Characterisation of extracellular vesicles (EVs) from dysfunctional endothelial cells following exposure to alcohol

A dissertation for the degree of M.Sc.

by

Susan Harman B.Sc, M.Sc

Under the supervision of Prof. Paul A. Cahill

School of Biotechnology

Dublin City University

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Publications

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Table of figures

List of tables

Table of Contents

Abbreviations

16K PRL	16-kDa N-terminal prolactin fragment
AB	Antibodies
ACTA2	Smooth muscle alpha actin 2
AFP	Alpha-fetoprotein
AKT	Protein kinase b
ALIX	Apoptosis-linked gene 2-interacting protein X
ANXA5	Epithelial cell adhesion molecule
ANXA5	Annexin A5
AS	Arteriosclerosis
ATM	Ataxia telangiectasia
BCLAF1	Bcl-2-associated transcription factor 1
BMD-SCs	Bone marrow-derived stem cells
BMS	Bare metal stent
BMS	Bare metal stent
CAD	Coronary Artery Disease
CD2AP	Endothelial actin-binding protein CD2-associated-protein
CETP	Cholesteryl ester transfer protein
cGMP	Cyclic guanosine monophosphate
cIMT	Carotid Intimal-Medial Thickness
CLPTM1	Palate-associated transmembrane protein 1
CM	Conditioned media
CNN1	Calponin 1
CNN3	Calponin 3
CPCs	Cardiac progenitor cells
CVD	Cardiovascular disease
CVD	Cardiovascular disease
DE	Differential expression
DES	Drug-eluting stents
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Essential Medium
EC	Endothelial cell
ECM	Extracellular matrix

ED	Endothelial dysfunction
EDRF	Endothelium-derived relaxing factor
EndMT	Endothelial cell transition
EndMT	Endothelial to mesenchymal transition
eNOS	endothelial nitric oxide synthase
ESCRT	Endosomal sorting complex required for transport
ESCs	Embryonic stem cells
ET-1	Endothelin-1
EtOH	Ethanol
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FDR	False discovery rate
FLOT1	Flotillin
GALNS	N-acetylgalactosamine-6-sulfatase
GC	Guanylate cyclase
GM130	Golgi matrix protein 130
GO	Gene Ontology
GSK3β	Glycogen synthase kinase 3β
HAEC	Human aortic endothelial cell
HAEC-derived EVs under 25mM EtOH conditions	25-EXO
HAEC-derived EVs under 50mM EtOH conditions	50-EXO
HAEC-derived EVs under healthy conditions	H-EXO
HDL	High-density lipoprotein
Hh	Hedgehog
HHIP	Hedgehog Interacting Protein
HiPSC	Human-induced Pluripotent Cells
HPCs	Hematopoietic progenitor cells
hSMC	Human Aortic Smooth Muscle Cell
HUVECs	Human umbilical vein endothelial cells

ICAM1	Intercellular adhesion molecule 1
ICC	Immunocytochemistry
IGF-I	Insulin-like growth factor I
IGHA1	Immunoglobulin heavy constant alpha 1
IL-1β	Interleukin 1 beta
ILV	Intraluminal vesicles
IMT	Intimal medial thickening
IRADEs	Insulin resistance adipocyte-derived exosomes
ISR	In stent restenosis
iST	in-StageTip
JAM	Junctional adhesion molecule
KRT2	Keratin complex-2
LAP	Lipid accumulation product
LC/MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein
mESCs	Mouse embryonic stem cells
mG	Membrane-targeted Green fluorescent protein
MICU2	Mitochondria Ca^{2+} uptake protein 2
miRNA	micro RNA
miRNA	Myocardial infarction
MLCK	Myosin light chain kinase
Mrna	Messenger RNA
MS/MS	Tandem mass spectrometry,
MSCs	Mesenchymal stem cell
mT	Tandem dimer Tomato
MVB	Multivesicular body
MVs	Microvesicles

MVSC	Multipotent Vascular Stem Cells
Myh11	Myosin heavy chain 11
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NC	Non-conditioned media
NCD	noncommunicable diseases
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
OS	Oxidative stress
ox-LDL	Oxidized low-density lipoprotein
PA	Phosphatidic acid
PCI	Percutaneous coronary intervention
PCI	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PGI_2	Prostacyclin
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PIGR	Polymeric immunoglobulin receptor
PKC	Protein kinase C
PLD2	Phospholipase D2
PM	Plasma membrane
PPCM	Peripartum cardiomyopathy
pre-PIT	pre-pathological intimal thickening
PRPF40A	Pre-mRNA processing factor 40A
PRX 1	Peroxiredoxin 1
PS	Sphingomyelin, phosphatidylserine
PSMB10	Proteasome subunit beta-10
PSMB3	Proteasome subunit beta type-3
PTM	Posttranslational modifications

qRT-PCR	Reverse-Transcription Polymerase Chain Reaction
R18	Octadecylrhodamine B chloride
RNAi	RNA interference
ROCK2	Rho-associated protein kinase 2
Sca1+	Stem cell antigen-1 positive
scRNA-seq	single-cell RNA sequence analysis
SHs	Sonic hedgehog
siRNA	Short interfering
SMase2	Neutral sphingomyelinase 2
SMC	Smooth Muscle Cell
SM-MHC	Smooth muscle-myosin heavy chain
SMO	Smoothened
SMTN	Smoothelin
SM-α-actin	Smooth muscle α-actin
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNEPs	Spontaneous NE Progenitor Stem Cells
SOX10	SRY-Box Transcription Factor -10
SOX17	SRY-Box Transcription Factor -17
STAT	Signal transducers and activators of transcription
TAGLN	Transgelin
TDI	Time Delay Integration
TFAM	Mitochondrial transcription factor A
TG	Triglycerides
TGF-β	Transforming growth factor β
TIRF	Total internal reflection fluorescence
TNFα	tumor necrosis factor alphaTNFα
TRAIL	TNF-related apoptosis-inducing ligand
TSG101	Tumor susceptibility gene 101
UBE2NL	Ubiquitin-conjugating enzyme E2N-like
USP39	Ubiquitin-specific peptidase 39
VAMP7	Vesicle-associated membrane protein 7
VCAM-1	Cell adhesion molecule
VEC	Endothelial VE-cadherin

vECs	Vascular endothelial cells
VEGF	Vascular endothelial growth factors
VLDL	Very low-density lipoproteins
vSMC	Vascular Smooth Muscle Cell
VVO	Vesiculo-vacuolar organelle
WHO	World Health Organisation
αSMA	Alpha smooth muscle actin

Units of measurement

^{o}C	Degrees Celsius
cm	centimetre
g	grams
hr	hour
L	litre
M	molar
mg	milligram
min	minutes
mL	millilitres
mM	millimolar
nm	nanometre
nM	nanomolar
rpm	revolutions per minute
sec	second
µL	microlitre
µg	microgram
µm	micrometer
µM	micromolar
x g	G-force

Abstract

Characterisation of extracellular vesicles from dysfunctional endothelial cells following exposure to alcohol

Susan Harman

Endothelial cell (EC) dysfunction is classically associated with subclinical atherosclerosis and the development of atheroprone lesions. Extracellular vesicles (EVs) are biological nanoparticles secreted by most cell types that contain a complex mix of cell-type specific signalling proteins and nucleic acids. At high concentrations, alcohol is a known risk factor for atherosclerosis but may be protective at lower concentrations.

In this study, we first investigated whether alcohol (EtOH) at varying concentrations can alter the number and/or phenotype of EVs released from endothelial cells before the proteomic profile of endothelial cell-derived EVs was assessed. Human aortic endothelial (HAEC) and smooth aortic vascular muscle cells (HuSMCs) were first characterised for lineage specific markers before endothelial cell dysfunction was induced following treatment of HAECs with low and high doses of EtOH. EVs released from HAEC were isolated from conditioned cell media, enumerated and characterised using dynamic light scattering (DLS), immunoblot and Amnis™ Cell Stream FACS analysis for EV biomarkers before the proteomic profile of the EVs was evaluated using Liquid chromatography massspectrometry (LC-MS) analysis. The data generated suggest that the number of endothelial derived-EVs released following endothelial dysfunction increases following exposure of cells to different concentrations of EtOH. Moreover, the protein cargo within these EVs varies, even though the EVs originated from the same cell source. Proteomic and bioinformatic analysis revealed that protein metabolism and cell growth and/or maintenance are the key pathways and biological processes associated with these EVs and provides further insight into elucidating their putative role following endothelial dysfunction. Lastly, EVs from dysfunctional HAECs had little effect on the growth and migration of HuSMCs but did promote HuSMC de-differentiation following treatment with 25-EXO. These data highlight the potential important role for EVs released following endothelial dysfunction on the underlying smooth muscle cell population that may contribute to arteriosclerotic lesion formation

Chapter 1 Introduction

1.1 Cardiovascular disease

According to the World Health Organisation (WHO), cardiovascular disease (CVD) are a group of disorders of the heart and blood vessels. Heart attacks and strokes are usually acute events and are largely caused by a blockage that prevents blood from flowing to the heart or brain. CVD typically results from arterial remodelling, which results in the thickening of the blood vessel wall. This refers to the many structural and functional changes within the vascular wall that occurs in response to injury, ageing or disease (Van Varik et al., 2012). CVD may develop in patients with a genetic predisposition or can develop over time throughout their lifetime through a process known as AS (Agarwal, 2001). CVD remains the leading cause of global morbidity, taking an estimated 17.9 million lives a year, a number that is expected to grow to greater than 23.6 million by 2030. In Ireland, approximately 10,000 people die each year from CVD (Irish Heart Foundation, 2018). Of all the noncommunicable diseases (NCDs) such as cancer and diabetes, CVD currently accounts for nearly half of NCDs. NCDs have overtaken communicable diseases as the world's major disease burden (Joseph et al., 2017). CVDs are not only detrimental to the health of a population but also put a massive financial burden on the healthcare systems and on the government. In 2010, the estimated global cost of CVD was $863 billion, and it is estimated to rise to $1044 billion by 2030 (Betts et al., 2020). It is clear from the evidence and worrisome trends that we need to urgently address the increasing burden of CVD. It is critical that we reduce these numbers and improve the quality of life through the prevention and treatment of this progressive disease.

1.2 The Vasculature

The vasculature is a network of blood vessels that connect the heart with all other organs and tissues within the body. The vascular system is divided into two major parts, systemic circulation, and pulmonary circulation. It supplies oxygen-rich blood and nutrients by transport of the arteries and arterioles, from the heart to the organs and tissues. While it also carries de-oxygenated blood back to the heart by transport of the venules and veins. Capillaries have the function of exchanging gases and transferring nutrients between blood and tissues. In total, there are five classes of blood vessels, all briefly mentioned above. Arteries transport blood under high pressure, and for this reason they have strong vascular walls. The arterioles connect the arteries and capillaries. They also have a strong wall which can contact and dilate severalfold, giving it the capability of altering blood flow. Capillaries are the smallest type of

blood vessel. Their walls are very thin and have many minuscule capillary pores permeable to water and other molecules. Veins act as a reservoir of blood and transport low-pressure blood from the venules to the heart. Compared to arteries, their blood vessel walls are much thinner. However, they are muscular enough to expand and contract. Lastly, the venules collect blood from the capillaries and carry them to the veins. Many venules can unite to form a vein (Black & Garbutt, 2002)

Blood vessels, except capillaries, are made of three layers: the intima (*tunica intima)*, media (*tunica media)* and adventitia (*tunica adventitia*). As mentioned before, arteries and veins have a variable thickness, so the composition of these layers varies between the two. Each region contains different amounts of smooth muscle cells and elastin. The intima, the innermost and thinnest layer, consists of a single layer of endothelial cells that cover the subendothelial layer of the basal lamina and is supported by the internal elastic lamina. Endothelial cells secrete many bioactive substances to control thrombosis, prevent adhesion of platelets and leukocytes, modulate vascular tone and act as a selective diffusion barrier (Waller et al., 1992; D. Wang et al., 2017). The internal elastic membrane separates the intima from the media, this is composed of elastic fibres. The subendothelial layer consists of vSMCs and an extracellular matrix. These cells have a low proliferation rate and are phenotypically stable. There is an increase in elastic fibres and cell layers more toward the media. When stimulated by proatherogenic factors, tumour necrosis factor-alpha (TNF-α) like oxidized lipids and Interleukin 1 beta (IL-1β) this causes vSMCs to proliferate and generate high amounts of extracellular matrix (ECM), forming pre-pathological intimal thickening (pre-PIT) (M. Cheng et al., 2019; Milutinović et al., 2020). The media contains predominantly smooth muscle cells and elastin fibres as well as ECM, collagen, and proteoglycans. This layer is much thicker in arteries than in veins. Fibre composition also varies in that vein contain fewer elastic fibres. In larger arteries, these layers are more highly organised due to their functions and important role in the movement of large volumes of blood. The external elastic lamina is also common in this region to give structural support (Tellides & Pober, 2015; Zorc-Pleskovič et al., 2018). vSMC in the media usually make up to 40 layers and are arranged helically or circumferentially. Lastly, the adventitia, the outermost layer of the blood vessel. This is composed entirely of fibro-elastic connective tissue, consisting of fibroblasts being the main cell in the adventitial connective tissue. As well as progenitor cells and immune cells such as macrophages, T-cells, B-cells and dendritic cells (K. A. Campbell et al., 2012; D. Wang et al., 2017). It is also surrounded by external lamina which helps to anchor the blood vessels to surrounding tissues. This layer is thicker in veins,

to prevent the collapse of the blood vessel (Pugsley & Tabrizchi, 2000). The adventitia seems to participate in cell trafficking through the arterial wall and is involved in the signalling between the vSMCs, endothelial cells and the tissue environment. This layer is responsible for the regulation of lumen size, the inner and outer wall remodelling response and the repair mechanism in response to injury (Milutinović et al., 2020). In response to stress or injury, the fibroblasts in the adventitia begin to proliferate and differentiate into myofibroblasts and migrate towards the intima (F. Xu et al., 2007) They regulate the growth of endothelial cells and vSMCs by secreting factors and recruit progenitor and inflammatory cells to the vessel (F. Xu et al., 2015).

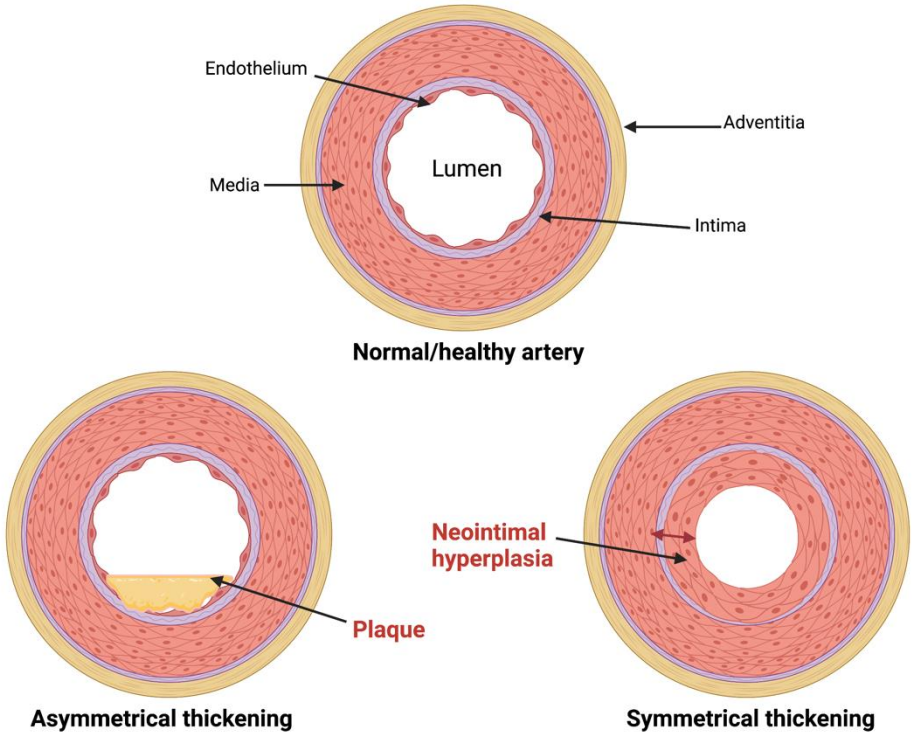


Figure 1 Structure of a normal artery, vs an artery with atherosclerotic and arteriosclerotic lesions.Arteries are composed of three layers; including the intima, media, and adventitia. Here we have a normal artery at the top, shown in comparison to a diseased artery; one depicting AS, asymmetrical thickening on the left-hand side and the other depicting arteriosclerosis which is symmetrical on the right-hand side. Image Adapted from templates, by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

CVD includes a wide variety of diseases, however, the most prominent pathologic condition that is affecting our population in the millions is coronary artery disease (CAD), known as arteriosclerosis. This is defined as vascular remodelling, resulting in the thickening of the artery, either symmetrically (non- lipid dependent) or asymmetrically (lipid dependent). There

are three recognized types of atherosclerosis, namely AS, arteriolosclerosis, (Figure 1) and Monckeberg medial calcific sclerosis (Fishbein ∗ & Fishbein, 2015). Mönckeberg Medial Calcific Sclerosis, which refers to a calcification of the medial arterial layer of small and medium sized arteries, which leads to a reduction of elasticity and increased blood pressure (Lanzer et al., 2014).

