7$ purinergic receptor pathway. The results of this project showed for the first time that nutrional changes can directly affect MV release and underlined the protective influence of berries on vascular health.

In the PROFLOW clinical study, we observed a negative relationship between coronary flow reserve (CFR), a parameter depicting blood flow in the heart, and levels of endothelial and platet MVs, circulating in patients with CVD. Proteomic profiling demonstrated similar connection for CFR and several vesicular biomarkers. The outcome of this clinical study pointed at a potential application of MVs for diagnosis of vascular dysfunction.

Lastly, we showed that atherosclerotic plaque released MVs, following balloon angioplasty. The results revealed that the vesicles originated from several types of cells and exhibited a pro-atherogenic ‘pattern’ of proteins that represented the pathological processes within the cardiovascular system that can lead to the atheroma formation. We also demonstrated an advantage of analysing isolated EVs, compared to crude plasma samples.

The outcome of the studies included in this thesis points at the important role of MVs in the cardiovascular system. Study I and II underlined the importance of exercise and nutrition for prevention of CVD through decrease in MVs, whereas the Western world is still characterized with physical inactivity and imbalanced diet. Study III and IV focused on potential application of MVs as biomarkers or targets of therapeutics, however it needs further, thorough research and possibly, personalized approach.

Key words Microvesicles, Cardiovascular disease, Atherosclerosis, Biomarkers, Acoustic trapping

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'Education is the most powerful weapon,
which you can use to change the world'
Nelson Mandela

'For a man to conquer himself
is the first and noblest of all victories'
Plato

To my Family

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II. **Paulina Bryl-Górecka**, Ramasri Sathanoori, Lilith Arevström, Rikard Landberg, Cecilia Bergh, Mikael Evander, Björn Olde, Thomas Laurell, Ole Fröbert and David Erlinge. *Bilberry supplementation after myocardial infarction decreases microvesicles in blood and affects endothelial vesiculation.* Mol Nutr Food Res 2020, 64(20):e2000108; doi: 10.1002/mnfr.202000108.

III. **Paulina Bryl-Górecka**, Kreema James, Kristina Torngren, Inger Haraldsson, Li-Ming Gan, Sara Svedlund, Björn Olde, Thomas Laurell, Elmir Omerovic and David Erlinge. *Microvesicles in plasma reflect coronary flow reserve in patients with cardiovascular disease.* Am J Physiol Heart Circ Physiol 2021; doi: 10.1152/ajpheart.00869.2020.

IV. **Paulina Bryl-Górecka**, Kreema James, Björn Olde, Thomas Laurell, David Erlinge. *Characterization of extracellular vesicles released from atherosclerotic plaque following balloon angioplasty*. Manuscript unpublished.

Popular Summary

Cardiovascular disease (CVD) is one of the leading causes of death worldwide. Major risk factors are both lifestyle-related, as well as non-modifiable. Atherosclerosis is a chronic pathology of the arteries that is demonstrated as a plaque built-up within the vessel. Such deposition can lead to a reduction in blood flow and finally, myocardial infarction. Although many types of anti-atherogenic medications have been already developed, it is important to also address modifiable risk factors connected with lifestyle and nutrition, to prevent or slow down the CVD progression.

Microvesicles (MVs) are a type of so called extracellular vesicles that originate from different types of cells and are released to the environment. MVs found in the circulation are predominantly released from blood cells or endothelial cell layer in the vessels, and are mainly stimulated by pathological, pro-inflammatory conditions. MVs carry many different classes of molecules and thus are a very interesting source of potential biomarkers – indicators of a disease. To analyse circulating MVs, we optimized a new method for MV isolation from plasma, called acoustic trapping. It is a new technology that employs acoustic standing wave that 'traps' vesicles.

In Paper I we showed that acoustic trapping is comparable to standard protocols and could potentially be used for vesicle-based diagnostics. The results of a clinical study revealed that exercise has a beneficial effect on the vasculature through decreasing a release of MVs from endothelium. The analysis also indicated that physical activity changes the protein content of the vesicles.

Bilberries, or Nordic blueberries, are considered beneficial for patients with CVD. Thus, in Paper II we investigated if there was an effect of bilberry powder supplementation on circulating MVs in myocardial infarction patients. Eight weeks of dietary intervention significantly decreased the concentration of endothelial and platelet-derived MVs. *In vitro* culturing of endothelial cells demonstrated that bilberry extract decreased vesicle release that was related to the $P2X_7$ receptor. This project underlines that nutrional changes can affect MV release and confirm the protective impact of berries on cardiovascular health.

In Paper III, we observed a negative correlation between coronary flow reserve, a parameter depicting blood flow in the heart, and levels of endothelial and platet MVs, circulating in patients with CVD. There was a similar connection for CFR and

several proteins, transported in MVs. The outcome of this clinical study points at a potential application of MVs for diagnosis of vascular dysfunction.

In paper IV we showed that atherosclerotic plaque releases MVs, following balloon angioplasty that originated from several types of cells. The vesicles exhibited a pathological 'pattern' of proteins that represent the processes within the cardiovascular system that lead to the formation of atheroma.

In summary, the outcome of the studies included in this thesis points at the important role of MVs in the cardiovascular system and their potential application as biomarkers.

Populär Sammanfattning

Kardiovaskulär sjukdom (CVD) är en av de vanligaste dödsorsakerna i världen. De största riskfaktorerna är både livsstilsrelaterade och kroniska. Åderförkalkning är en kronisk patologi i artärerna som kännetecknas av plackbildning i blodkärlen. Denna förändring kan i sin tur leda till en minskning av blodflödet och slutligen till hjärtinfarkt. Även om många typer av anti-aterogena läkemedel redan har utvecklats, så är det viktigt att även ta itu med riskfaktorer kopplade till livsstil och näring för att förhindra eller sakta ner progressionen av CVD.

Mikrovesiklar (MV) är extracellulära vesiklar som frigörs från celler till omgivningen genom avknoppning från plasmamembranet. MV som finns i cirkulationen frigörs huvudsakligen från blodkroppar eller från kärlens endotelcellskikt vilket i sin tur kan stimuleras av patologiska, proinflammatoriska tillstånd. MV innehåller många olika typer av molekyler och är därför en mycket intressant källa till potentiella biomarkörer – dvs indikatorer på en sjukdom. För att analysera cirkulerande MV:er optimerade vi en ny metod för MV-isolering från plasma, kallad ”acoustic trapping”. Det är en ny teknik som använder en akustisk stående våg som "fångar" mikrovesiklar.

I Arbete I visade vi att ”acoustic trapping” är jämförbar med standardmetoder och potentiellt kan användas för vesikelbaserad diagnostik. Resultaten av en klinisk studie visade att motion har en gynnsam effekt på kärlsystemet genom att minska frisättningen av MV från endotelet. Analysen visade också att fysisk aktivitet förändrar proteininnehållet i vesiklarna.

Blåbär anses vara fördelaktiga för patienter med CVD. Således undersökte vi i Arbete II om ett diet tillägg av blåbärspulver har effekt på cirkulerande MV hos hjärtinfarktpatienter. Vi fann att åtta veckors dietintervention signifikant minskade koncentrationen av MV från endotel och trombocyter. Blåbärsextrakt minskade frisättningen av vesiklar från odlade endotelceller. Detta projekt visar att näringsförändringar kan påverka MV-frisättning och bekräftar den skyddande effekten av blåbär på hjärt-kärlhälsan.

I Arbete III observerade vi en negativ korrelation mellan koronarflödesreserv, en parameter som visar blodflödet i hjärtat och nivåer av endotel- och trombocyt-MV, hos patienter med CVD. Det fanns liknande kopplingar mellan CFR och flera

olika vesikel-proteiner. Resultatet av denna kliniska studie pekar på en potentiell tillämpning av MV för diagnos av vaskulär dysfunktion.

I Arbete IV visade vi att ballongangioplastik orsakar att aterosklerotiska plack frisätter MV, och att dessa härstammar från flera typer av celler. Vesiklarna uppvisade ett patologiskt ”mönster” av proteiner som alla representerar processer som leder till bildandet av aterom.

Sammanfattningsvis pekar resultaten, som ingår i denna avhandling, på MV:s viktiga roll i det kardiovaskulära systemet och deras potentiella tillämpning som biomarkörer.

Podsumowanie popularnonaukowe

Choroby układu sercowo-naczyniowego są jedną z głównych przyczyn zgonów na świecie. Czynniki ryzyka tej grupy chorób są zarówno związane ze stylem życia, jak i niemodyfikowalne. Miażdżyca to choroba zwyrodnieniowa tętnic, polegająca na wytworzeniu blaszki miażdżycowej wewnątrz naczynia krwionośnego. Proces ten może doprowadzić do zmniejszenia przepływu krwi, a w skrajnych przypadkach – zawału mięśnia sercowego. Pomimo istnienia wielu typów leków przeciwmiażdżycowych, należy zadbać przede wszystkim o modyfikowalne czynniki ryzyka, związane ze stylem życia i odżywianiem, w celu zapobiegania i spowolnienia rozwoju chorób układu krążenia.

Mikropęcherzyki to rodzaj tzw. pęcherzyków zewnątrzkomórkowych, wytwarzanych z błony komórkowej, które są uwalniane przez komórki i wysyłane do otoczenia. Proces ten ma miejsce głównie w patologicznych warunkach prozapalnych. Mikropęcherzyki krążące we krwi pochodzą przede wszystkim z płytek krwi, leukocytów i erytrocytów, a także śródbłonka, czyli warstwy komórek wyścielających naczynia krwionośne. Mikropęcherzyki transportują wiele różnych rodzajów związków chemicznych i z tego względu są interesującym źródłem potencjalnych biomarkerów – molekularnych wskaźników choroby. Aby zbadać mikropęcherzyki krążące we krwi, opracowaliśmy nową metodę o nazwie „pułapkowanie akustyczne", pozwalającą na ich pozyskanie z osocza. Technologia ta wykorzystuje tzw. stojącą falę akustyczną, która umożliwia „złapanie" i oczyszczenie pęcherzyków.

W publikacji nr 1 pokazaliśmy, że pułapkowanie akustyczne osiąga porównywalne wyniki, jak standardowe protokoły do izolacji mikropęcherzyków i w przyszłości mogłaby być potencjalnie użyta w celach diagnostycznych. Rezultaty testu klinicznego dowiodły, że aktywność fizyczna ma pozytywny wpływ na naczynia krwionośne poprzez zmniejszanie wydzielania mikropęcherzyków przez śródbłonek. Wyniki analizy wskazały również na wpływ ćwiczeń na zmiany w zawartości białek, obecnych w pęcherzykach.

Działanie borówki czarnej, popularnie zwanej jagodą, uznawane jest za korzystne w przypadku chorób sercowo-naczyniowych. Z tego względu, w publikacji nr 2 badaliśmy wpływ suplementacji proszkiem z owoców tej rośliny na mikropęcherzyki we krwi pacjentów po przebytym zawale serca. Ośmiotygodniowa interwencja dietetyczna znacząco zmniejszyła poziom

mikropęcherzyków pochodzących ze śródbłonka oraz płytek krwi. Badania *in vitro* na wyizolowanych komórkach śródbłonka ukazały, że ekstrakt z borówki czarnej zmniejszył produkcję pęcherzyków zewnątrzkomórkowych, co było powiązane z receptorem $P2X_7$. Wyniki tego projektu podkreślają wpływ diety na uwalnianie mikropęcherzyków i potwierdzają pozytywny efekt jagód na układ krążenia.

W publikacji nr 3 zaobserwowaliśmy odwrotną korelację między tzw. rezerwą wieńcową, parametrem obrazującym przepływ w sercu, a poziomami mikropęcherzyków pochodzących z płytek krwi i śródbłonka u pacjentów ze zdiagnozowaną chorobą układu krążenia. Podobny związek miał miejsce również w przypadku rezerwy wieńcowej oraz kilkoma białkami, transportowanymi przez mikropęcherzyki. Rezultaty tego badania wskazują na potencjalne zastosowanie mikropęcherzyków w celu diagnozy dysfunkcji śródbłonka.

W publikacji nr 4 ukazaliśmy, że po zabiegu angioplastyki tętnic, płytka miażdżycowa uwalniała mikropęcherzyki, pochodzące z rożnych rodzajów komórek. Pęcherzyki z blaszki miażdżycowej charakteryzowały się również patologicznym wzorcem białek, co oddaje procesy toczące się w naczyniach krwionośnych, które doprowadzają do rozwoju miażdżycy.

Podsumowując, wyniki naszych badań wskazują na ważną rolę mikropęcherzyków w funkcjonowaniu układu sercowo-naczyniowego i ich potencjalne zastosowanie jako biomarkerów.

Introduction

Cardiovascular disease

Cardiovascular disease (CVD) is a term grouping diseases related to circulatory system, mainly blood vessels and the heart. CVD is a serious global burden, as it accounts for approximately one third of deaths worldwide.[1] Major risk factors comprise smoking, obesity, genetics, hypertension, diabetes, high cholesterol levels, physical inactivity, alcohol consumption and air pollution. In the past years, mainly due to an increase in CVD treatment options, a reduction in morbidity and mortality has been observed. Examples of applied medications include statins, beta-blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, warfarin, platelet aggregation-regulating compounds such as aspirin, clopidogrel or newer: prasugrel and ticagrelor.[1] However, modifiable risk factors should be primarily addressed by modification of lifestyle (e.g. regular exercise) and diet.

One of the diseases belonging to the CVD group is coronary artery disease (CAD), also known as ischemic heart disease.[2] It is connected with accumulation of atherosclerotic plaque in the heart arteries that leads to a decrease in the blood flow. CAD includes stable and unstable angina, cardiac arrest and finally, myocardial infarction or heart attack (Fig. 1), characterized by heart tissue damage due to very low oxygen levels and release of troponin into the circulation. Other types of CVD include e.g. heart failure, stroke, cardiomyopathy, hypertensive heart disease and aortic aneurysm. While not all CVD types are connected with atherosclerotic plaques, CAD is directly related to this vascular pathology.

Endothelial dysfunction and atherosclerosis

Endothelial dysfunction is the first step towards development and progression of atherosclerosis and further CAD. It involves several molecular events, such as decrease in nitric oxide (NO) production, release of pro-inflammatory and pro-thrombotic molecules, increased platelet aggregation that lead to a decline in vascular relaxation and subsequent reduction in blood flow in coronary arteries.[3,4]

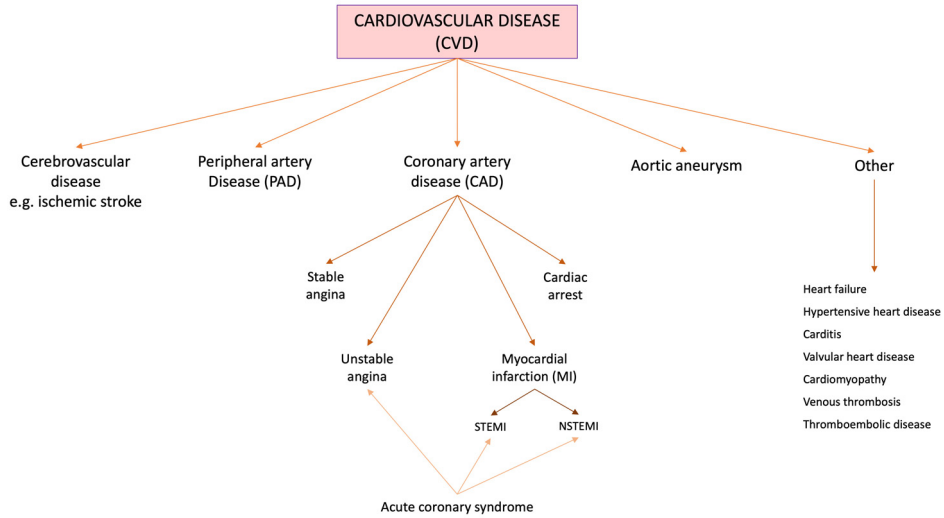

Fig. 1 Types of cardiovascular diseases, based on Olvera Lopez et al.[2] Abbreviations: STEMI – ST elevation myocardial infarction, NSTEMI – non-ST elevation myocardial infarction.

Transition from endothelial dysfunction to a chronic disease called atherosclerosis is caused by interplay between several molecules that switch the vasculature to increasingly pathological status. Interestingly, atherosclerosis is sometimes referred to as 'inflammatory disease', reflecting the complexity of this process.[5] The occurring molecular events include e.g. high levels of LDL in blood, accumulation of oxidized lipids in the vascular wall, leukocyte adhesion to the endothelial layer, proliferation of vascular smooth muscle cells (SMCs), extracellular matrix deposition, as well as calcium accumulation that finally lead to atherosclerotic plaque formation in the vessel, decrease of the vessel lumen and deterioration of blood flow (Fig. 2).[6] Substantial blood flow limitation in the arteries is manifested as arterial stenosis that is an advanced stadium of the disease.[4,7]

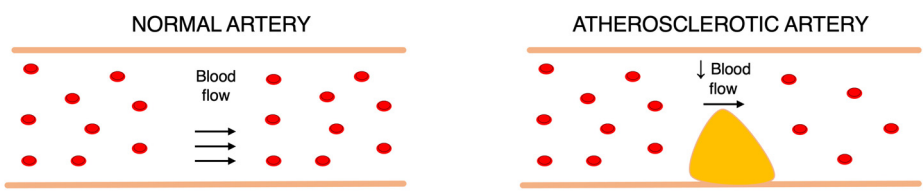

Fig. 2 Schematic representation on how the presence of atherosclerotic plaque decreases arterial blood flow.

