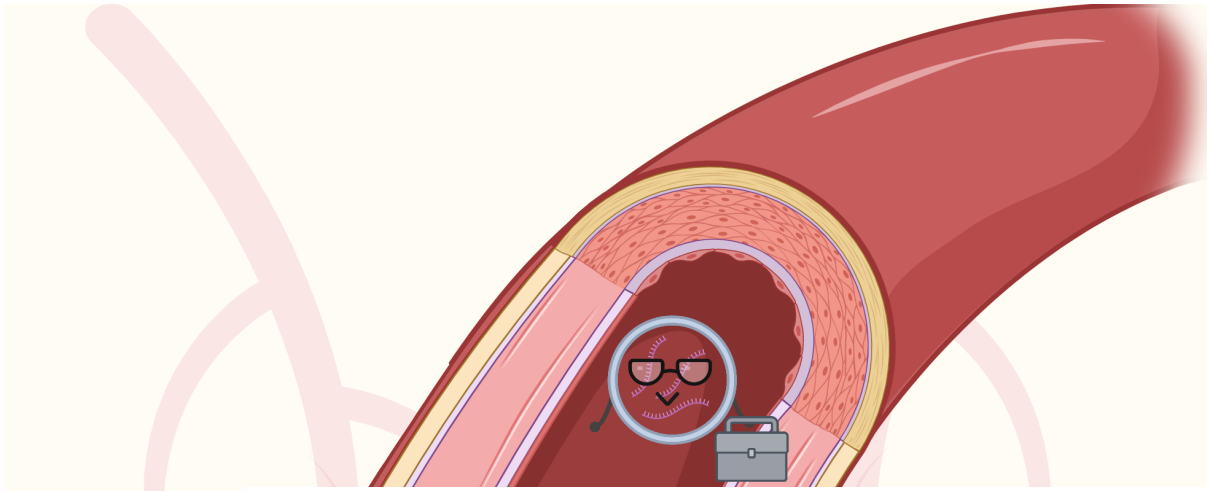
# Micro-RNA: The “Micro”-managers of cardiovascular diseases - Role of extracellular vesicles-mediated miRNAs in cardiovascular diseases

James, Kreema

2022

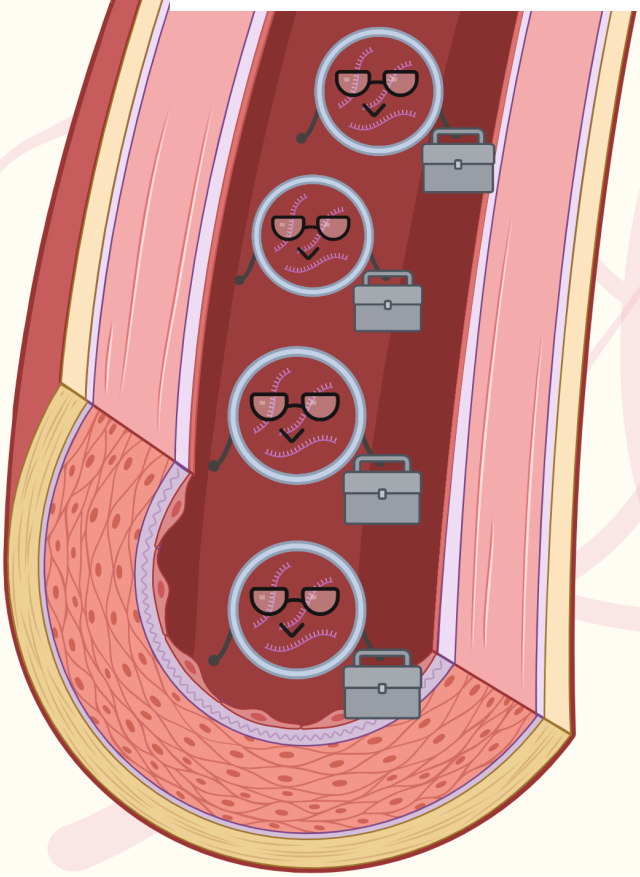
*Document Version:*
Publisher's PDF, also known as Version of record



# Micro-RNA: The "Micro"-managers of cardiovascular diseases

## Role of extracellular vesicles-mediated miRNAs in cardiovascular diseases

KREEMA JAMES
DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



# Micro-RNA: The “Micro”-managers of cardiovascular diseases

# Micro-RNA: The “Micro”-managers of cardiovascular diseases

Role of extracellular vesicles-mediated miRNAs in cardiovascular diseases

Kreema James


LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University, Department of Clinical Sciences, to be publicly defended on 15th of December at 08.30 in Segerfalksalen, Biomedical Centre (BMC), Lund, Sweden

*Faculty opponent*
Dr. Lars Maegdefessel

Department of Cardiovascular Medicine

Karolinska Institutet

| Organization | Document name |
|---|---|
| LUND UNIVERSITY<br>Faculty of Medicine<br>Department of Clinical Sciences<br>Author: Kreema James | Doctoral Dissertation<br>**Date of issue**<br>2022-12-15<br>Sponsoring organization |


**Title and subtitle:** Micro-RNA: The "Micro"-managers of cardiovascular diseases- Role of EV-mediated miRNAs in cardiovascular diseases


Cardiovascular disease (CVD) is the leading cause of death globally, impacted by both extrinsic and intrinsic factors. The disease at the molecular level, however, is governed by inflammation and characterized by endothelial dysfunction. Over the last decade extracellular vesicles (EVs) have emerged as crucial mediators of intercellular communication especially under varied pathophysiological conditions including CVD.

EVs are known to carry several molecules of biological significance such as proteins, ligands, micro-RNAs, receptors, etc. The studies in this thesis focused on the micro-RNA content of circulating EVs in relation to endothelial dysfunction and atherosclerosis measured by novel and reliable clinical parameters. We identified three significant miRNAs from the circulating EVs from three different cohorts. These single stranded non-coding RNAs function by silencing protein coding mRNAs.

miR-224-5p was associated with coronary flow reserve and was identified as a biomarker for a sub-type of hepatic EVs that lead to inflammation by upregulating ICAM-1. While the miRNAs, miR-451a and miR-16-5p were had lower expression in patients with vulnerable plaques with a negative association to platelet EVs. Further investigations revealed that the significant downregulation of these miRNAs in the circulating EVs of the patients was associated with an increase in inflammation and several anti-inflammatory proteins including adhesion molecules. Similarly, the miR-451a demonstrated its significance in an exploratory study involving EVs from ruptured coronary plaques and was observed to be associated to apoptosis related proteins along with adhesion molecules.

The studies shed a light on the uptake of EVs and EV miRNA by recipient cells in the circulation especially the first barrier of endothelial cells and its effect on downstream translation. The studies also highlight the importance of micro-RNAs in the circulation as as prognostic and diagnostic biomarkers of CVD.



**Key words:** cardiovascular diseases, extracellular vesicles, micro-RNA

Classification system and/or index terms (if any)

| Supplementary bibliographical information | | **Language:** English |
|---|---|---|
| **ISSN and key title:** 1652-8220. | | **ISBN:** 978-91-8021-332-5 |
| Recipient's notes | **Number of pages** 67 | Price |
| | Security classification | |

# Micro-RNA: The “Micro”-managers of cardiovascular diseases

Role of extracellular vesicles-mediated miRNAs in cardiovascular diseases

Kreema James


LUND
UNIVERSITY

Cover photo by Kreema James created with BioRender



Faculty of Medicine
Department of Clinical Sciences

ISBN 978-91-8021-332-5
ISSN 1652-8220
Printed in Sweden by Media-Tryck, Lund University
Lund 2022

*My mission in life is not merely to survive, but to thrive; and to do so with some passion, some compassion, some humour, and some style*

*-Maya Angelou*

*To Ammachi and Kunjumol Aunty*

*The Lord is my Shepherd, there is nothing I shall want*
*Pslams:23*

# Table of Contents

# Abstract

Cardiovascular disease (CVD) is the leading cause of death globally, impacted by both extrinsic and intrinsic factors. The disease at the molecular level, however, is governed by inflammation and characterized by endothelial dysfunction. Over the last decade extracellular vesicles (EV) have emerged as crucial mediators of intercellular communication especially under varied pathophysiological conditions including CVD.

EVs are known to carry several molecules of biological significance such as proteins, ligands, micro-RNAs, receptors, etc. The studies in this thesis focused on the micro-RNA content of circulating EVs in relation to endothelial dysfunction and atherosclerosis measured by novel and reliable clinical parameters. We identified three significant miRNAs from the circulating EVs from three different cohorts. These single stranded non-coding RNAs function by silencing protein coding mRNAs.

miR-224-5p was associated with coronary flow reserve and was identified as a biomarker for a sub-type of hepatic EVs that lead to inflammation by upregulating ICAM-1. While the miRNAs, miR-451a and miR-16-5p were had lower expression in patients with vulnerable plaques with a negative association to platelet EVs. Further investigations revealed that the significant downregulation of these miRNAs in the circulating EVs of the patients was associated with an increase in inflammation and several anti-inflammatory proteins including adhesion molecules. Similarly, the miR-451a demonstrated its significance in an exploratory study involving EVs from ruptured coronary plaques and was observed to be associated to apoptosis related proteins along with adhesion molecules.

The studies shed a light on the uptake of EVs and EV miRNA by recipient cells in the circulation especially the first barrier of endothelial cells and its effect on downstream translation. The studies also highlight the importance of micro-RNAs in the circulation as prognostic and diagnostic biomarkers of CVD.

# Popular Science Summary

One in every five deaths is caused by heart disease. Many unhealthy habits such as lifestyle and smoking increase the risk of heart diseases. These diseases are caused by the thickening and hardening of blood vessels as well as accumulation of bad cholesterol in these blood vessels. The phenomenon is known as atherosclerosis. This eventually obstructs the blood flow leading to a myocardial infarction or heart attack and the obstruction is most often caused by a plaque. Through our research we wanted to reveal new mechanisms of how these diseases are developed and how we can detect them earlier through simple blood tests, so that we can prevent heart attacks and sudden death.

We hence studied blood samples of patients with cardiac diseases measuring small carriers released from various cells in the body known as extracellular vesicles. These vesicles mediate communication between different cell types of the body, especially under inflammation and can reach even to the smallest of blood vessels and contribute to microvascular disease. Platelets are a type of blood cell, that react to injury or bleeding by accumulating in the site of vascular injury. In our studies, we saw that the number of these vesicles, especially the ones originating from platelets, increase in the circulation of patients with heart diseases.

Vesicles carry various relevant messenger molecules released under disease conditions and may contribute to the development of the disease. One of these messenger molecules that we pursued further was micro-RNA, a small RNA molecule that can regulate production of the proteins it targets. Proteins are large, complex molecules that are required for structural as well as mechanical functions of cells. Lack of certain proteins can hinder proper functioning and maintenance of cells, and at the same time, excess of harmful proteins can worsen the disease condition.

In one of our studies, we observed that some of these micro-RNAs, represent their cell of origin and communicate between two different organ systems through the circulation affecting the rate of blood flow in small vessels. While, in another study we saw the micro-RNAs such as miR-16-5p and miR-451a, are reduced in the circulating vesicles of patients with plaques which upon rupture can cause myocardial infarction. Their reduction leads to the excess production of proteins that cause inflammation and formation of plaque, resulting in the progression of

cardiac diseases. The lack of miR-16-5p was even observed to increase cholesterol levels in patients with poor cardiac health.

Furthermore, an interesting finding was when we observed the same micro-RNA, miR-451a, to be released from ruptured plaque in patients undergoing a routine balloon dilatation of the plaque. Hence, strengthening our collective findings of its role in the progression of atherosclerosis in patients by manipulating its circulating proteins.

These studies, show us the intricate mechanisms of the cardiovascular system at the molecular level. With further studies and effective ways to apply these promising findings, the results could help diagnose and be used as therapeutics for cardiac diseases.

# List of Papers

1. Microvesicles in plasma reflect coronary flow reserve in patients with cardiovascular disease

P. Bryl-Gorecka, **K. James,** K. Torngren, I. Haraldsson, L. M., Gan, S. Svedlund, B. Olde, T. Laurell, E. Omerovic & D. Erlinge

American Journal of Physiology Heart Circulation Physiology 2021 Vol. 320, 5, H2147-h2160

2. Increased expression of miR-224-5p in circulating extracellular vesicles of patients with reduced coronary flow reserve

**K. James,** P. Bryl-Gorecka, B. Olde, O. Gidlof, K. Torngren and D. Erlinge

BMC Cardiovascular Disorders 2022 Vol. 22 Issue 1 Pages 321

3. Lower expression of anti-inflammatory micro-RNAs in the circulating extracellular vesicles of patients with high lipid-rich plaque burden

**K. James,** P. Bryl-Gorecka, T. Sharma, B. Olde, O. Gidlöf, A. Maehara, 3, M. Matsumura, O. Ben-Yehuda, M. Maeng, G.S. Mintz, S. K. James, Z. A. Ali, G. Stone, David Erlinge

4. Profiling extracellular vesicle microRNA released from atherosclerotic plaques

**K. James,** P. Bryl-Gorecka, B. Olde, O. Gidlöf, S. Koul, D. Erlinge

# Abbreviations

| | |
|---|---|
| CVD | Cardiovascular diseases |
| MI | Myocardial infarction |
| CAD | Coronary Artery Disease |
| MACE | Major adverse cardiac events |
| EVs | Extracellular vesicles |
| MVE | Multivesicular endosomes |
| ILV | Intraluminal vesicles |
| miRNAs | Micro-RNAs |
| pri-miR | Primary miRNA |
| AGO2 | argonaute RISC catalytic component 2 |
| Abs | Antibodies |
| FCM | Flow cytometry |
| CFR | Coronary flow reserve |
| DPBS | Distilled phosphate buffer solution |
| RT | Room temperature |
| NIRS. | Near infrared spectroscopy |
| IVUS | Intravenous ultrasound |
| LCBI | Lipid core bundle index |
| PB | Plaque burden |
| PCI | Percutaneous coronary intervention |
| SMC | Smooth muscle cells |
| PBMC | Peripheral blood mononuclear cells |
| HCAEC | Human coronary artery endothelial cells |
| HMEC | Human microvascular endothelial cells |
| oxLDL | Oxidized Low Density Lipid |

# Introduction

**Cardiovascular diseases** have prevailed as a major cause of morbidity and mortality in developed countries and increasingly in developing countries (1, 2). Various factors contribute to the manifestation of CVD including lifestyle, diet, and genetics (3). Clinically, CVD is defined by impaired vascular flow due to vasoconstriction, plaque formation and rupture leading to myocardial infarction (*fig.1*), major adverse cardiac events and even death (4).

The studies in our thesis have focused on two molecular components involved in CVD i.e., extracellular vesicles and their transport of micro-RNA in the context of **endothelial dysfunction and atherosclerosis**.