1.2.1 Atherosclerosis

Atherosclerosis (AS), a specific type of arteriosclerosis, otherwise known as asymmetric vascular remodelling, is a chronic inflammatory disease. The arterial lumen diameter is reduced, and the arterial wall is thickened due to the asymmetrical accumulation of cholesterol, lipids, macrophages and vSMCs in the endothelium. These components make up a plaque known as atheroma (R. T. Lee & Libby, 1997). A fibrous cap forms over this plaque, thereby if left untreated, this can rupture and lead to a sudden cardiac arrest that can be fatal (Figure 2) (Ross, 1986). AS is an ongoing progressive development. At as early as 11-12 years, a fatty streak develops, at 15-30 years a fibrous plaque develops at the same sites as the fatty streaks, suggesting that fatty streaks evolve into the atheroma (Ridker et al., 2009). Endothelial injury is a key mediator in the development of AS. Nitric oxide which is catalysed by endothelial nitric oxide synthase (eNOS), is a potent vasodilator and is essential for vessel remodelling and good blood flow. When there is damage to the endothelium, the level of nitric oxide (NO) production is greatly reduced. The endothelium becomes more permeable to lipoproteins and the endothelial cells express various adhesion molecules such as Monocyte chemoattractant protein (MCP-1) and vascular cell adhesion molecule (VCAM-1), which play a role in binding, by allowing the monocytes and T-lymphocytes to infiltrate the permeable barrier. After their adhesion to endothelial cells, this causes the monocytes to migrate into the sub endothelial space and undergo the process of differentiation into macrophages. Once at the site, macrophages ingest the fatty streaks becoming foam cells. These foam cells produce reactive oxygen species and cytokines which contribute to the proliferation of SMCs which leads to an enhanced inflammatory process and causes ECM degradation (Aziz, 2016; Cunningham & Gotlieb, 2005; Newby et al., n.d.; Stocker & Keaney, 2004). Among the many key risk factors of AS are age, family history, smoking, Diabetes mellitus, hypertension, alcohol and Obesity (Quick & Cotton, 1982).

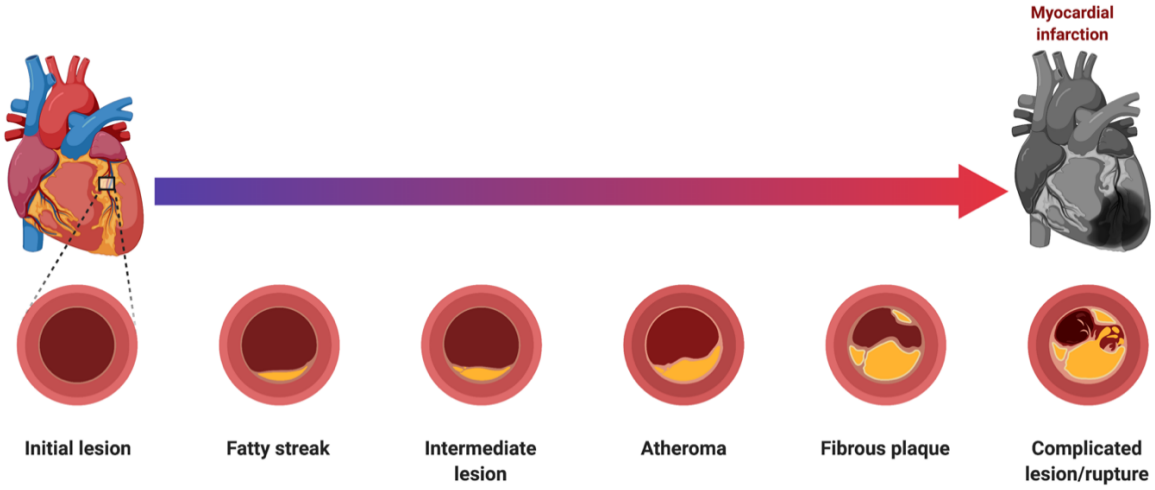


Figure 2 Progression of AS, resulting in myocardial infarction. Firstly, we have an artery with normal histology undergoing the initial macrophage infiltration. A fatty streak develops due to intracellular lipid accumulation. Intracellular and extracellular lipids continue to accumulate, creating an intermediate lesion. *This leads to* raised lesion called the atheroma forms, that protrudes into the vessel lumen. New fatty streaks form, multiple lipid cores and fibrotic and calcific layers form the fibrous plaque. Finally leading to disruptive surfaces from plaque rupture, thrombosis, and blockage of blood flow. Image Adapted from "AS Progression", by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

1.2.2 Arteriosclerosis

Arteriolosclerosis otherwise known as the symmetrical vascular remodelling is the thickening of the intimal and medial layers of an artery. A hallmark of arteriosclerosis is the formation of a fibrous lesion known as a neointima. This is due to the accumulation of vSMC-like cells resulting in intima-media thickness (IMT) (Bennett et al., 2016). This neointima formation leads to the continuous remodelling of the vessel wall, causing the lumen to narrow, reducing elasticity, and preventing normal blood flow, leading to stroke or myocardial infarction. Vessel injury as a result of stenting and ageing are the main causes of arteriosclerosis (Fishbein & Fishbein, 2009). The origin of neointimal cells is not fully known, however, research does exist for potential sources of these cells. De-differentiation of smooth muscle cells has been accepted as a classic theory for decades for their major role in intimal thickening after vascular injury (G. K. Owens, 1995). Resident vascular stem cells, circulating bone-marrow-derived stem cells, and endothelial cell transition (EndMT) are also potential sources of these neointima cells (Falei Yuan et al., 2017).

1.3 Current treatments of cardiovascular diseases

Treatment for coronary artery disease usually involves lifestyle changes and, if necessary, drugs and certain medical procedures tailored to the severity and stage of progression of the disease. In severe cases of CVD cases, a more aggressive and invasive approach is needed. One of these methods is a bypass graft. This involves the surgeon taking a blood vessel from another part of the body and using it to allow blood to flow around the blocked coronary artery (J. H. Alexander & Smith, 2016). An alternative treatment is balloon angioplasty. This uses a fine catheter tube, which punctures the skin and is inserted through the blood vessel making its way through the obstructed artery to the site of plaque formation. The balloon is inflated to break down the plaque and widen the lumen to increase blood flow. More recently a bare-metal stent (BMS) is placed inside the artery to reduce elastic recoil and more acute luminal gain than balloon dilation and fastens down the plaque and tissue that might otherwise compromise flow, therefore stabilising acute procedural results (Byrne et al., 2017). A stent is a small piece of metal mesh used to prevent artery narrowing. These types of procedures are called endovascular treatment, however with these treatments come challenges, most notably the occurrence of in-stent restenosis (Haery et al., 2004).

1.3.1 In-stent restenosis

Even with technological advances and an improved understanding of the restenosis process, following bare-metal stent (BMS) implantation, the overall rate of in-stent restenosis remains high. When a stent is inserted in the artery wall, this causes the endothelial lining to rupture because of mechanical stress (Figure 3). This leads to platelets and macrophages to play a critical role via vSMC migration and proliferation in the intima to produce the neointimal hyperplasia. There is an increased extracellular matrix formation that appears to form most of the neointimal hyperplasia (Haery et al., 2004). This remodelling is caused by the constant proliferation and migration of these vSMC-like cells. In contrast to the original atherosclerotic event, which is both clinically and pathologically similar, this event is actually significantly different at the molecular level (Hoffmann et al., 1996). This is due to the expression of alpha-smooth muscle actin of neointimal tissue increasing from 26% on day 7 to 98% on day 30 following vessel injury (Christen et al., 2001). The neointimal lesion is fibrous in nature and made up of these vSMC-like cells, rather than a lipid-based plaque. Unfortunately, a gradual re-blocking of the stented area reoccurs over the course of 3 and 12 months after placement of a stent, requiring re-intervention, occurs in approximately 20 % of cases using bare-metal stents

. This limitation of BMS leads to the progressive loss of the arterial lumen inside the stent and in-stent restenosis (ISR) decreased the efficacy of this technique.

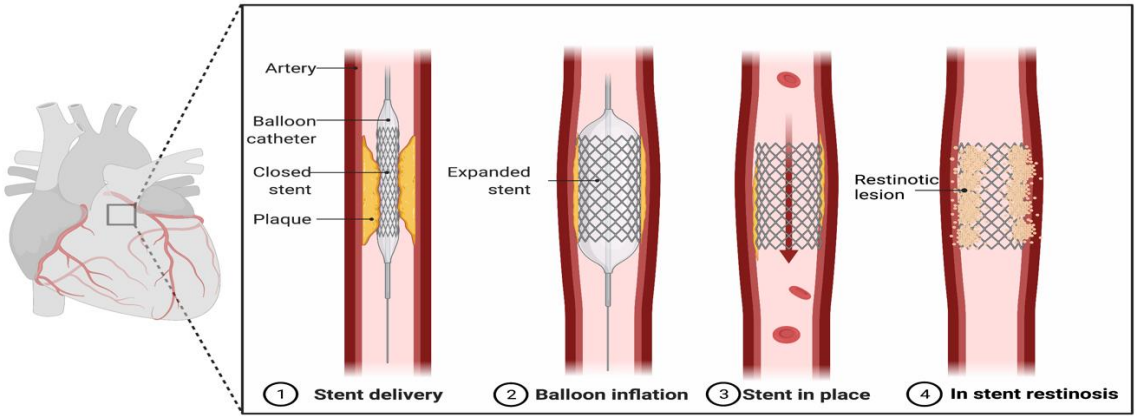


Figure 3 In-stent restenosis. This image shows the use of Balloon angioplasty which widens the lumen of an artery, that has been narrowed due to the build-up of plaque. A metal mesh stent is inserted around the balloon to prevent elastic recoil of the lumen. However, this can injure the artery wall which leads to neointima forming leading to in-stent restenosis. Image Adapted from "Percutaneous Coronary Intervention", by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

To combat this issue of ISR from the use of BMS, the first generation of drug-eluting stents (DES) were developed. These stents are coated with particular drugs that are released at the site of stenting to prevent ISR from occurring (Farooq et al., 2011). There are several types of drug coatings used for DES such as anti-proliferative, anti-mitotic and anti-thrombotic drugs with varying degrees of efficacy. However, this does not come without its challenges. Due to the rapid delivery of the drugs, the anti-proliferative and anti-thrombotic effects only last a number of days or weeks. These are just postponing the inevitable event of ISR in most cases (Yin et al., 2014) Although they did reduce ISR, there was a worrying increase in late stent thrombosis. A step forward in percutaneous coronary intervention (PCI) was achieved with the second generation of DES, this involved more biocompatible or biodegradable polymers, different designs, stent platforms and drug release formulation leading to a decrease in ISR (Torrado et al., 2018). Their ability to release the drug in a slow and long-term manner and their ability to penetrate the tissue have shown to be much more promising in preventing ISR. Although this technology has significantly reduced the occurrence of ISR, it is still essential

we gain a better understanding of the molecular mechanisms involved in neointimal formation and ISR.

1.4 The Neointima

PCIs have become widely adopted for the treatment of coronary AS. However, we are now familiar that restenosis still limits the long-term outcome of PCI. The vascular remodelling process is characterised by the functional changes in the cells within the vessel wall. One of the main pathophysiological hallmarks in neointima formation is the excessive proliferation of vSMC (Dzau et al., 2002). Studies have shown that neointimal cells express markers of vSMCs, so it is believed that the SMCs resident in the medial layer migrates into the subendothelial region, start to proliferate and form ECM, contributing to the formation of the neointima. The source of these vSMC-like cells has been a long-standing assumption that they originate from de-differentiated SMCs in the medial layer (Owens, 1995; Owens et al., 2004) However, other research has shown that other cell sources may include vascular resident stem cells, circulating bone marrow cells (Shimizu et al., 2001) and endothelial cells undergoing a process called endothelial to mesenchymal transition (Figure 4), (Cooley et al., 2014)(Frid et al., 2002).

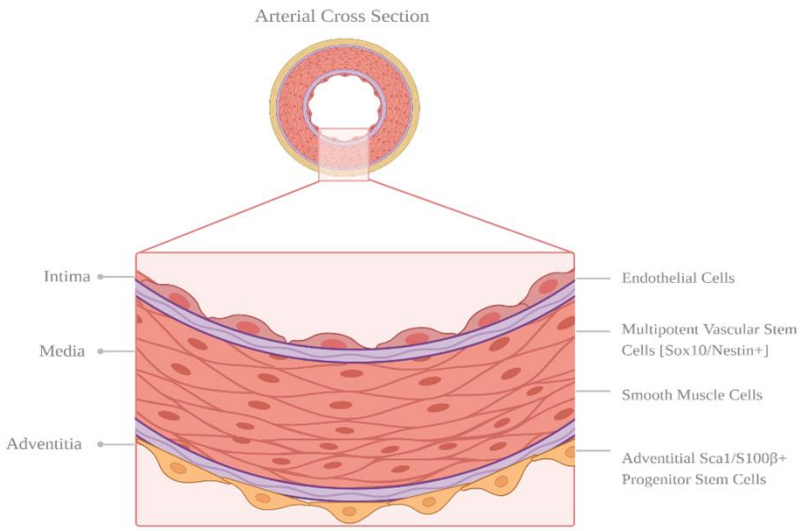


Figure 4 Structural composition of a healthy arterial wall. The structural composition of a healthy arterial wall and it's distinct cell populations that reside within it. The innermost layer, the intima,

comprises a single layer of ECs known as the endothelium. The middle layer, the intima, contains differentiated vSMCs giving the artery structural function due to their contractile phenotype. The outermost layer, the adventitia, contains many cell populations, including immune cells, an adventitial progenitor stem cell population and fibroblasts (Created with BioRender.com)

1.4.1 Dedifferentiated smooth muscle cells

SMCs cells are highly specialized cells whose fundamental function is contraction. The mature SMCs or terminally differentiated SMCs proliferate at a low rate and express contractile proteins and signalling molecules which are required for their function of contraction (Oishi et al., 2002). SMCs express different markers at different stages of maturation. The two marker proteins that provide the best characterisation of a mature contractile SMC phenotype are smooth muscle-myosin heavy chain (SM-MHC) and smoothelin **(**SMTN). These differentiated or contractile SMCs are thought to be the precursors of the newly formed SMCs-like cells that are characteristic of the neointima due to their non-terminally differentiated phenotype. Differentiated SMCs have been shown in many studies to have a high level of plasticity and can undergo reversible changes in phenotype in response to changes in local environmental cues that normally regulate phenotype (Owens, 1995). Studies carried out by Gary Owens research group have supported this theory on several occasions, reporting that the down-regulation of SMC markers, such as smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α-actin (SM-α-actin) in neointimal cells post vessel ligation, which led these cells to be described as SMC-like as opposed to SMCs (Owens et al., 2004). Upon vascular injury, these SMCs are capable of undergoing dedifferentiation as well as a process called phenotypic switching, whereby they are subjected to transient modification of the phenotype to a highly synthetic phenotype. It was shown to occur in vSMC following vessel injury with SMCs spontaneously switching to a synthetic SMC phenotype with higher levels of proliferation and inflammatory cell recruitment (Rensen et al., 2007).

Lineage tracing studies are a powerful tool to trace the lineage of a given cell and determine its origin. Adults' cells can be traced due to the expression of a fluorescent protein such as membrane-targeted tandem dimer Tomato (mT) or membrane-targeted green fluorescent protein (mG) under the control of the tissue-specific promoter, in the case of SMC lineage tracing this would be SM-MCH. Nemenoff et al., study performed a lineage tracing study where clear evidence was shown that differentiated SMCs undergo phenotypic modulation in response to wire-induced injury of the femoral artery using tamoxifen-inducible SM MHC-CreER^{T2}/Rosa26-floxStop/βGal mice. This study showed that cells in the neointima arose from

a cell that, prior to vessel ligation, was positive for Myh11 expression. Overall they concluded that contractile SMCs play a multifaceted role in generating the neointima by contributing to forming the majority of neointimal cells, recruiting inflammatory cells and playing a role in adventitial remodelling (Nemenoff et al., 2011). The Herring et al., (2014) study used a genetic fate mapping technique with tamoxifen regulated smooth muscle-specific cre recombinase and a dual colour cre-dependent reporter gene. They concluded that neointimal SMCs that arise following carotid artery ligation is largely derived from a subpopulation of differentiated medial vSMCs (Herring et al., 2014). Chappell et al., 2016 expanded on this and demonstrated extensive proliferation in the low proportion of high plastic vSMCs which resulted in the build-up of vSMC to accumulate post-injury in atherosclerotic regions. They also showed that cell proliferation occurs in the medial layer of the artery (Chappell et al., 2016).

However, there has been criticism of the lack of relevant controls and a lack of established parameters. Reinert, *et al* (2012) highlighted that the precise length of time that tamoxifen can induce the nuclear translocation of CreER(Tm) is not well defined and hard to assess. There is a possibility that tamoxifen remains in the tissue, therefore may result in labelling cells that are newly differentiated for up to four weeks after injury, as well as labelling cells with the active promotor that are of interest prior to injury (Reinert et al., 2012). This has caused challenges in the interpretation of these results. This study along with others, where by using MYH11-cre mice for lineage tracing showing further complicates the story with conflicting results indicating the SMCs are not the only major contributors to the neointima formation (Herring et al., 2014; Reinert et al., 2012).

1.4.2 Endothelial cells

The endothelial barrier is important for the regulation of cells and cytokines leaking from the blood and for maintaining vascular homeostasis. Endothelial dysfunction (ED) plays a major contribution in human diseases, in particular arteriosclerosis (Cahill & Redmond, 2016; Khaddaj Mallat et al., 2017). The most common pathogenesis of vascular diseases is the loss of integrity of the endothelial barrier, the infiltration of inflammatory cells and disordered endothelial proliferation (D. Chistiakov et al., 2015). Studies have shown that ECs have the ability to undergo a process termed endothelial to mesenchymal transition (EndMT) when the ECs are exposed to chronic stress and inflammatory factors (J. G. Cho et al., 2018; Pérez et al., 2017). Many studies show evidence that ECs contribute to vascular SMCs and cardiac fibroblasts through endothelial to mesenchymal transition (EndMT). EndMT is a process

characterised by the loss of endothelial markers such as VE-cadherin and CD3, and the acquisitions of specific markers of mesenchymal stem cells such as alpha-smooth muscle actin (αSMA), N-cadherin, and calponin is acquired (Frid et al., 2002; Krenning et al., 2008; Krüger-Genge et al., 2019). During EndMT, the tight cell-cell junctions of ECs become disrupted causing them to lose their morphological features of a cobblestone-like structure. They reorganise their cytoskeleton and become more spindle-shaped, and have morphological features of that of a fibroblast (Ma et al., 2020). The acquisition of these mesenchymal-specific cell markers plays a role in regulating the function of endothelial cells and structural remodelling of the blood vessels (Souilhol et al., 2018). As already discussed, the AS is a result of vascular remodelling. EndMT can be a cause of ED which can lead to vascular remodelling. However, it has been supported by some studies that EndMT may be reversible (Okada & Kalluri, 2005a, 2005b). The contributory roles of EndMT in atherosclerotic vascular remodelling have been supported by many studies (Evrard et al., 2016; Mahmoud et al., 2017; Moonen et al., 2015). Cooley et al (2014) study group were able to demonstrate that over 50% of the cells within the neointima were EC-derived following the study of neointimal hyperplasia following murine vein graft remodelling. They also showed that TGF-β-Smad2/3-Slug signalling pathway plays a major role in regulating EndMT (Cooley et al., 2014). Transforming growth factor β (TGF-β) multifunctional cytokine secreted by many tissues and is recognised as one of the best studied inducers of EndMT. Another study carried out by Yang et al (2016) determined that only a small population of EC-derived cells were present in the sub endothelial region, suggesting very little evidence for EndMT (P. Yang et al., 2016).