Atheroma composition

Atherosclerotic plaque, or atheroma, has a diverse composition that is generally divided into the following sections: a necrotic core, containing macrophages and cholesterol crystals, as well as a fibrous cap, consisting of extracellular matrix, SMCs and leukocytes (Fig. 3). The lesions are considered as vulnerable, when the cap is thin or stable, when the cap is thick due to abundant presence of SMCs.[8]

In severe cases, such as arterial occlusion and MI, the plaque is ruptured that results in an exposure to the bloodstream of prothrombotic material, derived from the atheroma and further, formation of a thrombus (Fig. 3).[8]

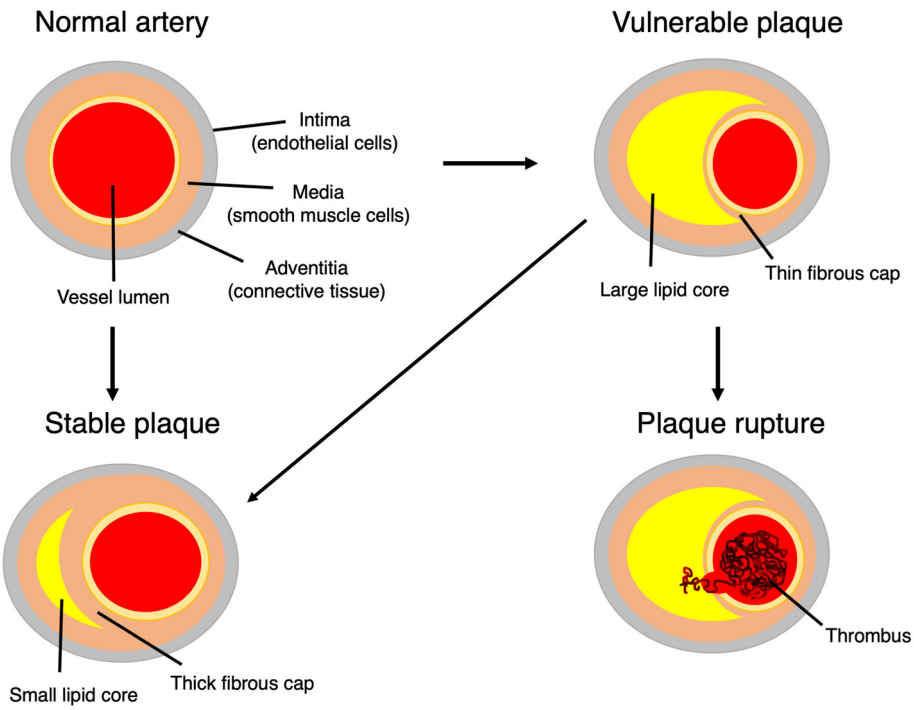

Fig. 3 Schematic representation of atheroma composition and progression.

A standard method to increase the lumen of the narrowed vessel and improve the blood flow is balloon angioplasty.[9] The procedure applies a catheter with a deflated balloon that is placed in the artery and moved to the respective site of the plaque. Once the balloon reaches the atheroma, it becomes inflated and compresses the plaque, thus increasing blood flow (Fig. 4).

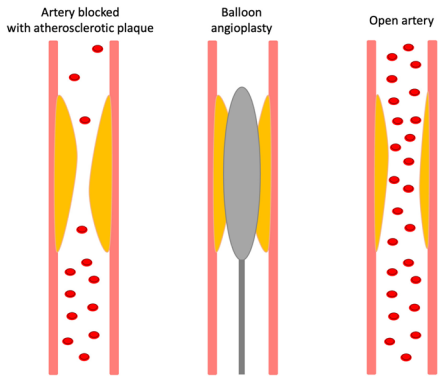

Fig. 4 Schematic representation of balloon angioplasty procedure.

Effects of nutrition on cardiovascular health

Nutrition for prevention and treatment of CVD

Diet is one of the essential lifestyle-related factors, profoundly affecting human health. Proper nutrition is a weapon combating not only excessive body weight, but also preventing from several pathologies, especially diseases of affluence, including CVDs. Current recommendations from the American Heart Association for a healthy, heart-friendly diet include limiting intake of sweets, sodium, alcohol and animal-derived products rich in saturated fats, e.g. red meat and high-fat dairy; incorporation of substantial amounts of vegetables, fruits, whole grains, nuts, low fat dairy, fish and plant oils rich in polyunsaturated fatty acids (PUFAs).[10] Some of the officially recommended approaches that fulfil these criteria are Dietary Approach to stop Hypertension (DASH),[11,12] plant-based [13,14] and Mediterranean diets.[15,16] There is also a growing interest in nutritional supplementation for CVD prevention and additional treatment. For example, intake of standardized anti-inflammatory Omega-3 PUFAs, found naturally in e.g. fatty fish, flax seeds and canola oil, has recently gained attention as potential beneficial anti-CVD compound, although the results of meta-analyses have not been consistent so far.[17-21]

Bilberries for cardiovascular health

It has been reported that diet rich in berries exhibits protective effects on the cardiovascular system.[22,23] Majority of these beneficial properties are attributed to a high content of polyphenolic pigments called anthocyanins. The mechanism of action on CVD-related pathologies is based on e.g. decreasing local oxidative

stress,[24-26] improving NO production[27] or inflammation inhibition.[28] Summary of favourable influence of bilberry consumption is presented on Fig. 5. As bilberry (*Vaccinium myrtillus*) is a widely consumed berry species, its incorporation in the diet as a component or a supplement, appears to be a promising way of improving the status of CVD patients.

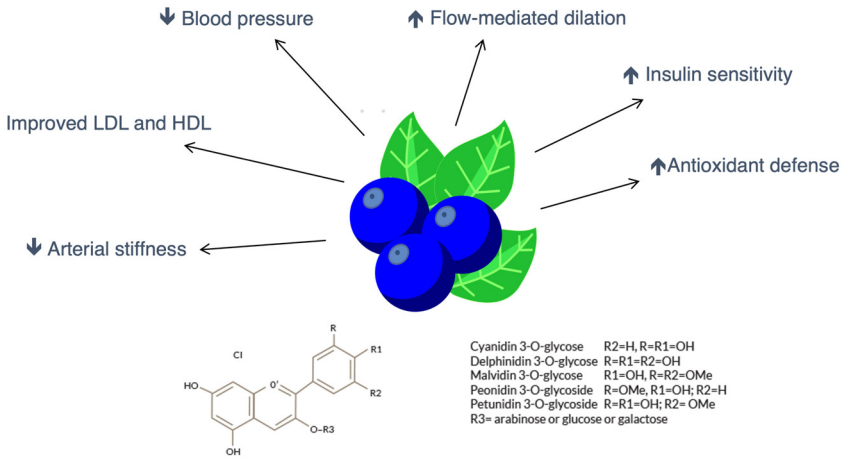

Fig. 5 Proposed benefits of bilberry dietary supplementation (upper panel), based on the current clinical and *in vivo* studies and structure of anthocyanins (lower panel), plant pigments connected with beneficial effects evoked by the bilberry intake. Based on Cutler et al.[29]

Effects of exercise on cardiovascular health

Physical activity is another factor, connected to lifestyle that influences health status. It is a tool to fight excess kilograms, however it is also crucial for maintaining healthy cardiovascular system, as decreased muscle mass and strength are known CVD risk factors. World Health Organisation recommends at least 150 min of moderate-to-vigorous physical activity each week or 20 min daily for adults.[30] Interestingly, a positive dose-response effect of the amount of exercise and vascular status has been observed.[31] It is important to note that in the Western world a sedentary lifestyle prevails and a so-called sarcopenic obesity, a state of simultaneous obesity with low muscle strength due to lack of training, is very common. Thus, an active lifestyle is a cornerstone of CVD prevention and treatment, especially in the elderly population.[30,32]

Regular training stimulates anti-atherosclerotic changes in endothelium, decreases blood pressure, improves insulin sensitivity, vasodilation and coronary flow reserve; is cardioprotective, anti-arrhythmic and anti-thrombotic.[30,32,33] Furthermore, it is beneficial for cardiac regeneration via stimulation

of angiogenesis. An important player in promoting vascular health is muscle tissue that releases myokines and other factors into the circulation, thus influencing the whole circulatory system.[30] These muscle-derived 'messengers' have gained more attention in the past decade and the knowledge in this field is still expanding.

Microvesicles

Definition

Microvesicles (MVs), sometimes referred to as microparticles or ectosomes, are a subtype of a larger group, called extracellular vesicles (EVs) that also comprises exosomes and apoptotic bodies.[34] This division is based on size or biogenesis process (Fig. 6). MV size ranges between 30 – 1000 nm and this type of EVs is produced by shedding of plasma membrane. The membrane blebbing involves numerous intracellular and membrane-bound proteins, as well as activation of different signalling pathways that depend on the cell type. Similar to MVs, apoptotic bodies are membrane-derived vesicles, however their size varies between 1 to 5 μm and the release occurs during apoptosis. Exosomes are the smallest vesicles, less than 100 nm in diameter that are released from the cell in a complex and controlled process of exocytosis, involving several transport-associated proteins and cytoskeletal remodelling.

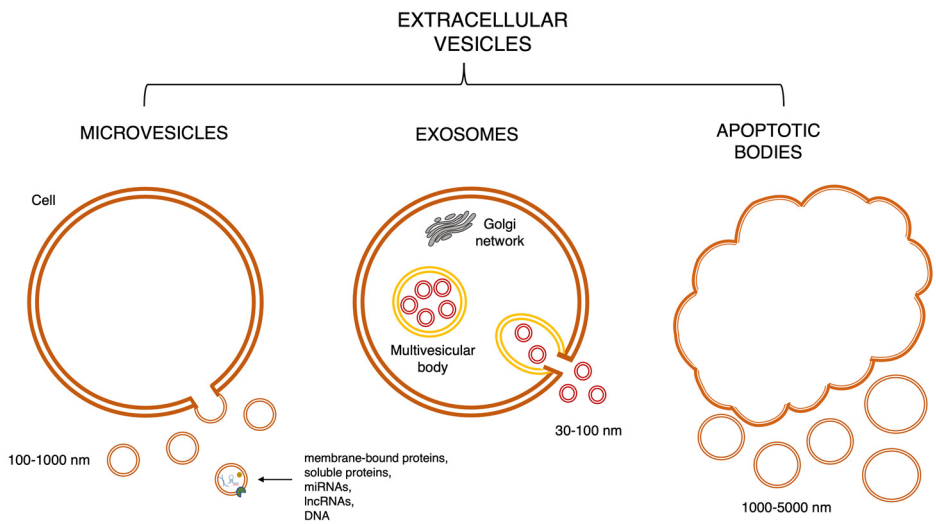

Fig. 6 Subtypes of extracellular vesicles, based on the size and biogenesis process.

Cellular origin of plasma microvesicles

The first plasma MVs were discovered by British physician Peter Wolf that specialised in blood coagulation.[35] In 1967 he published an article 'The nature and significance of platelet products in human plasma' that described the presence of so called 'platelet dust' and its procoagulant activity. From then on, the research about plasma MVs has developed and the knowledge about these small, but very important cellular particles has been substantially broadened.

The majority of plasma MVs stem from cellular components circulating in blood, such as platelets, erythrocytes and leukocytes.[34,36] Furthermore, a very important source of plasma MVs are vascular endothelial cells (ECs), especially in the context of endothelial dysfunction and atherosclerosis progression. Pro-inflammatory and pro-coagulant conditions trigger EC activation and a release of MVs, presenting E-selectin (CD62E) on their surface.[37] Similarly, proatherosclerotic conditions also lead to shedding of platelet MVs.[38]

In vivo experiments in rodents demonstrated physiological removal of circulating MVs by organs such as liver and spleen. The exact mechanism of MV clearance is yet unknown, however the available data proposes lipid- and protein-directed phagocytosis that depends on the recipient cell, as well as the donor cell of MVs.[39]

As MVs are membrane-derived vesicles, they carry biologically active molecules on their surface,[40,41] such as receptors and phospholipids, originating from the mother cell. MVs also transport a molecular cargo in their lumen: proteins,[42-45] miRNAs[46] and long non-coding RNAs (Fig. 6).[47] This enrichment of different classes of particles, present both inside and on the membrane, underlines the value of MVs in mechanistic studies, cardiovascular biomarker discovery and potential diagnostic application in the future.[48,49]

Role in development of atherosclerosis

Low levels of MVs are also detected in blood of healthy people that suggests an existence of vesicle release in physiological conditions. Nevertheless, there are strong indications that elevated levels of circulating MVs are the hallmarks of vascular dysfunction and CVD.[50] Endothelial and platelet MVs have been associated with a range of cardiovascular system pathologies, such as hypertension, CAD and MI.[34,36,51-54] Currently available data shows that MVs can affect cardiovascular system via many molecular mechanisms. For example, activated ECs release MVs, augmenting platelet aggregation *ex vivo* that occurs through a von Willebrand factor (vWF)-dependent crosslinking.[55] MVs can further facilitate pro-atherogenic processes by e.g. activating clotting cascade, promoting monocyte-endothelium interactions and regulating endothelial and vascular SMC proliferation.[56,57] As MVs are a rich source of potential biomarkers and are involved

in vascular pathologies, the projects included in this thesis have mainly been focused on vesicles of the MV subtype.

Methods of microvesicle isolation and detection

Characterization of circulating MVs in blood and so-called 'liquid biopsies' provide a novel approach in both pre-clinical research, as well as clinical diagnostics. However, MV isolation from patient plasma remains technically challenging. Most of the currently used isolation techniques (e.g. ultracentrifugation, iodixanol/sucrose gradient centrifugation, immunoaffinity beads) were first developed to isolate EVs, especially exosomes from culture medium used in the *in vitro* experiments. It is, however, possible to apply them for MV isolation from human plasma.[58] Nevertheless, these methods exhibit several disadvantages, especially with regards to limited-volume biobank samples, as they require substantial sample volumes[59,60] and can lead to loss of vesicles and EV integrity.[61-63] Thus, the traditional methods are challenging to use in the cardiovascular-related diagnostic context.

Traditionally, high-speed centrifugation has been used for plasma MV isolation. Compared to ultracentrifugation that is usually used for culture medium, this technique uses much lower than 100.000 x g rotor speed, ranging between 13.000 and 20.000 x g for flow cytometric or e.g. proteomic/miRNA analyses (Fig. 7). One of the aims of this thesis was to optimize a novel method for MV enrichment from plasma, called acoustic trapping that is a non-contact method, based on a microscale technology. The method applies acoustic standing wave instead of g force, thus preventing vesicle disruption and plasma aggregation. Furthermore, the volume of plasma needed for a successful MV isolation is very low, reaching 50 µl per sample for e.g. proteomic analysis. Another advantage of acoustic trapping is no need for staining before the isolation, so that the whole range of different MVs is isolated. A detailed technical description of the technique is given in the 'Materials and Methods' section and Article I. Both high-speed centrifugation and acoustic trapping are used for MV isolation from frozen patient samples, thus it is vital to ensure a proper handling of the samples and removal of blood cells prior freezing.[61] As numerous novel microscale approaches for EV isolation are currently appearing and different lab-on-a-chip devices are being developed for liquid biopsies,[64-67] several interesting discoveries in the field should be expected in the future.

Flow cytometry is a widely-applied method for measuring concentration and analysis of cellular origin of plasma MVs.[68] It is based on a staining of MVs with fluorescently-labelled antibodies, directed against proteins, present on the outer membrane of the vesicles.[69] Most of the currently available research on MVs has been done on conventional flow cytometers that usually enable measuring vesicles not smaller than 500-200 nm.[70] However, new branches, called high-sensitive (sometimes also referred to as micro or nano) flow cytometry[71-73] and imaging flow

cytometry[74,75] have recently appeared and are developing rapidly. This thesis presents data obtained both on a conventional (Study I. II and III), as well as a micro (Study III and IV) flow cytometer.

Other methods for MV detection and enumeration that allow to measure other physical parameters of the vesicles, involve more or less-known techniques such as electron and atomic force microscopy, nanoparticle tracking analysis, zeta potential analysis, dynamic light scattering and tunable resistive pulse sensing (Fig. 8). It is important to note that all above-mentioned techniques have strengths and weaknesses, especially with regards to potential application in clinical diagnostics and the best approaches for MV isolation and analyses are yet to be determined.

