The effects of hypercholesterolaemia and atherosclerosis on circulating CD63+ extracellular vesicle levels

PhD thesis

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Budapest
2025

1. Introduction

Atherosclerotic cardiovascular disease (ACVD) is the leading global cause of mortality, primarily due to coronary and carotid arteries causing myocardial infarction (MI) and strokes. A critical factor in ACVD is the accumulation of low-density lipoprotein (LDL), which oxidizes forming oxidized LDL (ox-LDL), promoting monocyte recruitment, and their differentiation to macrophages. Macrophages may subsequently transform into foam cells, leading to plaque formation.

Extracellular vesicles (EVs) are membrane bound nano particles packaged with vital cell material, which are released from all known cells and play a crucial role in cellular communication. EVs are characterized in a multitude of ways, currently, either by size (large or small) or biogenic pathway. Based on the origin of an EV, they may have one or more of the following surface markers, CD9, CD63, or CD81, with more specific markers based on their cell of origin. With our knowledge of a diverse heterogeneity of EVs growing rapidly, the fast-developing EV field has shown EV involvement in most physiological and pathological prosses, including disease exacerbation. EV researchers now utilize our understanding of EV for diagnostics, prognostics as well

as in therapeutics and engineering for drug development. Emerging research continues to reveal more evidence for the associations between EVs and lipoproteins including, but not limited to, chylomicrons, very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL). This sparked interest in the field with the possibility of EV involvement in dyscholesterolaemia. This led us to hypothesize an EV contribution to ACVD.

In this project we assess circulating EV levels in hypercholesterolaemic mice as well as in mice with CVD development. Here the hypercholesterolaemic mouse model LDL-receptor (LDLR) knockout (KO) as well as the hypocholesterolaemic mouse model proprotein convertase subtilisin/kexin type 9 (PCSK9) KO were used. A wild-type (WT) mice group was used as a control. Mice were either fed mice HFD or left to age, in order to observe the effects of diet and aging on CVD. We also assessed the EV levels in patients with normo- or hypercholesterolaemia.

2. Objectives

Our principal objectives in this work were as shown below.

- To investigate the relationship between circulating EVs, and lipoproteins.
- To explore whether circulating EV levels correlate with the presence, and extent of atherosclerosis.
- To examine how circulating EV levels vary with age in-vivo.
- To assess the potential of CD63+, CD81+, and annexin V+ EVs as prognostic biomarkers for ACVD, as well as to consider how aging may impede this correlation.

3. Methods

3.1 *In vivo* & clinical testing:

Wild-type (WT), PCSK9$^{-/-}$, and LDLR$^{-/-}$ mice were studied at adolescence (11-weeks), after 12 weeks of high-fat diet (HFD), and at old age (22-months). Human samples: platelet-free plasma (PFP) samples were collected from normocholesterolaemic and hypercholesterolaemic patients.

3.2 Key techniques

Mice total cholesterol (TC), LDL, and HDL cholesterol levels were quantified using a colorimetric assay. Here, human cholesterol levels were measured at the Városmajor Clinic using a chemistry analyzer (DxC 700 AU, Beckman).

For mouse cardiovascular function: echocardiography and Doppler imaging was used to assess parameters including ejection fraction, cardiac output, fractional shortening, E/e' ratio (index to evaluate the LV filling pressure), and left ventricular wall thickness.

Aortic arches were removed after termination. The arches were stained with OilRed-O revealing plaque spots (red). Arteries were imaged using a stereomicroscope, and plaques quantified using ImageJ software.

Plasma large EVs (lEVs) and lEV sized lipoprotein levels were assessed using flow cytometry. MACSPlex bead-based exosome kits were used to qualify and further quantify EV levels and associated markers in both mice and human PFP samples.

3.3 *In vitro*

HUVECs were treated with lEVs isolated from human plasma. Quantitative PCR was used to evaluate gene expression levels of inflammatory markers in treated HUVECs. Here, untreated cells were used as a negative control and LPS was used as a positive control.