1.4.3 Resident vascular stem cells

It has always been accepted that the de-differentiation of SMCs plays an important role during vascular remodelling. However, evidence carried out by other studies has challenged this. The theory that resident stem cells in the vessel wall differentiate to give rise to SMC-like cells in the neointima has gained popularity. Over the past decade, several studies have shown evidence supporting the role of vascular resident stem cells undergoing myogenic differentiation. A population of stem cell antigen-1 positive (Sca1+) were found present in the adventitia. These Sca1+ cells were shown to differentiate through a myofibroblast intermediate into a SMC population which contributes to AS. Sca1+ cells in a murine model have been shown to differentiate into SMCs both in vitro and *in vivo* (Hu et al., 2004; Qingzhong Xiao et al., 2007). It has been reported that the medial layer harbours a side population of Sca1+ cells as well as

$ABCG2^+$ side populations that have the capability to differentiate into smooth muscle (Sainz et al., 2006). In 2012, Tang et al identified a small niche of cells called multipotent vascular stem cells (MVSCs) residing in the medial layer of vessels. These MVSCs were $Sca1^-$ but were positive for several neural stem cell markers such as S100β, SRY-Box Transcription Factor-10 (SOX10), SRY-Box Transcription Factor -17 (SOX17) and Nestin. These MVSCs are cloneable, have telomerase activity, and can differentiate into mesenchymal stem cells (MSC)-like cells and neural cells that can differentiate into SMCs. They carried out lineage tracing with smooth muscle myosin heavy chain as a marker to show that Multipotent Vascular Stem Cells (MVSCs) and proliferative SMCs are not generated through the process of de-differentiation of mature SMCs. This study showed evidence that differentiation of MVSCs contributes to vascular remodelling and diseases (Tang et al., 2012). However, Nguyen et al. (2013) pointed out the major limitations of the model system used by Tang et al and how the study lacks *in vivo* lineage tracing evidence that supports the MVSC model, raising many concerns (Nguyen et al., 2013). They highlight that the model used by Tang et al. was not conditionally regulated and therefore cannot lineage tag more mature SMCs at a certain time point. The model could not be validated for labelling SMC-specific cells and determining which cell progeny they generated from. The conclusions drawn by Tang et al. are therefore compromised due to a lack of SMC lineage tracing as well as other flaws in the experimental design and analysis of data (Nguyen et al., 2013). Despite the contradictory results, the discovery of these MVSCs still poses an important question as to whether they play a role in the formation of the neointima.

1.4.4 Circulating Progenitors, and BM-derived Stem Cells

A lot of evidence through various studies suggests that the origin of neointimal cells as discussed above, can be derived from either de-differentiated SMCs, ECs and resident stem cells circulating, however, more findings suggest a fourth source; bone marrow-derived stem cells (BMD-SCs) and hematopoietic progenitor cells (HPCs). To evaluate the potential source of SMCs in neointimal hyperplasia, Sata et al. (2003) carried out a bone marrow transplant whereby the bone marrow of ROSA26-mice that express LacZ (BMTLacZJApoE-/- mice) was transplanted to control mice. Four weeks post-surgery, the mice were fed a western-type diet for eight weeks. It was observed that in the atherosclerotic lesion of the BMTLacZJApoE-/- mice, the smooth muscle actin-positive cells were shown to express LacZ, this suggested that their origin is from the bone marrow (Sata, 2003). Immunofluorescence double-staining of

lesions showed that the bone marrow-derived LacZ positive cells were CD31 and αSMA positive, suggesting that the bone marrow-derived cells differentiated in ECs and SMCs. A similar study carried out by Shimizu et al. (2001) carried out a bone marrow transplant of cells expressing β-galactosidase into aortic allograft recipients and indicated that the intimal cells were of bone marrow origin (Shimizu et al., 2001). However, studies have also shown that circulating cells, although they are recruited to the site of ligation, don't always give rise to neointimal cells (Hoofnagle et al., 2006).

1.5 Vascular smooth muscle cells

As we know, vSMCs are a major component of the vessel wall, they are responsible for regulating blood via their contractile functions and for maintaining vascular tone (Owens, 1995). vSMCs were revealed to retain remarkable cell plasticity. In normal arteries, vSMCs have spindle-like shape morphology and express a range of proteins such as MYH11, transgelin (TAGLN) and smooth muscle alpha actin 2 (ACTA2) which are required for their contractile characteristics (Campbell & Campbell, 2012; Jones et al., 1996). When vSMCs undergo vascular injury, these contractile marker gene expression are downregulated, which is partially mediated through a G/C repressor element (Regan et al., 2000). The identification of vSMCs within atherosclerotic lesions was based on the detection of these markers, indicating that cells of vSMC lacking in these contractile markers are not being identified. vSMCs possess remarkable phenotypic plasticity that allows them to rapidly adapt to changing environments, this plays an important role in the development of vascular diseases. The concept of phenotypic switching has been widely accepted. Platelet-derived growth factor (PDGF) and angiotensin II (Ang II) are the main factors that induce phenotypic switching of vSMCs from contractile to proliferative state. This has been demonstrated *in vivo* with inhibitors of PDGF-BB expression, function and receptor activation (Bilder et al., 1999; Ferns et al., 1991). This phenotypic change contributes to the vSMC migration to the intima and proliferation which facilitates the progression of AS (Gomez & Owens, 2012; MacK, 2011; Montezano et al., 2014; Z. Zhang et al., 2012). Acute vascular injury promotes the increase in synthesis and release of PDGF from many vascular cells like ECs, vSMCs and macrophages (Barrett & Benditt, 1987; Heldin & Westermark, 1999). There have also been studies to show that there is an increase in the expression of PDGF and its receptors which has been observed in the human coronary arteries after angioplasty (Tanizawa et al., 1996; Ueda et al., 1996). Several studies have also shown that vSMC phenotypic switching is determined by the expression levels and the phosphorylation state of insulin receptor substrate, which is a major intermediate component

shared by insulin/insulin-like growth factor I (IGF-I) signalling which promotes vSMC differentiation via activation of phosphatidylinositol 3-kinase (PI3K) and Akt (Hayashi et al., 2004; Low Wang et al., 2003; Martin et al., 2004, 2007). PDGF-stimulated vSMC response may involve signalling pathways and regulatory molecules such as protein kinase b (AKT) and mitogen-activated protein kinases (MAPKs) and matrix metalloproteinase (MMP) proteins (Braun-Dullaeus et al., 2004; Heldin & Westermark, 1999). vSMC proliferation is induced by PDGF via multiple signalling cascade pathways which include AKT phosphorylation and MAPKs (ERK1/2, JNK and p38MAPK) (Heldin & Westermark, 1999; Uzui et al., 2000). Following injury, vSMC pass from the G1-phase to S-phase cell cycle when treated with PDGF. Cell cycle progression is modulated through the activation of cyclin/CDK complexes (Braun-Dullaeus et al., 2004; S. J. Lee et al., 2016). The kinase activity of cyclin/CDK complexes is controlled by $p21^{WAF1}$ and $p27^{KIP1}$ which are negative cell cycle inhibitors, which are members of the KIP family of cell cycle proteins, which inhibit several cyclin-dependent kinase complexes.

vSMCs are found in two distinct phenotypes: contractile and synthetic. Both phenotypic types have different morphological features. Contractile SMCs are recognised as being mature and are spindle-shaped and elongated whereas synthetic SMCs take on a more cobblestone shape and are less elongated. Synthetic SMCs possess a high amount of organelles that are involved in the process of protein synthesis, in the case of contractile SMCs, these organelles are largely replaced by contractile filaments (Rensen et al., 2007). Both contractile and synthetic SMCs have different migratory and proliferative characteristics, whereby synthetic SMCs display a higher growth and migratory rate compared to contractile cells which remain in a more dormant state (H. Hao et al., 2003).

1.5.1 Embryonic origin of vascular smooth cells

The process of gastrulation which forms the ectoderm, mesoderm and endoderm are one of the most defining events of embryogenesis. This process is marked by a groove called the primitive streak which forms in the posterior region of the epiblast at embryonic day 6.5 in mice (Tam & Behringer, 1997) and the start of the third week in humans (O'Rahilly & Müller, 2010). Cells in the most proximal end (in mice) and most posterior region (in man) of the primitive streak undergo EMT to form the extraembryonic mesoderm. The embryonic mesoderm and endoderm

are derived from the mid and distal portions of the primitive streak. Lineage tracing studies have shown that vSMCs are derived from multiple developmental origins. These include the secondary heart field, neural crest, proepicardium, somites and serosal mesothelium (Majesky, 2007). VSMCs localized in the aortic root are derived only from the secondary heart field (Waldo et al., 2005). vSMCs in the thoracic and abdominal aortae are of mesodermal origin (Waldo et al., 2005; Wasteson et al., 2008). vSMCs in the carotid arteries and aortic arch are derived from the neural crest (Jiang et al., 2000; Nakamura et al., 2006). vSMCs within coronary arteries are solely derived from the proepicardium (Mikawa & Gourdie, 1996). vSMCs in the ascending aorta are derived from the secondary heart field and as well as the neural crest (Sawada et al., 2017). Many studies have demonstrated that vSMCs from specific embryonic lineages can respond in a lineage-specific manner to stimuli such as the same environmental cues when examined in identical conditions (Cheung et al., 2012; Owens et al., 2010; Topouzis & Majesky, 1996). This suggests that the embryological origin of vSMCs can influence vSMC behaviour and their role in certain processes that contribute to AS such as phenotypic switching, proliferation, and migration. Unravelling the molecular mechanisms that are fundamental for the differentiation of vSMCs by better understanding the developmental origins is key to giving a better insight into the mechanisms of vascular disease.

1.6 The Endothelium

The endothelium was once believed to act as a thin surface of the vascular tree, with the main function of selective permeability to electrolytes and water. However, major advances and research have given us a much better understanding of the endothelium and it's much more complex functions. As we already know, the vascular endothelial cells (vECs) line all blood vessels and lymphatics in the body. The vascular endothelium is a layer of these closely connected vECs. It weighs approximately 1kg in an average patient and can cover 4000-7000 m^2. For a long time, the endothelium has been undervalued as a notable organ (Kitchens et al., 2013). The endothelium functions as a physical and semipermeable barrier, limiting the entry of cells, molecules, and plasma. It can control blood perfusion to ensure nutrient supply to meet the metabolic needs of tissues by regulating the vasomotor response to vasoactive metabolites or hormones (Yuan & Rigor, 2010). The semi-permeability characteristic of the endothelium that is present on the walls of capillaries allows the nutrients, cells, and plasma fluid to move to tissues and allows the metabolic by-products to be placed back into circulation. Therefore, is crucial for fluid and metabolic homeostasis. Organ function and tissue viability rely on the

regulation of endothelial barrier function. This control is achieved through the opening and closing of cell-cell junctions. Endothelial cells have specialised junctional regions such as adheren junctions and tight junctions which can act as sites of cell-cell attachment and also function as a signalling structure to regulate vascular homeostasis, communicate cell position and apoptosis. Junction complexes can engage growth factor receptors and signalling proteins which can directly trigger intracellular signals. They can also limit nuclear translocation by binding transcription factors to the cell membrane. There are a limited number of junctional molecules that are endothelial-specific. At tight junctions, endothelial adhesion is due to claudin5, which is a member of the claudin family (Nitta et al., 2003). Other adhesive proteins present at tight junctions are members of the junctional adhesion molecule (JAM) family and occludin. At adherens junctions, adhesion is mediated by vascular endothelial VE-cadherin (VEC), a transmembrane protein (Lampugnani et al., 1992). The Nectin-afadin complex is important in the organisation of both adherens junctions and tight junctions (Takahashi et al., 1999). Junctions are essential for maintaining the integrity and function of the vessel walls and any modifications to the intracellular signalling of junctional proteins or their organisation can have a negative effect on vascular homeostasis (Dejana, 2004).

1.6.1 Vascular permeability

Vascular permeability occurs when macromolecules and solutes transfer, into and out of the blood through the vessel wall, in an unstimulated environment. Small molecules (<40kDa) tend to extravasate sporadically (Egawa et al., 2013). However, active disruption to the vascular barrier is required for larger molecules to extravasate into the surrounding area. Post capillary venules are where the induced leakage usually takes place (Majno et al., 1969), however larger venules and capillaries can also leak (Roberts & Palade, 1995). Many pathophysiologic and physiologic can lead to acute and chronic changes in endothelial permeability. Acute inflammatory mediators such as histamine and thrombin can impact the endothelium, stimulating it to open its intercellular junctions (Lum & Malik, 1996). The signalling involved in mediating these responses includes protein kinase C (PKC) specific phosphorylation of linking proteins at the cell-cell and cell-matrix junctions. This increased phosphorylation leads to cell rounding, actin re-organisation and increased paracellular transport (Lum & Malik, 1996). Another critical event is the activation of Myosin light chain kinase (MLCK) by

inflammatory mediators resulting in actin-myosin contraction which leads to the retraction of endothelial cells (Lum & Malik, 1996). Endotoxin, Lipopolysaccharide (LPS) causes endothelial hyperpermeability by stimulating the activity of the small GTPase Rho A and its effector Rho Kinase (ROCK). Increased levels of LPS cause phosphorylation and activation of $pp60^{Src}$ which is a member of the Src family kinase. This event disrupts the endothelial barrier and plays a critical role in LPS-mediated vascular dysfunction (Joshi et al., 2014). Increased vascular permeability refers to structural and functional changes in the vessel wall, which allows the passing of cells, plasma and macromolecules into the intima, which occurs at the start of AS, which is mainly due to changes in the endothelial barrier function (Rahimi, 2017). ED initiates a dysregulation in transendothelial migration, which leads to a build-up of deposits of cells and molecules in the intima. This results in an increase in the size of the intima and inflammation, which features in early AS (De Caterina R, 2007).

Within the vasculature, there a two main types of microenvironmental cues that can induce remodeling of the vascular endothelium, cyclic strain and shear stress. Shear stress, effects endothelial cells exclusively and pertains to the force of the consistant blood flow over the endothelium. The endothelium can determine shear stress as a mechanotransducive force, in which signals are changed from mechanical to biological signals which affects the intracellular pathways and patterns of gene expression (D. A. Chistiakov et al., 2017). Cyclic strain refers to the force placed on blood vessles due to pressure gradients which results in alterations in EC morphology and orientation and blood vessel wall distention and tension (X. M. Liu et al., 2013).

1.6.2 Endothelial regulation of vascular tone

A healthy endothelium is essential for the regulation of vascular tone and structure, as well as exerting fibrinolytic, anticoagulant, and anti-platelet properties. The vascular tone is maintained by the release of vasodilator and vasoconstrictor substances. NO is one of the major vasodilative substances that is released by the endothelium, which was originally discovered and identified as an endothelium-derived relaxing factor (EDRF) in 1980 (Furchgott & Zawadzki, 1980). Bradykinin and prostacyclin (PGI_2) are also examples of endothelium-derived vasodilators (Drexler, 1998). NO is known to play an important role in the physiological regulation of the CVD since aberrant bioavailability or production of NO

accompanies diseases such as AS and hypertension (Moncada & Higgs, 2006). NO, and L-citrulline is produced by L-arginine via the enzyme, endothelial nitric oxide synthase (eNOS). Other distinct genes that encode NOS isozymes that catalyse the production of NO include Neuronal NOS (nNOS) and cytokine-inducible NOS (iNOS) (Andrew & Mayer, 1999). NO production from L-arginine by eNOS requires many co-factors such as flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4), calmodulin, flavin mononucleotide (FMN) and iron protoporphyrin (Alderton et al., 2001; Förstermann & Münzel, 2006). This conversion of L-arginine to NO by eNOS can be carried out through two independent steps. The first step requires calcium-calmodulin for the hydroxylation of L-arginine, leading to the formation of N^G-hydroxy-L-arginine, this reaction is accelerated by BH_4. The second step, which leads to the generation of NO and L-citrulline, involves the oxidation of the intermediate, using O_2 and NADPH. This step is also accelerated by BH_4 and is calcium-calmodulin-dependent (Andrew & Mayer, 1999). Increased intercellular Ca^{++} in response to shear stress or vasodilator agonists causes the inhibitor caveolin to be displaced from calmodulin activating eNOS. NO diffuses to the vSMCs and causes relaxation by activating guanylate cyclase (GC), this, in turn, causes an increase in intracellular cyclic guanosine monophosphate (cGMP) (Félétou, 2011). NO, in addition to causing vasodilation, has beneficial roles such as antiproliferative and anti-inflammatory properties that inhibit the atherosclerotic process. ECs and platelets that produce NO have an antithrombotic effect, whereby platelet activation is reduced (Gkaliagkousi & Ferro, 2011). One of the most potent endogenous vasoconstrictors identified to date is endothelin-1 (ET-1). Angiotensin is also another vasoconstrictor substance produced by the endothelium (Sowers, 2002). Angiotensin II can act as a pro-oxidant which stimulates ET-1 production as well as acting as a vasoconstrictor. Plaque can form as a result of ET-1 and angiotensin II being released, as the vasoconstrictors promote the proliferation of SMCs (Drexler, 1998). ET-1 has been shown to contribute to the increased vascular tone of atherosclerotic plaque (Kinlay et al., 2001). Damage to the endothelium causes an imbalance between vasodilation and vasoconstriction, which instigates processes that promote AS, for example, the generation of cytokines and increased endothelial permeability (Ross, 1999). Reduced activity and production of NO can be one of the premature signs of AS. Chronic consumptions of EtOH has been suggested to increase endothelium-dependant relaxation (Hatake et al., 1994; Williams et al., 1990), and stimulate vascular prostacyclin formation (Wakabayashi, 2014) and desensitise response to vasoconstrictive action of phenylephrine (Strickland & Wooles, 1988). Acute ethanol treatment on ECs has been shown to enhance agonist-stimulated nitric oxide (NO) release (Davda et al., 1993), increase blood flow and

decrease coronary artery resistances in humans (Cigarroa et al., 1990). However negative influences of EtOH on arterial tone regulation have been observed, as chronic EtOH ingestion has been demonstrated to increase vascular contractility (Hatton et al., 1992; Sahna et al., 2000). Acute EtOH ingestion has been shown to inhibit endothelium-dependent relaxation, whereas chronic EtOH ingestion has no effect. (Hatake et al., 1991). It has also been shown to inhibit both cyclic GMP-independent and -independent relaxation mechanisms in rat arteries (Hatake et al., 1993; Katsuhiko Hatake et al., 1989).