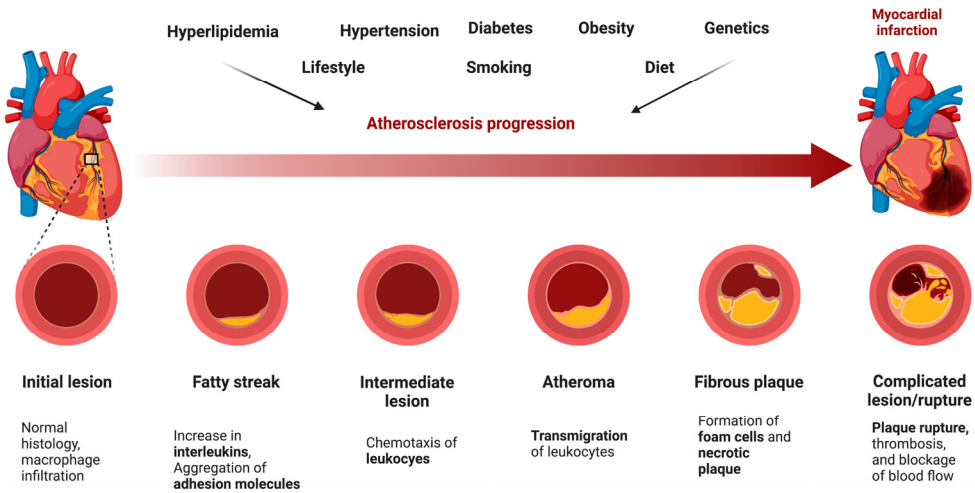



***Fig. 1:*** *The illustration graphically demonstrates the clinical as well as molecular progression of atherosclerosis manifesting into a Myocardial Infarction. The illustration is created on BioRender.*

# Endothelial dysfunction

**Endothelial dysfunction** disrupts homeostasis, the primary function of the endothelium which overtime leads to atherosclerosis and myocardial infarction (*fig.1*), two major characteristics of the cohorts we studied (5).

At the molecular level, most clinical risk-factors of CVD such as hyperlipidemia, diabetes, obesity, acute coronary syndrome are defined by an underlying **proinflammatory** cascade leading to an increase in the levels of cytokines, activation of adhesion molecules leading to increased vascular permeability, lipid accumulation, apoptosis and endothelial dysfunction (6, 7).

# Extracellular vesicles

**Extracellular vesicles** are lipid bilayer bound membrane particles known for their role in inter-cellular communication. EVs are classified into three sub-types of namely exosomes, microvesicles and apoptotic bodies depending on their size and biogenesis. *For our studies we will focus on exosomes and microvesicles and use the abbreviation EVs to address them.*

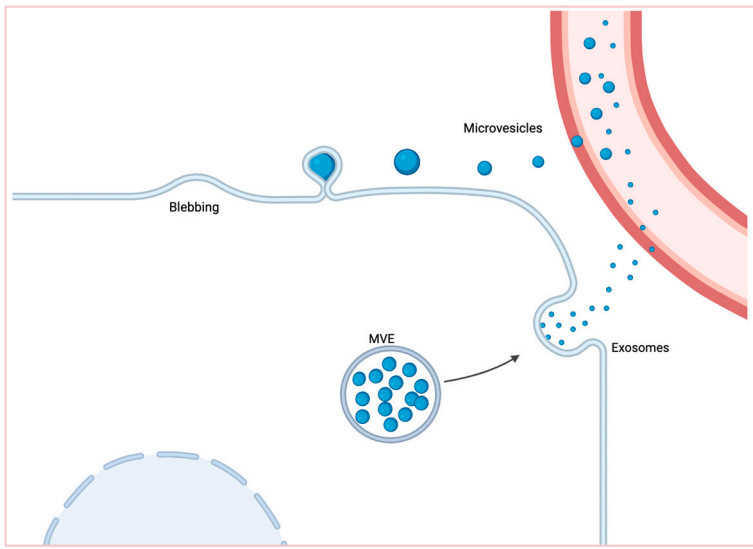



***Fig. 2:*** *The illustration demonstrates the generation of microvesicles (50-500 nm) by blebbing and exosomes (50-150 nm) through MVE fusion to plasma membrane, released into the circulating plasma. The illustration is created on BioRender.*

**Exosomes** are 50-150 nm in size, formed as intraluminal vesicles (ILVs) within the lumen of multivesicular endosomes (MVEs) and released by the fusion of the MVE to the plasma membrane releasing the ILVs then known as exosomes. While **microvesicles** (MVs) are 50-500 nm particles formed by the blebbing of the plasma membrane releasing the MVs (8) (*fig.2*).

Since the vesicles released outside the cell are an extension of the donor cell, they represent their cell of origin and its microenvironment. These vesicles can further enter a recipient cell by mechanisms such as clathrin-dependent and independent endocytosis, phagocytosis as well as enzyme dependent permeabilization (9). EVs have been studied extensively in the last decade as crucial modulators of cardiovascular diseases (10).

# Micro-RNA

EVs are packaged with mechanistic specificity and can affect their recipient cells. Our papers will focus on the micro-RNAs transferred by EVs under an inflammatory micro-environment (11). **Micro-RNAs** are about 22 nucleotide long, single-stranded non-coding RNAs and are formed either canonically or non-canonically, with the canonical pathway being the most dominant.

The **canonical** pathway (*fig.3*) involves the transcription of a pri-miRNA from the respective genes followed by the formation of a pre-miR by a microprocessor complex made of DGCR8 and ribonuclease III enzyme, Drosha. The pre-miRNA is then transported by exportin 5/ RanGTP complex to the cytoplasm wherein it is processed into the mature miRNA by the RNase III endonuclease, Dicer.

The **non-canonical** pathways (*fig.3*) on the other hand use different combinations of the canonical pathways and can be Drosha or Dicer independent. However, the pre-miRNAs require AGO2 to form a mature miRNA in the non-canonical pathways (12).

The **mature miRNA** induces mRNA silencing through the miRNA-induced silencing (miRISC) complex that consists of AGO and a guide strand. This complex is highly specific due to its complementarity to the miRNA response elements (MRE) on the target mRNA. This complementarity leads to translational inhibition or decay of the mRNA (12) (*fig.3*).

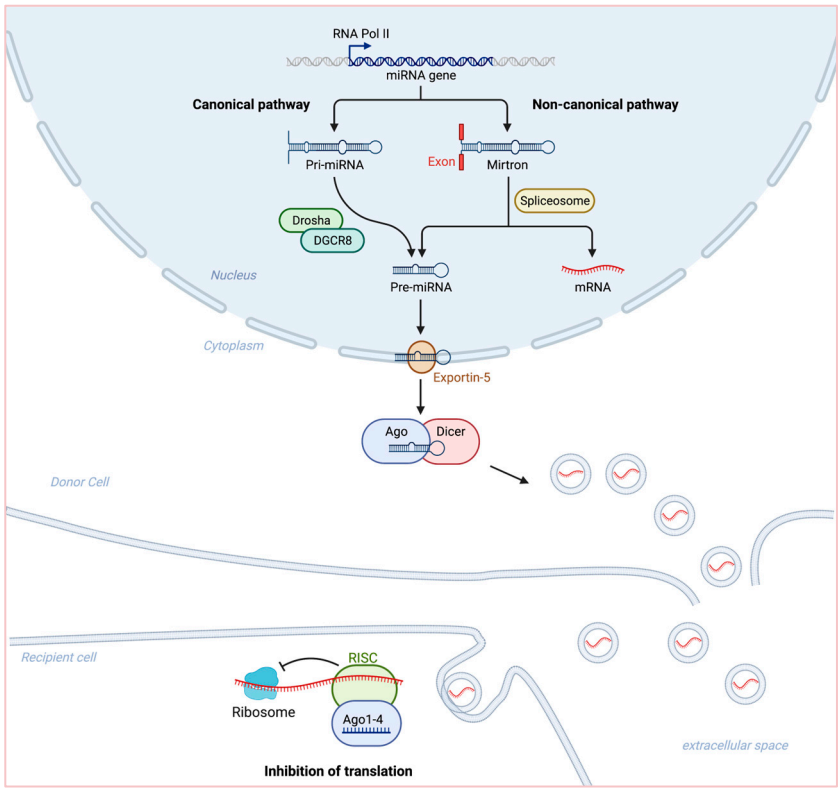



***Fig. 3:*** *The illustration shows the canonical and non-canonical biogenesis of an miRNA and its possible regulation of translation in its recipient cell. The illustration is created on BioRender*

The release of miRNA into the extracellular milieu is a highly regulated process and affects the recipient cells. Several studies have shown the effect of extracellular miRNAs, as chemical messengers in cell-cell interaction (12). Studies have shown that exosomes and vesicles predominantly carry miRNAs in serum and saliva. The encapsulation of miRNAs by vesicles or other proteins, increases their stability outside the cell and can be excellent biomarkers for diseases (13). The release of miRNA in exosomes is shown to be a ceramide dependent process and affects the target cells (14). The effect of EV-miRNA has been studied in the pathophysiology of CVD (11).

However, a relative gap in understanding exists in the role and function of EV-mediated miRNA in endothelial dysfunction and atherosclerosis leading to CVD and the papers in this thesis aim to bridge this gap.

# Aims of the thesis

The following are the general aims of the thesis addressed and investigated in the broader sense in all the four manuscripts included in this thesis.

- Understanding novel molecular mechanisms involved in the manifestation of cardiovascular diseases
- The role of extracellular vesicles and its cargo in the pathophysiology of cardiovascular diseases assessed by different parameters
- The role of EV mediated micro-RNA in the cardiovascular micro-environment in patients with endothelial dysfunction
- The clinical relevance of extracellular vesicles and its micro-RNA content as prognostic as well as diagnostic biomarkers of the atherosclerotic disease

# General Methods

## Extracellular vesicle isolation

The projects in this thesis revolved around the isolation of extracellular vesicles from human blood plasma, to explore its content and its effect on the pathophysiology of cardiovascular diseases. Various methods exist to separate EVs from biological fluids, for our experiments and cohorts we employed the following methods.

### Ultracentrifugation

The gold standard of EV separation was used to isolate and enrich EVs from culture medium for our in-vitro studies. The method involves the application of centrifugal force at 100,000xG at 4°C for one and a half hour to obtain EVs from about 20ml of sample. The method led to a pool of EVs resuspended in DPBS, used for further detection and recipient cell interaction. **Ultracentrifugation** though an effective method is time-consuming and insufficient for smaller volumes such as bio-banked samples, making it difficult to use for larger cohorts.

### Acoustic Trapping

We hence used a novel method to isolate EVs for all our cohorts studied in this thesis, known as acoustic trapping. The method was developed at Lund University by Thomas Laurell and then branched out into AcouSort Ab at Medicon Village, Lund, Sweden. Their automated platform known as **Acoutrap** (*fig.4*) was used to isolate EVs from bio-banked plasma samples with volumes as low as 50µl.

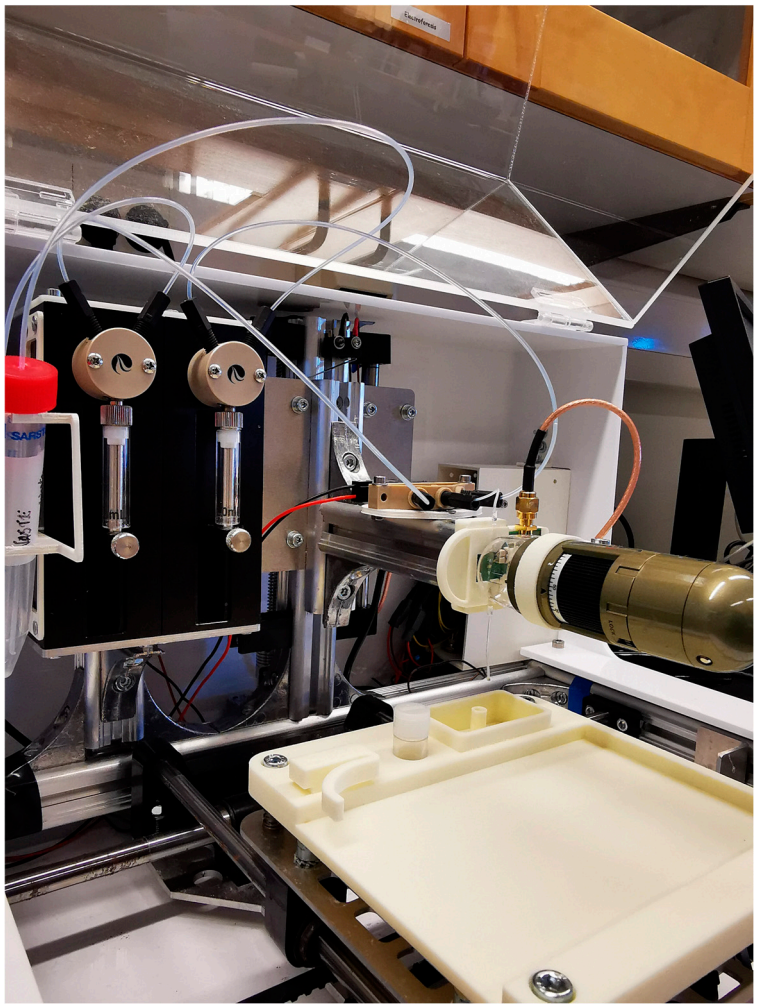


***Fig. 4:*** *AcouTrap by AcouSort Ab, Sweden, an automated platform used as the primary method of EV isolation for all our studies*

The script-based device utilizes acoustic waves (ultrasound standing waves) generated by a transducer in a microfluidic capillary, creating an acoustic trap to detain 12µm polystyrene beads (Sigma Aldrich) (*fig.5.A*). The microparticles or EVs are then captured by these larger seed particles and are subsequently washed and released in DPBS for further processing (*fig.5.B*).

Utilization of AcouTrap permitted the use of small samples volumes from larger cohorts with minimum processing time, and with an efficacy similar to ultracentrifugation.

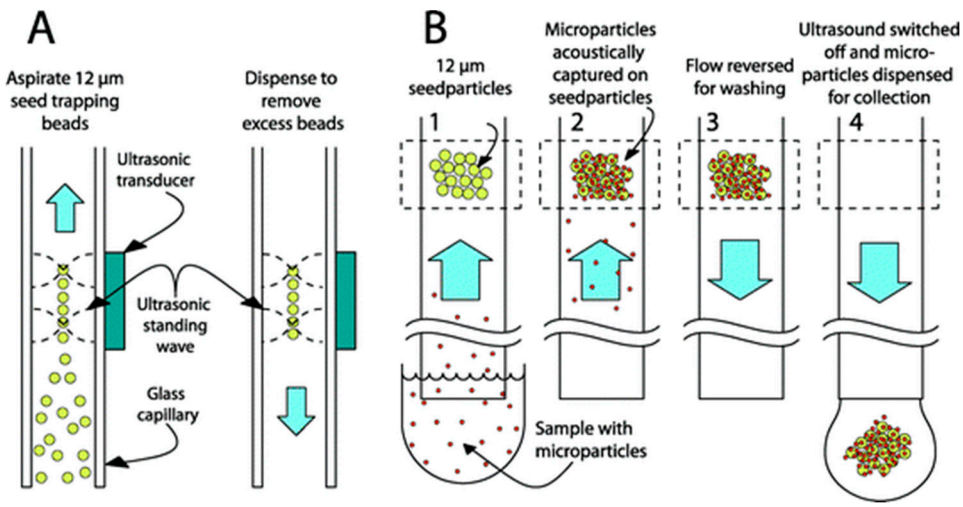



***Fig. 5:*** The fig demonstrates the detailed process of EV isolation using AcouTrap. A) shows the microfluidic capillary and generation of acoustic standing waves while B) shows the capture and collection of EVs from the sample aspirated by Evander.et.al (https://doi.org/10.1039/C5LC00290G)

The isolated EVs were further subjected to characterization for both their cellular origin and for their biological cargo using the following techniques.