3.4 Statistics

GraphPad Prism was used to analyze data. normality was assessed via the Shapiro-Wilk test, and appropriate parametric or non-parametric tests (t-tests, Mann-Whitney U, or Wilcoxon signed-rank) were applied.

4. Results

4.1 Cholesterol levels and body mass in mice

Compared to WT animals, PCSK9$^{-/-}$ mice exhibited consistently reduced cholesterol levels, while LDLR$^{-/-}$ mice showed elevated cholesterol levels.

HFD led to increased total cholesterol (TC) and LDL-cholesterol (LDL-C) levels in all animal models, but particularly in LDLR$^{-/-}$ mice.

Compared to WT, body mass in PCSK9$^{-/-}$ mice increased significantly despite low cholesterol.

4.2 Cardiovascular function and atherosclerosis

PCSK9$^{-/-}$ mice retained cardiovascular function under HFD conditions, with improved ejection fraction, cardiac output, fractional shortening, and E/e' compared to their WT counterparts. HFD did not cause advantageous or adverse effects in LDLR mice. Overall cardiovascular function was impacted by HFD, but no significant change to plaques were found in the aorta.

Aged LDLR$^{-/-}$ mice exhibited significant atherosclerotic plaque formation compared to WT mice, suggesting prolonged cholesterol elevation in aging populations impacts

plaque accumulation more than short term HFD alone.

4.3 lEVs & lEV sized lipoproteins in mice

Both annexin V+ and CD63+ circulating lEV levels increased in LDLR$^{-/-}$ and PCSK9$^{-/-}$ mice after HFD compared to WT mice. At old age both knock-out models (compared to WT), despite contrasting cholesterol profiles showed increased annexin V+ circulating lEVs.

Based on annexin V+ and CD63+ events, circulating lEV levels decreased across all three mouse groups after HFD, where cholesterol was increased. CD81+ lEVs showed no alterations.

In young mice, fasting animals showed lower cholesterol than prandial. Whether fasted or in immediate post-prandial state, no changes to EV levels were observed in mice.

ApoB showed little to no change between mice groups. No change in ApoB levels were observed after HFD or at old age in mice.

4.4 lEVs & lEV sized lipoproteins in humans

Hypercholesterolaemic patients showed significantly elevated TC and LDL levels as measured by the clinic. CD63+ lEV levels were significantly reduced in

hypercholesterolaemic patients, while annexin V and CD81+ lEV levels remained unchanged.

ApoB and ApoE levels (within the lEV gating) were both significantly elevated in hypercholesterolaemic patients, following a direct correlation to cholesterol levels.

4.5 Further EV analysis

The MACSplex bead-based exosome kits for mice and humans was used to reveal other potential EV markers based on EV (CD9, CD63, and CD81) capture. The kit revealed CD29 and CD62P as appearing prominent in blood plasma in both species.

Single EV measurements of both CD29 and CD62P markers using our EV gating in flow cytometry showed no significant alterations between groups. This was the case in both mice and humans.

4.6 Gene expression in HUVECs

In HUVECs, EVs from hypercholesterolaemic patients induced upregulation of markers CD36, HMOX1 and PPARg, they also induced a reduction in VEGFA expression levels. Other genes measured showed no impactful shifts in expression.

lEVs from normocholesterolaemic patients showed no impactful changes to gene expression in HUVECs of any genes assessed here.

5. Discussion

This study brings further evidence to the complex interplay between EVs and cholesterol in the context of cardiovascular health. We show for the first time an inverse relationship between EVs and cholesterol, and a direct relationship between EVs and cardiovascular health. Specifically, CD63+ lEVs. Despite opposing cholesterol levels, $LDLR^{-/-}$ and $PCSK9^{-/-}$ mice both show increased EV levels compared to WT animals. This suggests a potential role of cholesterol transport of EVs via other (non-LDLR) cholesterol related receptors such as the LRP1 or perhaps a scavenger receptor, for instance SCARB1.