1.6.3 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules that have a pivotal role as second messengers with cells and are derived from oxygen metabolism (Phaniendra ref). Members of the ROS family include alkoxyl radical (RO·), superoxide (O_2-), hydroxyl radicals (OH·), peroxyl radical (ROO·) hydrogen peroxide (H_2O_2), peroxynitrite (ONOO−), hypochlorous acid (HOCl) and ozone (O_3). ROS is essential for signalling molecules that maintain vascular homeostasis. An increase in ROS can lead to oxidative stress (OS) which can contribute to vascular disease progression. Vascular cell homeostasis is maintained by ROS by controlling the fate and phenotype of many types of cells for example; SMCs, ECs and stem/progenitor cells (Kattoor et al., 2017). OS is usually caused by an imbalance in the oxidant and antioxidant mechanisms. Alterations between antioxidants such as heme oxygenase, superoxide dismutase and glutathione peroxidase and oxidant enzymes such as oxidase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (Incalza et al., 2018). Regulation of the production of ROS from the endothelium by shear stress has been reported. The signalling response due to the abrupt decrease of shear that can occur during ischemia can lead to membrane depolarization which triggers PI3K/Akt activation causing NADPH oxidase activation and generation of ROS (Chatterjee et al., 2012). Contradicting these results, shear stress has been shown to upregulate the expression of peroxiredoxin 1 (PRX 1), which is an antioxidant. It plays an important role shear-dependent regulation of AS (Mowbray et al., 2008). Many studies have shown that flow patterns are critical in regulating ROS/NO balance. Both pro-atherogenic (nonunidirectional or turbulent) shear stress and anti-atherogenic shear stress (unidirectional laminar or steady) can stimulate ROS production. Anti-atherogenic shear stress usually generated lower ROS levels and greater bioavailability of NO, whereas pro-atherogenic results in higher levels of ROS and lower bioavailability of NO (Hsieh et al., 2014).

One of the primary causes of ED is the imbalance between antioxidant defence systems and the generation of ROS. ED is characterised by a decrease in NO bioactivity and usually occurs in the initial development of AS (Davignon & Ganz, 2004). As we already know, the endothelium maintains the balance of vasoconstriction and vasodilatation, stimulates fibrinolysis, thrombogenesis and adhesion and aggregation of platelets and promotes proliferation and migration of SMCs. NO is greatly involved in all of these processes, directly and indirectly, and any disruption to these tightly regulated balances can lead to ED. In CVDs, oxidative stress (OS) is the most common denominator causing ED and can be related to the reduced production of NO and its decrease in bioavailability (Félétou & Vanhoutte, 2006). The generation of superoxide anion and hydrogen peroxide in the vasculature induced by EtOH is associated with ED, hypertension and vasoconstriction (Passaglia et al., 2015). ROS generation by EtOH is important to its pathophysiology in the vascular system, as EtOH is metabolised to acetaldehyde in the liver by alcohol dehydrogenase (Yongke Lu & Cederbaum, 2008). Acetaldehyde is oxidised to acetate by acetaldehyde dehydrogenase which results in ROS generation and reduced NO levels (Deng et al., 2020). Some studies have shown that enzyme NADPH oxidases play a role in dysfunctions induced by chronic EtOH consumption in many tissue types (De Minicis & Brenner, 2008; Kono et al., 2000; V et al., 2006). Increased vascular OS as a result of EtOH consumption is related to the activation of NADPH oxidase and this molecular mechanism is associated with an increase in blood pressure caused by chronic EtOH ingestion. NADPH oxidase is the main source of ROS in ECs and SMCs (Touyz & Briones, 2011) and is considered a key factor in vascular dysfunctions induced by EtOH. It is speculated that OS plays a key role in the pathogenesis of many EtOH related disorders. OS and the generation of ROS is a result of EtOH metabolism in the vascular system (J et al., 2005). EtOH has also been shown to cause aortic vSMC proliferation. This is due to an increase in Ox-LDL-mediated OS and elevated levels of homocysteine which leads to changes in the aortic wall that is proatherogenic (Shirpoor et al., 2013).

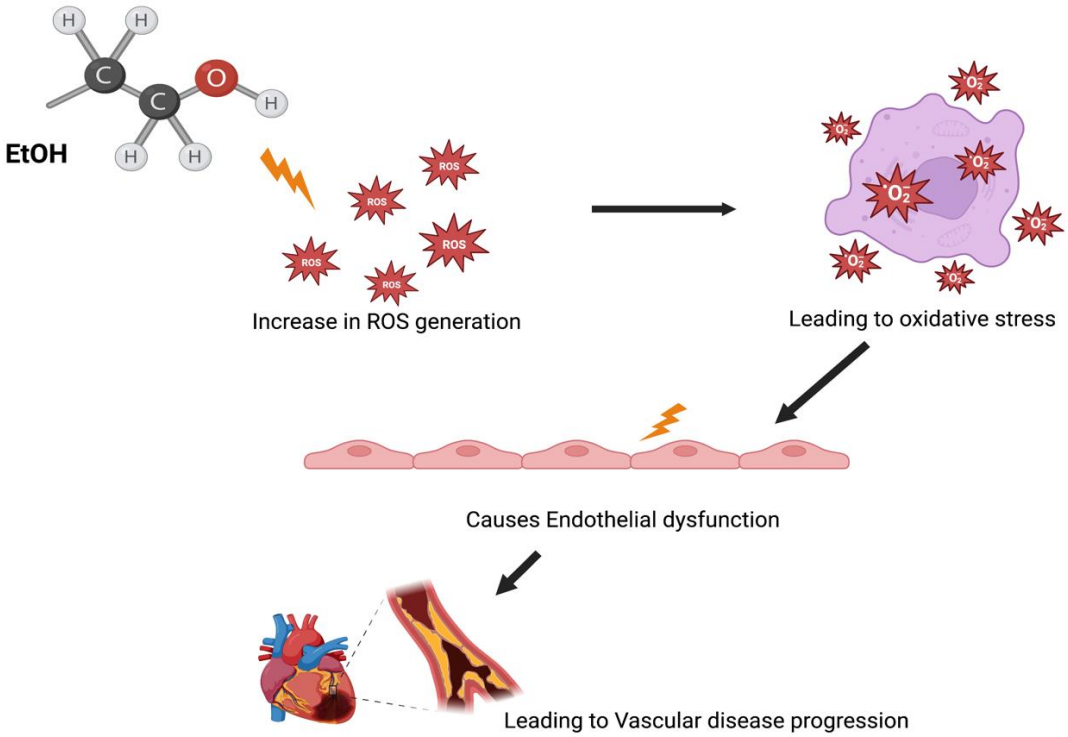


Figure 5 The relationship between EtOH and ROS. Ethanol causes an increase in ROS which can lead to oxidative stress (OS) which can contribute to vascular disease progression due to endothelial dusfynction. (Created with BioRender.com).

1.7 Extracellular Vesicles (EVs)

EVs are cell-derived, membrane-bound lipid vesicles which are shed from the PM. They are spherical in shape and have proteolipid bilayer membrane and have secretory properties. They range in size from 20-1000nm (Momen-Heravi et al., 2018). '*MISEV2018 recommends the use of 'extracellular vesicle' as the best generic terminology for use in publications, in part due to the challenges in confirming the biogenesis mechanisms of exosomes, microvesicles, and other particles, and in part due to the vague and varied uses of other terms'*(Doyle & Wang, 2019). EVs are released by most eukaryotic cell types, these minuscule bubbles of information are used for intracellular communication and for removing cellular waste. EVs express proteins on their membrane and contain other lipids and proteins as well as genetic information contained in mRNAs, miRNAs and DNA. Based on their size, composition, biogenesis and functions (Figure 5). EVs can be easily classified into microvesicles (MVs), exosomes and apoptotic bodies (J. Crenshaw et al., 2019; L. Yuan & Li, 2019). Firstly, we have MVs which are formed by budding directly off the PM and are usually 100-1000nm. MVs are usually enriched with proteins such as P-selectin glycoprotein Ib, arrestin and integrins (Heijnen et al., 1999). MVs

tend to have more proteins that have undergone a covalent processing event called posttranslational modifications (PTM), phosphoproteins and glycoproteins (Palmisano et al., 2012). MVs are released by cells under physiological conditions. The production of MVs increase when external physiological cues such as hypoxia, cell injury, shear stress, oxidative stress and proinflammatory stimulant activate cells (Hugel et al., 2005; VanWijk et al., 2003). As mentioned, MVs are formed through budding of the PM and by outward protrusion. This is initiated by an overload of intracellular cytosolic calcium that activates a calcium sensitive protease called calpain. Activated Calpain then detaches membrane proteins and causes proteolysis of cytoskeletal proteins (Pasquet et al., 1996). This leads to remodeling of the cytoskeleton by cleaving the actin protein which allows blebbing of MVs. MVs are released from the PM on caveolae domains or lipids rafts. A lipid bilayer makes up the the PM which is where phosphatidylserine resides. Phospholipid asymmetry is controlled by enzymes scramblase, floppase and flippase (Daleke, 2003). Floppase is activated following cellular activation via an increase of cytosolic calcium. This process allows for lipid movement to the outer membrane. Scramblase activation enables lipid movement in a bi-directionally. However flippase is inactivated resulting in dropping of negatively charged phosphatidykserine to the outer leaflet (Turturici et al., 2014).

Cells dying, undergo apoptosis and release apoptotic bodies from the PM, which vary in size between 50 – 2000 nm. These vesicles lack glycoproteins and possess DNA-binding histones (Théry et al., 2001). Lastly and most importantly, Exosomes are the smallest type of EV and are usually present in bodily fluids (Figure 6). Exosomes were believed to have a basic function as cellular waste disposal; however, extensive research has revealed their various functions such as intercellular communication, antigen presentation, immune response and many more. Exosomes derived from cancer cells have been shown to play a role in tumour metastasis and proven to carry cargo that initiates cell proliferation and angiogenesis (Momen-Heravi et al., 2018). Exosomes have also been shown to have a therapeutic purpose, as they have the ability to cross the blood-brain barrier and have low immunogenicity. This has led them to be interrogated for use in targeted drug delivery therapy (Crenshaw et al., 2019). Exosomes isolated from the cerebrospinal fluid can be used to diagnose neurodegenerative disorders and more specifically to diagnose prion diseases. Exosomes have also been isolated from urine and have served as biomarkers for prostate cancer (Andaloussi et al., 2013). Exosomes are widely considered to be a critical candidate for the role of biomarkers for detecting disease and making a prognosis. Exosomes show a great potential tool for clinical application, including liquid

biopsy. Their combination of various contents can serve as biomarkers, providing a more accurate diagnosis and prognosis for an endless list of diseases. However, there are still some barriers between basic versus clinical practice (Zhou et al., 2020). Firstly, due to exosomes being present in almost all bodily fluids, it is essential to have an extraction and classification method of exosomes to cover all the different types of liquids available. Accurate measurements of exosomes and their level of concentration in clinical samples are essential. This has been proven difficult, as isolating pure exosomes from both biological and through cell culture media is complex. When using low biofluid volume, it is critical to have more efficient methods in place to ensure there is a high yield and purity of exosomes, in order to be able to apply them as biomarkers (Kurian et al., 2021).

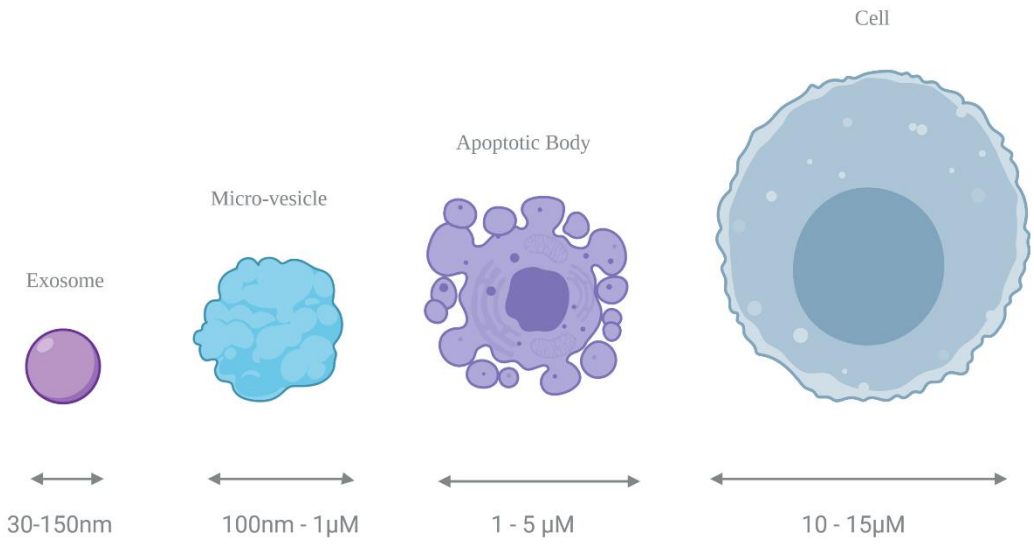


Figure 6 Comparison of size ranges of EVs. The smallest EV is the exosomes, ranging between 30-150nm. MVs are slightly larger ranging from 100nm to 1µM in diameter. Apoptotic bodies are the largest and most heterogeneous, ranging anywhere between 1 – 5µM. (Created with BioRender.com).

1.7.1 A History of exosomes

Exosomes were first discovered in 1983, coincidently, they were discovered by two independent research groups that released their publications only a week apart from each other (Harding et al., 1983; Pan & Johnstone, 1983). They both reported the movement of transferrin receptors (TfRs) and tracked them moving from the PM into maturing reticulocytes. They discovered that cells take up these transferrin receptors, and pack them into small vesicles. It

was thought that these vesicles were then trafficked to lysosomes and destroyed. However, they were released out of the mature reticulocytes and into the extracellular space and termed as we know them now, 'exosomes' (Johnstone, 2006). There are a variety of techniques available in order to isolate exosomes and characterise them based on their quantity, size and morphology. Isolation and purification methods include; differential ultracentrifugation, sucrose gradient centrifugation, size-exclusion chromatography filtration, ExoQuick™ and microfluidics (Momen-Heravi et al., 2013; Yakimchuk, 2015). Exosomes can be characterised using western blot, ramen spectroscopy, flow cytometry, nano site and electron microscopy (Baranyai et al., 2015; H. K. Kim et al., 2002; Mehdiani et al., 2015; Szatanek et al., 2017; Tirinato et al., 2012).

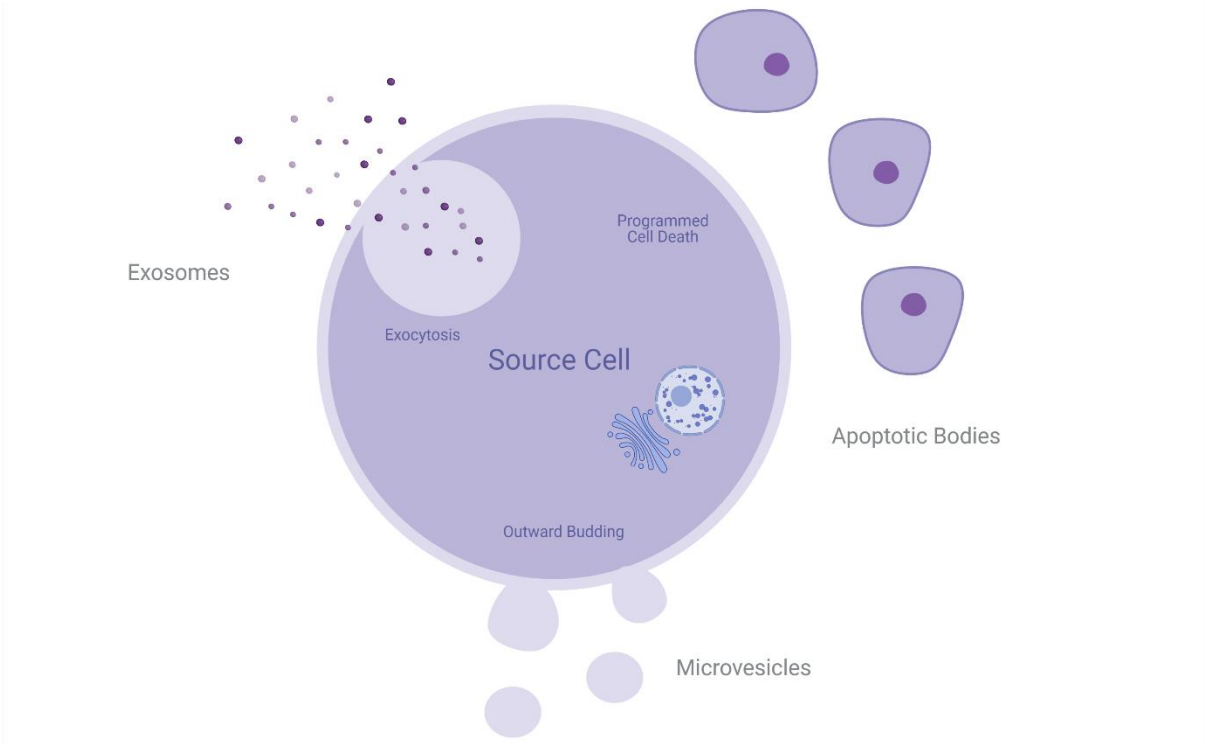


Figure 7 Subtypes of EVs and mode of formation. There are three subtypes of EVs that can be classified based on their mode of biogenesis. EVs are released from the cell via exocytosis. MVs are secreted by the outward budding of the plasma membrane. Apoptotic bodies are released by dying cells via apoptosis. (Created with BioRender.com).