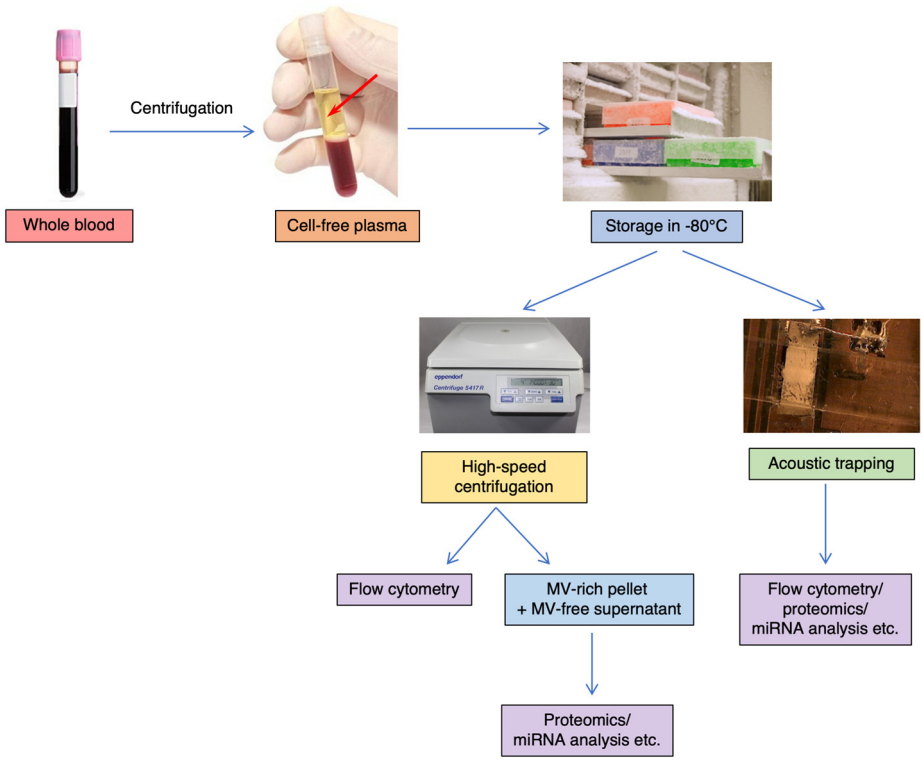

Fig. 7 Different methods of microvesicle isolation from plasma, presented in this thesis.

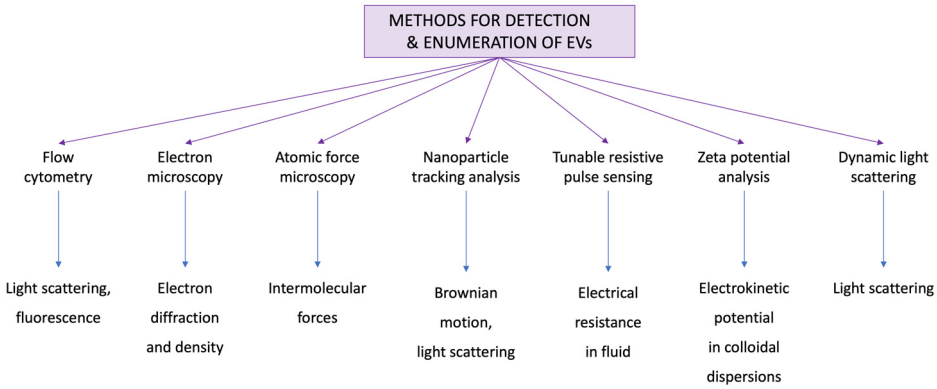

Fig. 8 Examples of methods used for extracellular vesicle analysis, with given measured physicochemical properties.[68,75-82]

Materials and Methods

Acoustic trapping (Papers I-IV)

As mentioned in the Introduction, acoustic trapping is a novel method for MV enrichment from plasma samples. The platform (AcouTrap, AcouSort AB) consists of a robotic unit, suitable for use of 96-well plates, a glass capillary, a transducer and pumps used for sample aspiration and washing steps (Fig. 9). Briefly, the procedure involves 'seed particle' aspiration and washing, plasma sample aspiration and MV trapping within the seed particle cluster, washing and elution of the MV suspension.

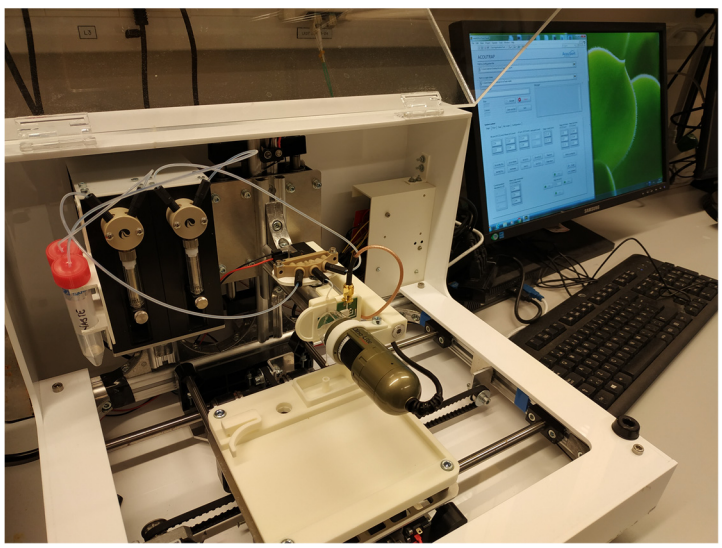

Fig. 9 Acoustic trapping platform at the Molecular Cardiology laboratory.

A rectangular glass capillary was used and a 4 MHz PZT transducer was attached through a thin layer of glycerol (Paper I and II) or glue (Paper III and IV) to the outside of the capillary. Both glycerol and glue ensure uniform acoustic coupling over time, without evaporation. The transducer generates a local acoustic standing wave in the capillary that enables particles to be retained or 'trapped' in a noncontact way. Because primary acoustic radiation force is dependent on the

particle volume, smaller particles (below 2 μm), e.g. MVs, cannot be directly retained in the acoustic trap, as the force is not sufficient for particle focusing. Larger particles, however, can be retained against the flow, thus 12 μm polystyrene beads were used as 'seed particles'. After aspiration of the diluted plasma sample, the scattered sound field between the seed particles and MVs present in the sample induces enrichment of MVs within the seed particle cluster. The cluster is further washed and subsequently released in a defined volume for further analyses, e.g. proteomic or miRNA profiling. For detailed graphical explanation of acoustic trapping working principles, please refer to the experimental section in Paper I.

High-speed centrifugation (Paper I)

To compare acoustic trapping with standard isolation protocol for flow cytometric analysis of MVs, plasma samples were centrifuged at 1600 x g for 15 min at RT, followed by 13 000 x g for 2 min. 80% of the supernatant was aspirated and diluted with DPBS for subsequent staining with fluorochrome-conjugated antibodies (Abs). For proteomic profiling, patient samples were centrifuged at 1600 x g for 15 min at RT and 100 μl of plasma was further centrifuged at 20 000 x g for 60 min at RT, resulting in two fractions: MV-rich pellet and MV-free supernatant. Detailed explanation of the workflow and method comparison is included in Paper I.

Flow cytometry (Papers I-IV)

Flow cytometry (FCM) is a method that uses optical characteristics of the cells or vesicles for real-time visualisation and enumeration. MVs stained with fluorescently labelled antibodies are injected into the cytometer, positioned in a central fluid stream (hydrodynamic focusing), illuminated with a laser beam and further detected.

In Papers I-III Accuri C6 flow cytometer (BD Accuri) was used for enumerating platelet and endothelial MVs isolated with acoustic trapping or high-speed centrifugation. Polystyrene size standard beads (0.1–3 μm) were used to establish a flow cytometric 'MV gate', corresponding to the size of MVs. 100 μl of MV suspension was incubated with PE-conjugated anti-CD62E (E-selectin) or CD42a (glycoprotein IX) Abs for 30 min at RT. 20 000 events were collected in the MV gate and the obtained data is shown as the number of fluorescently labelled MVs/μl. Fluorescence-based gates were established using respective Ab isotype controls. ApoGee Micro flow cytometer (Apogee Flow Systems) was used in Papers III and IV. Size-calibrated silica beads (ApogeeMix) were used

to calibrate the MV gate. For Papers III (BAFF analysis section) and IV, patient plasma was diluted 1:200 with DPBS and 200 µl was stained with 2 µl of Abs for 30 min in RT. 110 µl was aspirated and analysed by the cytometer and the obtained data is represented as the number of fluorescently labelled MVs/µl. For additional gating strategies or detailed antibody description, please refer to the experimental sections or supplementary information of the respective publications.

Proximity Extension Assay (Papers I, III, IV)

To perform proteomic profiling of MVs, isolated and lysed MVs were analysed with Cardiovascular II and III Proseek Multiplex 196 x 96 panels (Olink Proteomics). This proximity extension assay (PEA) allows to simultaneously analyze 92 known or potential cardiovascular biomarkers in 96 samples, using 0.5-1 µg/µl protein. Each of the panels was evaluated by the manufacturer for specificity, precision, sensitivity, dynamic range, matrix effects and interference. The method involves three steps: incubation, extension and detection (Fig. 10). In the incubation phase, two specific antibody probes with oligonucleotide tags bind in close proximity to the target protein. In the extension phase, a PCR target sequence is formed that allows DNA polymerization during quantitative real-time PCR (qRT-PCR). The last step is a detection of the amplified sequence. The generated qRT-PCR data is further transformed to relative quantification in log2-scale as Normalized Protein eXpression (NPX). Detailed description controls used for the analysis, as well as equations used for NPX calculation are provided on the manufacturer's website (www.olink.com). The analysis was performed at Clinical Biomarkers Facility at the Science for Life Laboratory, Uppsala University.

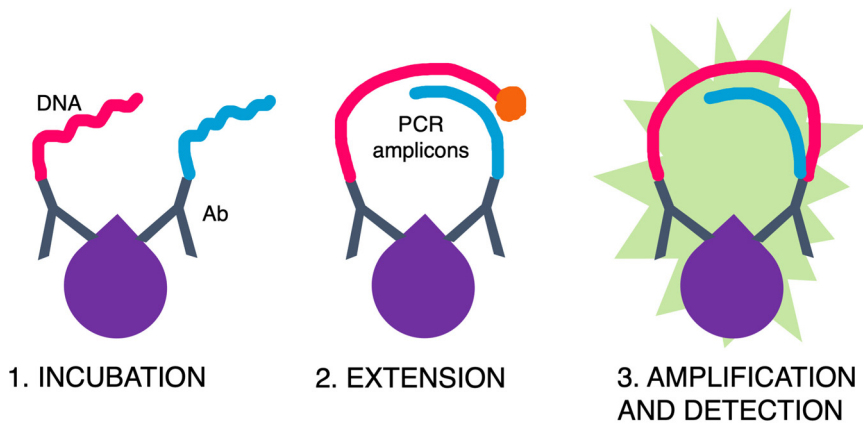

Fig. 10 Principle of Olink proximity extension assay.

Transmission Electron Microscopy (Paper I)

To visualize EVs in our sample set, transmission electron microscopy (TEM) was applied. This technique creates an image by transmitting a beam of electrons through an ultrathin specimen. Briefly, to obtain a TEM image of EVs, plasma sample was diluted with DPBS, centrifuged and washed. EV-rich pellet was fixed with paraformaldehyde in Sorensen's phosphate buffer solution. The fixed pellet was resuspended in DPBS and stained with mouse anti-human CD62E Ab. A glow discharge carbon-coated 400 mesh grid was blocked with BSA. The sample was then placed on the grid and the staining was performed with goat anti-mouse Ab conjugated with 10 nm of gold. After series of washing with PBS, the samples were fixed with glutaraldehyde, stained with uranyl acetate and dried. The imaging was done on FEI Tecnai Biotwin TEM.

Nanoparticle Tracking Analysis (Papers I and II)

Nanoparticle Tracking Analysis (NTA) is a method that allows to visualise, enumerate and assess the size distribution of EVs. It calculates the diameter of particles suspended in liquids based on Brownian motion. The analysis of isolated EVs was performed using NanoSight LM10 (Malvern) equipped with a 488 nm blue laser. EVs were stained with PE-conjugated CD62E or CD42a Abs and diluted for fluorescence-based NTA. Ab-only and PBS controls were used to ensure measurement of the vesicles.

Endothelial vascular cell culture (Paper II)

Human umbilical vein endothelial cells (HUVECs), pooled from multiple donors, were purchased from Life Technologies. Three different HUVEC lots below passage 4 were used in the *in vitro* experiments. The cells were seeded in dishes covered with attachment factor and cultured in Medium 200 with gentamycin/amphotericin and low serum growth supplement (LSGS, final serum concentration 2%), containing hydrocortisone, epidermal growth factor, basic fibroblast growth factor and heparin (Thermo Fisher Scientific).

Calcein assay (Paper II)

In study II we examined the effect of bilberry extract (BE) on endothelial vesicle release, hypothetically linked to the $P2X_7$ purinergic receptor. To study the vesiculation, ECs were stained with calcein AM, a hydrophobic dye that emits fluorescence inside the intact cells or EVs due to removal of acetomethoxy groups by endogenous intracellular esterase activity (Fig. 11). The green fluorescent signal can be further measured with e.g. fluorometer. Calcein is a reliable dye to measure vesicle release, as it does not emit fluorescence outside the cells or EVs due to its degradation, thus does not give false positive or unspecific signal like some lipophilic dyes do.[83]

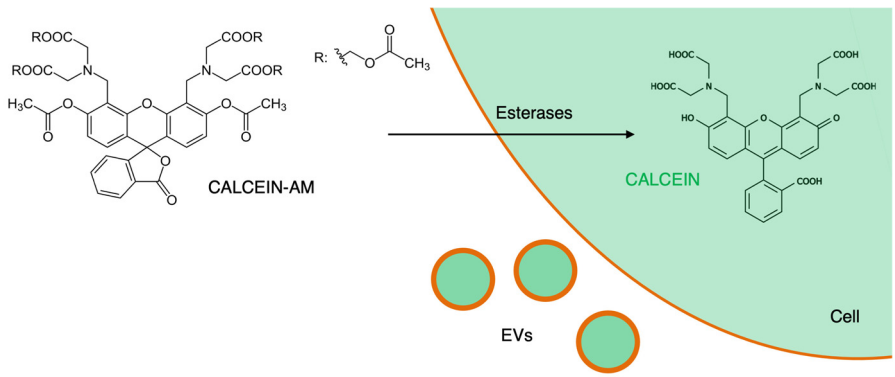

Fig. 11 Principle of calcein staining.

Compound effect on endothelial cells (Paper II)

To assess the effect of different concentrations of BE on viability and proliferation of HUVECs, trypan blue was used. The highest tested concentration of 1000 µg/ml of BE was chosen for further experiments due to the lack of toxic effects on the cells. HUVECs were seeded in 12-well dishes and cultured for 24 h. 1 mg/ml BE-containing medium was prepared from 100 mg/ml stock. The mixture or control medium were incubated with confluent cells for 24 h. 300 µm of $P2X_7$ agonist bzATP (Sigma-Aldrich) and 100 nm of $P2X_7$ antagonist AZ11645373 (Sigma-Aldrich) were prepared from stock solutions in the culture medium. Calcein AM (Thermo Fisher Scientific) stock was prepared in DMSO and diluted in HBSS to obtain 2 µm working solution. The cells were washed with HBSS, 1 ml of calcein solution was added per well and the cells were incubated at 37°C for 15 min. After washing, the diluted compounds or fresh culture medium were added to the calcein-stained cells and incubated for the next 24 h. The medium was further

collected, centrifuged to remove cellular debris and analysed using CLARIOstar plate reader (BMG Labtech). The workflow of the vesiculation measurement is presented on Fig. 12. All values were normalized to that of the control cells.

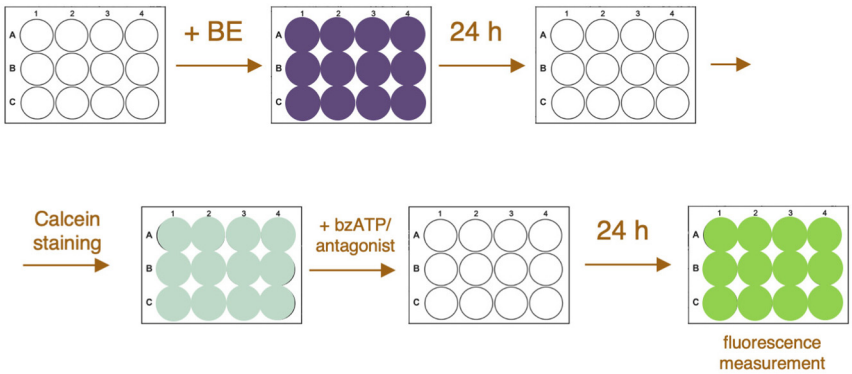

Fig. 12 Workflow of endothelial vesiculation analysis.

RNA extraction, reverse transcription and quantitative PCR (Paper II)

To measure gene expression, a real-time or quantitative polymerase chain reaction (qPCR) was performed on isolated RNA. The first step applies reverse transcriptase (RT) to create complementary DNA from RNA. (RT-PCR). The second step is based on a real-time measurement of a target DNA sequence amplification by DNA polymerase (qPCR) that reflects the expression of the gene of interest.