# Extracellular vesicle Characterization

For all the papers in this book the EV population and its cargo was characterized by the following methods detailed.

## Flow Cytometry

**Flow cytometry** was used for the characterization of EVs and for their association to various cellular origins as well as for the characterization and validation of their interaction with recipient cells. The used procedure was standard and was performed with the ApoGee A60 cytometer (Apogee Flow Systems, Hemel Hempstead, UK).

The samples were incubated with the required antibodies (Abs) for 30 minutes at room temperature with constant shaking and then directly analysed. Samples were diluted 1:200 for plasma samples and cultured cells while EV pellets obtained by ultracentrifugation were resuspended in 200 µl. Abs were centrifuged prior to incubation at 20,000 G for 30 mins at 4°C to remove aggregates. The instrument was calibrated with size calibration silica beads (Apogee Mix, Apogee Flow Systems) and antibody isotype controls.

## Micro-RNA profiling

Total RNA was extracted from EVs according to the manufacturer's protocol (**Plasm/ Serum RNA purification mini kit, Norgen Biotek**) and were then processed further for micro-RNA profiling .The following three methods were used to characterize micro-RNAs in EVs according to the experimental design.

*Multiplex PCR*

The total RNA samples from paper II and paper IV were profiled for 190 miRNAs using the **TaqMan Advanced miRNA Human Serum/Plasma Card**, a 384-well microfluidic system and analysed with the QuantStudio 12k Flex (*fig.6*). The assay has endogenous and exogeneous controls (ath-miR-159a & cel-miR-39-3p). Prior to the multiplex q-PCR the RNA was converted into c-DNA and uniformly amplified according to manufacturer's protocol (TaqMan Advanced cDNA Synthesis Kit). The miRNAs expression was determined by calculating the Relative fold change (2^-ddCT) and the data was normalized using global mean for each sample.

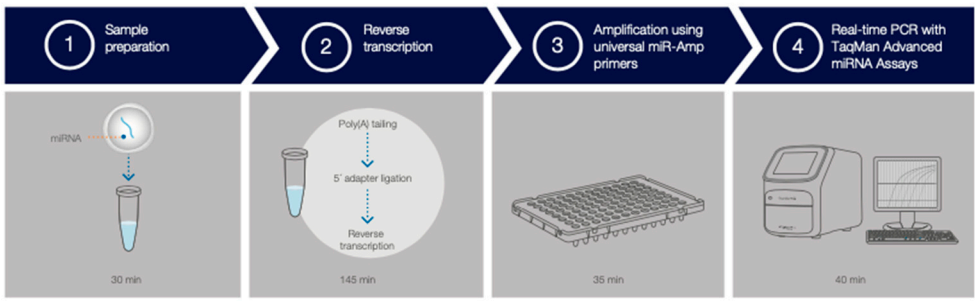



***Fig. 6:*** *A schematic process of the q-PCR based assay from TaqMan advanced miRNA assay.*

*Multiplex FCM*

We turned to a more direct approach with a novel method developed by Abcam that involved a multiplex assay analysed by FCM as the endpoint, known as the **FirePlex miRNA assay**. The Cardiology V2 panel was chosen for our samples with 65 micro-RNAs per well. The miRNAs were captured directly from the crude samples (acoustically trapped vesicles) by labelling them and subjected to universal amplification (*fig.7.a*). The amplified samples were recaptured and analysed by FCM (*fig.7.b*). The data was normalized by global normalization using internal controls (miR-17-5p, miR-20b-5p, miR-93-5p and miR-16-5p).

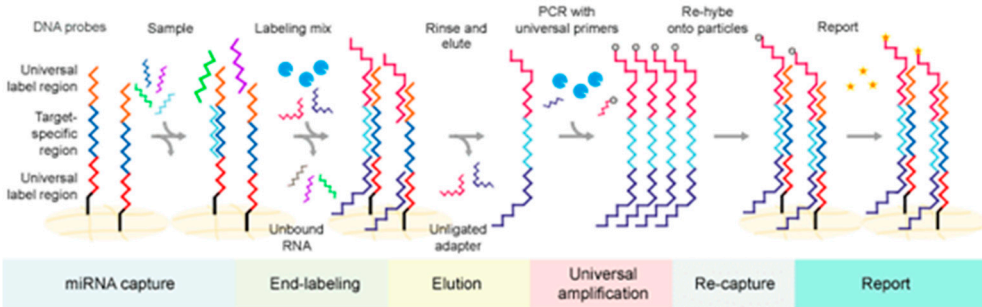



***Fig. 7.a:*** *The molecular workflow for the fireplex assay for miRNA by AbCam*

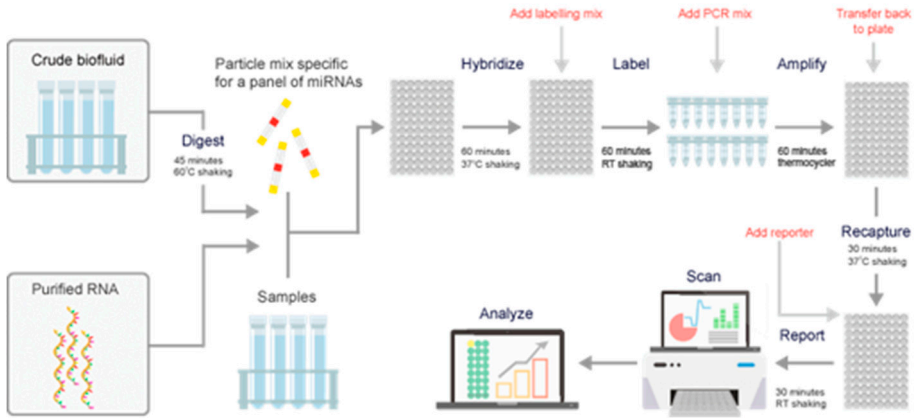



***Fig.7.b:*** *A diagramatic workflow of the fireplex miRNA assay by AbCam*

*Real-time PCR*

The expression of the identified miRNAs was validated using real-time PCR both in EVs and in cells. The **qPCR** was performed for the selected micro-RNAs, according to manufacturer's instructions (miRCURY LNA miRNA PCR Assay) with sn-U6 as the reference gene.

## Proximity Extension Assay

Protein content in EVs and circulating plasma demonstrates the translational downstream effect of the micro-RNAs. We used the CVD panel II and III for **multiplex proximity extension assay** by Olink Proteomics for paper I, III and IV.

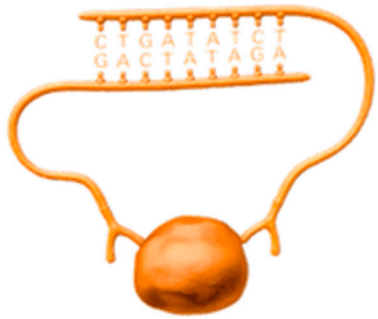


***Fig. 8:*** *A representative diagram of the proximity extension assay by Olink Proteomics*

The samples both vesicles and plasma were lysed with RIPA buffer (Thermo Fisher Scientific) and the protein concentration was measured with BCA assay (Thermo Fisher Scientific) and further adjusted to 0.7 µg/µl for all samples. The proximity extension assay binds unique DNA tags to the protein using antibodies targeting two different epitopes. The hybridization of the dual tags upon coming into close proximity of each other (*fig.8*) leads to a signal, read-out by q-PCR. The samples are then analyzed to give the normalized protein expression (NPX).

# Statistical Analysis

The statistical analysis in all the papers was performed on GraphPad Prism and in paper-III some tests were performed on Stata. The data in these papers were subjected to a normality test before determining the applied test i.e., parametric, or non-parametric. For regression models' appropriate model was chosen i.e., logistic for binary and linear for continuous variables.

For the **micro-RNA data** collected the relative fold change was determined by 2^-ddCT and then compared between the sample groups by either t-test (simple, one per row or Wilcoxon's signed rank test) or ANOVA(one or two-way) depending on the experimental design. For micro-RNA data false discovery rate was applied (Benjamini, Krieger and Yekutieli) for the multiple variables assessed. Proteomic and flow cytometric data were analyzed similarly. The micro-RNA data was also associated to the data from the flow cytometric analysis of EVs as well as the proteomic data with regression models and correlation matrix. Paper IV used dependent and paired testing.

The **clinical data** from our cohorts was used to characterize the patients using descriptive statistics as well to distinguish between patient groups. The patient data

was associated to the micro-RNA data using correlation matrix and regression models (logistic or linear).

## Plasma preparation and ethical statement

The **blood samples** for all the cohorts in our study were collected in EDTA tubes except for paper-III where the samples were collected in citrate tubes as well. The samples were processed within 30 min of collection and plasma was isolated by centrifuging twice at 1570G for 10 min and then stored at -80°C. The frozen and bio-banked plasma samples were thawed for further experimentation at RT and never re-frozen.

Informed written and verbal **consent** was undertaken in agreement with the International Conference on Harmonization Good Clinical Practice guidelines for all the studies. The studies were reviewed and accepted by the respective institutional ethical committee while patient data and samples were handled in accordance with the Declaration of Helsinki.

# Cohort Characteristics

## Prospective Evaluation of Coronary Flow Reserve (PROFLOW)

The **PROFLOW** cohort comprised of 619 patients identified in the Swedish Coronary Angiography and Angioplasty Registry (SCAAR) by the Department of Cardiology at Skåne University Hospital in Lund and Sahlgrenska University Hospital in Göteborg, Sweden. All the patients had prior type-1 MI (>3 months and <5 years) and distinguished based on Coronary Flow Reserve (CFR). CFR was measured in the identified and defined Left Anterior Descending artery (LAD) as the ratio between the hyperaemic and baseline flow velocity values at rest and after induction with adenosine (140ug/kg/min) with 3.5-MHz colour Doppler in the interventricular sulcus in a modified 2-chamber view.

**Table.1: Patient characteristics**

| Parameters | All Patients | High CFR | Low CFR |
|---|---|---|---|
| *n* | 220 | 108 | 112 |
| CFR, means ± SD | 2.96 | 3.41* | 2.45 |
| Age, yr | 67 ± 6.3 | 67 ± 6.4 | 67 ± 6.1 |
| Height, cm | 174 ± 8.7 | 174.5 ± 8.4 | 172.9 ± 8.8 |
| Weight, kg | 83 ± 13.3 | 83.4 ± 13.4 | 82.5 ± 13.2 |
| Men, *n* (%) | 183 (83) | 93 (86) | 90 (80) |
| Women, *n* (%) | 37 (17) | 15 (14) | 22 (20) |
| Hypertension, *n* (%) | 124 (56) | 61 (56) | 63 (56) |
| Diabetes, *n* (%) | 53 (24) | 23 (22) | 30 (27) |
| Dyslipidemia, *n* (%) | 99 (61) | 49 (45) | 50 (45) |
| Angina pectoris, *n* (%) | 39 (17) | 15 (14) | 24 (21) |
| NSTEMI, *n* (%) | 198 (89) | 94 (86) | 104 (92) |
| STEMI, *n* (%) | 24 (11) | 15 (14) | 9 (8) |
| Stenosis†, *n* (%) | 13 (6) | 5 (5) | 8 (7) |
| Presence of plaque, *ns*‡ (%) | 162 (74) | 74 (68) | 88 (78) |
| Current smokers, *n* (%) | 20 (9) | 7 (6) | 13 (11) |
| Previous smokers, *n* (%) | 125 (57) | 61 (56) | 63 (56) |
| Low-density lipoprotein, mmol/L | 2.1 ± 0.8 | 2.1 ± 0.8 | 2.1 ± 0.8 |
| Triglycerides, mmol/L | 1.1 ± 0.6 | 1.1 ± 0.6 | 1.1 ± 0.6 |
| C-reactive protein, mg/L, means ± SD | 1.7 ± 2.0 | 1.6 ± 2.0 | 1.7 ± 2.1 |
| Neutrophils, x$10^3$/$mm^3$, means ± SD | 4.0 ± 1.2 | 3.9 ± 1.1 | 4.0 ± 1.3 |
| GFR, mL/min/1.73 $m^3$, means ± SD | 85.5 ± 24.0 | 85.9 ± 22.4 | 85.1 ± 25.3 |
| Medication | | | |
| Statins, *n* (%) | 201 (91) | 98 (91) | 102 (91) |
| Acetylsalicylic acid, *n* (%) | 212 (96) | 105 (97) | 108 (96) |
| B-blocker, *n* (%) | 174 (79) | 84 (78) | 90 (80) |
| Diuretics, *n* (%) | 40 (18) | 21 (19) | 19 (18) |
| P2Y12 receptor antagonists, *n* (%) | 60 (27) | 31 (29) | 30 (27) |
| ACEi, *n* (%) | 126 (57) | 58 (53) | 68 (61) |
| ARB, *n* (%) | 59 (27) | 28 (26) | 31 (28) |
| CCB, *n* (%) | 34 (15) | 20 (18) | 14 (12) |

For the study of circulating EVs we chose 220 patients for proteomics (paper I) and from those, 120 patients for micro-RNA profiling (paper II), from Lund segregated into two groups based on a median CFR threshold of 2.9. The patients with CFR

≥2.9 were assigned to the High CFR group, whereas the patients with CFR ≤2.9 were assigned to the Low CFR group. The patient characteristics are in the table above.