Herein, we mainly focus on exosomes as they introduce the advantages of liquid biopsy and their application as a potential complement to personalised medicine particularly in CVDs. There are many prospects of exosomes in clinical applications (Zhou et al., 2020). Exosomes are present in nearly all bodily fluids therefore show significant superiority over other potential sources of liquid biopsy. They are secreted by all living cells and contain important biological

information from their parent cells and they possess a lipid membrane bilayer which gives them high stability when encapsulated. As exosomes express specific proteins such as CD63, CD81, CD82 and CD9, their identifaction is clear (R. Xu et al., 2016). Every cell secretes unique exosomes with characteristics and contents that are themselves unique. The cell origin is extremely important for the pontial harmful or beneficial characteristic of these sectreted exosomes. Exosomes released from cancerous cells are more likely to lead to the progression of cancer. Exosomes released from stem cells are likely to promote regeneration and anti-inflammatory properties like their parent cells. Various conditions and factors can cause an increase in exosome production. Under certain conditions, cells grown in culture tend to secrete more exosomes, typically under stress conditions such as hypoxia (C. Shao et al., n.d.). Intracellular calcium, Ceramide metabolism and endoplasmic reticulum stress, all reflecting the metabolic state of the originating cell, play a role in exosome biogenesis and release (Maligianni et al., 2022).

1.7.2 Biogenesis of Exosomes

Exosome biogenesis begins when the invagination of the endosomal membrane occurs and a multivesicular body (MVB) forms inside the endosome as a result (H. Shao et al., 2018). Following this, proteins and nucleic acids are formed into these MVBs (Raiborg & Stenmark, 2009). These MVBs undergo maturation, and the cargo they have encapsulated is sorted. They can fuse with lysosomes and become degraded. In the endolysosomal degradative route, proteins from the cell membrane are packaged into intraluminal vesicles (ILVs) which fuse with lysosomes and destroy proteins through the release of enzymes. They can also be secreted as exosomes into the extracellular space when the MVB fuses with the parental cell membrane (Samanta et al., 2018) (Figure 7). Many studies have shown that exosomes are formed through two major pathways that are highly regulated by multiple signal transduction cascades. These pathways are (ESCRT)-dependent and ESCRT-independent. Two important proteins, the endosomal sorting complex required for transport (ESCRT) and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes are essential for exosome formation. The ESCRT-dependent pathway involves four separate protein ESCRTs that make up this complex protein machinery, these are ESCRT-o, ESCRT-I, ESCRT-II and ESCRT-III complexes. All four interact with each other to contribute to the MVB formation and sorting of cargo. ESCRT-o complex interacts with ESCRT-I and ESCRT-II complexes. The membrane

becomes deformed when it undergoes ubiquitination, this process involved the addition of ubiquitin, a protein, to the lysine residues of target proteins. These ubiquitin tags are picked up by the ESCRT protein complex and form a neck which leads to a budding membrane. The total complexes previously formed will then interact with ESCRT-III which in turn interacts with Vps4 t, which forms ILVs. The ESCRT-III then detaches from the MVB membrane after interacting with Vps4 (Henne et al., 2011). The second mechanism is termed the (ESCRT)-independent pathway. It was proven that exosomes still formed when ESCRT protein was depleted, suggesting that ESCRT proteins were not essential for the formation of MVB (Stuffers et al., 2009). This particular pathway uses a lipid microdomain, termed lipid rafts contained within the PM and uses proteins such as tetraspanins (Stuffers et al., 2009). This pathway is driven by the presence of certain lipids, in particular those that are able to form shapes of cones and inverse cones. Exosome membranes contain cholesterol, sphingomyelin, phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylinositol (PI), ceramides, glycosphingolipids and many more lipids (Skotland et al., 2017). Exosomes are enriched with PS and PE, which promotes exosome biogenesis and secretion by increasing membrane budding efficiency. PA has also been shown to promote exosome biogenesis and Phospholipase D2 (PLD2) supports the endosomal budding of exosomal cargos (Luis Egea-Jimenez et al., 2020). Along with all these lipids, ceramide also plays a pivotal role in regulating the biogenesis of exosomes and imposing curvature on membranes which is critical for the budding of exosome vesicles into multivesicular endosomes (Trajkovic et al., 2008). It was first discovered that exosome biogenesis via ESCRT-independent mechanism required the generation of sphingolipid ceramide to transfer exosome-associated domains into the lumen of the endosome. This then caused negative curvature on the membranes, thereby promoting budding (Goñi & Alonso, 2009). Inhibition of the enzyme neutral sphingomyelinase 2 (nSMase2) that forms ceremide from sphingomyelin has been shown to impair exosome biogenesis. (Trajkovic et al., 2008). Therefore, this process highlights the key role that exosomal lipids play in this ceramide dependent mechanism.

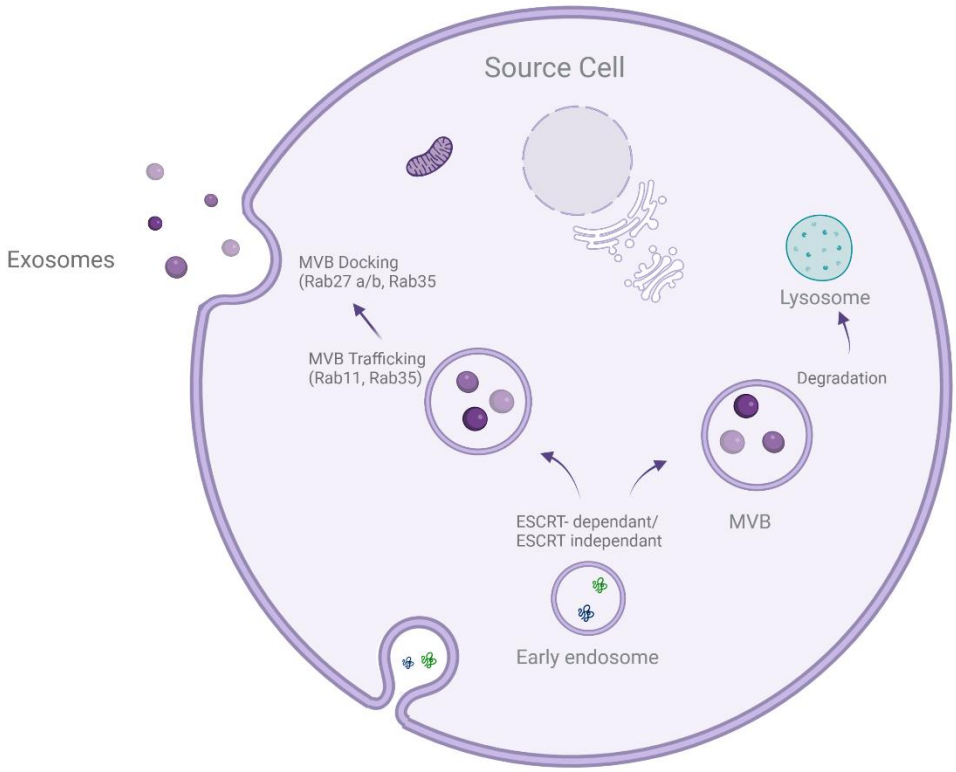


Figure 8 Exosome biogenesis. Exosome biogenesis can be conducted in an ESCRT-dependent/independent manner. The internalisation of the PM initiates the formation of exosomes by endocytosis. MVBs are produced via the fusion of early endosomes. Upon maturation, MVBs are directed to the PM via a tight Rab GTPase-dependent multistep process. SNARE-mediated plasma membrane fusion event occurs which is followed by secretion into the extracellular space. (Created with BioRender.com)

1.7.3 Exosome structure and cargo

As we already know, exosomes, upon secretion, deliver their cargo to distant or adjacent cells and play a key role in regulating gene expression and modification of phenotypes. Therefore the contents contained within the exosomes are a crucial factor in the functional characteristics of this particular vesicle (Tian et al., 2010). Exosomes contain many different proteins that mirror the content of the cell they originally came from (Boriachek et al., 2018). Many exosomes from a variety of cell types share common proteins in their cargo. As previously mentioned, these include ESCRT, a membrane transport protein, as well as other proteins such as metabolic and signalling proteins (Ibrahim & Marbán, 2016). The most common exosomal proteins are tetraspanins, including CD63, CD81, CD82 and CD9. These are responsible for fusion, cell penetration and invasion. These tetraspanins are often used as specific markers for exosomes. However, they have been detected on cell surfaces and may be markers for other

vesicles such as MVBs indicating they are part of PM-derived vesicles (Jankovičová et al., 2020). Other proteins that are common include Rab family GTPases, MVB-associated protein (Alix-1), adhesion molecules (CD45 and CD11b), heat shock proteins (hsp60) and many more (C. Liu & Su, 2019). ExoCarta is an exosome database that provides all information on exosomal contents that have been identified in multiple organisms. As many as 10,000 proteins have been discovered and characterised in relation to the cargo within the exosomes (http://exocarta.org/; accessed on 1st June 2021). As previously mentioned, EVs are also characterised by the presence of lipids such as PA, PI, PS and ceramides, which play a role in structure stability as well as intercellular signalling (Skotland et al., 2017). EVs can also encapsulate various nucleic acids including messenger RNA (mRNA), long non-coding RNA (lnRNA), micro RNA (miRNA), mitochondrial DNA and genomic DNA (Valadi et al., 2007).

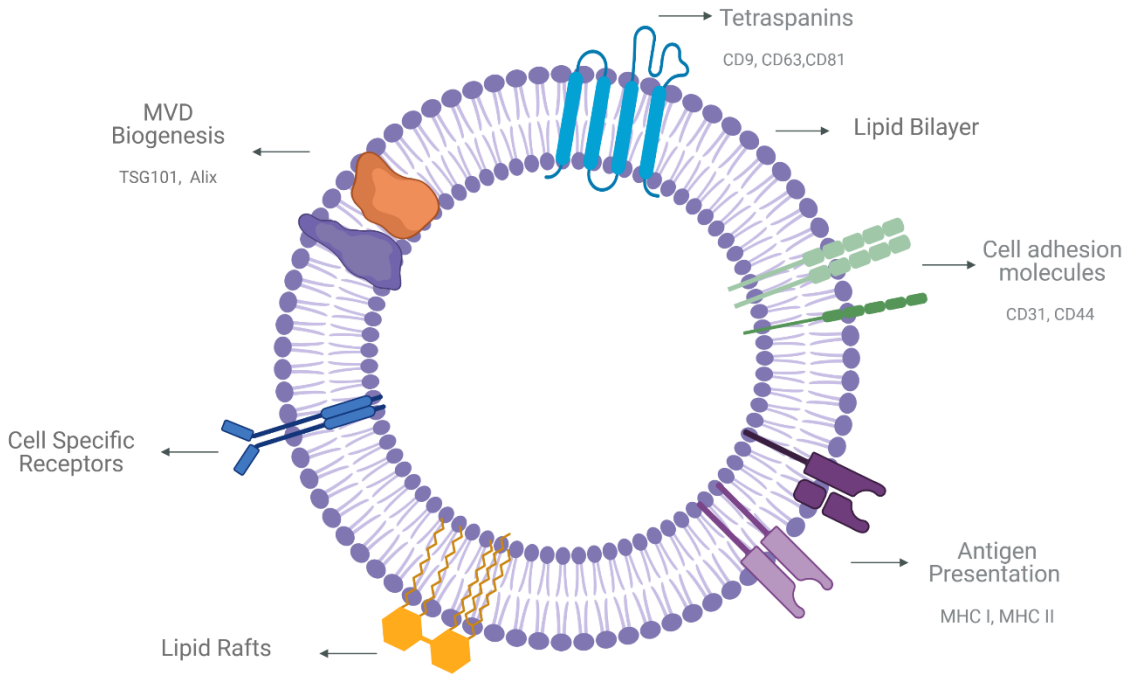


Figure 9 Structure and molecular components of exosomes. Exosomes structure includes a PM-derived phospholipid bilayer containing cytosol components from the cell of origin. The cell of origin, their cell state (healthy or stressed) and extracellular stimuli are what contribute to an exosome's composition. There are various proteins (tetraspanins), lipids, and miRNAs characteristic of exosomes, which are depicted above. (Created with BioRender.com)

1.7.4 MVB transportation, Exosome release and uptake

For exosomes to be released, there is a variety of cellular steps that need to be accomplished. ILVs must be formed in MVBs, these MVBs then must be transported to the PM, once they have reached the PM, the MVBs then fuse with the PM. Many proteins are involved in these

steps including Rabs, SNAREs, and coat proteins, all teaming together to mediate the transport, tether and fusion of these MVBs to the PM (H. Cai et al., 2007). Using RNA interference (RNAi), five Rab GTPases and other associated proteins were shown to be involved in trafficking mature MVB and promoting exosome secretion in HeLa cells (Ostrowski et al., 2010). It was found that exosome secretion decreased following the knockdown of Rab2b, Rab5a, Rab9a, Rab27a and Rab27b. In particular, they showed that RAB27a and RAB27b prevented the docking of MVBs to the PM through the use of total internal reflection fluorescence (TIRF) microscopy (Ostrowski et al., 2010). Another important protein, Rab GTPase-activating protein (Rab-GAP) TBC1D10 was found to contribute to exosome function and regulate exosome secretion (Hsu et al., 2010). Rab35, which is implicated in exosome secretion is also activated by TBC1D10 (Hsu et al., 2010). Rab11 has also been shown to be involved in exosome production (Beckett et al., 2013; Koles et al., 2012). The are many Rab family members that are involved with exosome secretion, it is important to note that the suppression of an individual Rab will not block this process due to a possible reserve system (McGough & Vincent, 2016). SNARE proteins play a role in the release of ILVs as exosomes by facilitating the fusion of these vesicles with the PM or membrane of different organelles. Three or four SNARES are required to form a SNARE complex, which forms a structural motif of four coiled-coil helices (Hessvik & Llorente, 2018). SNAREs are classified as Q-SNAREs or R-SNARES. When fusion occurs on the PM, generally one R-SNARE, usually Vesicle SNARES (vSNAREs) which are located on the MVB are recognised by three Q-SNARES, generally target SNARES (tSNAREs) which are located on the PM, all combining to drive this fusion (H. Cai et al., 2007). Another R-SNARE protein, vesicle-associated membrane protein 7 (VAMP7) has been shown to be involved in the mediation of exosome secretion in K562 cells (Fader et al., 2009). They also show that when VAMP7 which has a long N-terminal extension of 90 amino acids, is overexpressed, this inhibits SNARE complex formation and causes a decrease in the secretion of exosomes. They found that as a result of this overexpression, there was an increase of enlarged MVBs that were distributed adjacent to the cell periphery, leading to the probability that the fusion step was inhibited leading to an impaired PM (Fader et al., 2009). Over the years, many other SNARE proteins such as Syntaxin 1A and YKT6 have been suggested to play a role in the release of exosomes (Gross et al., 2012; Koles et al., 2012). However, to this date, no independent SNARE protein is known to solely be involved in the release of exosomes.

There are two different mechanisms in which acceptor cells uptake exosomes. The first mechanism involves the exosomes being internalized by fusing directly with the PM of the recipient cell, causing the vesicle to release its cargo into the cytosol (Montecalvo et al., 2012; Parolini et al., 2009). The hemi fusion stalk, which is a membrane structure, where two membranes connect to each, forms between the PM and the lipid bilayers of the exosomes, forming one structure. Rab and SNAREs facilitate this fusion (Chernomordik et al., 1987; Jahn & Südhof, 1999). Adhesion molecules, integrins and lipid raft domains that are present on the surface of the exosomes mediate the membrane fusion with the target cell (Mulcahy et al., 2014; Valapala & Vishwanatha, 2011). Studies have used a fluorescent probe, octadecyl rhodamine B chloride (R18) and introduced it to pre-existing membranes. This is a widely used method to distinguish endocytosis from fusion. The R18 is introduced into the bilayer of the exosomes at self-quenching concentrations, following fusion with the unlabelled membrane, the fluorescent probe becomes diluted, this follows with concomitant enhancement in fluorescence, which allows for detection of any membrane fusion (Nunes-Correia et al., 2002). Dendritic cells (Montecalvo et al., 2012) and tumour cells (Zheng et al., 2019) have demonstrated their ability to undergo membrane fusion. Some researchers have speculated that upon biophysical analysis of the membrane through lipid composition or fluidity, they showed there was an increase in rigidity and increase in sphingomyelin content in the exosomes when they were within the tumour microenvironment at a low pH. This environment was likely to have facilitated the increase in fusion efficiency (Parolini et al., 2009).