The cells were harvested with 700 µL QIAzol (Qiagen) and frozen at −80°C. RNA isolation was performed with miRNeasy kit with DNase digestion (Qiagen) as per manufacturer's instructions. RNA concentration and quality was assessed with NANODROP 2000C spectrophotometer. High Capacity RNA to cDNA kit (Thermo Fisher Scientific) was used for reverse transcription. The mRNA expression was assessed using TaqMan Fast Universal PCR Master Mixes (Thermo Fisher Scientific) with 20 ng template as per manufacturer's instructions. The TaqMan assay employs annealing of the template with dye-labelled probes. In case of no DNA polymerization, the dye is quenched and there is no fluorescence. However, when the DNA polymerase is active, it releases the dye from the quencher that results in the fluorescent signal that is proportional to the amount of the synthetized DNA. qPCR reaction was performed on StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). GAPDH was used as a housekeeping gene for the reference (Δ Ct) and the data was normalized to the

mean of the controls (ΔΔ Ct), according to the formula $2^{-\Delta\Delta Ct}$, giving the relative quantification (RQ) parameter.

Western Blot (Paper II)

Western Blot is a technique that allows to quantify protein expression based on the antibody binding. HUVECs were plated in 6-well plates and cultured for 24 h. 1 mg/ml BE was added and the cells were harvested and lysed with 200 µL RIPA buffer (Cell Signaling) containing phosphatase inhibitors. Protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific). 10 µg of protein was loaded on pre-cast polyacrylamide midi gels (Thermo Fisher Scientific) and separated with electrophoresis based on the molecular weight. Further, the proteins were transferred via iBlot 2 transferring system (Thermo Fisher Scientific) on a nitrocellulose membrane. To prevent non-specific binding of Abs, the membrane was blocked with protein-free blocking buffer (Thermo Fisher Scientific). Primary Abs against the proteins of interest were incubated with the membrane overnight and washed. Then, a secondary Ab, conjugated with horseradish peroxidase (HRP) and binding to the primary Ab was added. Visualisation was performed with Pico Chemiluminescent Assay (Thermo Fisher Scientific), containing HRP substrate, on Odyssey Fc (LI-COR) based on the densitometric properties of the membrane. β-actin was used as a reference protein. The phosphorylation levels of phospho-Akt (pAkt) and phospho-p38 (pp38) were normalised to the total levels of these proteins. The data is represented as normalised to the control.

Coronary flow reserve measurement (Paper III)

Coronary Flow Reserve (CFR) is a clinical parameter used for measuring vascular blood flow. It assesses both atherosclerotic lesions in the coronary arteries and microvascular dysfunction. As CFR represents blood delivery to the heart during higher demand,[84,85] high CFR values usually mean better coronary circulation (Fig. 13). Low CFR is a strong and independent predictor of cardiovascular events.[85-88] In Paper III, CFR was measured in LAD with transthoracic echocardiography by experienced operators at Skåne University Hospital and Sahlgrenska University Hospital. The above-mentioned method is an alternative to invasive methods and carries strong prognostic information.[85] In order to obtain maximal hyperaemia in coronary arteries, the procedure involved adenosine administration to patients. This purine nucleoside acts via A_{2A} G_s-protein coupled receptors, present in the vasculature that stimulate adenylyl cyclase.[89,90] cAMP further leads to SMC

hyperpolarization through potassium channels, subsequent endothelial-independent SMC relaxation and finally vasodilation of the coronary artery.[89]

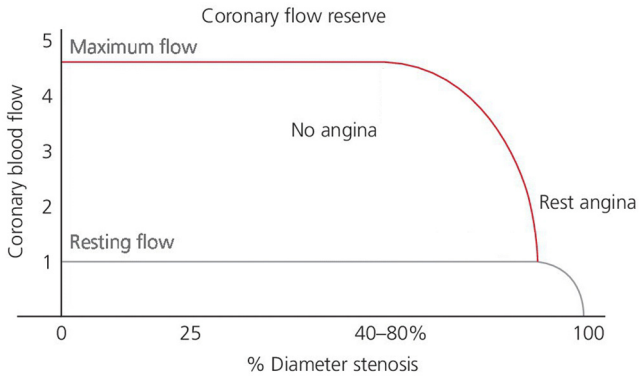

Fig. 13 Relationship between coronary flow reserve values and the level of stenosis. Based on https://thoracickey.com/24-fractional-flow-reserve/.

Statistics (Papers I-IV)

For all studies p-values <0.05 were considered as statistically significant. The normality of the data was determined using Shapiro test. Statistical analyses were performed using GraphPad Prism or STATA 14 software. In Papers I, III and IV, the analysis of proteomic profiling with Olink PEA was done on NPX (Normalized Protein Expression) values. Proteins with 50% ≤ values lower than the limit of detection were removed from the analysis and analysed with paired t-test or Wilcoxon signed-rank test. In study I, Wilcoxon signed rank test for paired data was used for FCM comparison between the time points (before and 1 h after exercise). In Paper II, Mann–Whitney U test was applied to compare differences between BE supplementation and no dietary intervention after 8 weeks. One-way ANOVA with false discovery rate (FDR) correction of Benjamini and Hochberg was used to analyse the *in vitro* effects of BE and compounds on ECs. In Paper III Spearman's coefficients were calculated to determine correlations. Multiple linear regression was used to measure the relationship between CFR and certain factors. In Paper IV, paired t-test or Wilcoxon-signed rank tests were used to analyse the differences in FCM and proteomic data between proximal and distal samples to the plaque. For detailed description of the applied statistical methods, please refer to the respective publications.

Aims and Hypotheses

The overall objective of this thesis was to study the role of MVs in cardiovascular-related pathologies, as well as to optimize the novel method for MV enrichment – acoustic trapping.

The specific objectives of each study are given below:

The primary aim of **Paper I** was to optimize the acoustic trapping machine for MV isolation from plasma and compare it to standard high-speed centrifugation protocols for flow cytometric and proteomic profiling. A clinical study was performed to also study the effect of exercise on the vasculature in the MV context.

Paper II investigated if there is an influence of bilberry extract dietary intervention in MI patients on MV levels in plasma. The *in vitro* part of the study aimed to explore the effect of bilberry extract on endothelial vesiculation, related to the $P2X_7$ receptor.

Paper III studied the relationship between coronary flow reserve, a parameter depicting blood flow in the heart and MV levels or vesicle-related biomarkers, circulating in patients with CVD.

Paper IV focused on exploring the composition and cellular origin of plaque-released EVs, following balloon angioplasty. It also examined if there was an advantage of analysing isolated EVs, compared to crude plasma samples.

Results and Discussion

Paper I

Plasma-derived MVs offer a promising approach in diagnostics of several clinical pathologies. As MV isolation by currently available methods is both technically challenging and requires large sample volumes,[61] we used an alternative method, based on an acoustic standing wave technology.

Optimization of acoustic trapping

The optimization of the acoustic trapping was established by monitoring the efficiency of the machine that was calculated based on the levels of CD42a+ platelet MVs (Fig. 14A), as the majority of circulating MVs is of platelet origin.[40] For this purpose, pooled frozen plasma from volunteers was used as an internal control to monitor the device stability. Our results showed that acoustic trapping exhibited high and stable MV recovery throughout the experiment. We further compared platelet MV isolation efficiency with a commonly used high-speed centrifugation protocol. As presented on Fig. 14B, the loss of MVs during isolation is substantially lower for acoustic trapping that can be related to non-contact principle of isolation and thus decreased aggregation/disruption of the vesicles. Improved design of the acoustic trapping machine increased MV recovery compared to earlier reports[91] and enhanced the long-term stability.

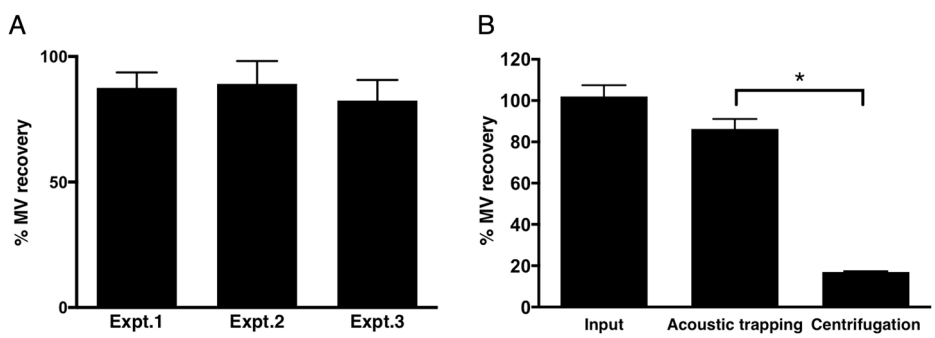

Fig. 14 Results of acoustic trapping optimization for plasma microvescicle isolation. A: Efficiency of acoustic trapping. B: Comparison of acoustic trapping and high-speed centrifugation recovery. Mean ± SEM. One-way ANOVA with FDR correction of Benjamini and Hochberg. *Statistically significant, $p<0.05$.

Exercise study worklflow

To add a clinical context to the comparison, a pilot study consisting of 20 subjects who underwent physical exercise was carried out. Plasma samples were obtained before and 1 h after the training. The complete study workflow is presented on Fig. 15. MVs were isolated both with acoustic trapping and standard high-speed centrifugation protocol.

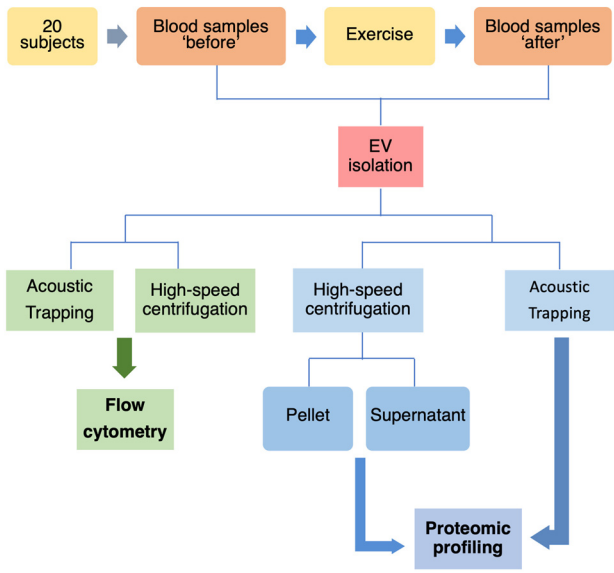

Fig. 15 Acoustic trapping and high-speed centrifugation comparison workflow.

Flow cytometric analysis of MVs

The methods were further compared by FCM. The analysis revealed a rapid decrease in CD62E+ (E-selectin) endothelial MVs 1 h after exercise on stationary bicycle that was, importantly, consistent for both methods (Fig. 16).

It has been described that physical exercise has an impact on MV concentration in blood. However, exercise protocols, MV isolation methods and analysed markers vary substantially between the studies, making the outcome comparisons challenging.[92-100] A study by Babbitt et al. also evaluated the levels of CD62E+ MVs, but after a long-term exercise intervention in African Americans.[95] The results showed that six months of aerobic exercise training significantly decreased circulating MVs derived from activated endothelium. One of the possible explanations of our and Babbitt's observations is a protective effect of exercise on human body, including the vasculature and inflammatory response[101-105] that is reflected in the levels of CD62E+ MVs in blood.

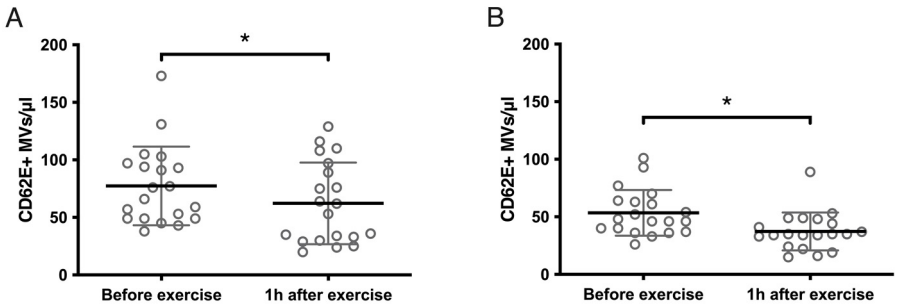

Fig. 16 Comparison of acoustic trapping (A) and high-speed centrifugation (B) for flow cytometric analysis of plasma microvesicles. Mean ± SD. Wilcoxon signed-rank test. *Statistically significant, p<0.05.

Nanoparticle tracking analysis of vesicles

NTA was another method, used for the comparison of the methods (Fig. 17). It revealed that both methods of isolation obtained similar results regarding EV size distribution, with CD62E+ vesicles primarily in the MV size range. Additionally, acoustic trapping yielded higher levels of CD62E+ EVs (Fig. 17B), compared to the centrifugation protocol (Fig. 17D), which is consistent with the results from the FCM analysis.

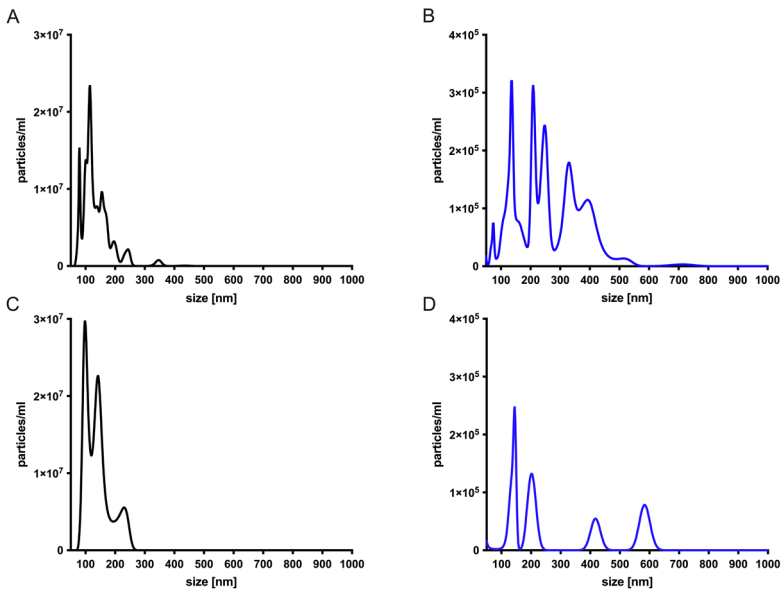

Fig. 17 Comparison of acoustic trapping (upper panel) and high-speed centrifugation (lower panel) with nanoparticle tracking analysis of plasma microvesicles. A, C – evaluation of total concentration of vesicles (unstained). B, D – evaluation of fluorescently labeled CD62E+ vesicles.

Proteomic profiling of MVs

We also compared the protein content of vesicles circulating before and 1 h after physical exercise, isolated with acoustic trapping and high-speed centrifugation. The proteomic analysis with Olink PEA cardiovascular panels showed 54 significantly changed proteins in the MV fraction (acoustic trapping and pellet fractions) in response to physical exercise, whereas the MV-free plasma proteome (supernatant) displayed only four differentially regulated proteins (Fig. 18). As the vast majority of differentially regulated proteins were present in the vesicles, this finding underlines the role of MVs in cellular communication and their potential application as plasma-derived biomarkers. STRING database search revealed that most of the changed proteins were associated with inflammatory response (e.g. CCL17, CCL24, CXCL1, CXCL16, IL-1RA, IL-18), angiogenesis (angiopoietin 1, TIE-2) and coagulation (PAR1, P-selectin, Src, vWF) that stands in line with the current knowledge about the effects of exercise on biochemical parameters.[102,106-118] Furthermore, most possible sources of the vesicular exercise-regulated proteins were ECs, leukocytes, muscle tissue and platelets.

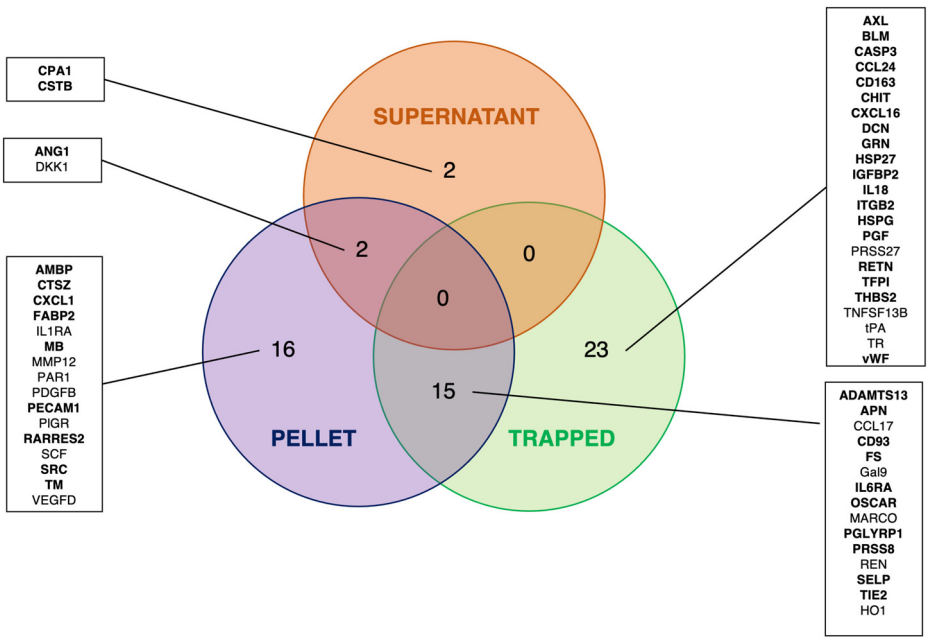

Fig. 18 Venn diagram showing comparison of acoustic trapping and high-speed centrifugation (microvesicle-rich pellet and microvesicle-free supernatant), based on the proteomic profiling of exercise-regulated proteins. Numbers in the circles represent significantly changed proteins after the exercise**.** Bolded proteins have been previously described as of vesicular origin, based on Vesiclepedia.[119] Abbreviations of all exercise-regulated proteins are explained in Paper I and its supplementary material.