## PROSPECT-II

The **PROSPECT-II** cohort consisted of 902 patients with MI enrolled irrespective of age and gender from Sweden, Denmark, and Norway. A 3-vessel NIRS-IVUS was conducted on non-culprit lesions to determine their maximum LCBI (4mm) and PB. For paper III, 100 patients were identified based on their measured maxLCBI and maxPB for further processing wherein the patients were divided in to high and low with median LCBI of 390 and median PB of 75%. The clinical parameters of the cohort as addressed in paper III are as follows:

**Table.2: Patient Characteristics**

| Characteristics | high LCBI & PB | low LCBI & PB | |
|---|---|---|---|
| | Median | Median | p-value |
| Lipid Core Burden Index (LCBI) | 715.5 | 24.5 | <0.000001 |
| Plaque Burden (%) | 78 | 58 | <0.000001 |
| Total cholesterol (mg/dL) | 205 | 190 | 0.03 |
| Triglycerides (mg/dL) | 136 | 124 | ns |
| Female (n) | 7 | 9 | |
| Male (n) | 41 | 39 | |
| Age (years) | 61.5 | 63 | |
| Body Mass Index (kg/m2) | 27 | 28 | ns |
| Smoker or Ex-smoker (n) | 26 | 24 | ns |
| Serum creatinine (mg/dL) | 1 | 1 | ns |
| Creatinine clearance (mL/min | 103 | 102 | ns |
| Haemoglobin (g/dL) | 15 | 15 | ns |
| Platelet count (109/L) | 236 | 238 | ns |
| WBC (109/L) | 9 | 9 | ns |
| High-sensitivity C-reactive protein (µg/mL) | 4 | 4 | ns |
| Haemoglobin A1c (%) | 6 | 6 | ns |

## Plaque EVs

This **exploratory study** addressed in paper IV, is derived from the standard invasive procedure of PCI where 20 patients with NSTEMI from Dept. of Cardiology, Lund were enrolled, irrespective of age and gender. During the procedure 20 ml of blood was drawn from the aorta proximal to the plaque, followed by dilation of the stenosis by balloon angioplasty. Another 20 ml of blood was drawn distal to the dilated plaque and centrifuged to obtain plasma. The patient characteristics are as follows:

**Table.3: Patient characteristics**

| Parameters | Patients (n=20) |
|---|---|
| Age, mean (years) | 66 |
| Men | 14 |
| Women | 6 |
| STEMI | 19 |
| NSTEMI | 1 |
| Stable angina | 0 |
| Coronary artery angioplasty (n) | LAD (9) |
| Occlusion | 12 |
| Diabetes Mellitus | 4 \| Dx (7) \| Cx (4) |
| Hypertension | 8 |
| Dyslipidaemia | 1 |
| Kidney disease | 2 |
| Current smokers | 3 |
| Previous smokers | 8 |
| Statins | 1 |
| Insulin | 0 |
| Oral anti-diabetic medication | 4 |
| P2Y12 antagonists during the procedure | 20 (Ticagrelor) |
| Glycoprotein IIb/IIIa inhibitors during the procedure | 0 |
| Ejection fraction, mean (%) | 50 |
| Max Troponin, mean (ng/L) | 3695 |
| HbA1c, mean (mmol/mol) | 39.7 |
| C-reactive protein, mean (mg/dL) | 9.3 |
| High density lipoprotein, mean (mmol/L) | 5.1 |
| Low density lipoprotein, mean (mmol/L) | 3.1 |
| Triglycerides, mean (mmol/L) | 1.7 |

# Results

The results in this thesis summary will focus on some of the main observations, emphasizing the role of EVs and their micro-RNA in the development of endothelial dysfunction.

## B-cell activating factor

The proteomic profiling of the EVs from the PROFLOW study showed several proteins that differed significantly between the Low and High CFR groups. BAFF (p=0.005) ( *fig.9*) of the TNF-family was explored further, due to its evident role in CAD and as a pharmacological target for anti-inflammatory therapeutics (15, 16).

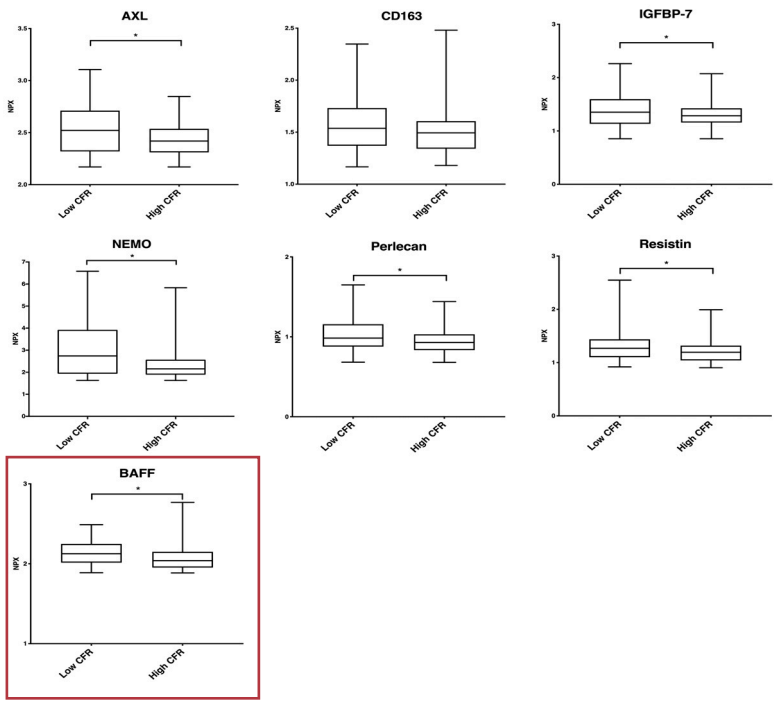



***Fig. 9:*** *The panel shows the boxplots of proteins that were significantly expressed in the circulating plasma of Low CFR patients. The highlighted plot, indicates the selection of the protein BAFF for further investigation*

## Origin of BAFF-EVs

*Plasma BAFF-EVs*

The circulating plasma of the PROFLOW cohort was subjected to a flow cytometric analysis (*fig.10*). The results showed that EVs with BAFF as their cargo originated in platelets (CD31+, CD42b+). It was further observed that CD31+ and CD42b+ EVs demonstrated positive co-staining for BAFF while showing no co-staining for endothelial (CD62E), erythrocyte (CD235a) and leukocyte origin (CD45 and CD16).

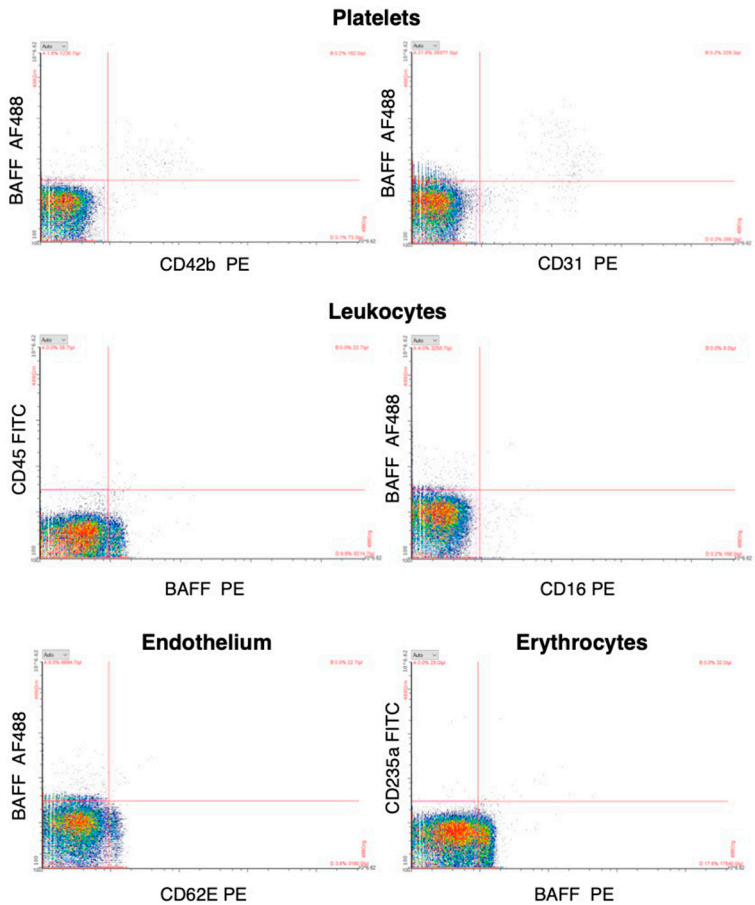



***Fig. 10:*** *The panel shows the presence of BAFF co-stained with EV of platelet, leukocyte, endothelial and erythroid origins respectively*

*BAFF-EV generation*

We, hence, performed an in-vitro assay to validate the origin of BAFF+ EVs from platelets under inflammatory conditions. Meg-01 cells were used to generate EVs with TNF-alpha (10ng/µl) stimulation and unstimulated cells were assessed as control. It was observed that the megakaryocyte cell-line Meg-01 generated higher levels of BAFF+ EVs under inflammatory conditions than control (*fig.11*).

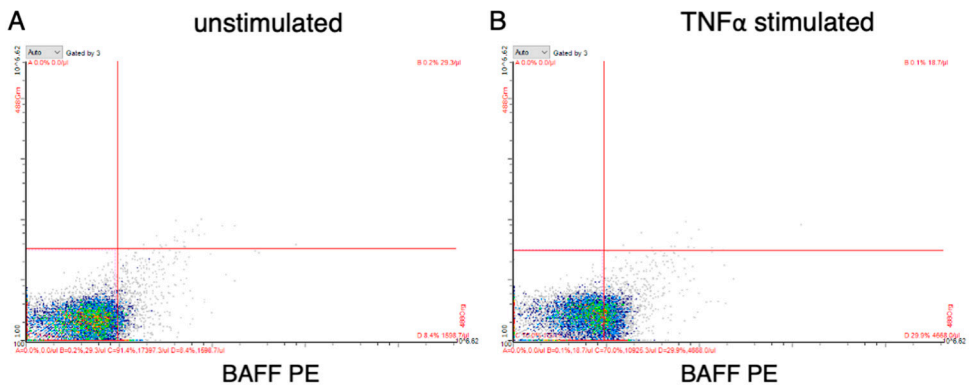



***Fig. 11:*** *The scatter plot (A) shows the presence of platelet EVs with BAFF without inflammatory stimulus, while (B) shows the effect of inflammatory stimulus leading to higher release of platelet EVs with BAFF in Meg-01 cells*

## Recipient cell interaction

We further wanted to observe the role of EVs in our cohort especially in the Low CFR group. EVs from patients of the two CFR-based groups were incubated with human microvascular endothelial cells (HMVEC) overnight, since endothelial cells constitute the first barrier that circulating EVs interact with. It was observed that the EVs from the Low CFR group i.e., the group with impaired coronary flow, generated a significant increase in the levels of the adhesion molecule ICAM-1 ($p<0.05$) (*fig.12*).

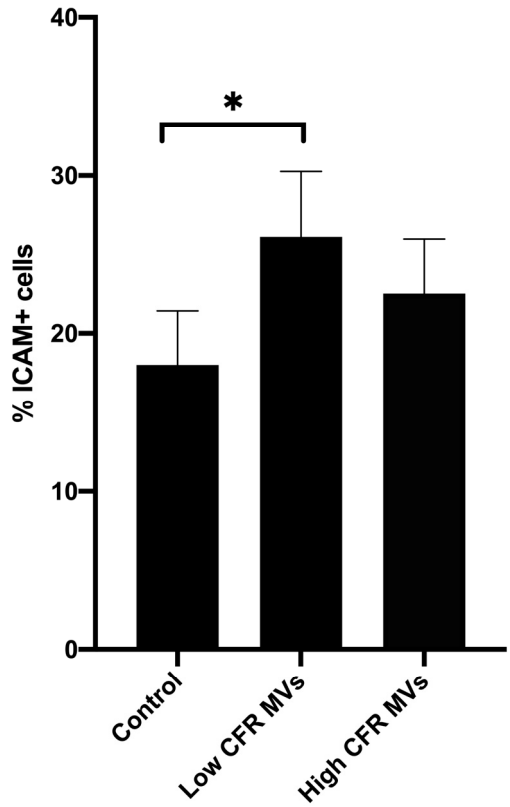



***Fig. 12:*** *The bar graph shows the percentage of ICAM-1 expression on endothelial cells after incubation with EVs from the low and high CFR groups compared to control cells.* ***The EVs in paper-I were addressed as MVs by the first author.***

# miR-224-5p

The multiplex micro-RNA profiling of the circulating EVs from the PROFLOW cohort showed a significant elevation of the miR-224-5p ($p<0.000001$) in the low CFR group (*fig.13*). The miRNA was further assessed for its origin and relevance both at the clinical and molecular level.

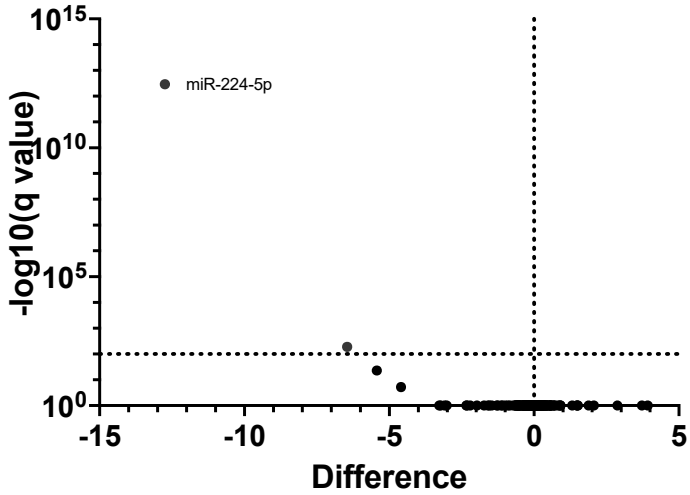



***Fig. 13:*** *The volcano plot shows the relevance of miR-224-5p in the low CFR group with respect to significance and relative fold-change*

## Association to clinical parameters

The miR-224-5p was first associated to the clinical parameters of the cohort i.e., CFR, BMI, age, hypertension, diabetes, hyperlipidaemia. It was observed that only the miR-224-5p showed an independent association to the primary criteria of the cohort; CFR ($p<0.001$) (*fig. 14*). The significant relationship was of an inverse nature indicating that the lower the CFR, the higher the expression of miR-224-5p.

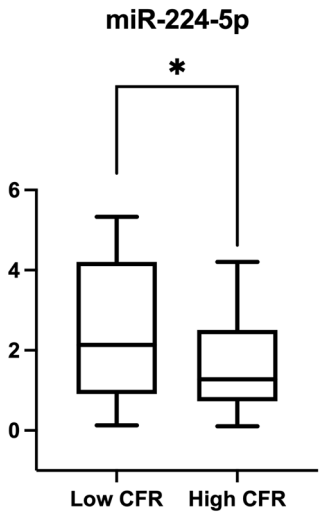



***Fig. 14:*** *The box-plot demonstrated significantly higher levels of miR-224-5p in the Low CFR group.*

## Cell-based assays

Hence, we further assessed the miR-224-5p at the molecular level using cell-based assays.

*Origin*

miR-224-5p was known to be of the hepatic origin as addressed in various studies especially in hepatocellular cancer (17). We hence decided to confirm whether the circulating EVs containing miR-224-5p in our cohort were of hepatic or cardiovascular origin. We assessed the same by comparing EVs generated from SMC, ECs, PBMCs, platelets and the liver cell-line HepG2 using q-PCR. It was observed that the miR-224-5p ($p<0.00001$) was of the hepatic origin (*fig.15*) as the miRNA was significantly released in EVs stimulated with TNF-alpha from the HepG2 cells compared to the other cell types.