$LDLR^{-/-}$ mice demonstrated that prolonged high LDL-C levels in aging populations, rather than short-term in younger populations (as seen in our HFD experiment), exacerbate plaque formation. We do see that 12-week HFD in mice can negatively impact other cardiac parameters, such as cardiac output and ejection fraction. This finding, along with multiple other reports, highlights the importance of proper diet in relation to cardiac health. In the last decade, more research has demonstrated genetics as a key factor in cardiovascular health, rather than lifestyle alone. Which in the current study, was also demonstrated with $LDLR^{-/-}$ animals. Based on

elevated plaque levels seen in senior LDLR$^{-/-}$ mice, we can postulate that although less likely in rodents, this genetic alteration may lead to MI or stroke in humans and in other primates.

In PCSK9$^{-/-}$ mice, despite having lower cholesterol levels, have exhibited increased body mass at all time points, compared to WT animals. This phenomenon is possibly linked to dysregulation of CD36, a key transporter of triglycerides. This link was shown in earlier publications and discussed in more depth by previous researchers. The effects caused by PCSK9 inhibition (even when stabilizing cholesterol) may lead to other health related detriments such as obesity and diabetes – which ultimately could lead back to CVD. This highlights once again the caution being applied to gene editing projects of the PCSK9 gene, which although received permission several years ago, no recruitment has yet been issued. Likely, ongoing and further research into the PSCK9 gene is needed before undertaking the more permanent approach to hypercholesterolaemia therapeutics.

We demonstrated that hypercholesterolaemic patients have altered EV profiles, suggesting EVs as diagnostic markers in CVD. Furthermore, when EVs from hypercholesterolaemic

patients were incubated with HUVECs gene expression levels were altered, with no observed changes to HUVECs incubated with normocholesterolaemic EVs. This allows us to further speculate an EV contribution to vascular inflammation, as some evidence was shown in our gene expression study.

Further work is needed to better understand the relationship between EVs and LDL. We have yet to answer how LDL elevation leads to the reduction of CD63+ lEVs. Finally, a more clinically relevant study with increased participant numbers may help establish EVs as relevant diagnostic markers for ACVD.

6. Conclusions

Circulating EV levels are influenced by cholesterol levels, age and cardiovascular health. More specifically EVs are shown to be inversely related to ACVD development. This makes EVs good potential biomarkers and possible therapeutic targets for ACVD. This is the first time an inverse relationship between CD63+ EVs and cholesterol levels has been shown. Further studies are needed to explore EV-based interactions and their translational potential in clinical settings. Finally, we highlight caution for PCSK9 based therapies, as these may lead to obesity and associated pathologies.

7. Summary

In 2016, EVs and lipoproteins were found to be associated in blood plasma, leading to widespread research on their co-isolation and contamination in all EV preperations. This prompted the hypothesis that EVs play a role in dyscholesterolaemia and the development of atherosclerosis. Using PCSK9$^{-/-}$ and LDLR$^{-/-}$ mouse models, we investigated cholesterol and EV levels, cardiovascular function, and plaque formation. Our results showed PCSK9$^{-/-}$ mice have lower LDL and overall cholesterol levels, while LDLR$^{-/-}$ mice had elevated levels. Annexin V and CD63-positive EVs were inversely related to cholesterol in mice and only CD63-positive EVs were inversely related to cholesterol in humans. PCSK9$^{-/-}$ mice demonstrated cardioprotective effects but these mice were also found to have greater body mass, suggesting caution for PCSK9 inhibitors due to potential weight gain. Overall, this work shows the diagnostic and prognostic potential for CD63+ lEVs, and the possibility for future EV based therapeutics for chronic hypercholesterolaemia and ACVD patients.

8 Bibliography of the candidate's publications

8.1. List of publications used for the PhD thesis:

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