Since the manuscript publication in 2018, several new original, as well as review reports regarding exercise and EVs have appeared.[100,120-125] Some researchers point at the role of muscle tissue as an endocrine organ and exercise-released exosomes as important reservoirs of different chemokines and growth factors, sometimes referred to as 'exerkines'.[120,122,123] This hypothesis is consistent with our findings from the proteomic study that identified exercise-regulated proteins of muscle tissue origin, such as myoglobin and follistatin. As described above, the NTA revealed a very small amount of EVs isolated with acoustic trapping with diameter below 100 nm that could be of exosome subtype of vesicles, transporting exerkines in the circulation during/after the physical exercise.

Transmission electron microscopy of vesicles

We also performed additional experiments to validate the presence of CD62E marker on the vesicles in our material. For this purpose, immunogold staining against CD62E was performed and analysed with TEM. It confirmed the presence of both the intact vesicles and the marker on the vesicle surface (Fig. 19).

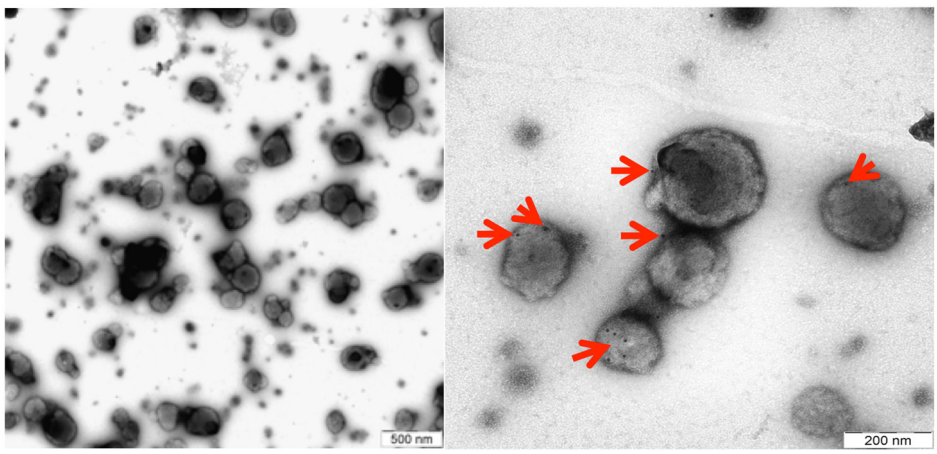

Fig. 19 CD62E immunogold staining for transmission electron microscopy. Red arrows indicate the presence of CD62E antigen.

In summary, results presented in Paper I showed that acoustic trapping is a fast and efficient method that is comparable with the standard centrifugation protocols for MV isolation. It has the advantage of using lower sample volumes and contact-free separation with higher yield. Furthermore, this study underlines the potential application of acoustic trapping in the clinical vesicle-based diagnostics. The results of the clinical study point at a protective effect of exercise on the vasculature.

Paper II

BEARSMART clinical study

In the BEARSMART study, MI patients received an 8-week bilberry extract (BE) dietary supplementation,[126] whereas the control group did not take any dietary supplementation (Fig. 20). MVs were isolated from the patient plasma with acoustic trapping. CD42a+ platelet MVs and CD62E+ endothelial MVs were measured with FCM.

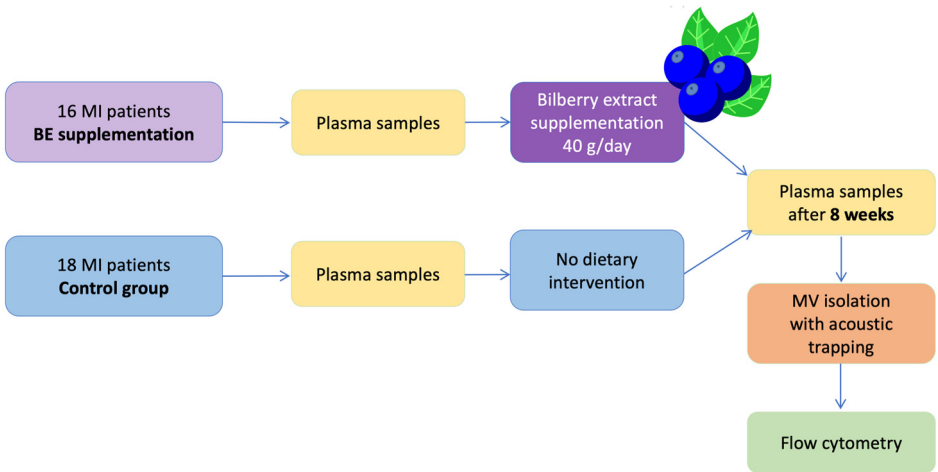

Fig. 20 Study II workflow – investigation of the effect of bilberry extract on circulating platelet and endothelial microvesicles.

Flow cytometric analysis of microvesicles

FCM analysis revealed that 8-week BE dietary intervention significantly decreased levels of both circulating platelet (Fig. 21A) and endothelial MVs (Fig. 21B), compared to the control group.

Anthocyanins are the major group of compounds present in bilberries that could be linked to the reduced vesiculation. These polyphenols reduce both platelet[23,127,128] and endothelial activation,[129,130] thus possibly the vesicle release. As MVs are related to the cardiovascular pathologies, our results demonstrate an important effect of dietary intervention with BE supplementation on MI patients.

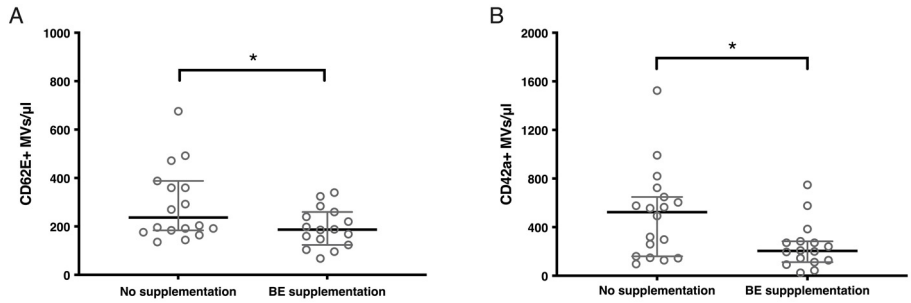

Fig. 21 Eight-week supplementation of bilberry extract (BE) significantly decreases circulating platelet (A) and endothelial (B) microvesicles. Median ± 95% CI. Mann-Whitney U test. *Statistically significant, p<0.05.

Nanoparticle tracking analysis of patient samples

We also performed NTA to determine vesicle size distribution in the samples (Fig. 22A). The majority of isolated vesicles were of 200-400 nm diameter, thus was in the range of MVs. The analysis also confirmed the presence of CD62E+ (Fig. 22B) and CD42a+ (Fig. 22C) vesicles in patient plasma.

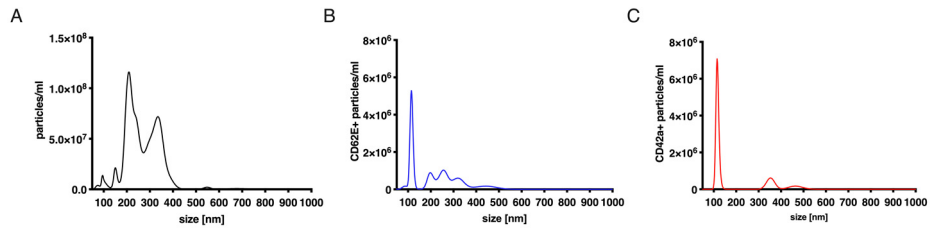

Fig. 22 Evaluation of size distribution of vesicles present in patient samples, isolated with acoustic trapping. A- size distribution of all vesicles (unstained). B- size distribution of vesicles originating from activated endothelium. C- size distrubution of platelet-derived vesicles.

Effect of bilberry extract on endothelial cells

As BE supplementation decreased circulating endothelial MVs, we further investigated the effect of BE solution on endothelial vesicle release, using HUVECs and calcein staining. Concentration of 1 mg/ml of BE was chosen due to the lack of toxic effects on the cells. Based on the literature, $P2X_7$ receptor was selected as a target for induction of vesiculation.[131] Western blot and RT-qPCR techniques were applied to explore the mechanisms of BE influence on ECs (Fig. 23).

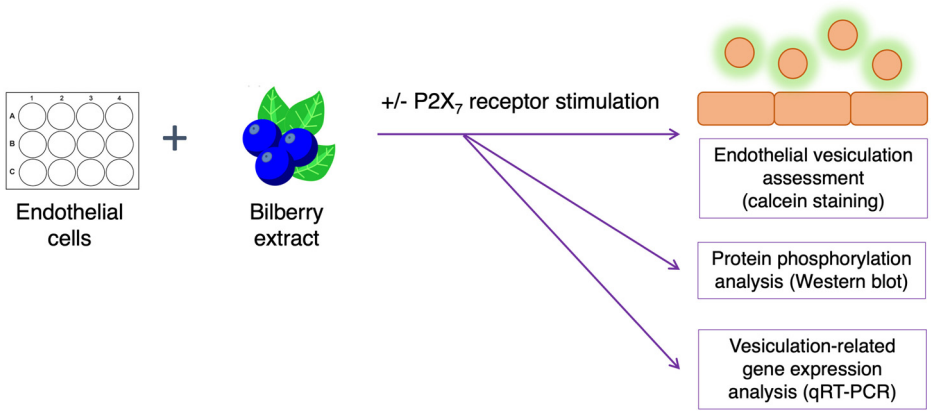

Fig. 23 Study II workflow – *in vitro* experimental part investigating the influence of bilberry extract on endothelial vesiculation, as well as the mechanisms underlying this effect.

Influence of bilberry extract on endothelial vesiculation

Our results show that *in vitro* treatment of ECs with BE decreased endothelial vesicle release (Fig. 24). $P2X_7$ receptor agonist, bzATP, increased the vesiculation compared to the control cells that was abrogated in the presence of $P2X_7$ receptor antagonist AZ11645373. Pre-incubation with BE further prevented bz-ATP evoked vesiculation. Thus, our data shows that BE reduces both unstimulated and $P2X_7$–stimulated vesicle release from ECs.

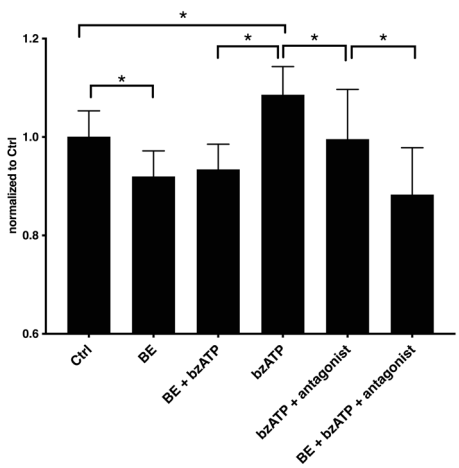

Fig. 24 Effect of bilberry extract (BE) on endothelial vesiculation. BE incubation significantly decreased vesicle release and bzATP increased the vesiculation compared to the control cells. Pre-incubation with BE prevented bz-ATP evoked vesiculation. Mean ± SD. One-way ANOVA with FDR correction of Benjamini and Hochberg. *Statistically significant, $p<0.05$.

Modulation of protein phosphorylation by bilberry extract

Based on the vesiculation results, Akt and p38 were selected as candidate proteins for further investigation, as both take part in $P2X_7$ signaling pathways, as well as vesicle release.[132-135] Furthermore, it has been previously reported that bilberry-derived compounds affect both Akt and p38 phosphorylation *in vitro* and *in vivo*.[136-139] Our results show that incubation of ECs with BE decreased Akt phosphorylation after 3 h (Fig. 25A), but not after 6 h (Fig. 25B). p38 phosphorylation, however, did not change at both time points (Fig. 25C,D). This suggests that p38 is not directly involved in the observed BE-related vesiculation decrease.

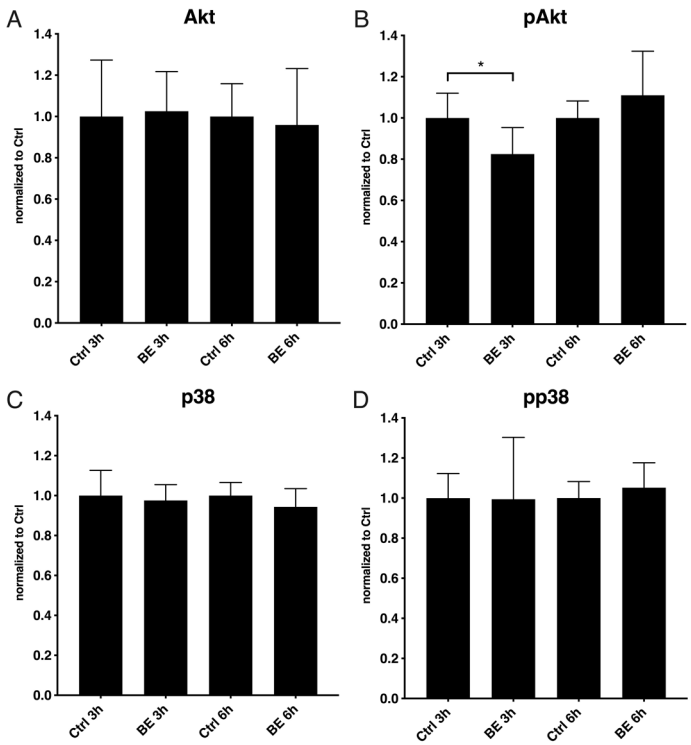

Fig. 25 Effect of bilberry extract (BE) on protein phosphorylation. BE incubation for 3 h significantly decreased Akt phosphorylation compared to the control cells. No effect was observed on p38 phosphorylation. Mean ± SD. One-way ANOVA with FDR correction of Benjamini and Hochberg. *Statistically significant, $p<0.05$.

Effect of bilberry extract on gene expression

Since it has been shown that Akt signaling pathway regulates $P2X_7$ expression in the neural cell line,[140] we also investigated if the observed decrease in the vesiculation is associated with BE effect on $P2X_7$ transcription. As seen on Fig. 26, BE treatment decreased $P2X_7$ gene expression that could explain a decrease in the

bzATP-stimulated vesiculation after pre-treatment with BE. A similar pattern was observed after bzATP incubation that can be explained by a negative-feedback loop and inhibition of $P2X_7$ transcription after the receptor stimulation.[141] However, BE decreased the receptor mRNA levels more profoundly than the agonist. Furthermore, treatment of HUVECs with BE and subsequent stimulation with bzATP also decreased $P2X_7$ expression.

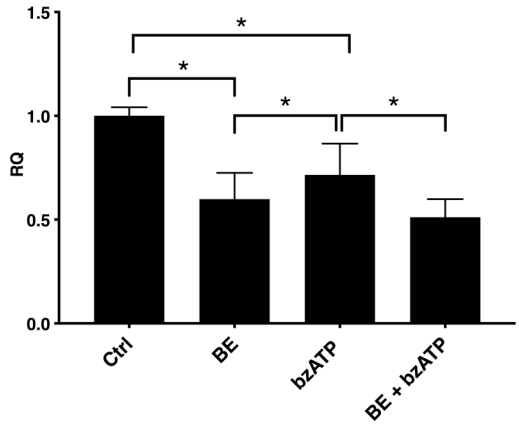

Fig. 26 Effect of bilberry extract (BE) on $P2X_7$ gene expression. BE incubation significantly decreased $P2X_7$ mRNA levels compared to the control cells. BzATP also decreased $P2X_7$ transcription. Mean ± SD. One-way ANOVA with FDR correction of Benjamini and Hochberg. *Statistically significant, $p<0.05$. RQ – relative quantification.

We further selected four vesiculation-related genes that could potentially be affected by BE. Rab27b and Rab27a control different stages of vesicle release,[142] SMPD1 regulates $P2X_7$-dependent vesiculation in glial cells[143] and ARF6 is involved in MV release from tumour cell line,[144] as well as from macrophages via $P2X_7$ activation.[145] Our results show that BE treatment decreased the expression of Rab27a and Rab27b genes (Fig. 27A,B). Pre-incubation with BE diminished the bzATP-induced increase in Rab27b, Rab27a and SMPD1 gene expression (Fig. 27A-C). The results were not conclusive for ARF6 expression, as its transcription decreased after all combinations of treatments (Fig. 27D).

Based on our findings, we propose a model, where BE and its major active constituents, anthocyanins, reduce Akt phosphorylation and further $P2X_7$ expression that subsequently decreases endothelial vesiculation. Importantly, we also believe that the *in vitro* observed effect of BE reflects the decrease in plasma endothelial MV concentration in BE-supplemented MI patients.