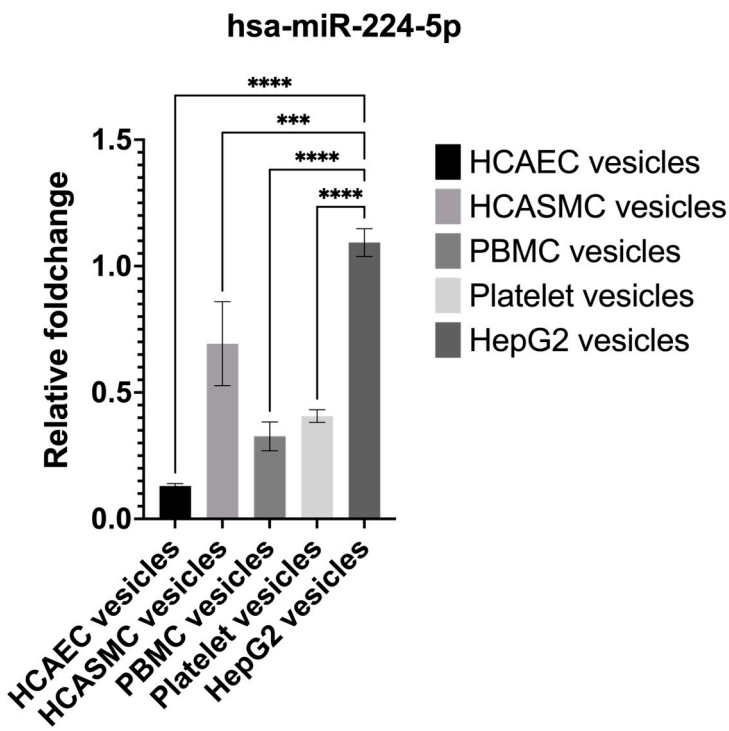



***Fig. 15:*** *The bar graph shows the significant release of EVs with the miR-224-5p upon inflammatory stimulus from HepG2 cells compared to other cell types. P< 0.00005=*****

*Liver EVs in the Cohort*

We, then, wanted to confirm that liver EVs (ASGPR+) were present in the circulating plasma of our cohort and that it could permeate the endothelial barrier (vanin-1+). However, some CD14+EVs of the monocytic lineage can also be ASGPR1+, hence we gated for CD14-/ASGPR1+ EVs. We observed that ASGPR1+/CD14- EVs were present in the circulating plasma of our cohort and that ASGPR1+ EVs also showed positive staining for Vanin-1 (*fig.16*).

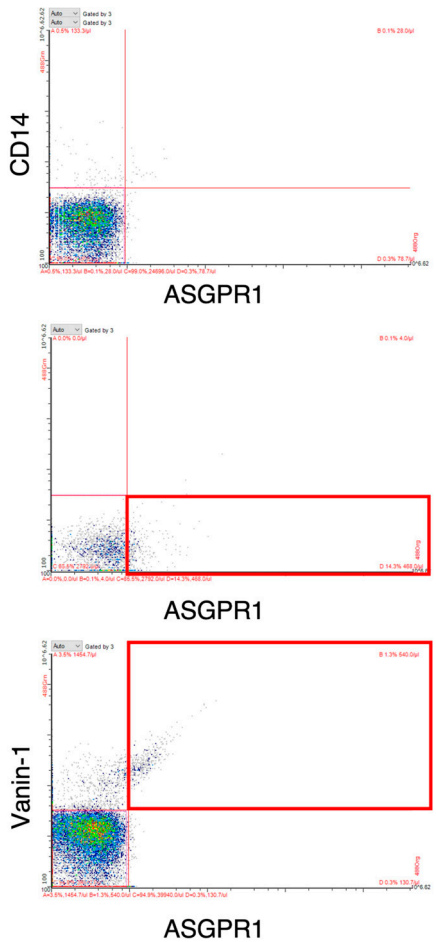



***Fig. 16:*** *The FCM scatter plot shows the significant presence of ASGPR-1+/CD14- hepatic EVs in the circulating plasma of the cohort in the uppermost panel while the lower panel shows the significant presence of ASGPR-1+/Vnn-1+ EVs. The middle panel shows the presence of only ASGPR-1+EVs in the cohort*

### Recipient cell interaction

The role of the micro-RNA was further studied using mimic and inhibitor for the miR-224-5p, in HepG2 cells. The detailed method is addressed in paper II. The EVs from the transfected cells were then transferred to endothelial cells to understand its biological relevance. We observed that the miR-224-5p was a marker of a sub-population of hepatic EVs and that the hepatic EVs, led to a significant increase in the levels of endothelial ICAM-1(*fig.17*).

e.

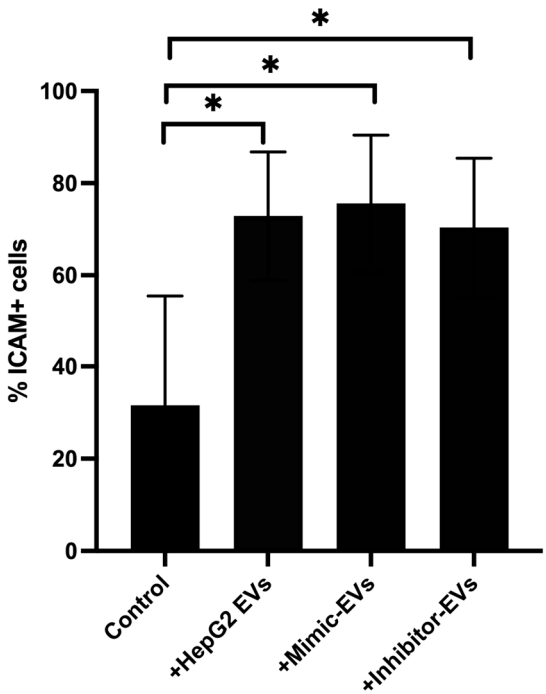



***Fig. 17:*** *The bar graph demonstrates the percentage increase in ICAM-1 in endothelial cells incubated with hepatic EVs, EVs with mimic for miR-224-5p and EVs with inhibitor for miR-224-5p compared to control cells*

# miR-16-5p

The miR-16-p was one of the significantly expressed miRNAs from the PROSPECT II cohort (p=0.0003) (*fig.18.A*), derived from the multiplex profiling of the circulating EVs. The miRNA was observed to be relatively downregulated in the patient group i.e., the high LCBI & PB group. It was further observed that the miRNA showed an inverse and significant relationship to megakaryocyte and platelet (CD42a+, CD62P+ & CD14+) EVs ($p<0.05$) as well as endothelial (CD62E+) EVs (*fig. 18.B*).

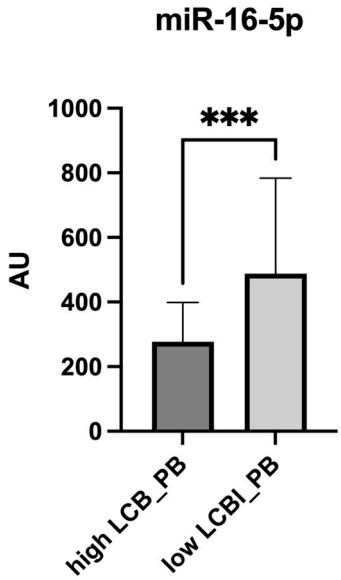



***Fig. 18.A:*** *The bar graph shows the significant expression of the miR-16-5p in the EVs of patients with Low LCBI and Low PB*

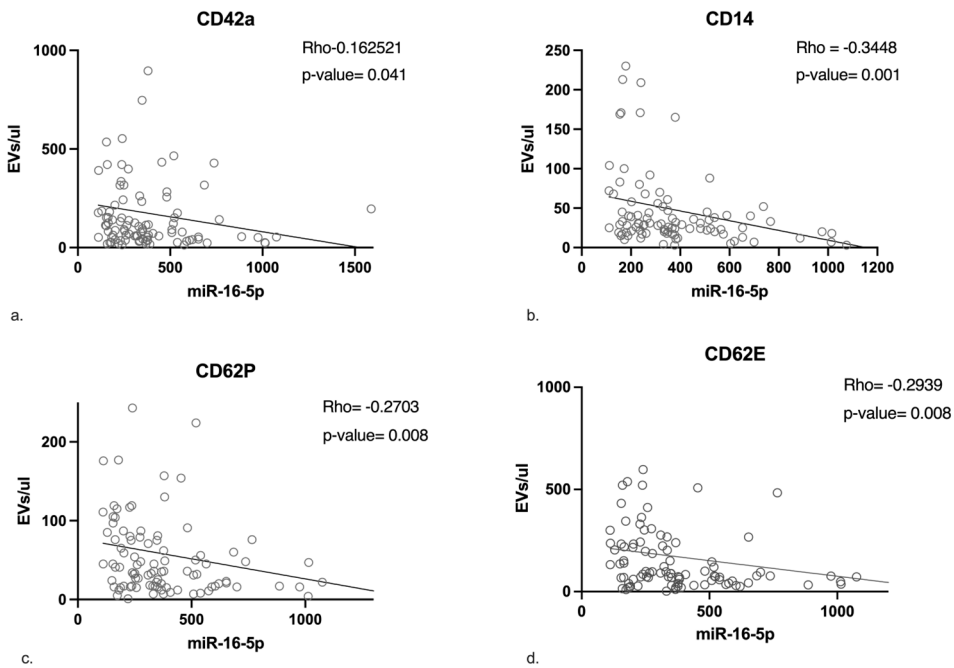



***Fig.18.B:*** *The scatterplots demonstrate the significant relationship between the miR-16-5p on the X-axis to CD42a (a), CD14 (b), CD62P (c) and CD62E (d) EVs on the Y-axis. The relationship is denoted by the respective line of regression and p-values.*

## Association to Clinical parameters

The miR-16-5p was further associated to the significant clinical parameters of the cohort i.e., LCBI, plaque burden and cholesterol. It was observed that the miR-16-5p showed a significantly inverse to all the three parameters of PB (*fig. 19.a*), LCBI (*fig. 19.b*) and cholesterol (*fig. 19.c*).

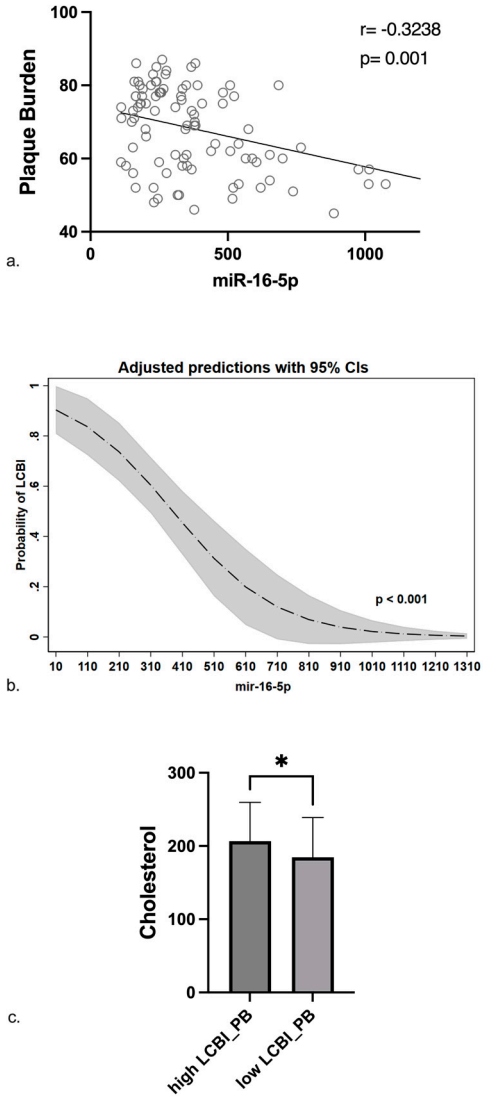



***Fig. 19:*** *The panel shows (a) the scatter plot demonstrating the relationship between miR-16-5p on the X-axis and plaque burden on Y-axis, (b) the probablity plot of the significant relationship of the miR-16-5p to LCBI and (c) demonstrates the bar graph of the significant difference in the total cholesterol between the two groups*

## In-silico Assessment

The miR-16-5p was then assessed in-silico to understand its role at the molecular level. It was observed that the miRNA targeted genes identified and validated for their role in inflammatory cascades such as Akt, IL, FGF families among others.

The targets from this unidirectional network (*fig.20*) were further analysed using the String database and it was confirmed that these genes resulted in proteins that play a role in several inflammatory diseases including CVDs indicated by their gene count in corresponding KEGG pathways.

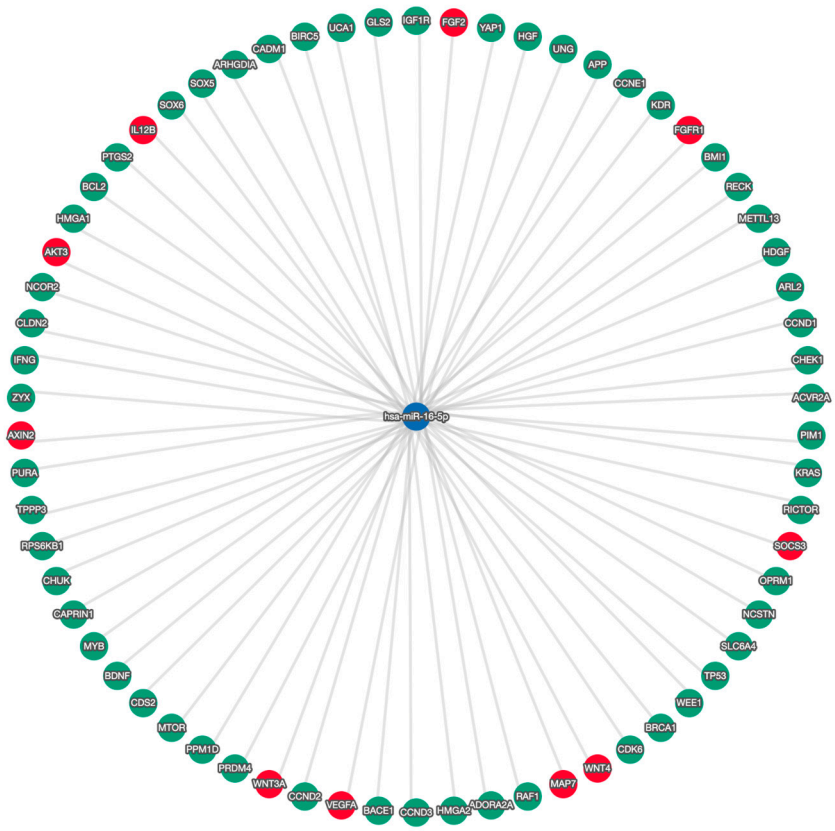



***Fig. 20:*** *The image shows the unidirectional relationship of the miR-16-5p to validated targets. The grey line signifies a negative relationship of the miR to the targets, while the gene targets in red are the ones associated with cardiovascular disease*

## Association to circulating proteins

The miRNA was then associated with the circulating plasma proteins of our cohort to understand the translational role of the miRNA in our patient groups. It was observed that the miR-16-5p showed a significant relationship to TIE-2 (p=0.01) (*fig.21.a*) and FGF-19 (p=0.004) (*fig.21.b*). The association suggested that the decrease in the miR-16-5p in the high LCBI and PB patients led to the decrease in angiopoietin receptor-I and fibroblast growth factor-19 in their circulating plasma.