The second mechanism is that exosomes are internalized via the endocytosis pathway (Tian et al., 2010). Exosomes can be internalised by; phagocytosis, lipid-raft mediated, clathrin-mediated endocytosis, micropinocytosis or caveolin-mediated endocytosis (Gurung et al., 2021). These different mechanisms for the uptake of exosomes can co-exist. Various endocytic pathways were shown to mediate exosomes to be internalised by ovarian tumour cells through many types of endocytic pathways (Escrevente et al., 2011). It was also found the uptake of PC12-derived exosomes by recipient bone marrow-derived mesenchymal stem cells was via micropinocytosis and clathrin-mediated endocytosis (Tian et al., 2014). Exosomes derived from K562 cells were shown to undergo cellular internalisation via phagocytosis but also were lipid raft dependent (Feng et al., 2010). The surface proteins and the specificity of the recipient cells as well as external factors can influence how exosomes are up taken (Clayton et al., 2004). Surface proteins present on exosomes can guide and specifically deliver to target cells for uptake. One research group showed that when rabies viral glycoprotein was enriched with

exosomes that carried a cargo of short interfering (si)RNA which were delivered to the brain in mice (Alvarez-Erviti et al., 2011).

1.7.6 Therapeutic and diagnostic potential of exosomes in CVD

There has been increasing evidence of the therapeutic potential of exosomes in CVD. Exosomes released from cardiac progenitor cells (CPCs) have been shown to reduce cell death in myocytes in an animal model for Acute myocardial infarction (AMI) (L. Chen et al., 2013), enhance endothelial cell migration, thus promoting angiogenesis (Dougherty et al., 2020; Vrijsen et al., 2010), and improve both acute and chronic cardiac function and decreased fibrosis (Gray et al., 2015). Many groups have shown evidence that post-AMI, stem cell-derived exosomes are involved in the increase in cardiac function. A variety of stem cells such as embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), and cardiac stem cells (CSCs) release exosomes that have been shown to display cardioprotective functions (Davidson & Yellon, 2018). Exosomes derived from Mouse embryonic stem cells (mESCs) have been shown to promote CPC survival, and neovascularization and reduce fibrosis (M. Khan et al., 2015). Cardiophere-derived cells have also been shown to secrete exosomes that improve acute and chronic cardiac function, reduce scarring, prevented adverse remodelling and enhance the proliferation of cardiomyocytes in the porcine MI animal model (Gallet et al., 2017). Exosomes derived from stem cells have regenerative and cardioprotective roles, however, one of the difficult challenges of these is a therapeutic delivery to the heart. *In vivo* tracking of murine melanoma, B16-BL6-derived exosomes post intravenous injection indicated that exosomes distributed to the liver and then to the lungs (Takahashi et al., 2013). In contrast, intramyocardial injection of exosomes has led to better results, however it is an invasive yet effective procedure. A novel method was created to overcome these invasive limitations, by using a cardiac homing peptide which is known to target the myocardial infarction (MI) heart, to tag exosomes. This increased cardiomyocyte proliferation and angiogenesis, decreased angiogenesis and suggested that this method increased the efficacy of intravenously delivered exosomes (Vandergriff et al., 2018). Most studies on exosomes are performed in vitro and/or in animal models, however research into exosomes and their characteristics in cardiovascular patients are still poorly understood. Exosome based therapy has huge benefits, however more research is essential to make it more clinically practical and more effective and less invasive delivery methods are essential to explore the clinical potential of exosomes (Bellin et al., 2019).

The cargo and quantity within the exosomes can differ under varying pathological CVDs. Exosomes have been suggested to be a novel and important diagnostic biomarker holding potential value. However as previously mentioned, the challenges in obtaining high quantities of purified exosomes for use as a diagnostic biomarker has it's challenges (Jansen & Li, 2017). Researchers have shown the impacts of mechanical cues such as shear stress and cyclic strain on EV production and cargo loading. HUVECs exposed to shear stress have been shown to increase miR-143/145 in HUVEC EVs by 30 fold when compaered to only 10 fold in cells. miR-143/145 which has been shown to control SMC phenotype (Hergenreider et al., 2012). It has also been shown that increasing shear stress duration can proportionally increase the formation of EVs from platelets (Miyazaki et al., 1996). It has also been demonstrated that fluid shear flow stimulation (35 dynes/cm2) can increase EV release from osteocyte-like MLO-Y4 cells by 20 times when compared to the control (Morrell et al., 2018). Shear stress is widely used in bioreactor systems to increase EVs (Syromiatnikova et al., 2022). Cyclic stretch has also been shown to induce vSMCs to secrete connective tissue growth factor and promotes endothelial progenitor cell differentiation and angiogenesis (J. Yan et al., 2020). Exosomes derived from bone marrow MSCs that have been exposed to cyclic mechanical stretch have been shown to inhibit RANKL-induced osteoclastogenesis via NF-κB signaling pathway (F. Xiao et al., 2021). Futher research into shear stress and cyclic strain on EVs production and bioactivity is needed to undertand the full impact of the mechanical cues on providing new insights into therapeutic strategies.

1.7.6 Endothelial-derived EVs

Endothelial-derived EVs are characterised by their expression of various EC-specific surface markers (CD31, PECAM and von Willebrand factor) (Desideri et al., 2021). There is growing evidence that vascular EC-derived EVs play an important role in physiological processes maintaining vascular development (Sheldon et al., 2010), vascular cell protection and EC regeneration (Abid Hussein et al., 2005), vascular homeostasis (Ridger et al., 2017), and pathological progression of vascular disease (Abid Hussein et al., 2005; Bellin et al., 2019). The endothelium is essential for maintaining vascular homeostasis and preventing the progression and development of AS. The endothelium's ability to respond to intrinsic physical stimuli enables it to carry out this important role. However, its response to CVD risk factors such as hyperglycaemia, LDL and nicotine affects the functional integrity of the endothelium. This is followed by mechanisms involved in subclinical AS leading to ED, which is observed in the early stages of AS (Park & Park, 2015). ED is characterised by decreased bioavailability

of vasodilators such as NO and/or endothelial-derived constricting factors. Endothelial activation is a result of ED, which is characterised by a shift in the action of the endothelium toward a proinflammatory state, procoagulant, reduced vasodilation and a proliferative state that promotes the development of AS (Rajendran et al., 2013). The ability of EVs to mediate cell-cell communication is a well explained process. Through the transfer of their cargo, including proteins and miRNA, exosomes have been proposed to contribute to the pathogenesis of AS (Boulanger et al., 2017). Based on their differences in size, biogenesis and content of different EC-EVs, they can exhibit different functional properties in AS. Transendothelial migration of monocytes is a fundamental process involved in the pathogenesis of AS. These cells migrate into the vessel wall where they differentiate into macrophages and uptake of oxLDL promotes foam cell formation, driving atherosclerotic lesion development (Bäck et al., 2019). Endothelial exosomes can control inflammation and regulate monocyte cell activation and migration. EVs released by quiescent HUVECs have been characterised in size of around 20-300nm in diameter and are determined to be exosomes which have anti-inflammatory effects (Njock et al., 2015). Intercellular communication between ECs and monocytes via EC-derived EVs exists with the transport of proteins such as heat shock protein 70. This protein within the exosome can induce monocyte activation and adhesion to ECs. (Paone et al., 2018). Some studies have demonstrated a transfer of miRNA between ECs and SMCs (Climent et al., 2015; Hergenreider et al., 2012). EVs released by ECs attenuated the SMC contractile phenotype, suggesting that exosome release from ECs directly regulates the SMC phenotype (X. Lin et al., 2016). EC-derived EVs have been shown to stimulate stimulated vascular cell adhesion molecule-1 VCAM-1 expression and promote leukocyte adhesion in vSMCs. These EVs also activated vSMC protein synthesis and senescence. Proteomic analysis of vSMCs following treatment with EC-derive EVs, revealed upregulation of many proteins such as high-mobility group box (HMGB) 1 and HMGB2 which a pro-inflammatory molecules. Suggesting that these EVs can activate signalling pathways that influence SMC phenotype (Boyer et al., 2020). One study showed that EtOH increases EC-derived EV vascularization bioactivity which could initiate alcohol-induced tumour angiogenesis (Lamichhane et al., 2017).

1.8 Sonic hedgehog protein

SHh is a type of morphogen that is part of the hedgehog protein family. SHh signalling has been known to play a role in cell survival, proliferation, and differentiation in many target tissues. After delivery of SHh to its target cell, signal transduction is initiated when HH binds

to Patched-1 and -2 (PTCH1, PTCH2) a transmembrane receptor, which leads to the inactivation of a second transmembrane receptor called Smoothened (SMO). SMO triggers a complex signalling cascade that leads to the activation of GLI1, GLI2 and GLI3 (Svärd et al., 2009). These activators induce the transcription of target genes that are involved in several molecular events. Some of these genes are components of the hedgehog (Hh) pathway such as PTCH and GLI (Marigo et al., 1996; Marigo & Tabin, 1996). Hedgehog interacting protein (HHIP) controls hedgehog signalling activity by binding and blocking the action of Hh proteins (Holtz et al., 2015; Kwong et al., 2014).

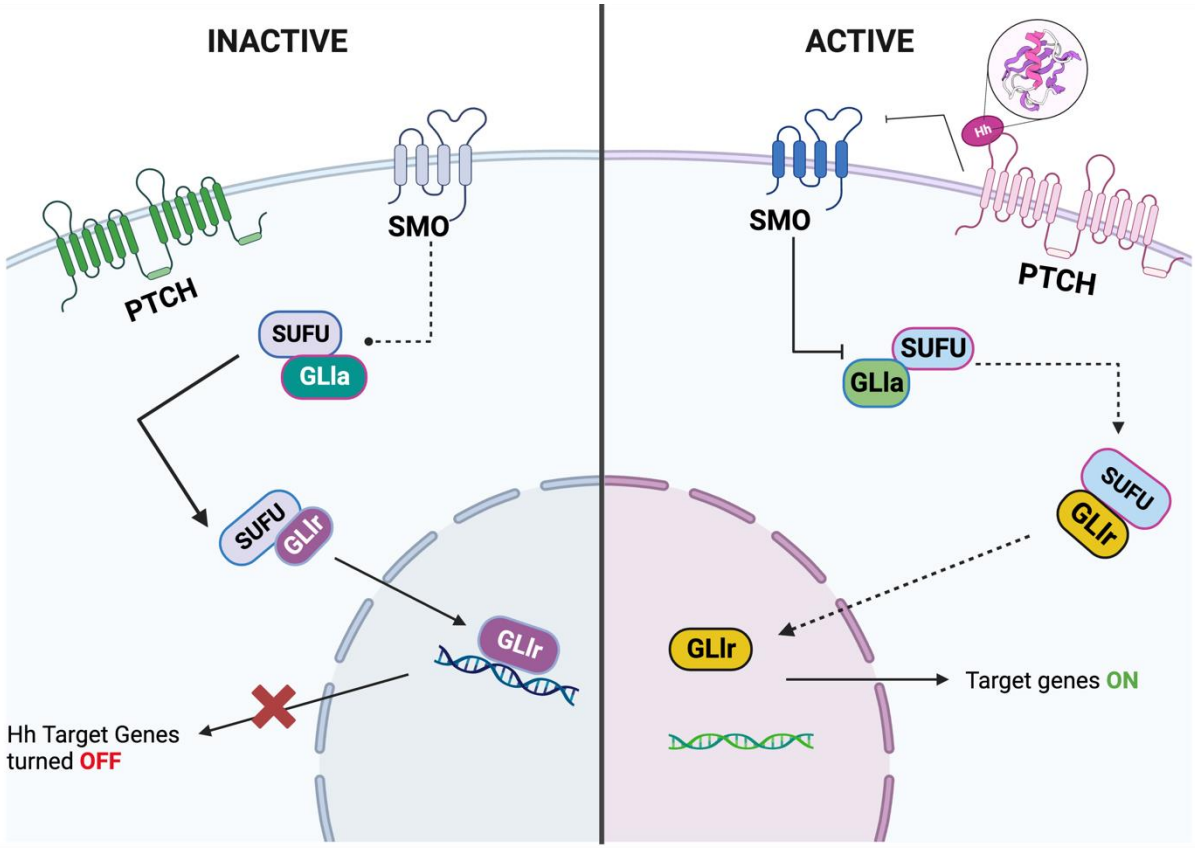


Figure 10 Hh signalling pathway. The membrane-bound Ptch receptor inhibits Smo when Hh is absent, this results in the Gli genes becoming repressed by forming a Gli repressor complex composed of Gli full-length proteins, suppressor of Fu, kinesin family member seven and costal-2. In comparison, the Hh signalling pathway is activated when the Hh ligand binds to Ptch receptor causing Smo to become phosphorylated, the promotes the Gli repressor complex to disassemble. This complex then localises in the nucleus activating target gene expression of Hh genes

Hh signalling is critical for normal embryonic development (Ingham & McMahon, 2001) and plays an important role in maintaining stem cell and progenitor cell population as well as tissue repair post-injury (Petrova & Joyner, 2014). Hh signalling is essential for early vascular development (Vokes et al., 2004) and remodelling (Byrd et al., 2002) as well as development and maintenance of the coronary vasculature (Lavine et al., 2008) and vessel maturation (Yao

et al., 2014). In adults, Hh signalling is involved in the maintenance of adult coronary vasculature and promotes ischemia-induced angiogenesis, including after MI (Lavine et al., 2008; Renault et al., 2013). Hh signalling and its role in AS .is not fully elucidated. Components of the hedgehog pathways have been shown to be expressed and detected in plaques, and inhibition of this signalling can block the binding of all three Hh proteins to PTCH1 increasing AS in *Apoe*$^{-/-}$ (apolipoprotein E deficient) mice (Beckers et al., 2007; Queiroz et al., 2012). Exosomes have been implicated in the release, diffusion, and signal transduction of Hh proteins. Cholesterol at the C terminus and palmitate on the N terminus are the two lipids that are contained within Hh proteins (Chamoun et al., 2001; J. J. Lee et al., 1994). Mutating the target cysteine can prevent Hh palmitoylation which leads to developmental defects, indicating that this palmitate modification is essential for signalling activity (Chamoun et al., 2001; M. H. Chen et al., 2004). Studies have shown that there is an increase in circulating SHh-EVs following apoptosis or T-cell activation (Martínez et al., 2006) following EC injury (AgouTii et al., 2007), following MI (Bueno-Betí et al., 2019) and during angiogenesis (Soleti et al., 2009). Another study also identified the secretion of HHIP-EVs from damaged ECs when released, HHIP-EVs bind to SHh-EVs preventing the activation of the Hh signalling pathway in target cells. This data suggest that inhibiting the generation of endothelial micro particles that carry HHIP or activating the SHh pathway could be a promising method of reducing endothelial damage (Nie et al., 2016).

1.9 Proteomics

Proteomics is a large-scale study of multiprotein systems within an organism, with the goal of identifying and understanding distinct proteins and their roles as a part of a larger networked system. Mass spectrometry is a powerful approach for identifying and quantifying the protein components of EVs. This approach helps toward understanding EV biogenesis and function, as well as distinguishing among different EV classes and subtypes, and identification of disease-specific markers. Due to the explosive interest in EVs, several research groups have employed the use of proteomic techniques to characterise and detect proteins within the cargo of EVs by applying gel electrophoresis (2-DE) and liquid chromatography-mass spectrometry (LC-MS/MS) or MALDI-TOF/TOF MS (Mallia et al., 2020). The LTQ-Orbitrap is one of the most widely used mass spectrometers and is superior for detecting and identifying peptides for the analysis of proteotypic peptides derived from tryptic digestions of proteins recovered from

the protein complement of the cell source, fluid or tissue being studied. The developments in software for data handling, interpretation and validation have enabled catalogues of these data to supply a map of the protein complement of a given organism (Greening et al., 2016). The information obtained for the validated peptide, using LTQ-Orbitrap, allowed for the development of rapid protein quantification based on the high sensitivity selection of the proteotypic peptide identified by its parent mass as well as its identifying dissociated fragments by the two mass filters of a triple quadrupole instrument and monitored over time provides for precise quantification.

In this study an Orbitrap Fusion Lumos Tribid Mass Spectrometer was used. This update in technology has been a great addition to the LTQ orbitrap family of detectors. It has a combination of a linear ion trap with an orbitrap mass detector that has improved greatly since its previous predecessors (Orbitrap Classic/XL). In order to modify the specific capability of the detector for whatever requirements necessary such as sensititivy, speed, resolution for precursor, fragmant ion scans and can also allow for various fragmentation types to produce complementary fragment information (Frese et al., 2011), especially for modified peptides (Jedrychowski et al., 2011). Changes in the design of the instrument, such as an addition of a quadrupole mass filter to an Orbitrap analyzer which features parallelization of ion isolation/accumulation and detection in this "Q Exactive" instrument (Michalski et al., 2011) increased speed and shortened the duty cycle due to the presence of a linear ion trap. In the Orbitrap Fusion Lumos, using a linear ion trap and a quadrupole, for ion isolation and fragment spectra acquisition retrospectively, have been combined which further enhances parallel data acquisition. The high degree of parallelization of various instrument operations are highlighted in the universal method which provides maximal peptide identification without method optimization for complex samples with an unknown concentration, this reduces the sample and instrument time requirements for routine peptide identification experiments, all which was developed by Thermo Fisher (Davis et al., 2017).

Thousands of proteins have been reported to be present within exosomes derived from many different cells of origin. Using Exocarta and EVpedia databases, allowed researchers to list the most detected exosome proteins that have been identified. For example, MVB synthesis proteins (ALIX and TSG101), heat shock proteins, membrane transporters, fusion proteins (annexins, GTPases, and flotillin), ESCRT proteins and tetraspanins (CD9, CD63 and CD81) (D. K. Kim et al., 2013; Simpson et al., 2012). One of the main limitations of proteomics for

EV analyses is the heterogeneity of EVs. Most of the current methods for EV isolation can yield mixed EV populations which can interfere with the differential analysis of small EV types. Biological variability also contributes significantly to EV heterogeneity. Relative enrichment of a protein in an EV population can be based on cell origin. It can also depend on the subcellular compartment from which these EVs originate. (Vagner et al., 2019). It has been demonstrated that EVs can be isolated from clinical samples (Lyu et al., 2021; Qiao et al., 2019). Proteomic analysis of (EVs) from the biological fluid is a powerful approach to discovering potential biomarkers for human diseases including cancers, as EVs secreted to biological fluids are originated from the affected tissue

1.10 Aims and Objectives

The primary aim of this study was to characterise, enumerate and phenotype extracellular vesicles (EVs) released from dysfunctional endothelial cells following exposure to alcohol using a human *in vitro* cell culture model. To achieve this goal, the following objectives were addressed:

- To develop an *in vitro* human cell model of HAEC and hSMC in culture and validate the expression of key lineage specific markers for ECs and SMCs
- To induce endothelial dysfunction of HAECs following ethanol treatment and characterise the number and phenotype of the EVs released using dynamic light scattering, immunoblot and Amnis ™ Cell Stream NanoFACS.