Furthermore, as BE is a complex mixture and the BE concentration used in the *in vitro* study was relatively high, other potential mechanisms could be involved in the reduction of circulating endothelial MVs. Anthocyanins or anthocyanin-

derived metabolites could potentially act locally as free radical scavengers[22,146] and thus prevent endothelial activation and vesiculation.[37]

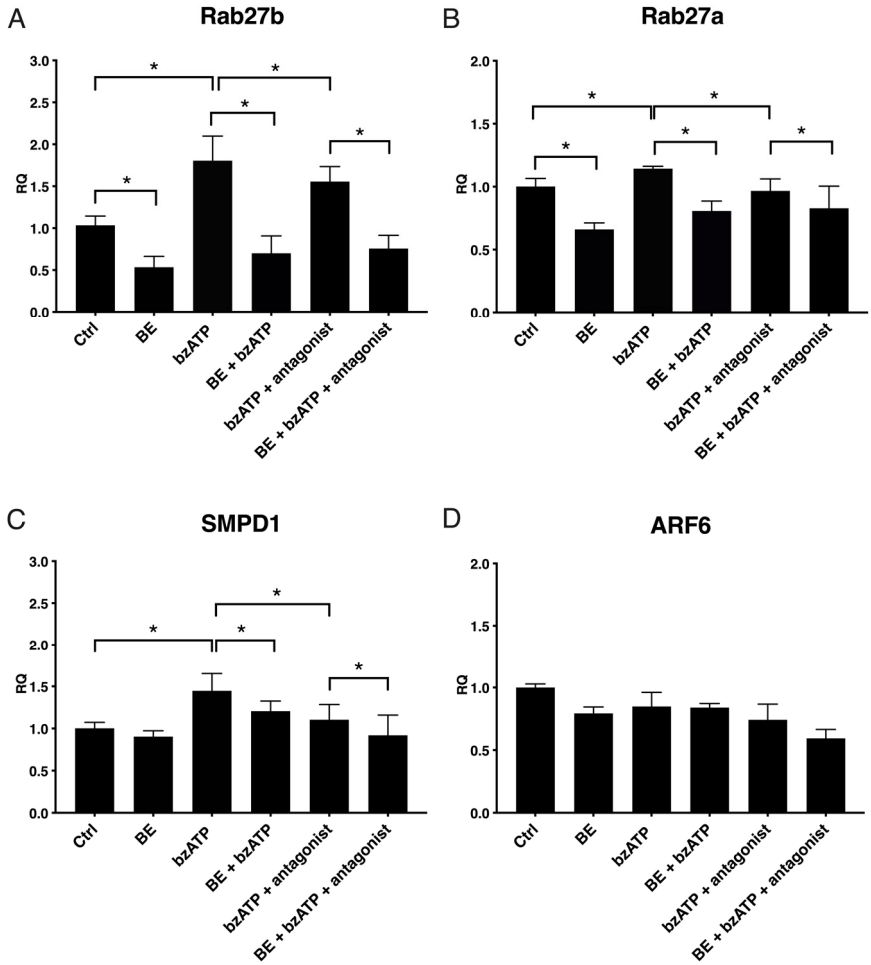

Fig. 27 Effect of bilberry extract (BE) on vesiculation-related gene expression. BE incubation significantly decreased mRNA levels of Rab27b and Rab27a (A, B). BzATP increased the transcription compared to the control cells (A, B, C). Pre-incubation with BE prevented bz-ATP evoked vesiculation (A, B, C). No effect was observed on ARF6 gene expression. Mean ± SD. One-way ANOVA with FDR correction of Benjamini and Hochberg. *Statistically significant, $p<0.05$. RQ – relative quantification

In summary, the results of Paper II demonstrated that BE dietary intervention improves the profile of circulating MVs in blood of CVD patients. Additionally, we show that BE evokes effects on endothelial vesiculation through several molecular mechanisms, connected with $P2X_7$ signaling pathway, such as Akt phosphorylation, $P2X_7$ and vesiculation-related gene expression. We reported for the first time that

dietary compounds have impact on vesiculation, both *in vitro* and *in vivo*. This supports the importance of our findings for potential dietary recommendations for cardiovascular patients, as well as a possible part of nutritional prevention of CVD.

Paper III

PROFLOW clinical study

The prospective, exploratory and open PROspective Evaluation of Coronary FLOW Reserve and Molecular Biomarkers in Patients with Established Coronary Artery Disease (PROFLOW) study has previously been described by Haraldsson et al.[147] It involved 619 high-risk patients with CAD, recruited by Department of Cardiology at Skåne University Hospital in Lund and Sahlgrenska University Hospital in Göteborg. Swedish Coronary Angiography and Angioplasty registry (SCAAR) was used to identify patients with high risk for future major adverse cardiovascular events. This study presents the data from 220 patients recruited in Lund. Patients were clustered into high and low CFR groups based on the median CFR value. CFR was measured in LAD with ultrasound system as described previously[147] and adenosine-assisted transthoracic echocardiography protocol was applied. MVs were isolated from plasma with acoustic trapping and analysed with FCM and Olink proteomic panels.

Coronary flow reserve and microvesicles

We investigated the relationship between platelet and endothelial MVs and CFR, as the currently available data points at their contribution to endothelial dysfunction and development of atherosclerosis. Our results show a negative correlation between CFR and platelet and endothelial MV levels in plasma (Fig. 28 B, D). MV levels were also compared in high and low CFR groups. According to our results, the low CFR group had significantly higher concentration of circulating platelet (Fig. 26A) and endothelial MVs (Fig. 26C), compared to the high CFR group. Furthermore, we found a positive correlation between platelet and endothelial MV levels (Fig. 29). We did not observe any relationship between MVs and risk factors, as well as disease or inflammatory burden.

We also applied a statistical approach, involving explanatory multiple linear regression, to investigate the relationship of CFR with patient characteristics such as BMI, smoking, age, diabetes, hypertension, dyslipidaemia, low density lipoprotein (LDL) levels and circulating platelet and endothelial MV concentrations. The results showed a statistical significance for platelet MV levels parameter estimate, which means that for each increase by 100 platelet MVs/µl in plasma, the CFR value decreases on average by 0.06.

The above results could be explained with the notion that circulating MVs could contribute to a decrease in CFR. It is known that MVs can affect vascular physiology and contribute to formation of atherosclerotic plaques.[34,39,46,148] The lower CFR values can also result from the presence of MVs released by already dysfunctional endothelium that further decrease NO production and vascular relaxation in healthy ECs.[149] Moreover, MVs generated by ageing ECs leadto progression of the plaque through increased calcification in the vessel.[39]

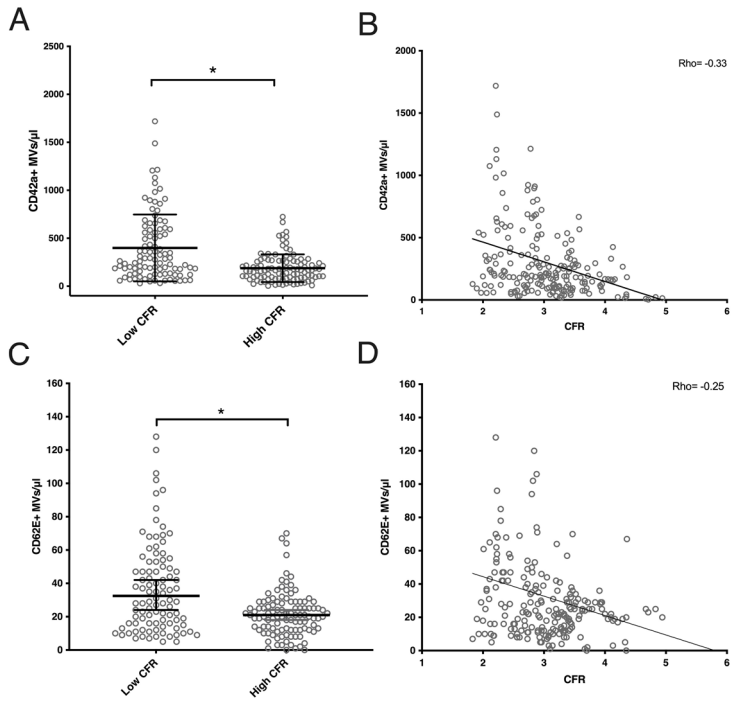

Fig. 28 Coronary flow reserve negatively correlates with circulating platelet and endothelial microvesicles. A, C: Patients with high CFR have lower concentration of circulating platelet (A) and endothelial (C) MVs, compared to the low CFR group. Median ± 95% CI. Mann-Whitney U test. *Statistically significant, p<0.05. B, D: Platelet (B) and endothelial (D) MVs in plasma negatively correlate with CFR, Rho - Spearman's coefficient, p <0.05.

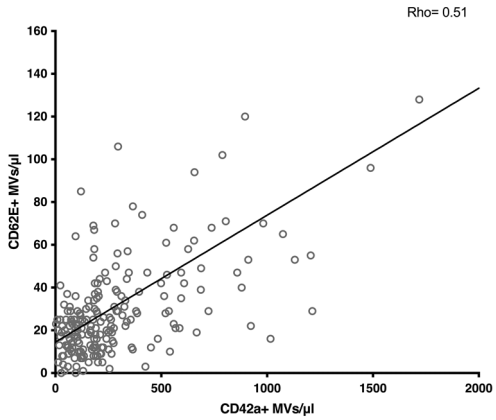

Fig. 29 Circulating endothelial microvesicle levels positively correlate with platelet microvesicles. Rho - Spearman's coefficient, $p<0.05$.

Proteomic analysis of microvesicles

As our data demonstrated that there is a relationship between CFR and MVs in plasma, the notion if this is reflected in the MV content was further explored. Olink CVD panels were used to perform a proteomic analysis on MVs enriched from patient plasma. The statistical analysis of CFR and NPX levels showed seven negatively correlating proteins (Fig. 30): NFκB essential modulator (NEMO), resistin, Tyrosine-protein kinase receptor UFO (AXL), perlecan, B-cell activating factor (BAFF), Insulin-like growth factor-binding protein 7 (IGFBP7) and CD163. No positively correlating proteins with CFR were found.

The comparison of high and low CFR groups revealed that the low CFR group exhibited higher concentration in the vesicles of nearly all above-mentioned proteins, with CD163 as an exception that did not reach statistical significance, despite a visible trend toward higher values in the low CFR group (Fig. 31).

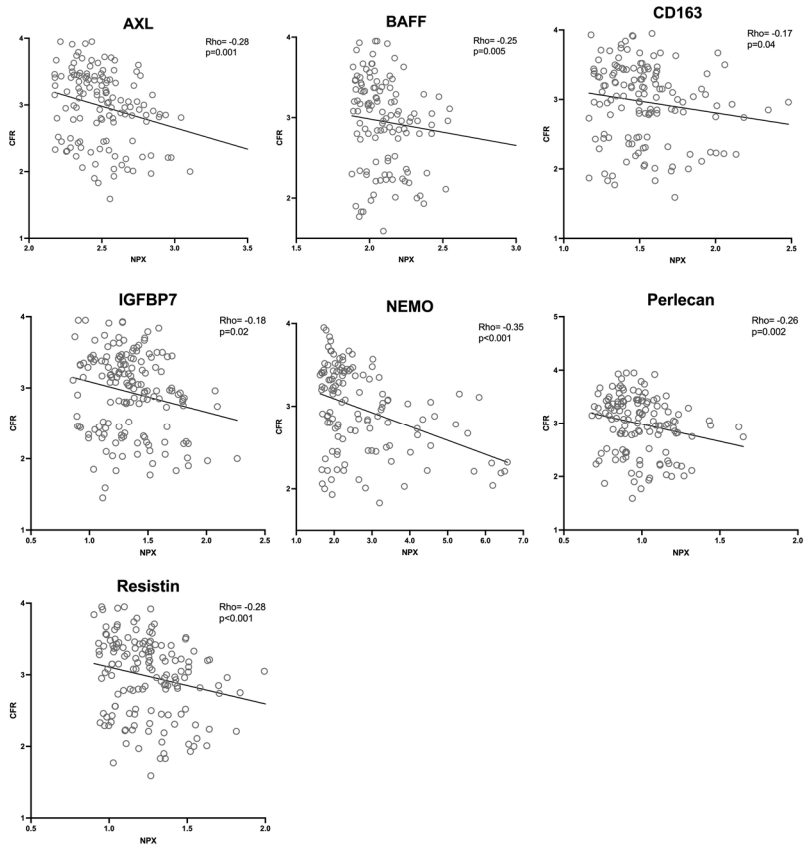

Fig. 30 Correlation plots showing results of proteomic analysis with Olink cardiovascular panels. Seven proteins negatively correlate with patient coronary flow reserve, based on the normalized protein levels (NPX), Rho – Spearman's coefficient, $p<0.05$.

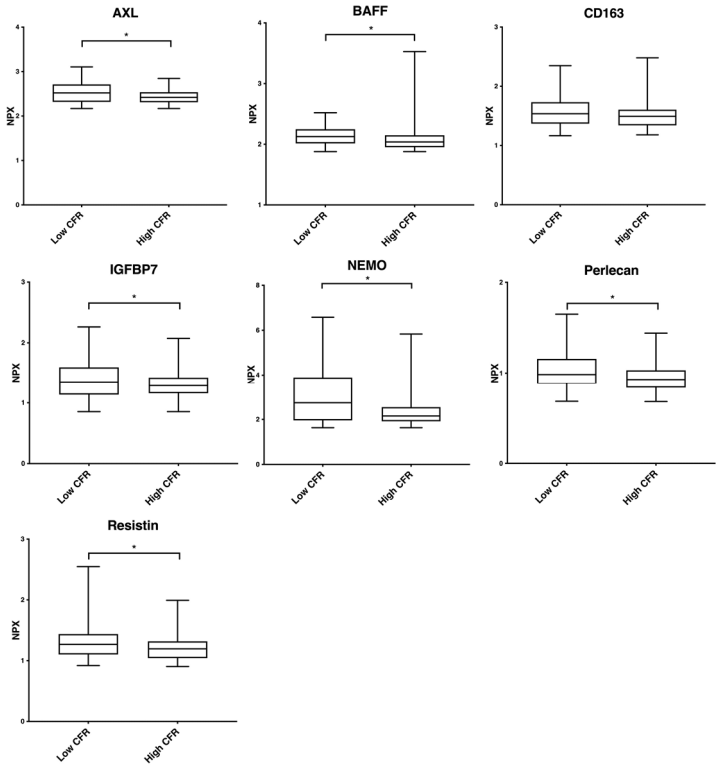

Fig. 31 Patients from the low CFR group are characterized with significantly higher levels of cardiovascular biomarkers levels, based on the normalized protein expression (NPX) values. Median ± range, Mann-Whitney U test for unpaired data. *Statistically significant, p<0.05.

According to the current knowledge, all of the significantly correlating proteins are involved in pathological and pro-atherosclerotic processes.[38,150-163] It is noteworthy that as most of the proteins have been measured as soluble forms in plasma, there is a possibility that they could have been partially measured as MV components. Thus, this study confirms for the first time that these proteins are present in the vesicular fraction of plasma.

Analysis of circulating BAFF-expressing microvesicles

One of the significant proteins, BAFF, is a protein that is directly involved in atherogenesis and its role is implicated in CAD.[148] It is also a pharmacological target for developing anti-inflammatory therapeutics.[164] Furthermore, its role has been implied in the progression of atherosclerosis in mice[150] and application of anti-BAFF receptor Ab reduces the lesions.[165] BAFF-related B-cell depletion in mice decreases cardiac inflammation and infarct size, as well as circulating levels of BAFF correlate with adverse effects in MI patients.[166]

Thus, we performed FCM analysis to identify cellular origin of circulating BAFF-expressing MVs. Platelet, leukocyte, endothelial and erythrocyte origin of BAFF+ MVs was explored. As seen on Fig. 32, our results showed that BAFF+ MVs in plasma co-stain with CD42b and CD31 markers and therefore have platelet origin. Our data revealed that leukocytes (CD45 and CD16 markers), activated endothelial cells (CD62E marker) or erythrocytes (CD235a marker) were not the major sources of circulating BAFF+ MVs (Fig. 32).