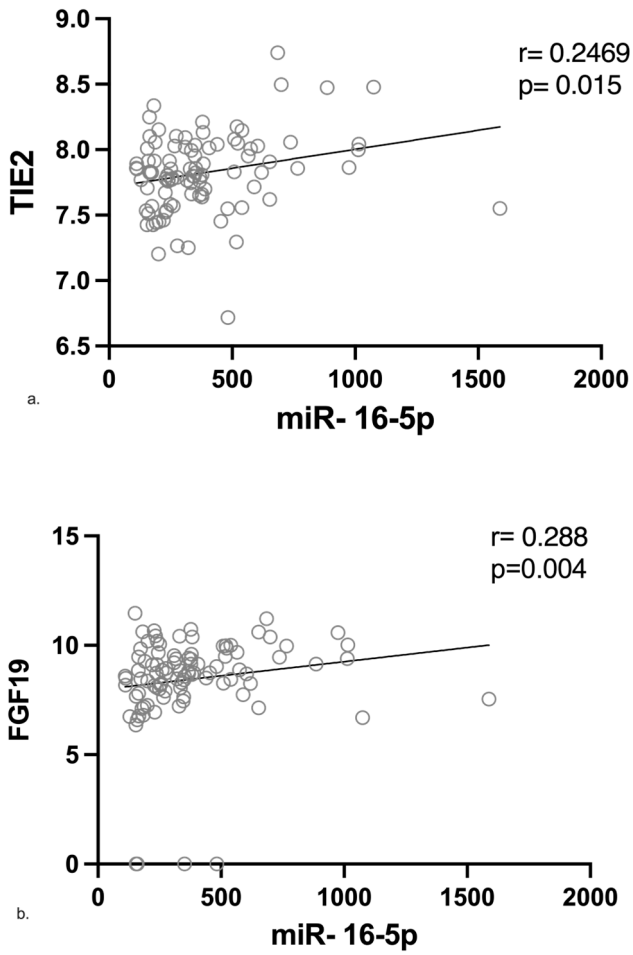



***Fig. 21:*** *The scatter plots show the significant association of the miR-16-5p on the X-axis to the circulating proteins TIE2 (a) and FGF-19 (b) on the Y-axis respectively. Their relationship is denoted by the line of regression and p-value*

# miR-451a

The miR-451a was one of the significant miRNAs in the circulating EVs of the PROSPECT II cohort ($p<0.0001$) and was the only significant miRNA in the Thrombus patients EVs ($p=0.00003$) (*fig.22.A*). The miR showed a significantly inverse relationship to platelet (CD42a+ & CD62P+) EVs (*fig.22.B*). These findings suggested a prominent role of miR-451a in the development of atherosclerosis.

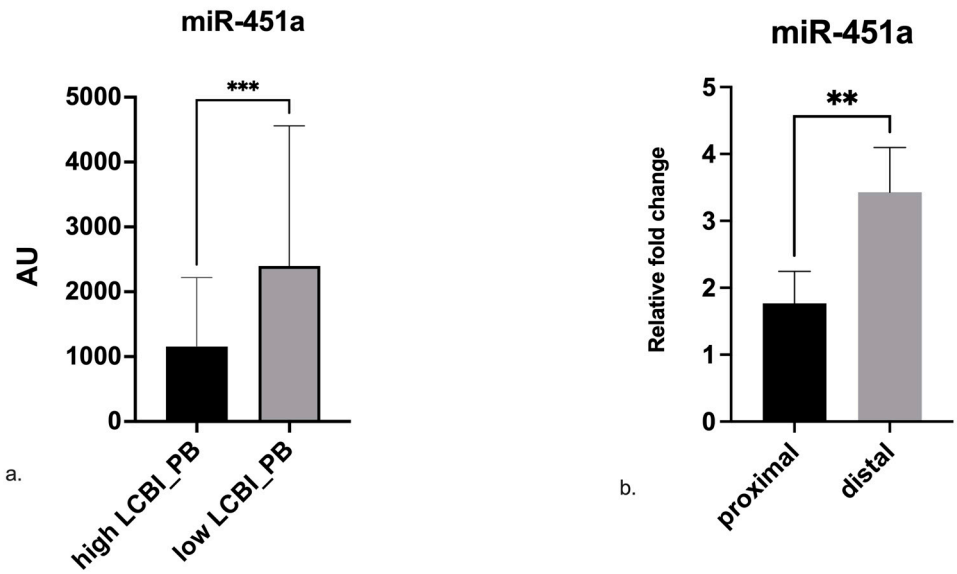



***Fig. 22.A:*** *The bar graphs demonstrate the expression of the miR-451a (a) in the Low LCBI and PB group in the Prospect-II cohort and (b) in the circulating EVs released from plaques after balloon angioplasty*

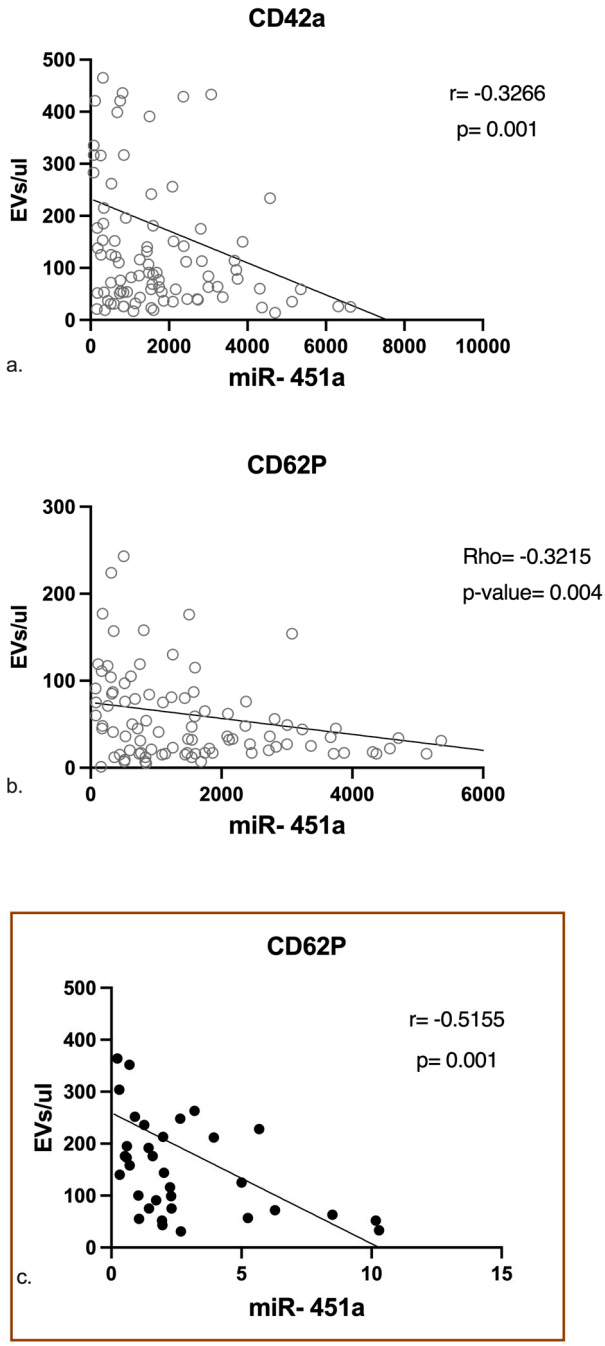



***Fig. 22.B:*** *The panel shows the relationship of the miR-451a to platelet EVs (a) & (b) in the Prospect-II cohort and (c) in the Plaque EV study.*

## Association to Clinical Parameters

We, hence, further associated the miR-451a to the significant clinical parameters of the cohorts i.e., LCBI, plaque burden and cholesterol of the PROSPECT-II cohort and BMI, diabetes, hypertension, age, gender, hyperlipidaemia in the Thrombus patients. It was observed that the miR-451a showed a significantly inverse relationship to LCBI and PB (*fig.23*), indicating that a decrease in the miR-451a would lead to the increase in plaque burden. The miR did not show association to any other parameters in both the cohorts.

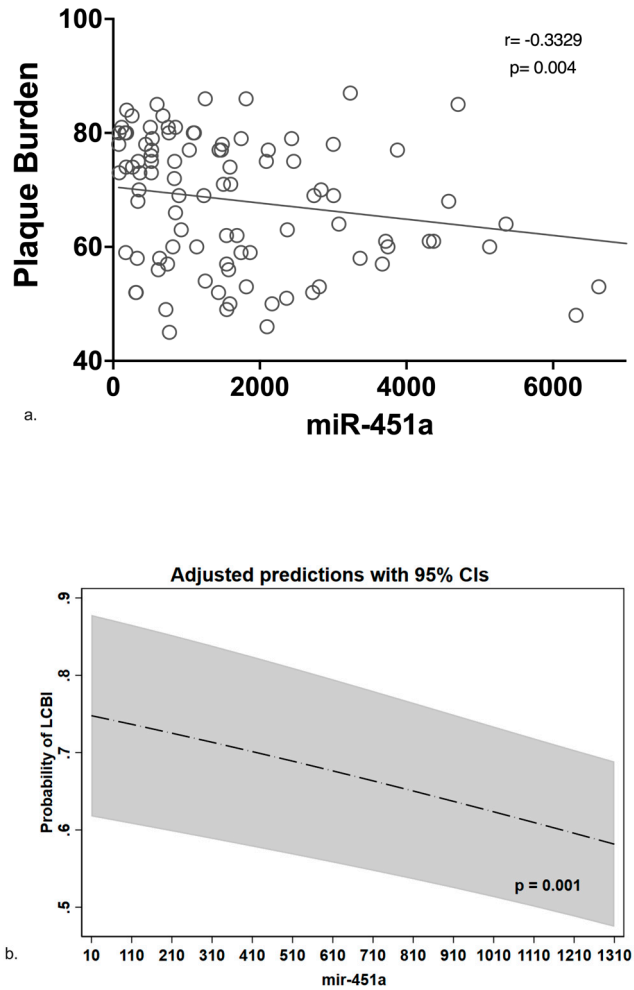



***Fig. 23:*** *The panel shows (a) the scatterplot of miR-451a on the X-axis with plaque burden on the Y-axis with the relationship denoted by the line of regression and p-value while (b) shows the probablity plot of between the miR-451a and LCBI*

## In-silico Assessment

The miR-451a was further assessed in-silico to understand its role at the molecular level. It was observed that the miR targeted genes identified and validated for their role in inflammatory cascades such as Akt, IL, MMP families among others.

The targets from this unidirectional network (*fig.24*) were further analysed using the String database and it was confirmed that these genes resulted in proteins that play a role in several inflammatory diseases including CVDs indicated by their gene count in the corresponding KEGG pathways.

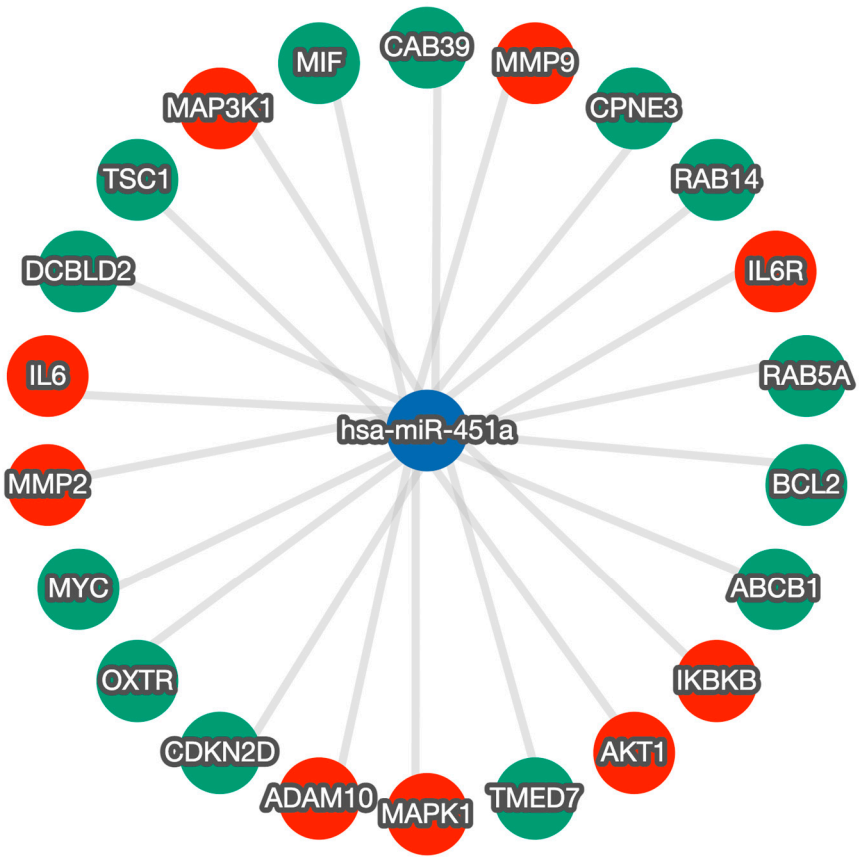



***Fig. 24:*** *The image shows the unidirectional relationship of the miR-451a to validated targets. The grey line signifies negative relationships of the miR to the targets, while the gene targets in red are the ones associated with cardiovascular disease.*

## Association to circulating proteins

*PROSPECT II*

The circulating proteins of the cohort were associated to the miRNA to assess the translational effect of the miRNA under an atherosclerotic environment. It was observed that the miR-451a significantly ($p<0.05$) correlated to the proteins (*fig.25.A*) caspase-3 (a), caspase-8 (b), insulin growth factor binding protein-1 (c) and intercellular adhesion molecule-3 (d). The proteins showed a negative association to the miR-451a, suggesting an increase of the respective protein in the circulation of patients with higher plaque burden with a decrease in the miRNA.