The secondary aim of this study was to determine the proteomic profile of extracellular vesicles (EVs) released from dysfunctional endothelial cells following exposure to alcohol and determine whether endothelial-derived EVs dictate the phenotype of hSMCs

To achieve this goal, the following objectives were addressed:

- To determine the proteomic cargo within EC-derived EVs following exposure of HAECs to alcohol using label-free LC/MS analysis
- To determine whether HAEC-derived EVs **f**ollowing exposure of HAECs to alcohol impact on the phenotype of hSMCs *in vitro.*
- To determine whether EC-derived EVs **f**ollowing exposure of HAECs to alcohol activates hedgehog signalling hSMCs *in vitro*

Chapter 2

Materials and Methods

2.1 Biological materials

All materials purchased for experiments that were commercially available were of high purity and were of cell culture standard where applicable.

2.1.1 Commercial cell lines and cell culture medium

Table 1 Commercial cell lines used in this study

Cells	Morphology	Cell origin	Description	Supplier
Mouse Aortic Endothelial cells	Cobblestone	Adult mouse aorta	Mouse Aortic Endothelial cells are isolated from Swiss mouse aorta. Passage 1, more than 500.000 viable cells	Innoprot
Human Aortic Endothelial cells	Cobblestone	Human ascending and descending aorta	Human Aortic Endothelial cells were isolated from ascending and descending aorta. Passage 2, more than 500.000 viable cells	Promocell
Human Aortic Smooth muscle cells	Spindle shape	Human ascending and descending aorta	Human Aortic smooth muscle cells were isolated from plaque-free regions of the aorta. Passage 2, more than 500.000 viable cells	Promocell

2.1.2 Cell Line Culture Medium used in this study

Table 2 Cell Line Culture Medium used in this study

Cell line	Basal medium	Serum	Supplements	Cryopreservation
Mouse Aortic Endothelial cell	Endothelial basal medium	5% FBS	% Endothelial cell growth supplement	10% Dimethyl sulfoxide (DMSO) 41639 (Sigma) 90% Complete medium
Human Aortic Endothelial cell	Endothelial cell growth media	5% Fetal Calf Serum	1. 90g / ml Heparin 1 µg / ml 2. Hydrocortisone 10 ng / ml 3. EGF 0.004 ml / ml 4. Endothelial cell growthsupplement 5. 1% P/S	10% DMSO 41639 (Sigma) 90% Complete medium
Human aortic Smooth Muscle cell	Dulbecco's Modified Eagle's Medium	5% FBS	1%P/S	10% DMSO 41639 (Sigma) 90% Complete medium

2.1.2 Primers used in this study

Table 3 Qiagen primers used in this study

Product	Target gene	Catalog no.
Hs_GAPDH_1_SG QuantiTect Primer Assay	GAPDH	QT00079247
Hs_HPRT1_1_SG QuantiTect Primer Assay	HPRT1	QT00059066
Hs_CNN1_1_SG QuantiTect Primer Assay	CNN1	QT00067718
Hs_Myh11_1_SG QuantiTect Primer Assay	Myh11	QT00069391
Hs_GLI1_1_SG QuantiTect Primer Assay	GLI1	QT00060501
Hs_GLI2_1_SG QuantiTect Primer Assay	GLI2	QT00018648
Hs_PECAM1_1_SG QuantiTect Primer Assay	Pecam1	QT00081172
Hs_ENOSF1_1_SG QuantiTect Primer Assay	eNOS	QT00059164
Mm_Hprt_1_SG QuantiTect Primer Assay	Hprt	QT00166768
Mm_Myh11_1_SG QuantiTect Primer Assay	Myh11	QT01060843
Mm_Hey1_1_SG QuantiTect Primer Assay	Hey1	QT00115094
Mm_Gli1_1_SG QuantiTect Primer Assay	Gli1	QT00173537
Mm_Gli2_1_SG QuantiTect Primer Assay	Gli2	QT00291711
Mm_Pecam1_1_SG QuantiTect Primer Assay	Pecam1	QT01052044
Mm_S100b_1_SG QuantiTect Primer Assay	S100b	QT00151536

2.1.3 Antibodies

Table 4 Antibodies used in this study

Product	Description	Application	Supplier	Catalogue no.
Anti-CD31 (EPR17260-2361)	Recombinant Rabbit Monoclonal antibody recognizes CD31	ICC	Abcam	ab222783
Anti-Calponin- -1 (EP798Y)	Rabbit monoclonal IgG to Nestin, EP798Y . Concentration 0.063 - 0.067 mg/ml	ICC	Abcam	ab46794
Anti s100B	Rabbit monoclonal [EP1576Y] to S100 beta - Astrocyte Marker	ICC/FACS	Abcam	ab52642
Alexa Fluor 488 Anti-Mouse	Goat polyclonal IgG (whole antibody) conjugated with Alexa Fluor® 488 fluorochrome. Cross-absorbed against human IgG and human serum prior to conjugation. Concentration 2.0 mg/mL	Secondary Antibody for IF and FC	Biosciences	A-11001
Alexa Fluor 488 Anti-Rabbit	Goat polyclonal IgG (whole antibody) conjugated with Alexa Fluor® 488 fluorochrome to rabbit IgG. Cross-adsorbed against human IgG, human serum, mouse IgG, mouse serum, and bovine serum.	Secondary Antibody for IF and FC	Biosciences	A- 11008)

2.1.4 Chemical and biological agents used in this study

Table 5 Chemical and biological agents used during this study

Supplier	Product and catalogue number
R&D Systems	Mouse SonicHedgehog/Shh N-Terminus Antibody (MAB4641)
Biotechne	Recombinant Human IgG1 Fc Protein, CF (110-HG-100) Recombinant Rat Jagged 1 Fc Chimera Protein, CF (599-JG-100)
ExBio	Anti-Hu CD-9 PE (IP-208-T025), Anti-Hu CD-81 (1P-558-T025), Anti-Hu CD63 (1P-632-C025)
Sigma	2-mercaptoethanol (M-7154), DAPT (D5942), DMSO (41639-500ML), L-Glutamine Solution (G7513), Penicillin-Streptomycin (P4333), Triton X-100 (T8787), Bovine serum albumin (A4503), Dulbecco's Modified Eagle's Medium -500ml (D6046), Fluoromount™ Aqueous Mounting Medium (F4680), TEMED (T7024), Glycine (G8898), Gelatin solution (G1393),RIPA (R0278), Transferrin (10652202001), RPMI-1640 (R8758), Trypsin-EDTA solution (T4174), DPBS without Ca+MG (D5773-10XIL), Ethanol (E7023-500ML-D), TE buffer (93283-100ml). TWEEN ®20(P1379)
Gibco	Fungizone® Antimycotic (20mL) (15290018),VEGF (M1168, Lot #0317), 2-Mercaptoethanol (50Mm)
SBI	ExoQuick-TC, 50ml (EXOTC50A-1-SBI, Lot 3180326-001), Exo-Check Exosome Antibody Array (EXORAY-8-SBI), ExoGlow-Membrane EV Labelling Kit (EXOGM600A-SBI)
VWR	Collagen IV mouse 1mg (734-0099)
Bio-Rad	Bio-Rad Protein Assay (Product #23235, Lot #C148587)
Cell biologics	VEGF (MT 168), Fetal Bovine Serum (M1168), Endothelial cell growth supplement (M1168). Epidermal growth factor (M1168,), Heparin (M1168), Hydrocortisone (MI 168), L-Glutamine (M1168), Antibiotic - Antimycotic Solution (M1168)
Qiagen	Rotor-Gene@SYBR Green RT-PCR Kit (204174)
Thermo Fisher scientific	Pierce ™ BCA protein assay kit, RNASE away spray 475ml bottle (10666421, lot #15051819
PreOmics GmbH	iST sample preparation (lot # P.O.00001

2.1.5 Plasticware used during this study

Table 6 Plasticware used during this study

Supplier	Catalogue no.
Lennox	5mL pre sterilized serological pipet (APP260.30,), 10ml. pre-sterilized serological pipet (APP260.40). 25ml pre-sterilized serological pipet (APP260.60), 15ml. conical centrifuge Tubes (ACF450/10X), 50ml. Centrifuge tubes (ACF450.30X), Minisart® NML 0.2um filter (2020-5), Minisart® NML 0.45um filter (2020-11)
Thermo scientific	Nunc ® EasYFlask™ 75cm2 Nunclon Delta Surface (156499), Nunc ® EasyFlask ™ 25cm2 Nunclon Delta Surface (156367), Cover Glass (12440S)

2.2 Methods

2.2.1 Cell Culture and Maintenance

All cell culture techniques were carried out aseptically in a Bioscience Air 2000 M.AC. Laminar flow cabinet. Cells were seeded in Nunc EasyFlask 75cm ^{2} or 25 cm ^{2} Nunclon Delta Surface culture wear and maintained in a Hera cell water jacket cell incubator at 37 C in an atmosphere humidified with 5% CO . Cells were visualised and imaged using an Olympus CK30 phase-contrast microscope at varying magnifications. All cell lines were cultured in the same fashion. Briefly, to subculture, the medium was aspirated from 70-80 % confluent cells and washed with DPBS which was subsequently removed. Cell dissociation was initiated using 1X Trypsin solution. Following dissociation, old media was added to dilute the effect of Trypsin. Fresh medium was introduced into new culture vessels (i.e. flasks or plates) and the appropriate seeding density was then added to each vessel (in accordance with the manufacturers' recommendations) and the vessel was placed at 37 ℃ 5 % CO2

2.2.2 Primary cell culture

2.2.2.1 Primary Mouse aortic endothelial cells

Primary derived mouse aortic endothelial cells (MAECs) were obtained from Innoprot (Cat no. P10427) and routinely grown in endothelium cell medium (Cat no. P60104) supplemented with 5% FBS, 1% endothelial cell growth supplement and 1% PS were grown and maintained as described in section 2.2.1.

2.2.2.2 Primary Human aortic endothelial cells

Primary-derived human aortic endothelial cells (HAECs) were obtained from Promocell GmBH (Heidelberg, Germany - Cat No. C-12271) and routinely grown in Promocell Endothelial Cell Growth Media MV (Cat No. C-22120) supplemented with 5% fetal calf serum, epidermal growth factor (10 ng/mL), hydrocortisone (1 µg/ml), 0.4% endothelial cell growth supplement/bovine hypothalamic extract, heparin (90 µg/mL), and 1% PS. All cells (passages 5–12) were grown and maintained as described in section 2.2.1.

2.2.2.3 Primary Human aortic smooth muscle cells

Primary human aortic smooth muscle cells (hSMCs) were purchased from Promocell Gmbh (Heidelberg, Germany). HASMCs were grown in DMEM, supplemented with 10% FBS (Sigma) and 1% PS. All cells (passages 5–12) were grown and maintained as described in section 2.2.1.

2.2.4 Trypsinisation of cells

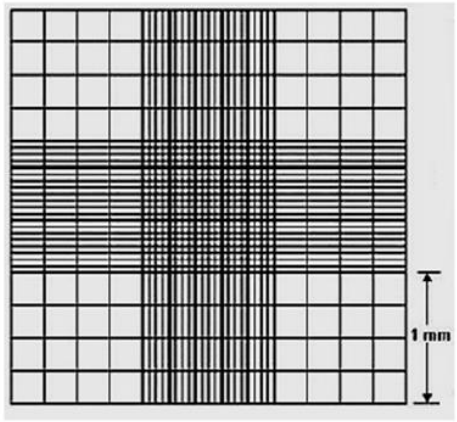
All cell lines used were adherent therefore trypsinisation was required to sub-culture the cells. Trypsin is a proteolytic enzyme that detaches the cells from the surface they are cultured on. The growth medium was removed from the cells by washing them twice with sterile PBS or hanks buffer to remove any remnants of FBS, which contains α-2-macroglobulin, a component of inhibits the action of trypsin. 1X trypsin was added to the flask which was incubated at 37°C for 2-5 minutes afterwards. A growth medium containing FBS was added to neutralise the cells. Cells were harvested by centrifugation at 1500 RPM for 5 minutes at room temperature. Following that resuspended in the appropriate volume of growth medium and counted using a haemocytometer. Cells were split at different ratios according to the specifications of individual cell lines. reduces damage to cells that can be caused by other enzymes present in some trypsin extracts.

2.2.5 Cryopreservation

To generate cryostocks, cells were pelleted following dilution of Trypsin with DPBS at 300 X G for 5 min and re-suspended in 90 % complete medium 10 % DMSO and transferred to cryovials. The cryovials were then labelled appropriately and placed into a Mr Frosty™ freezing container for 24 hrs in a -80 freezer. Cryovials were then removed and placed in long-term storage in liquid nitrogen. To reanimate, cells were thawed very quickly at 37 °C and transferred to a prewarmed complete medium in a centrifuge tube. The cells were then pelleted at 300 X G for 5 min, medium removed and then re-suspended in pre-warmed fresh medium. The cells were then seeded for culture.

2.2.6 Cell count

Following the process of trypsinisation, cells were re-suspended in media and counted using a haemocytometer to obtain the number of viable cells. 20µl cell suspension was mixed gently at 1:1 with 20µl of Trypan Blue. 20µl of this suspension was added to the haemocytometer. Trypan blue stains the cells which are not viable due to their leaky membrane. Viable healthy cells do not take up the dye and will appear bright under the microscope in comparison to the dead cells. Four of the chambers were counted excluding any cells which touched the line indicating the left side of the square and the top side of the square.



Above is an image of a Haemocytometer cell counting

The cell number was calculated using the formula:
(Cell Number)/4 * (Dilution Factor (2)) * 1x10,000 (area under cover slip mm) = Number of viable cells/ml

2.2.7 Cell Characterisation

2.2.7.1 Coverslip Preparation

Petri dishes were filled with IMS, coverslips were placed into the dish very carefully using sterile tweezers and left for 1 hour. The coverslips were removed and washed three times in sterile H20 Coverslips were then let to air dry in the laminar flow hood under UV light for an hour before use.

2.2.7.2 Immunocytochemistry (ICC)

Cells were seeded on sterile coverslips in a 6-well plate at a low density (5,000 cells/well). When cells were at the required confluency, cell culture media was aspirated and cells were washed with PBS. Cells were fixed by incubating with 3.7% formaldehyde at room temperature (RT) for 15 minutes. Cells were washed 3 times with PBST (PBS w/ 1% Tween-20). For intracellular staining, cells were permeabilised by incubating with 0.025% Triton X-100 at RT for 15 minutes. Cells were washed 3 times with PBST. Wells were blocked for 1hr at room temperature with PBS containing 5% BSA, 1% Tween-20 and 0.3M glycine. Samples were incubated at 4^{O}C overnight with the primary antibody diluted to an appropriate concentration with blocking buffer. Cells were washed 3 times following incubation with PBST. Samples were incubated with the secondary antibody (diluted in blocking buffer for 1hr and protected from light. Cells were washed 3 times with PBST. Cells were incubated with DAPI (diluted to 2µg/mL) for 15 minutes at RT to stain the nucleus of the cells. Cells were washed 3 times with PBST. Coverslips were then placed onto glass slides using mounting media and stored at 4°C for 24 hours to dry. An Olympus CK30 microscope with Cell F software was used to capture images. Three different magnifications were used 10X, 20X, 60X and 100X. Prior to imaging, a threshold of the background of negativity was defined using secondary antibody control and exposure rates limited to rule out any false positives. A minimum of five images from the Olympus CK30 microscopy per experimental group (minimum n=3) were analysed using ImageJ software.

2.2.8 Polymerase Chain Reaction (PCR)

2.2.8.1 RNA Isolation

RNA was extracted from cells using the Promega Reliaprep RNA Miniprep System. Kit instructions were followed using the technical manual provided (TM370, Revised 11/12). Prior to isolation, cells were washed and scraped into PBS. Cells were pelleted by centrifugation at 1500 rpm for 5 minutes. The cell pellet was immediately resuspended in an appropriate volume of BL+TG lysis buffer depending on the number of cells present. Isopropanol was then added to the sample which was then vortexed and transferred to a clean Reliaprep Minicolumn in a collection tube. This was then centrifuged at 13.000xg for 30 seconds. An RNA wash solution which contains ethanol was added and the tube was centrifuged again at 13.000xg for 30 seconds. DNase solution was made up of yellow core buffer, DNase I enzyme and MnC and

added to the column and incubated for 15min, to degrade any DNA present. The column was washed using the wash solution provided and centrifuged at 13.500 X g for 15 seconds. After 3 more wash steps. an appropriate volume of nuclease-free water was added to the column. The tube was centrifuged at high speed to elute the purified RNA. The resulting purified RNA was then measured for quantity and purity and stored at-80°C.

2.2.8.2 Quantitation of nucleic acid concentration and quality assessment

The concentration of RNA or DNA was determined using the Nanodrop ND- 1000 Spectrophotometer (Thermo Scientific). The instrument was initiated with molecular grade water and blanked using elution buffer. A sample of 1µl of the sample was measured at wavelengths of 260 and 280 nm. Quantitation of DNA/RNA was automatically given in the concentration of ng/ µL. The purity of the RNA sample was examined by calculating the A 260/280 ratios. For nucleic acids, the acceptable A260/A230 is 1.8, which indicates pure DNA, whilst the ratio of 2.0 indicates pure RNA.