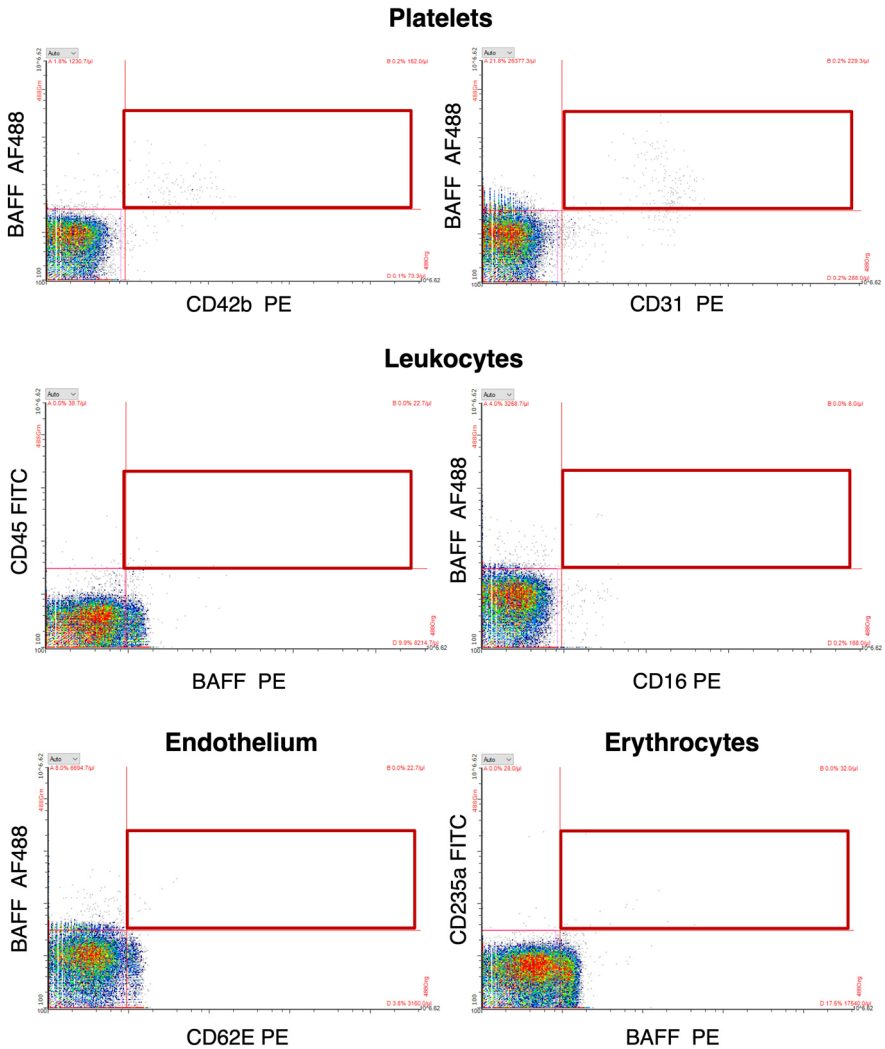

Fig. 32 Investigation of cellular origin of BAFF+ microvesicles in plasma. Patient plasma was stained both with BAFF and platelet (upper panel), leukocyte (middle paned), endothelial (lower panel, left) and erythrocyte (lower panel, right) markers. Dot plots show that circulating BAFF+ MVs represent a fraction of platelet MVs.

As small fraction of CD31 is also expressed by ECs, we co-stained CD31 with platelet marker CD42b and endothelial marker CD144 (VE-cadherin) (Fig. 33). The staining confirmed platelet (Fig. 33A) and not endothelial origin (Fig. 33B) of BAFF+ CD31+ MVs.

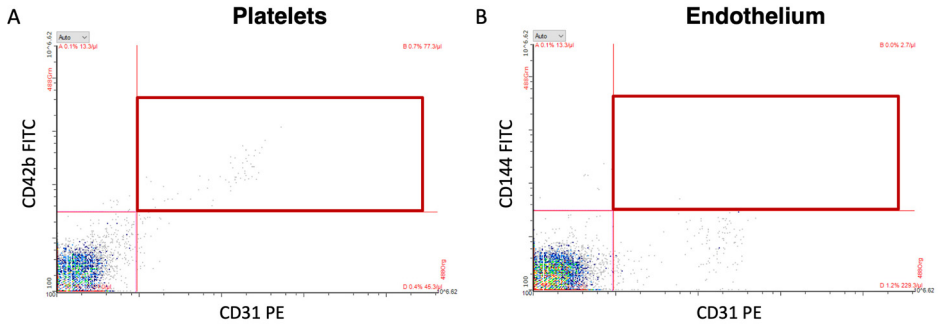

Fig. 33 Co-staining of CD31 marker with platelet (A) and endothelial (B) markers to determine major CD31 cellular origin in plasma. Dot plots show that CD31+ MVs are mainly of platelet origin, thus confirm that BAFF-expressing MVs are a fraction of platelet MVs.

To further explore circulating BAFF+ MV, we investigated the expression on their surface of CD36 that is an oxidized LDL receptor, involved in pro-atherogenic processes[167] (Fig. 34). The staining demonstrated that a fraction of BAFF+ MVs expressed CD36 and the marker was of platelet (Fig. 34B) and not endothelial (Fig. 34C), leukocyte (Fig. 32D) or erythrocyte (Fig. 34E) origin.

Previous reports considered leukocytes as the main cellular source of BAFF in plasma.[164] However, our data demonstrated that the vast majority of circulating BAFF+ MVs is a subpopulation of platelet MVs. Our findings are supported by the results of other groups who discovered BAFF expression on platelets[168] and megakaryocytes.[169] Furthermore, as BAFF+ MVs also carry pro-atherosclerotic CD36, they represent a fraction of MVs involved in pro-inflammatory and pro-atherogenic processes.

Atherosclerosis is sometimes described as an inflammatory disease,[5] thus to further explore if pro-inflammatory conditions are connected with BAFF+ MV release, we stimulated a megakaryocyte cell line with TNFα, a cytokine related to endothelial dysfunction.[170] Addition of TNFα to the culture medium increased the production of BAFF-expressing MVs by MEG01 cells (Fig. 35B), compared to the lack of stimulation (Fig. 35A). The above results could partially explain the higher levels BAFF in MVs from the low CFR group. BAFF appears to be a promising drug target for treatment of vascular dysfunction, as therapeutics against it already exist.

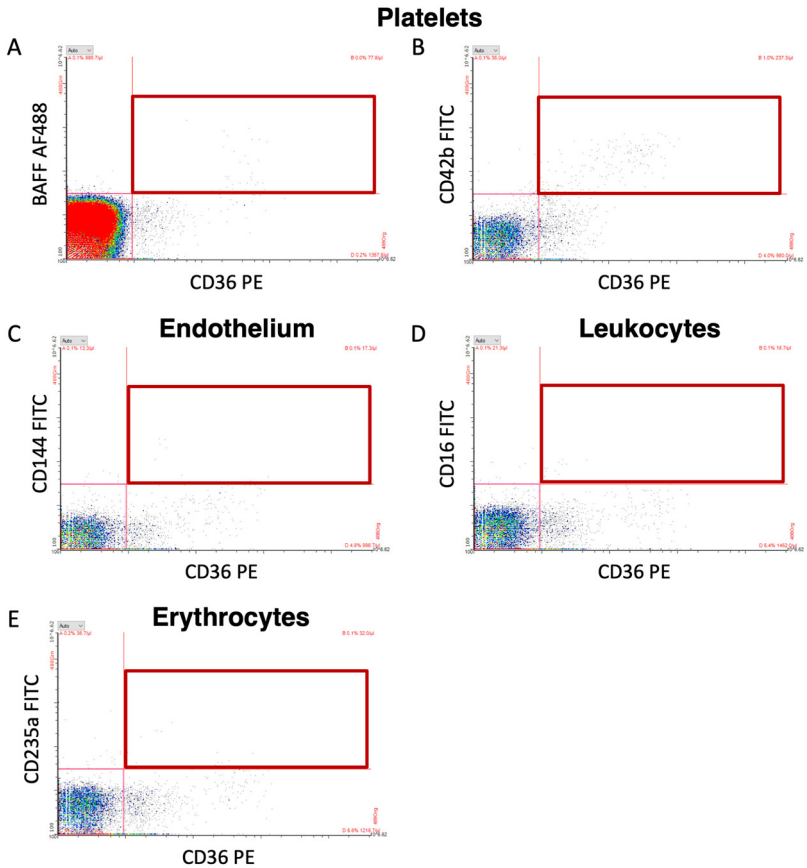

Fig. 34 Analysis of CD36 marker expression on BAFF+ microvesicles (A) and co-staining with platelet (B), endothelial (C), leukocyte (D) and erythrocyte (E) markers to determine its cellular origin in plasma. Dot plots demonstrate that CD36+ MVs are mainly of platelet origin.

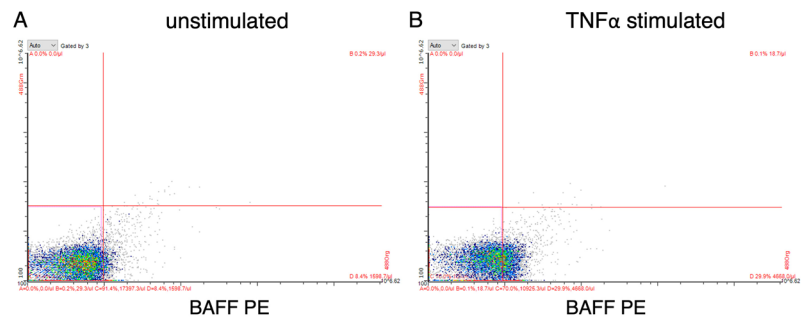

Fig. 35 Pro-inflammatory conditions stimulate a release of BAFF+ microvesicles from megakaryocyte cell line. MEG01 cells were stimulated with TNFα that substantially increased BAFF+ MV release (B), compared to the unstimulated cells (A).

In summary, in Paper III we investigated if there was a connection between CFR values and circulating MVs. The low CFR group was characterized with significantly increased concentration of platelet and endothelial MV levels in plasma. Proteomic analysis of biomarker cargo in MVs showed that AXL, CD163, IGFBP-7, NEMO, resistin, BAFF and perlecan levels negatively correlated with CFR. All of the above-mentioned proteins are involved in inflammatory and pro-atherogenic processes, thus they reflect the pathological vascular status of the patients from the low CFR group and have potential clinical value as biomarkers of deterioration of vascular status. Moreover, anti-BAFF therapeutics could be potentially explored for treatment against CVD.

Paper IV

'Plaque-released MVs' clinical study

The study consisted of 20 participants with a mean age of 66 years that were recruited at Department of Cardiology, Skåne University Hospital in Lund. All patients had a diagnosed coronary arterial stenosis and had undergone balloon angioplasty. Blood samples were derived from aortic root (proximal to the plaque), reflecting circulating MVs or the coronary artery part distal to the plaque, containing plaque-released MVs, following balloon angioplasty.

Flow cytometric analysis of plaque-released MVs

We applied FCM to explore if atherosclerotic plaque contains MVs that are released after balloon angioplasty. Our analysis showed that total concentration of MVs derived from part distal to the plaque was significantly higher, compared to the MV levels present in the circulation (Fig. 36). Although a substantial increase in total MV concentration after balloon angioplasty was observed, no subtype of MVs increased significantly more than the other.

We further investigated the cellular origin of plaque-derived MVs with FCM. Several cellular origins of MVs have been detected (Fig. 37): platelet (CD42b+, CD62P+, CD36+, BAFF+), endothelial (CD62E+, CD201+), leukocyte (CD45+, CD16+, CD14+, CD204+), SMC (CD140b+) and erythrocyte (CD235a+).

It has been implicated that the above-mentioned MVs are hallmarks of CVD and atherosclerosis[34,52,149,171] and their markers play several roles in pro-inflammatory and pro-atherosclerotic processes.[34,172-175] Generally, high concentration of MVs in blood, expressing proteins involved in progression of atherosclerosis, are related to a degree of CVD and points at a pathological environment within the vascular system.

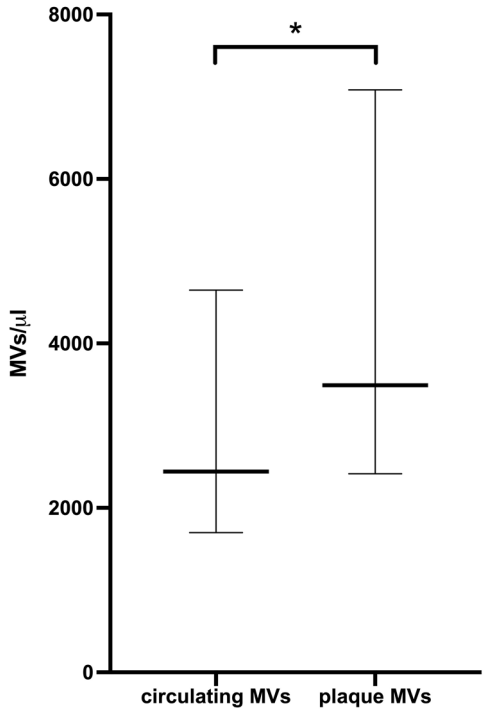

Fig. 36 Flow cytometric analysis of a total MV release from atherosclerotic plaque, following balloon angioplasty. Median ± 95% CI. Wilcoxon signed rank test for paired data. * Statistically significant, p<0.05

Proteomic analysis of plaque-released EVs

To explore a protein cargo of plaque-released MVs after balloon angioplasty, we performed an analysis with Olink CVD panels. Additional aim of this study was to examine if there was an advantage of analysing enriched EVs. Thus, we compared the proteomic results from EVs isolated with acoustic trapping with the data obtained from crude plasma samples that also contain soluble, non-vesicular proteins.

In case of enriched vesicles, 29 proteins had significantly higher concentration in plaque released-MVs, compared to circulating MVs (Table 1). Analysis of crude plasma samples revealed 49 proteins with significantly higher levels in samples derived from distal part to the plaque (Table 1). There was an overlap of 12 significant proteins between enriched MVs and plasma samples (Fig. 38, Table 1).

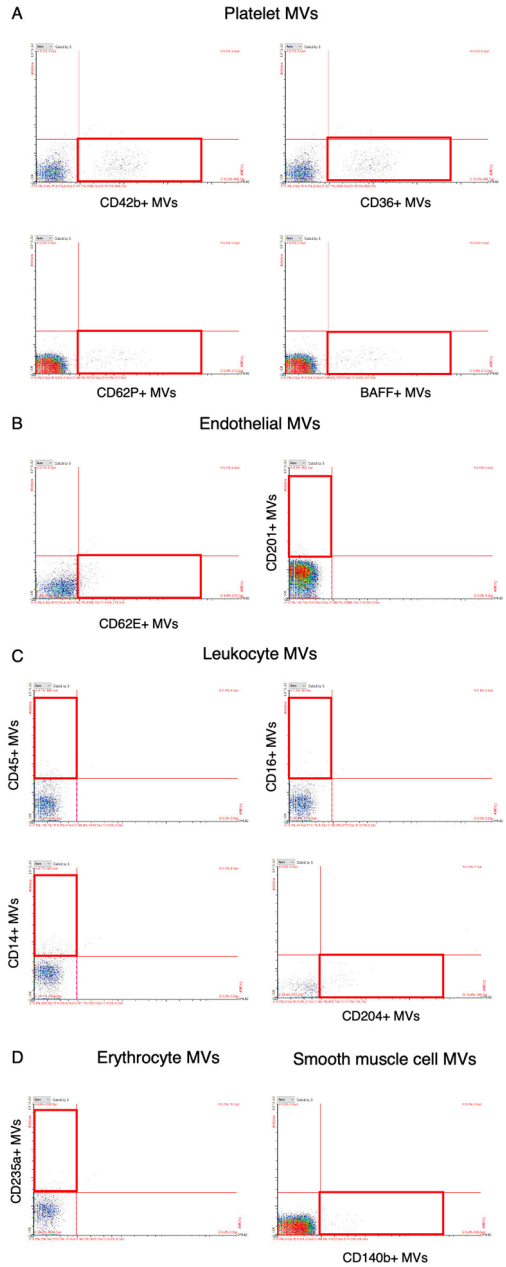

Fig. 37 Analysis of cellular origin of plaque-released MVs with flow cytometry. Plasma samples were stained with Abs against platelet markers: CD42b, CD36, CD62P, BAFF (A), endothelial markers: CD62E, CD201 (B), leukocyte markers: CD45, CD16, CD14, CD204 (C), erythrocyte marker CD235a and SMC marker CD140b (D).

Table 1. List of proteins with significantly higher levels in plaque-released Evs and distal plasma samples, idetified with Olink proteomic panels.