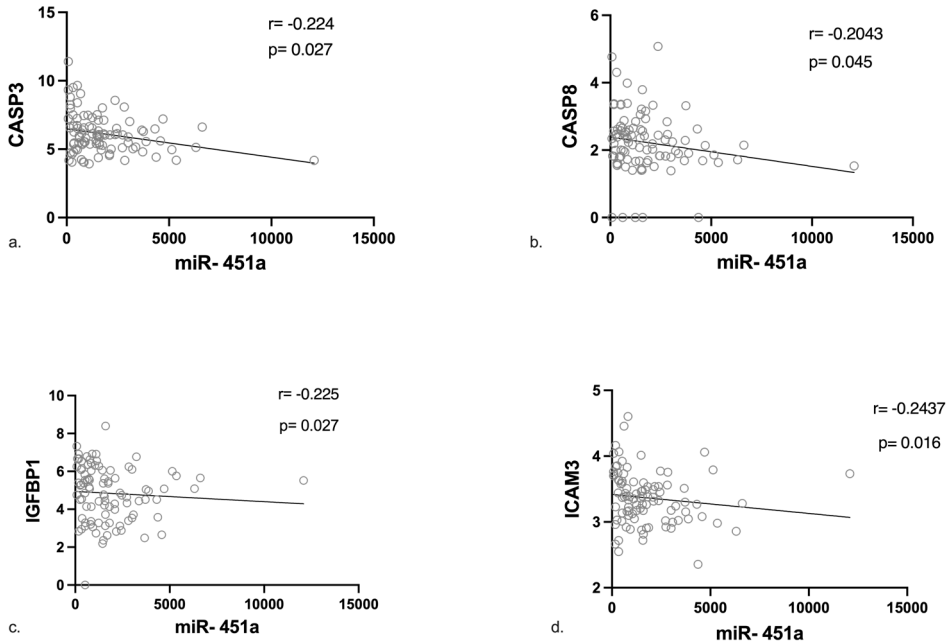



***Fig. 25.A:*** *The scatterplots show the association of miR-451a on the X-axis, in the Prospect-II cohort and it correlation to the circulating proteins Caspase-3 (a), Caspase-8 (b), IGFBP1 (c) and ICAM3 (d) on the Y-axis respectively. The relationship is shown by the line of regression and significant p-values.*

*Release from ruptured coronary plaques*

Similarly, the circulating proteins (*fig.25.B*) activated leukocyte adhesion molecule (a) , platelet endothelial cell adhesion molecule (b), inteleukin-17 (c) & 2 (d) receptor alpha chain, intercellular adhesion molecule-2 and interleukin-1 receptor type-1(f) were observed to be significantly correlated to the miR-451a in the samples distal to ruptured coronary plaques from patients. It suggested that the decrease in the miR-451a proximal to the plaque led to an increase in the respective

proteins leading to possible plaque formation and further release into the distal circulation upon dilation of the stenosis by balloon angioplasty.

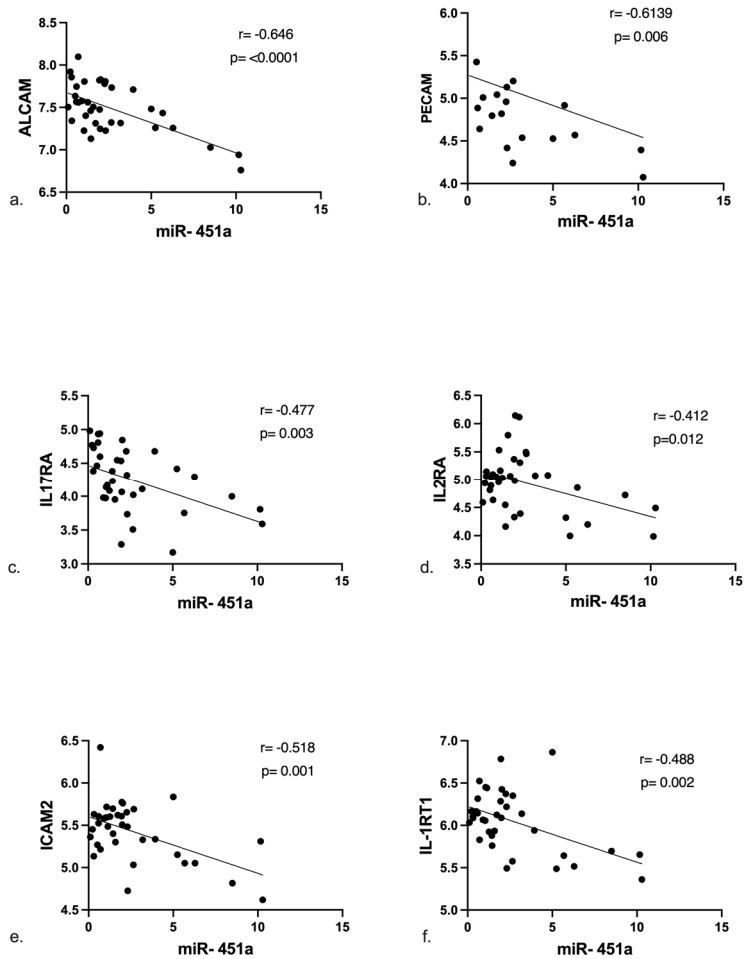



***Fig. 25.B***: *The scatterplots show the association of miR-451a on the X-axis, in the plaque EV study and it correlation to the circulating proteins ALCAM (a), PECAM (b), IL17RA (c), IL2RA (d), ICAM2 (e) and IL-1RT1 (f) on the Y-axis respectively. The relationship is shown by the line of regression and significant p-values.*

# Discussion

The atherosclerotic cascade begins with hyperlipidemia, followed by the oxidization or modification of the accumulated lipids, leading to an activated endothelium. Adhesion molecules and interleukins function by initiating chemoattraction on the surface of the activated endothelium leading to the accumulation and adhesion of leukocytes on the primary lesion (18). The interleukin receptors are cell surface receptors required for the activation of the interleukins modulating inflammation (19). Once adhered, it leads to the transmigration of leukocytes into the intimal wall, increasing the endothelial permeability (20). This leads to a series of reactions ranging from decrease in the local NO levels to atheroma formation, defining the various stages of endothelial dysfunction and CVD (7). This leads to impaired vascular flow and eventual rupture of the necrotic plaque causing an MI.

Over the years micro-RNAs transported by vesicles have been studied for their role in cardiovascular diseases (21). Circulating micro-RNAs including the ones transported by EVs have been known to enter the endothelial barrier and affect the recipient cells leading to alteration or silencing of targeted mRNA and consequently the downstream protein (22). The discussion below will focus on understanding the detected EVs and EV-miRNAs in our cohorts and their relationship to the pathophysiology of impaired vascular health.

## Circulating EVs and endothelial dysfunction

One of the recurrent observations in our cohorts was the presence of **platelet EVs** and their significant relationship to the risk factors i.e., CFR, high plaque burden and LCBI as well as the release of plaque after balloon angioplasty. It is known that microvesicles were first discovered as platelet dust and since then platelet and macrophage EVs have been known to play a crucial role in the pathophysiology of CVD (23).

Circulating EVs have also been known to impact the expression of adhesion molecules including ICAM-1 (24, 25). We observed the same in the paper-I were megakaryocyte EVs containing BAFF induced an increased expression of ICAM-1. Adverse effects after MI have been correlated to the increase in circulating levels of

BAFF(26), as BAFF functions by activating B-cell differentiation and survival, aggravating lesion formation (27).

However, EVs of platelet origin were not the only EVs to impact endothelial dysfunction. We recognized an increase in a sub-population of hepatic EVs in the paper II, identified by miR-224-5p. Similar to platelet EVs, these **hepatic EVs** also led to the increase in ICAM-1. Hepatic EVs have been studied as carriers of Arginase-I to the endothelium leading to increase in ICAM-1 and diapedesis (28).

An increase in platelet EVs was also observed in the paper III and IV, with a significantly inverse association to the miR-16-5p and miR-451a, suggesting their decrease in the patient groups is associated to increase in platelet EVs. We speculate that their decrease led to megakaryocyte releasing higher levels of platelet EVs contributing to an atherosclerotic environment, wherein platelet EVs initiate endothelial dysfunction.

# EV-microRNA and endothelial dysfunction

**Micro-RNAs** have been studied for their role in the pathophysiology of endothelial dysfunction and CVD (29). EVs carrying micro-RNAs have been identified as key regulators of molecular mechanisms affecting downstream translation and reflect their donor and host environments (8).

**miR-224-5p** was an excellent example of the type of miRNAs reflecting their donor cell. This makes it an ideal candidate as a biomarker for detection of hepatic EVs that aid progression of CVDs by impairing vascular flow. The liver as an organ and its hepatokines have been known to mediate inflammation while having an association to cardiovascular diseases (30-32). miR-224-5p brings to the spotlight the crosstalk between the hepatic and cardiovascular systems, through a sub-population of hepatic EVs under inflammatory stimuli.

**miR-16-5p** showed a significant relationship to cholesterol, a canonical component in the progression of atherosclerosis. The accumulation of cholesterol and its eventual oxidization, leads to an atherogenic environment. The circulating proteins showing significant associations to miR-16-5p were **FGF-19**, a therapeutic protein studied in the prevention of atherosclerosis by promoting HDL biogenesis and cholesterol efflux (33, 34) as well as **TIE2**, that protects the coronary artery from oxLDL to maintain vascular health (35, 36). Hence, suggesting that the decrease in miR-16-5p leads to accumulation of cholesterol and decrease in FGF-19 and TIE-2 leading to endothelial dysfunction. miR-16-5p has also been studied as an inhibitor of Sorting Nexins 16 (SNX16), an indicator of atherosclerosis, that leads to the inhibition of oxLDL pyroptosis (37). It has also been shown that the miR-16-5p

inhibits the expression of thioredoxin-interacting protein (TXNIP), an activator of the NLRP3 inflammasome in patients with atherosclerosis (38).

**miR-451a** is an interesting miRNA identified, as it was detected by two different methods, in two different cohorts representing early and late stages of atherosclerosis. A highly conserved miRNA, miR-451a is known to be of the erythroid origin (39). It inversely correlated to plaque burden and was present in the released plaque. The in-silico assessment of the miR-451a showed that it targeted mRNAs of an inflammatory proteins and consequently affected the respective pathway. miR-451a has been studied in mice models, to exacerbate lipotoxicity caused by a high-fat diet (40). miR-451a has also been known to downregulate neutrophil chemotaxis while its downregulation leads to cytokine storming in Covid-19 (41, 42).

The effect of the miR-451a was evident from its correlation to two important molecule groups that navigate endothelial dysfunction, i.e., the adhesion molecules and interleukin receptors. **ICAM-2 & 3, ALCAM** and **PECAM** are known for their role in increased vascular permeability caused aggregation of adhesion molecules, leading to leukocyte transmigration; one of the first steps towards endothelial dysfunction(43-46). This permeability further exposes the atheroma to interleukins and an increase in their receptors is observed (47). **IL-17, IL-2** as well as **IL-1** have been known to be elevated in atherosclerosis and require their respective receptors to function by recruiting leukocytes and impairing endothelial function (48-50).

**Caspase-3 & 8** as well as **IGFBP1** also showed an inverse relationship to the miRNA. Caspase-3 & 8 lead to apoptosis, the defining characteristic of the necrotic plaque (51). While IGFBP1, plays a crucial role in the development of cardiovascular diseases and is predictor of cardiovascular morbidity (52, 53).

The combined results suggest an increase in inflammatory proteins in the circulating profile of patients with high plaque burden as well as in the aorta proximal to the plaque with the downregulation of the miR-451a while an increase in cholesterol with the downregulation of miR-16-5p. It sheds a light on their significance as minimally invasive and biomarkers for the development and progression of endothelial dysfunction and cardiovascular diseases. Clinically, the miR-451a has already been shown as a diagnostic and prognostic biomarker for atherosclerosis, while miR-16-5p has been studied as a biomarker for secondary cardiovascular events(54, 55). Our results, add to the current knowledge on these miRNAs and their importance as biomarkers.

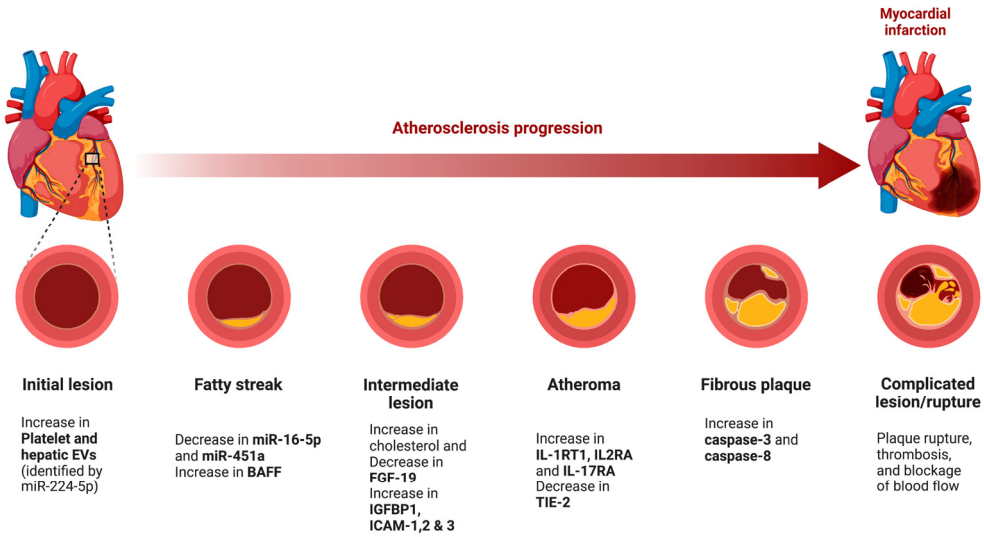



***Fig. 26:*** *The graphical representation summarizes the possible impact of the identified miRNAs and their correlated circulating proteins in the progression of atherosclerosis*

The overall findings addressed in this thesis, provides an insight into the varied role and functions of EV-micro-RNAs with respect to progression of endothelial dysfunction and development of cardiovascular diseases.

# Future aspects

The studies in paper III and IV open up for future possible studies.

- The studies have identified two crucial miRNAs i.e., miR-451a and miR-16-5p as a prognostic as well as diagnostic marker for atherosclerosis
- Further in-vitro investigation into the miRNAs origins and functions could provide a more precise insight into its biological function
- A validation of their targets identified by the statistical and in-silico analysis would strengthen the findings at the molecular level
- Future studies indicate the application of miR-16-5p and miR-451a as potential therapeutics in the treatment of atherosclerosis and as biomarkers.
- If it can be confirmed that miR-16-5p and miR-451a inhibit development of atherosclerosis, it would be possible to develop stabilized variants that could be tested as therapeutic agents

# Highlights of the thesis

- miR-224-5p enabled us to identify sub-population of hepatic-EVs that are associated with endothelial dysfunction
- The downregulation of the miR-16-5p and especially miR-451a presents possible biomarkers for plaque formation and atherosclerosis
- The essential role of EV micro-RNAs as molecular switches regulating disease related pathways
- EV micro-RNA in the circulation can be potentially applied as minimally invasive and early biomarkers for the impairment of vascular health and development of CVDs

# Acknowledgements

Thank you, **David**, for taking a chance on a girl all the way in India and making her a part of the Department of Molecular Cardiology. I am forever, grateful for the opportunity you provided and for making my time in Clinical sciences so comfortable.

I would also like to extend my gratitude to the **Faculty of Medicine**, Lund University, and the **Research Education Board** for their acceptance and support throughout the duration of my doctoral education.

**Björn**, words cannot summarize my gratitude for everything that you have taught and for your constant support through professional as well as personal hurdles. Thank you for everything.

**Olof**, thank you, for your insights and supervision throughout the last 4 and half years. The wisdom you imparted will always stay with me.

**Siv and Monica**, I do not know how I could have ever survived the lab without you. Thank you for managing the smaller details that have made this larger picture. Thank you, **Rebecca**, with all the help with Statistics and the ever so pleasant conversations.

**Paulina**, I cannot thank you enough for what seemed like very short 2 years of this journey, made so easy whether it be working together or just talking in the kitchen. I am so grateful for your support and faith in me.

Everybody on **BMC D12** that are still there and those who are not, thank you for making D12 so welcoming all these years. Especially the **BMC Divas** (you'll know who you are) **and Fatima** for the love and laughter during the countless fika breaks and lunches. **Prem** and **Li**, our short conversations and smile-exchanges on the corridors have been a comfort on the lowest days.

**Manar** and **Neha**, thank you so much for being my confidants and biggest supporters at D12. I am eternally grateful for the memories that I will cherish forever and the kindness you have shown at the darkest of times.

When I thought life at the faculty couldn't get any better **MDR** came along, and I am so glad it did. I have had some of the best time of my life with the most splendid conversations with the most exceptional people I have ever met. I am so grateful to have got to know you **Shelby, Vasiliki, Vibha, Sanjay** (thank you for my back

cover picture)**, Divya, Ganesh, Emil, Jamirah, and Radhika**. **Christopher**, I cannot contemplate going through my year as the Chair without your help and support. **Esther**, from being friends over zoom to IRL, I am grateful for your friendship and unwavering support. Thank you all for being my rock at the Faculty of Medicine.

**Sakshi**, I think I will need an entire book to thank you but I am going to try to summarize. Thank you for being my friend, Roomba, roommate (aka Lund Husband) and a ray of hope since I have known you. Thank you for having faith in me when I didn't and most of all for being the statistics expert and scientific genius that I could always rely on.

**Daria**, thank you, for being my first friend in an unknown land and sharing my love for travel and Christmas. Thank you for being a ray of sunshine and a ball of joy even on the dullest of days.

**Aishwarya**, I am indebted to you for all the love, support, and kindness that you have showed me for the last 10 years as your best friend and even more as family. You are my family by choice and I am so thankful that you are my person.

**Pranay**, thank you for being the voice of reason and sanity in my life. I am so thankful for your unwavering kindness and faith in me. For being there whenever I needed you and reminding me of my strength to survive.

**Kanishka** and **Priyanka** thank you for having my back for the last 15 years. I am so grateful to have you both and to be able to share this bond irrespective of the distance between us. You will always be my Power Puff girls.

I am grateful to my roots, to all my **school as well as college friends and teachers** without whom I wouldn't be the person I am today.

**Jayshree Aunty, Sridhar Uncle, Ananya, Alfred, and Yash,** thank you for being my family in Pune , taking care of me and always wishing well for me. It is the cumulation of your love and prayers that I am here and for that I am grateful.

**Pallavi, Pintesh, Ashfaque and Lubna,** thank you for being my Swedish family, thank you for always lending a helping hand and making me feel I had people I could rely on. **Anna,** you will always be my baby, I am so proud of you and the woman you have become, serving your country and family. Thank you for all your love and support. Thank you, **Janani, Twinkle and Heena** for being such kind and helpful friends, your kindness has meant a lot to me.

**Mummy**, **Pappa** and **Meha**, I may not say this enough but I love you. Thank you for always praying for me and supporting me through all the decisions I have made. I hope you are proud of me and I will strive to keep making you proud forever. None of this would have been possible without your blessings and prayers and for that I cannot thank you enough.

**Ammachi** and **Kunjumol aunty**, I was not able to thank you before you left this world but I know I have everything I need because you are both up there with your rosaries in hand praying for me. I wholeheartedly dedicate this PhD to you and your love. **Appapan**, **Ammachi**, **Baa** and **Dada** hope wherever you are, you are smiling down on me and are proud of me.

**Simba**, you cannot read this, but you have saved me from the darkness. Thank you for choosing me as your pawrent and lighting up my world with your furry personality. You make me the happiest person on the planet.

**Shivi**, I do not know where to begin thanking you, for your love and being my partner in crime. Thank you for being my strength every single day, thank you for making me laugh when I didn't want to and thank you for loving me in my entirety. I am eternally grateful for everything big and small that you do for me and especially for the constant support you have shown towards my ambitions and dreams. Love you forever and always.

As they say, every person you meet has a part to play in how your life unfolds and hence, I would like to acknowledge every single person that has crossed my path in the last 4 years. I am grateful for even the faintest of smile, for the smallest of conversations as well as for the smallest of thought and teachings. If you are reading this, know that I , **Thank You**.

# References


1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation. 2015;131(4):e29-322.
2. Mendis SP, P.; Norrving, B. Global Atlas on Cardiovascular Disease Prevention and Control. (WHO: Geneva, Switzerland, 2011.).
3. Aschermann M, Sedlácek K, Aschermann O, Linhart A. [Molecular genetics of myocardial infarction]. Vnitr Lek. 2007;53(4):348-53.
4. Zhu Y, Xian X, Wang Z, Bi Y, Chen Q, Han X, et al. Research Progress on the Relationship between Atherosclerosis and Inflammation. Biomolecules. 2018;8(3):80.
5. Davignon J, Ganz P. Role of Endothelial Dysfunction in Atherosclerosis. Circulation. 2004;109(23_suppl_1):III-27-III-32.
6. Endemann DH, Schiffrin EL. Endothelial Dysfunction. Journal of the American Society of Nephrology. 2004;15(8):1983-92.
7. Libby P, Ridker PM, Maseri A. Inflammation and Atherosclerosis. Circulation. 2002;105(9):1135-43.
8. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol. 2018;19(4):213-28.
9. Mulcahy LA, Pink RC, Carter DRF. Routes and mechanisms of extracellular vesicle uptake. Journal of Extracellular Vesicles. 2014;3(1):24641.
10. Boulanger CM, Loyer X, Rautou P-E, Amabile N. Extracellular vesicles in coronary artery disease. Nature Reviews Cardiology. 2017;14(5):259-72.
11. Das S, Halushka MK. Extracellular vesicle microRNA transfer in cardiovascular disease. Cardiovascular Pathology. 2015;24(4):199-206.
12. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Frontiers in Endocrinology. 2018;9.
13. Gallo A, Tandon M, Alevizos I, Illei GG. The Majority of MicroRNAs Detectable in Serum and Saliva Is Concentrated in Exosomes. PLOS ONE. 2012;7(3):e30679.
14. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells*♦. Journal of Biological Chemistry. 2010;285(23):17442-52.
15. Samy E, Wax S, Huard B, Hess H, Schneider P. Targeting BAFF and APRIL in systemic lupus erythematosus and other antibody-associated diseases. International Reviews of Immunology. 2017;36(1):3-19.

16. Natorska J, Marek G, Sadowski J, Undas A. Presence of B cells within aortic valves in patients with aortic stenosis: Relation to severity of the disease. Journal of Cardiology. 2016;67(1):80-5.
17. Ma D, Tao X, Gao F, Fan C, Wu D. miR-224 functions as an onco-miRNA in hepatocellular carcinoma cells by activating AKT signaling. Oncol Lett. 2012;4(3):483-8.
18. Etzioni A. Adhesion Molecules-Their Role in Health and Disease. Pediatric Research. 1996;39(2):191-8.
19. Carbone ML, Failla CM. Interleukin role in the regulation of endothelial cell pathological activation. Vasc Biol. 2021;3(1):R96-R105.
20. Libby P. The changing landscape of atherosclerosis. Nature. 2021;592(7855):524-33.
21. Kalayinia S, Arjmand F, Maleki M, Malakootian M, Singh CP. MicroRNAs: roles in cardiovascular development and disease. Cardiovascular Pathology. 2021;50:107296.
22. Pfeifer P, Werner N, Jansen F. Role and Function of MicroRNAs in Extracellular Vesicles in Cardiovascular Biology. BioMed Research International. 2015;2015:161393.
23. Zaldivia MTK, McFadyen JD, Lim B, Wang X, Peter K. Platelet-Derived Microvesicles in Cardiovascular Diseases. Frontiers in cardiovascular medicine. 2017;4:74-.
24. Hosseinkhani B, van den Akker NMS, Molin DGM, Michiels L. (Sub)populations of extracellular vesicles released by TNF-α –triggered human endothelial cells promote vascular inflammation and monocyte migration. Journal of Extracellular Vesicles. 2020;9(1):1801153.
25. Hosseinkhani B, Kuypers S, van den Akker NMS, Molin DGM, Michiels L. Extracellular Vesicles Work as a Functional Inflammatory Mediator Between Vascular Endothelial Cells and Immune Cells. Frontiers in Immunology. 2018;9.
26. Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guérin C, et al. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. Nature Medicine. 2013;19(10):1273-80.
27. Kyaw T, Cui P, Tay C, Kanellakis P, Hosseini H, Liu E, et al. BAFF Receptor mAb Treatment Ameliorates Development and Progression of Atherosclerosis in Hyperlipidemic ApoE−/− Mice. PLOS ONE. 2013;8(4):e60430.
28. Zhu C, Yu Y, Montani JP, Ming XF, Yang Z. Arginase-I enhances vascular endothelial inflammation and senescence through eNOS-uncoupling. BMC Res Notes. 2017;10(1):82.
29. Jansen F, Yang X, Proebsting S, Hoelscher M, Przybilla D, Baumann K, et al. MicroRNA Expression in Circulating Microvesicles Predicts Cardiovascular Events in Patients With Coronary Artery Disease. Journal of the American Heart Association. 2014;3(6):e001249.
30. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. Clinical Gastroenterology and Hepatology. 2012;10(6):646-50.

31. Lonardo A, Sookoian S, Pirola CJ, Targher G. Non-alcoholic fatty liver disease and risk of cardiovascular disease. Metabolism. 2016;65(8):1136-50.
32. Huang K, Bai H. Liver hepatokines and peroxisomes as therapeutic targets for cardiovascular diseases. Future Cardiology.0(0):null.
33. Zhou M, Learned RM, Rossi SJ, Tian H, DePaoli AM, Ling L. Therapeutic FGF19 promotes HDL biogenesis and transhepatic cholesterol efflux to prevent atherosclerosis [S]. Journal of Lipid Research. 2019;60(3):550-65.
34. Hao Y, Zhou J, Zhou M, Ma X, Lu Z, Gao M, et al. Serum Levels of Fibroblast Growth Factor 19 Are Inversely Associated with Coronary Artery Disease in Chinese Individuals. PLOS ONE. 2013;8(8):e72345.
35. Baffert F, Le T, Thurston G, McDonald DM. Angiopoietin-1 decreases plasma leakage by reducing number and size of endothelial gaps in venules. Am J Physiol Heart Circ Physiol. 2006;290(1):H107-18.
36. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med. 2000;6(4):460-3.
37. Katsumata Y, Terada J, Matsumura T, Koshikawa K, Sakao S, Tomiyoshi G, et al. Circulating Anti-Sorting Nexins 16 Antibodies as an Emerging Biomarker of Coronary Artery Disease in Patients with Obstructive Sleep Apnea. Diagnostics. 2020;10(2):71.
38. Li B, Zhang Z, Fu Y. Anti-inflammatory effects of artesunate on atherosclerosis via miR-16-5p and TXNIP regulation of the NLRP3 inflammasome. Ann Transl Med. 2021;9(20):1558-.
39. Oxendine Harp K, Bashi A, Botchway F, Dei-Adomakoh Y, Iqbal SA, Wilson MD, et al. MicroRNAs miR-451a and Let-7i-5p Profiles in Circulating Exosomes Vary among Individuals with Different Sickle Hemoglobin Genotypes and Malaria. Journal of Clinical Medicine. 2022;11(3):500.
40. Kuwabara Y, Horie T, Baba O, Watanabe S, Nishiga M, Usami S, et al. MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway. Circulation Research. 2015;116(2):279-88.
41. Murata K, Yoshitomi H, Furu M, Ishikawa M, Shibuya H, Ito H, et al. MicroRNA-451 Down-Regulates Neutrophil Chemotaxis via p38 MAPK. Arthritis & Rheumatology. 2014;66(3):549-59.
42. Yang P, Zhao Y, Li J, Liu C, Zhu L, Zhang J, et al. Downregulated miR-451a as a feature of the plasma cfRNA landscape reveals regulatory networks of IL-6/IL-6R-associated cytokine storms in COVID-19 patients. Cell Mol Immunol. 2021;18(4):1064-6.
43. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis. 2003;170(2):191-203.
44. Ueland T, Åkerblom A, Ghukasyan T, Michelsen AE, Becker RC, Bertilsson M, et al. ALCAM predicts future cardiovascular death in acute coronary syndromes: Insights from the PLATO trial. Atherosclerosis. 2020;293:35-41.

45. Arif N, Zinnhardt M, Nyamay'Antu A, Teber D, Brückner R, Schaefer K, et al. PECAM-1 supports leukocyte diapedesis by tension-dependent dephosphorylation of VE-cadherin. Embo j. 2021;40(9):e106113.
46. Woodfin A, Voisin MB, Nourshargh S. PECAM-1: a multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol. 2007;27(12):2514-23.
47. von der Thüsen JH, Kuiper J, van Berkel TJ, Biessen EA. Interleukins in atherosclerosis: molecular pathways and therapeutic potential. Pharmacol Rev. 2003;55(1):133-66.
48. Taleb S, Tedgui A, Mallat Z. IL-17 and Th17 Cells in Atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2015;35(2):258-64.
49. Nordlohne J, von Vietinghoff S. Interleukin 17A in atherosclerosis – Regulation and pathophysiologic effector function. Cytokine. 2019;122:154089.
50. Durda P, Sabourin J, Lange EM, Nalls MA, Mychaleckyj JC, Jenny NS, et al. Plasma Levels of Soluble Interleukin-2 Receptor &#x3b1. Arteriosclerosis, Thrombosis, and Vascular Biology. 2015;35(10):2246-53.
51. Wang Y, Karki R, Zheng M, Kancharana B, Lee S, Kesavardhana S, et al. Cutting Edge: Caspase-8 Is a Linchpin in Caspase-3 and Gasdermin D Activation to Control Cell Death, Cytokine Release, and Host Defense during Influenza A Virus Infection. J Immunol. 2021;207(10):2411-6.
52. Ezzat VA, Duncan ER, Wheatcroft SB, Kearney MT. The role of IGF-I and its binding proteins in the development of type 2 diabetes and cardiovascular disease. Diabetes Obes Metab. 2008;10(3):198-211.
53. Wallander M, Norhammar A, Malmberg K, Ohrvik J, Rydén L, Brismar K. IGF binding protein 1 predicts cardiovascular morbidity and mortality in patients with acute myocardial infarction and type 2 diabetes. Diabetes Care. 2007;30(9):2343-8.
54. Badacz R, Kleczyński P, Legutko J, Żmudka K, Gacoń J, Przewłocki T, et al. Expression of miR-1-3p, miR-16-5p and miR-122-5p as Possible Risk Factors of Secondary Cardiovascular Events. Biomedicines. 2021;9(8):1055.
55. Wang B, Duan X, Xu Q, Li Y. Diagnostic and prognostic significance of miR-451a in patients with atherosclerosis. Vascular. 2021:17085381211058571.

Printed by Media-Tryck, Lund 2022 NORDIC SWAN ECOLABEL 3041 0903

**KREEMA JAMES,** is an MTech in Biotechnology from Dr. D. Y. Patil Biotechnology and Bioinformatic Institute, Pune, India. She has presented her work on extracellular vesicles at the Department of Molecular Cardiology through this thesis. She aims to keep pursuing her love for science that helps answer the unanswered.

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2022:170
ISBN 978-91-8021-332-5
ISSN 1652-8220