Abbreviation	Protein name	Enriched in plaque-released EVs	Enriched in distal plasma samples
ACE2	Angiotensin-converting enzyme 2		+
ADM	Pro-adrenomedullin		+
AGRP	Agouti-related protein		+
AMBP	Alpha-1-microglobulin/bikunin precursor		+
AZU1	Azurocidin	+	
BOC	Brother of CDO		+
CCL3	c-c motif chemokine 3 (MIP1)		+
CD163	Scavenger receptor cysteine-rich type 1 protein M130	+	
CD4	T-cell surface glycoprotein cluster of differentiation 4		+
CD93	Complement component C1q receptor	+	
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8 (CD66b)		+
CHI3L1	Chitinase-3-like protein 1	+	
CPB1	Carboxypeptidase B	+	
CTRC	Chymotrypsin-C		+
CTSL	Procathepsin L		+
DCN	Decorin		+
FABP2	Fatty acid-binding protein, intestinal		+
FGF21	Fibroblast growth factor 21		+
FS	Follistatin	+	
Gal-9	Galectin-9	+	+
GDF-2	Growth/differentiation factor 2/BMP-9		+
GIF	Cobalamin binding intrinsic factor		+
GRN	Progranulin	+	
HAOX1	Hydroxyacid oxidase 1		+
HO-1	Heme oxygenase-1	+	+
IGFBP-7	Insulin-like growth factor-binding protein 7	+	
IL-6RA	Interleukin-6 receptor subunit alpha	+	
IL16	Pro-interleukin-16		+
IL1RA	Interleukin-1 receptor antagonist		+
IL1RL2	Interleukin-1 receptor-like 2		+
IL4RA	Interleukin-4 receptor subunit alpha		+
IL6	Interleukin-6		+
KIM1	Hepatitis A virus cellular receptor 1 (HAVCR1, TIM1)		+
LEP	Leptin		+
LOX-1	Lectin-like oxidized LDL receptor 1	+	+
LPL	Lipoprotein lipase	+	
MARCO	Macrophage receptor MARCO	+	+
MB	Myoglobin	+	
MERTK	Tyrosine-protein kinase Mer		+
MMP12	Matrix metalloproteinase-12	+	+
OPN	Osteopontin	+	
OSCAR	Osteoclast-associated immunoglobulin-like receptor	+	+
PARP-1	Poly (ADP-ribose) polymerase 1		+
PD-L2	Programmed cell death 1 ligand 2		+
PGLYRP1	Peptidoglycan recognition protein 1	+	
PLC	Perlecan	+	
PlgR	Polymeric immunoglobulin receptor		+

PRELP	Prolargin	+	
PRSS8	Prostasin	+	+
RAGE	Receptor for advanced glycosylation end products	+	+
REN	Renin		+
SCF	Kit ligand/Stem cell factor		+
SELE	E-selectin	+	
SERPINA12	Serpin A12/Vaspin		+
SPON2	Spondin-2	+	+
STK4	Serine/threonine-protein kinase 4/Mst1		+
TF	Tissue Factor		+
TGM2	Protein-glutamine gamma-glutamyltransferase 2	+	+
THBS2	Thrombospondin-2	+	+
TIE2	Angiopoietin-1 receptor		+
TM	Thrombomodulin		+
TNFRSF13B	Tumor necrosis factor receptor superfamily member 13B (TACI)	+	+
TNFSF13B	B-cell activating factor (BAFF)	+	
TRAIL-R2	Tumor necrosis factor receptor superfamily member 10B		+
VEGFD	Vascular endothelial growth factor D		+
XCL1	Lymphotactin		+

This study was of an exploratory kind, thus the proteomic analysis aimed to study the biomarker patterns in plaque-released MVs, rather than concentrating on particular proteins. We compared the results obtained from isolated MVs (vesicular fraction) with plasma samples (vesicular + soluble fractions) to investigate if there was any advantage of MV purification.

Isolated plaque-released MVs, compared to circulating MVs, were characterised with significantly higher levels of proteins involved in progression of atherosclerosis, e.g. inflammation (RAGE, CHI3L1, E-selectin, PGLYRP1, galectin-9, progranulin, azurocidin, IL-6 receptor, BAFF and BAFF receptor – TNFRSF13B, OSCAR),[176-186] macrophage activation (CD163, CD93, MARCO),[163,187,188] oxidized lipid uptake (LOX-1),[189] foam cell formation (follistatin),[190] extracellular matrix remodelling (MMP12, TGM2, spondin-2, thrombospondin-2, prolargin, perlecan, osteopontin, CHI3L1, IGFBP7),[160,191-199] proteolysis (prostasin, CPB1)[200,201] and muscle tissue biology (myoglobin).[202]

Distal plasma samples, containing both soluble and vesicle-derived proteins, were characterized with significantly higher levels of proteins related to e.g. inflammation (IL-6, IL-16, IL-1RA, IL-4 receptor, IL-1RL2, RAGE, CCL3, CD4, TNFRSF13B, leptin, CEACAM8, OSCAR, PD-L2, PlgR, XCL1, galectin-9, STK4),[176,178,182,183,186,203-215] activation of macrophages (MARCO, KIM1, MERTK),[188,216,217] endothelial dysfunction (leptin, GDF2),[215,218] calcification (GDF2),[219] coagulation (TF, thrombomodulin),[220,221] biology of fatty acids (FABP2, LOX1, HAOX1, TIE2),[189,222-224] extracellular matrix (decorin, spondin-2, thrombospondin-2, TGM2),[191,192,194,225] apoptosis (TRAIL-R2, STK4),[226,227]

proteolysis (MMP12, chymotrypsin-C, procathepsin L, prostasin),[200,228,229] angiogenesis (TIE2, VEGFD),[224,230] antioxidant activity (HO1, AMBP),[231,232] muscle tissue (BOC),[233] DNA repair (PARP1),[234] proliferation (SCF)[235] and feeding behaviour (AGRP, FGF21).[236-238] Interestingly, we observed an increase in distal plasma samples of vasoconstrictors ACE2[239] and proadrenomedullin,[240-243] as well as a potent vasoconstrictor, renin.[244]

Bioinformatic analysis of isolated EVs and plasma

We further explored the cargo of plaque-released MVs with STRING database search (Fig. 41). The biological process analysis showed that vesicle preparations, containing atheroma-derived MVs, were generally related to pro-atherogenic processes, such as stress and inflammatory responses (macrophage activation, chemotaxis, cytokine production, cell adhesion, leukocyte migration), as well as regulation of SMC biology and lipid signaling (Fig. 39A). A high number of proteins enriched in plaque-released MVs was involved in vesicle-mediated transport, thus pointing at their vesicular origin (Fig. 39A). This finding could also be connected with an overall higher level of MVs, as shown by FCM. Similar results were obtained after analysing significantly higher proteins in crude, distal plasma samples (Fig. 39D).

The majority of proteins higher in plaque-released MVs were mainly derived from plasma membrane, cytoplasmic vesicles and secretory granules that further corroborates their MV origin (Fig. 39B). Importantly, the results of cellular component analysis for crude plasma did not reveal vesicle-related terms for significant proteins in distal samples (Fig. 39E). This discrepancy underlines an advantage of vesicle isolation, instead analysing crude plasma samples, for studying MV-derived proteins, as the sensitivity of detecting the differences in protein concentration may be too low in crude plasma samples.

The cellular origin analysis revealed that proteins with significantly higher levels in plaque-released MVs were predominantly derived from macrophages, ECs, SMCs, neutrophils, platelets and other sources (Fig. 39C), similarly to the FCM results. Augmented concentration of macrophage-derived molecules in plaque-released MVs implies a significant role of these cells in progression of atherosclerosis.[7,245] Analogous picture was presented by results from analysis of distal plasma samples (Fig. 39F).

In conclusion, we believe that the above results confirm that atherosclerotic plaque releases EVs that present a proatherogenic proteomic ‘pattern’ that represents the pathological molecular events within the cardiovascular system, as well as the health status of the patients.

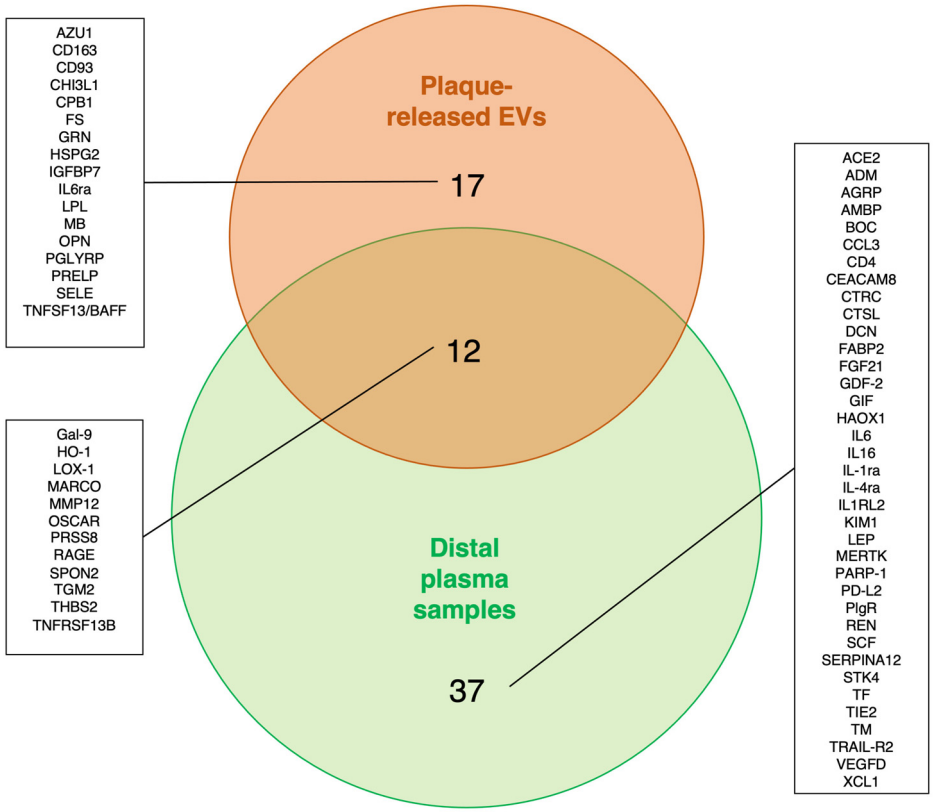

Fig. 38 proteomic content comparison of isolated MVs and crude plasma samples. The numbers reflect significantly higher proteins ($p<0.05$), which are listed in the boxes. Protein abbreviations are explained in Table 1.

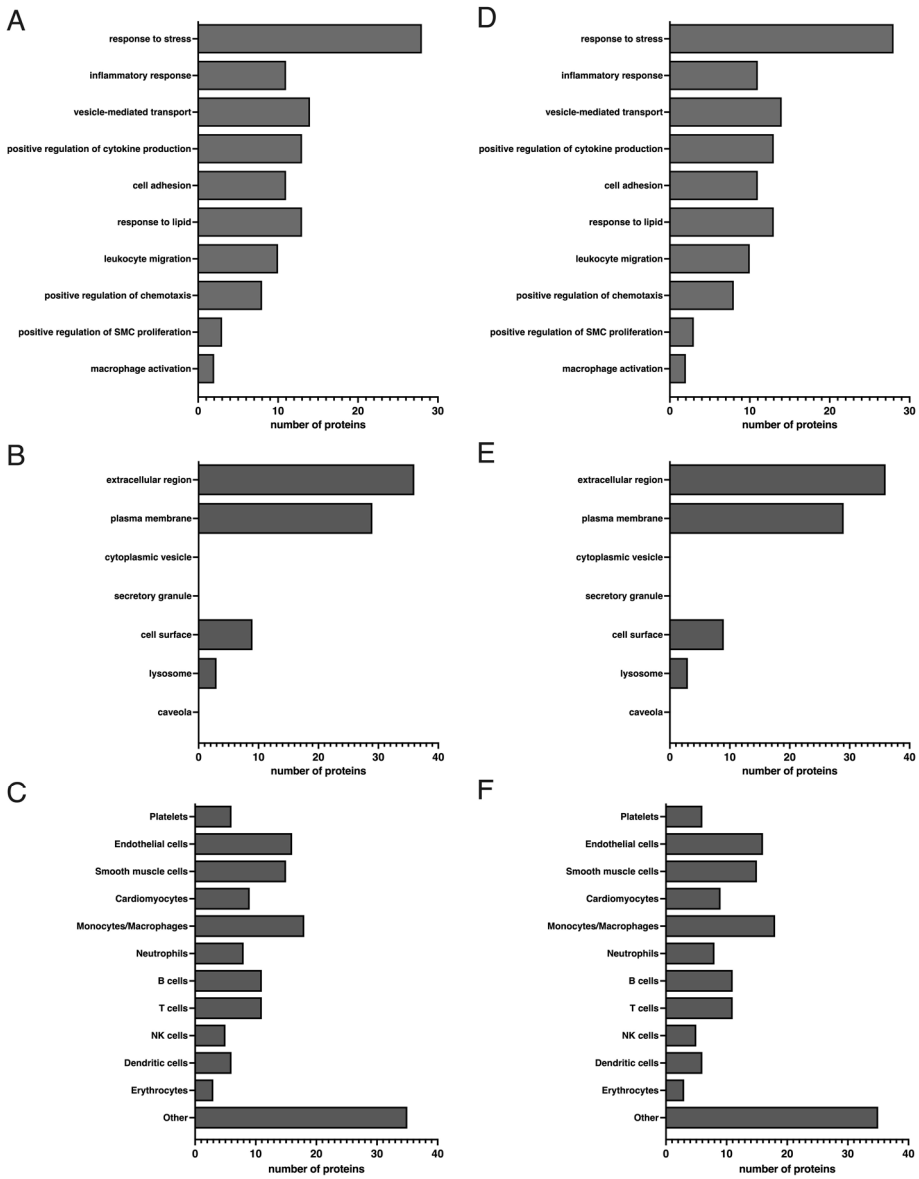

Fig. 39 Results of biological process, cellular compartment and tissue/cellular origin analyses for isolated MVs (left panel) and plasma (right panel). The graphs were created based on significantly higher proteins in distal samples, identified with proteomic panels. Biological process and cellular compartments analyses were performed with STRING. Tissue origin was assessed with The Human Protein Atlas and The Platelet Web.

Summary and Perspectives

The overall aim of this thesis was to investigate the role of MVs in the physiology and pathology of the cardiovascular system. To answer our questions, both clinical studies and *in vitro* experiments were performed.

In Paper I we showed that a novel method for vesicle isolation, acoustic trapping, is a valid method for MV isolation that could potentially be applied in clinical diagnostics. Furthermore, the results of the clinical study also demonstrated a protective effect of exercise on the vasculature, including decrease of MV release from the endothelium.

Paper II revealed that dietary supplementation with bilberry extract exhibit favourable effect on MI patient health status, by decreasing the concentration of platelet and endothelial MVs in blood. Additionally, we discovered that bilberry extract positively affects endothelial cells by reducing the vesiculation via several molecular mechanisms, connected with $P2X_7$ receptor. This study reports for the first time that dietary compounds have an effect on vesicle release, both *in vitro* and *in vivo*. These findings are important for potential future dietary recommendations for patients with CVD, as well as disease prevention with nutrition.

Paper III showed that there is a relationship between coronary flow reserve and MVs in blood. The low CFR group, thus the group with a decreased blood flow, exhibited significantly higher levels of platelet and endothelial MVs. The group was also characterized with increased concentration of several pro-atherosclerotic proteins in the vesicles. These results reflect the pathological status of cardiovascular system of the patients and have a potential clinical value as biomarkers of deterioration of endothelial function. It also raises the possibility that anti-BAFF therapeutics could be further explored as treatment against CVD.

In Study IV we demonstrated that atherosclerotic plaque releases MVs, following balloon angioplasty. The vesicles originate from several types of cells and present a pro-atherogenic ‘pattern’ of proteins. These results represent the pathological processes within the cardiovascular system that lead to the formation of atherosclerotic plaque.

The outcome of the studies included in this thesis points at the important role of MVs in the cardiovascular system. Study I and II underlined the importance of exercise and nutrition for prevention of CVD through decrease in MVs, whereas the

Western world is still characterized with physical inactivity and diet with low nutritional value. Study III and IV focused on potential application of MVs as biomarkers or targets of therapeutics, however it needs further, thorough research and possibly, personalized approach.

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Gratitude is the beautiful form of happiness: this is its cause and its result.

Walter Dirks

Exactly six years ago I got a chance to take part in a long, exciting, sometimes exhausting, but very meaningful educational journey – I became a PhD student at Lund University.

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Podziękowania

Kiedy czasem zdam sobie sprawę z olbrzymich konsekwencji całkiem małych rzeczy… Nie mogę odeprzeć myśli… że małych rzeczy nie ma.

Bruce Barton

Myślę, że gdy wiele lat temu usłyszałam słowa: *Paulinka, pamiętaj, uczysz się dla siebie*, nikt nie pomyślałby, że sprawy zajdą tak daleko, że w wieku trzydziestu lat będę bronić doktoratu na Uniwersytecie w Lund w Szwecji. Niestety nie wszystkie moi bliscy doczekali tej chwili. Jest wiele bliskich mi osób, którym chciałabym podziękować za to, że jestem tu, gdzie jestem i muszę przyznać, że pisanie tych słów wiele dla mnie znaczy.

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przysłowie wietnamskie

Przede wszystkim dziękuję moim Rodzicom, którzy zawsze we mnie wierzyli, wspierali mnie i nigdy nie bali się moich, czasem dziwacznych, pomysłów na przyszły zawód. Od piosenkarki i weterynarza (era przedszkolna), przez filologa i teatrologa (gimnazjum), wreszcie psychologa (liceum). Na tym oczywiście nie poprzestałam przyprawiać ich o kołatanie serca, no bo po maturach postanowiłam jednak studiować biologię, no a potem biochemię, a jeszcze później w ogóle mi się zachciało jakiś kardiologicznych doktoratów ;) Nigdy nie usłyszałam od Was, że coś jest dla mnie za trudne, że nie warto próbować, a jednocześnie zawsze wykazywaliście zrozumienie i nie stawialiście poprzeczki zbyt wysoko. Dzięki temu jestem teraz szczęśliwym człowiekiem, założyłam wspaniałą rodzinę i cieszę się udanym życiem osobistym i zawodowym. Wasza rola w moim sukcesie jest nie do przecenienia.

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Microvesicle signaling in cardiovascular biology under normal and pathobiological conditions

This PhD thesis is a compilation of my work at the Molecular Cardiology group, Lund University, Sweden. The main goal of the publications included in this thesis was to measure and characterize microvesicles present in blood of patients with cardiovascular disease, as well as optimize a novel, non-contact method for vesicle isolation from plasma, called acoustic trapping.

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