2.2.8.3 RNA Storage

RNA samples were stored in sterile PBS at -80°C. RNA samples were kept on ice at all times when outside -80°C.

2.2.9 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

This technique was performed using the Rotor-Gene RG-3000 M light cycler from Corbett Research and the Rotor-Gene SYBR Green RT-PCR Kit (QIAGEN) as per the manufacturer's instructions. RNA was isolated as described in section 2.2.7.2. RNA was diluted to a final concentration required (1-10 ng) using DEPC water. Sample RNA was first converted to cDNA by Reverse Transcriptase. Gene-specific primers and a Taq Polymerase enzyme then facilitated the amplification of a particular target gene over a number of temperature cycles. The complex formed between the cDNA and SYBR Green Dye led to the emission of a fluorescent signal. Therefore, fluorescence intensity was directly proportional to the starting amount of gene-specific mRNA in a given sample. A ubiquitously expressed reference gene (HPRT for stem cells or GAPDH for terminally differentiated cells) was used to indicate the expression levels of the target gene in the sample cell population. Samples were run in triplicate

for each primer set and a control tube containing no RT was included to confirm the absence of contaminating genomic material.

Table 7 qRT-PCR reaction tube setup with Qiagen SYBR Green Kit

Component	Volume
2x Rotor gene SYBR green RT-PCR master mix Quanti Tech Primers Reverse Transcriptase Nuclease-free H2O Template RNA (1-10 ng/ul.)	12.5 ul 2.5 ul 0.25 ul 4.75 ul 5 ul
Total	25 ul

Table 8 qRT-PCR programme setup with Qiagen SYBR Green Kit

Step	Temperature	Time	Cycles
Reverse Transcription	55°C	10 min	1
Polymerase Activation	95°C	5 min	1
Denaturation	95°C	5 secs	40
Annealing and Extension	60°C	10 secs	

2.2.9 Analysis of EVs

2.2.9.1 Conditioned media harvesting

HAECs were pre-treated with serum deprivation. The percentage of serum was decreased from 5% and dropped by 0.1% over a period of time until reaching 0% serum (every 24 hours until growth in 0% serum was achieved). This allows us to eliminate FBS-derived contamination

which can introduce unwanted exogenous FBS-derived EVs and other nanoparticles and ensure cells were G0 quiescence and their cell cycles synchronised. Once the cells reached 80-90% confluency in a T75cm^2, HAEC cell culture media was removed, the cells were washed three times with PBS, and cells were replaced with complete cell culture media with no treatment, 25mM ethanol and 50mM ethanol (which contains 0% serum). Approximately 10ml of conditioned media was then collected and centrifuged at 2000rpm for 5 minutes to remove cellular material, and stored at -80°C in preparation for experiments. Our lab has previously shown that fresh vs frozen conditioned cell culture made no difference on the immuno-phenotype of isolated exosomes. Other research has also shown than similer qualities of exosomal markers were found in samples of exosomes stored at different temperatures, at 4 °C for 24 h and then at −80 °C, samples immediately stored at −80 °C and fresh samples urine used immediately (Cheruvanky et al., 2007). Optimal storage conditions is essential for exosome function and integrity. Current evidence indicates that –80 °C is the most optimal condition for storing biofluids and isolated exosomes (Fumin Yuan et al., 2021).

2.2.9.2 Isolation of EVs from conditioned media

ExoQuick™ reagent was used to isolate EVs as per the manufacturer's protocol. All centrifugation steps were done at 4°C. Briefly, 10 ml of conditioned media (CM) was centrifuged at 3,000g for 15 minutes removing cells and cellular debris. The supernatants were then transferred to a sterile 50ml tube, and appropriate amounts of ExoQuick™ reagent were added to CM. Samples were mixed by inverting several times, followed by overnight incubation at 4°C for 24 hours. Following incubation, samples were centrifuged for 1,500 x g for 30 minutes. The resulting supernatants were aspirated, and second centrifugation at 1,500 x g for 5 mins was carried out. The supernatant was then aspirated, and the resulting pellet was re-suspended in sterile DPBS or ddH20 or cell culture media depending on the downstream application. Note: Functional experiments which involved treatment with EVs from HAEC CM were carried out immediately after EV isolation. EVs were stained using ExoGlow and tetraspanins, CD9, CD63 and CD81 and analysed using the AmnisTM CellStream TM flow cytometer.

2.2.9.3 Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed using the Malvern Zetasizer Nano. This technique is laser-based which determines the size distributions of isolated EVs. It sends a monochromatic beam through a polarised sample sending light scatters in all directions and this scattering intensity is recorded and measured to determine the size of particles in the EV solution. Samples were diluted by adding 100ul of isolated EVs into 900 ul of ultrapure water and filtered through a 0.45μM filter into a disposable plastic cuvette and covered with parafilm to prevent the entry of dust particles. 12 reads at 30 seconds each were performed using standard settings Refractive Index = 1.331, viscosity = 0.89, temperature = 25°C.

2.2.9.3 Cell Stream flow cytometer

The Amnis™ CellStream Flow Cytometer is a system that contains camera technology which provides Time Delay Integration (TDI) and camera technology to deliver sensitivity and expandability beyond what is possible with traditional flow cytometers, with the ability to view cells as they are analysed in real-time for quality control and troubleshooting. This instrument has high sensitivity submicron particle detector. One of the major advantages of CellStream™ is that it can detect and discriminate particles as small as 0.3 μm. The camera converts low-resolution cell images into high throughput intensity data enabling enhanced fluorescence sensitivity, and superior detection of cells and submicron particles.

2.2.9.4 BCA protein assay to determine the concentration of exosomes.

Pierce™ BCA Protein Assay Kit was used to determine the total EV protein concentration. Kit instructions were followed for carrying out the BCA assay. Briefly, 25μL of each sample or standard was added to each well of a 96-well plate. 200μL of Working Reagent was added to each well. The plate was then incubated at 37^{O}C for 30 minutes. Absorbance was read at 562nm in a plate reader. Sample readings were compared against a standard curve of BSA to determine protein concentration.

2.2.9.5 Fluorescent labelling to visualise exogenous EVs using ExoGlow-Membrane™ EV labelling kit

ExoGlow-Membrane™ EV Labelling Kit (EXOGP100A-1, SBI) was used to robustly and specifically label EV membranes per the manufacturer's instructions. Reaction buffer and labelling reaction were mixed until the dye was dissolved to make the labelling reaction

buffer.100µg of EVs was mixed with labelling reaction buffer and incubated (protected from light) for 30 minutes at RT. Free unlabelled dye was removed using PD SpinTrap G-25 columns. A single 1 min spin was carried out for dye removal. Labelled EVs were then analysed on the Amnis ™ CellStream flow cytometer. This was set to 'small particle detection for the detection of potential EVs. Control samples were collected for unstained EVs, Antibody only, PBS, and HAEC-EVs labelled with antibodies to verify the detection of EVs. A gate was set up using an SSC vs FSC plot to identify "potential EVs". Using this "potential EVs" gated population antibody + EVs Antibody expression was displayed on a two-variable plot using Frequency vs Intensity.

2.2.9.6 Fluorescent labelling of EVs for tetraspanins CD9, CD63 and CD81

EVs were labelled with antibodies, anti-CD9, anti-CD63, and anti-CD81. All antibodies were pre-conjugated to either FITC, PE or APC. Antibodies (AB) were prepared by diluting with appropriate volumes of PBS to make an AB mixture and centrifuged for 10 min at 17,000g. EV samples were diluted with AB mixture in appropriate volumes and allowed to incubate in the dark at RT for 2 hours. Labelled EVs were then analysed on the Amnis™ Cell Stream nanoFACs as described in section 2.2.9.5.

2.2.9.7 Antibody Array

Exo-Check™ Antibody Array (EXORAY210A-8) was used to detect exosome-associated proteins in isolated EVs. Protein concentration was determined using a BCA protein assay. 100ug/ml of protein was used for the arrays. The manufacturer's protocol was followed to detect EV protein cargo including EpCAM, TSG101ANXA5, ICAM, ALIX, GM130, FL0T-1, CD81 and CD63. Protein blots were developed with TMB solution (Sigma Aldrich) for 5-10 minutes.

2.2.10 Proteomics

2.2.10.1 Proteomic Sample Collection and Preparation

EVs were isolated as described in section 2.2.9.2. The iST kit (iST 8x P.O.00001) was used to prepare samples for mass spectrometry analysis as per the manufacturer's protocol. This procedure involves the following steps, lyse, digest and purify.

Briefly, LYSE component was added to 1-100 µg of protein sample and placed on a heating block (95°C; 1,000 rpm; 10 min). The sample was transferred to a cartridge and cooled down. RESUSPEND component was added to DIGEST component and shaken (RT; 500 rpm; 10 min. The DIGEST component was added to the cartridge and placed in a pre-heated heating block (37°C; 500 rpm; 1-3 hours). STOP component was added to the cartridge and shaken (RT; 500 rpm). Cartridge was then centrifuged (3,800 rcf; 1-3 min). WASH 1 was added to the cartridge, centrifuged and flow-through discarded. WASH 2 was added to the cartridge and centrifuged and flow-through discarded. ELUTE component was added to the cartridge and centrifuged, this flow-through was kept. The sample was then placed into an Eppendorf and placed in a vacuum evaporator (45°C; until completely dry). LC-LOAD was added to re-solubilise the sample, and a 1 g/L concentration was obtained. Samples were stored at -80°C.

2.2.10.2 Liquid chromatography–mass spectrometry (LC/MS) analysis

Mass spectrometry was performed by Mr Michael Henry. Nano LC-MS/MS analysis was carried out using an Orbitrap Fusion Lumos Tribid Mass Spectrometer (Thermo Fisher Scientific). The data generated was searched using the algorithm SEQUEST to identify peptides and proteins. As outlined in detail (Murphy et al., 2018), briefly the digested samples were loaded onto the trapping column at a flow rate of 25 µL/min with 2% (v/v) acetonitrile (ACN), 0.1% (v/v) trifluoroacetic acid (TFA) for 3 min before being resolved onto an analytical column. Binary gradients solvent A (0.1% (v/v) formic acid in LC-MS grade water) and 2-27.5% solvent B (80% (v/v) ACN, 0.08% (v/v) formic acid in LC-MS grade water) were used to elute the peptides for 110 mins at a flow rate of 300 nL/min. 1.9 kV voltage was applied for peptide ionisation and a capillary temperature of 320 °C was used. Using an Orbitrap mass analyser, a targeted automatic gain control (AGC) and a maximum injection time of 50 ms, data-dependent acquisition with full scans were performed. The top-speed acquisition algorithm determined the number of selected precursor ions for fragmentation. The Quadruple isolated the precursor ions that were selected with a width of 1.6Da. Peptides that had a charge of 2 + to 6 + were analysed. Higher energy collision-induced dissociation (HCD) was used to fragment the precursor ions. The linear ion trap measured the MS/MS ions.

2.2.10.3 Protein profiling by label-free liquid chromatography-tandem mass spectrometry

Qualitative data analysis was used to characterise the proteome of EVs. MS raw files from label-free liquid chromatography mass spectrometry (LC-MS) analysis were processed using the Proteome Discoverer 1.4 (Thermo Fisher Scientific) software with Sequest HT as the search engine and the *Homo sapiens* UniProtKB-SwissProt sequence database. The following search criteria were used for protein identification (1) peptide mass tolerance of 10 ppm, (2) MS/MS mass tolerance set to 0.6 Da, and (3) included up to two missed cleavages which allow for full tryptic digestion, (4) a fixed modification of carbamidomethylation and (5) variable modification set as methionine oxidation. Peptides were filtered using a minimum XCorr score of 1.5 for + 1, 2.0 for + 2, 2.25 for + 3 and 2.5 for + 4 charge states, with peptide probability set to high confidence (Murphy et al., 2018).

Progenesis QI for Proteomics software (version 3.1; Non-Linear Dynamics, a Waters Company, Newcastle upon Tyne, UK) was used for the quantitative analysis of comparing fractions of EVs to each other. The runs were aligned to a reference run to allow for potential drifts in retention time. The following settings were used to filter peptide features: (1) peptide features with ANOVA ≤ 0.05 between experimental groups, (2) mass peaks with charge states from + 1 to + 4 and (3) greater than one isotope per peptide (Murphy et al., 2018). All exported MS/MS spectra generated a Mascot generic file (mgf) was used for peptide and protein identification via Proteome Discoverer 2.1 (Thermo Scientific) using Sequest HT against the UniProtKB-SwissProt *Homo sapien* database. The following search criteria were used for protein identification (1) peptide mass tolerance of 10 ppm, (2) MS/MS mass tolerance set to 0.6 Da, and (3) included up to two missed cleavages which allow for full tryptic digestion, (4) a fixed modification of carbamidomethylation and (5) variable modification set as methionine oxidation. Only highly confident peptides with an FDR of ≤0.01 as determined by Percolator validation in Proteome Discoverer were allowed (O'Sullivan et al., 2017) for re-importation back into Progenesis LC-MS software as a PepXML file. The following parameters were applied to assign proteins as positively identified: (1) an ANOVA score between experimental groups of ≤ 0.05, (2) proteins with ≥ 2 unique peptides matched and (3) a fold change ≥ 1.5. The normalised abundances of differentially abundant proteins determined using Progenesis QI for Proteomics were transferred as a text file into Perseus- Max Quant and the data were log2 transformed. Z-score was used to perform hierarchical clustering on normalised intensity values using Euclidean distance and average linkage to cluster both samples and proteins. The

final protein ratios were then generated which uses Log 2-fold change and ANOVA *p*-value to identify regulated significant proteins. Proteins were plotted on a volcano plot which allowed them to be considered for our screen. The x-axis is the logFC and the y-axis in the ANOVA p-value, which required every protein to be quantified in at least two of the biological replicates to calculate the variance.

2.2.11 Data Analysis and Statistics

The mean and standard deviation (or standard error margin) was calculated for all of the samples replicates in every experiment and these values were used to plot the data. In order to determine the statistical significance of the data, several tests were carried out. The type of significance test used was determined by whether or not the data were normally distributed or not. The D'Agostino-Pearson omnibus normality test was used to analyse the spread of the data.

In the case of data containing two samples, a paired or unpaired t-test (depending on the matching of replicates) with Welch's correction was performed on normally distributed data. For non-parametric (skewed) data, the Mann-Whitney (paired) or Wilcoxon (unpaired) test was carried out to calculate statistical significance. For data containing more than two samples, ANOVA testing was used to perform multiple comparisons between all samples. This could be carried out using an ordinary one-way (one parameter to compare) or two-way (two parameters to compare) ANOVA for normalised data, or the Kruskal-Wallis test for non-parametric data. This would reveal the level of statistical significance for both the data as a whole and also between each sample.

Chapter 3

Characterisation of endothelial cell-derived EVs following exposure of cells to alcohol.

3.1 Introduction

AS is a chronic progressive inflammatory disease widely regarded as one of the leading causes of death worldwide. A hallmark of AS is the accumulation of vSMC-like cells leading to intimal medial thickening (IMT), lipid accumulation, plaque formation and the obstruction to blood flow that may result in a heart attack or stroke (Bennett et al., 2016). Based on pathological observations in human vessels, adaptive lesions, which are present from birth confirm that early 'transitional' lesions enriched with SMC-like cells are routinely observed in atherosclerotic-prone regions of arteries driving subclinical AS, lipid retention, pathologic intimal thickening and the formation of a developed plaque (Sakamoto et al., 2018). Various cell types are believed to be involved in the development of AS, such as resident stem cells, macrophages, platelets, SMCs and ECs (Pryma et al., 2019). The origin of neointimal vSMC-like cells is controversial however lineage tracing and single-cell RNA sequence analysis (scRNA-seq) have revealed strong evidence in many types of animal models for the involvement of a rare population of differentiated myosin heavy chain 11 (Myh11) medial SMC that are stem cell antigen-1 positive (Sca1+) that de-differentiate and undergo phenotypic switching (Chappell et al., 2016; Dobnikar et al., 2018). vSMCs display remarkable plasticity and do not terminally differentiate. They can undergo a phenotypic switch from quiescent-contractile to migratory-proliferative and synthetic. In response to vascular injury, this phenotypic switch can occur, whereby contractile/differentiated vSMCs switch to a dedifferentiated phenotype, which leads to an increase in the synthesis of extracellular matrix, an increase in proliferation and migration and a decrease in the expression of contractile markers (Frismantiene et al., 2018). The response to vascular injury and response to retention are the two widely accepted theories that support the idea of ED. This is an important early event that is profoundly implicated in the development of cardiovascular disease (CVD) through the development of atheroprone lesions. (Kaperonis et al., 2006; Ross, 1999). Studying the complex pathophysiology of CVD comes with many challenges. The lack of access to human tissues as the point of which it is obtained is at the post mortum stage due to ethical reasons. To overcome this issue, many in vitro and in vivo models have been developed to better understand our knowledge in progression and detection of CVD. Animals such as mice and rats are selected for disease modelling for research due to being cost effective, their minimal maintainence and fast reproduction rate. Use of in vitro cell culture models provides us with an easily accessable platform used to research the various aspects of the pathogenisus of AS, including vascular cell response to pathological stimuli (Mathur et al., 2016). Another

common in vitro cell culture models is the use of primary human cells which are derived from living tissues, such as vSMC and ECs (Poursaleh et al., 2019; Thormodsson & Olafsson, 2005).

Over the past two decades, research has focused on addressing the risk factors of CVD as a means of lowering the risk of developing this life-threatening disease which is currently the world-leading cause of death (31% of all worldwide deaths) (Chiva-blanch & Badimon, 2020; Saeed et al., 2017). Alcohol consumption is a common trigger of CVD and is the main cause of death in males aged between 15-49 years (O'Keefe et al., 2014). Alcohol has been considered to have a biphasic impact on the cardiovascular system, depending on the consumption levels, type of alcohol consumed and drinking patterns. When consumed in moderation, research has found this to be a negative risk factor for AS, beneficial (Ronksley et al., 2011) or less harmless to our cardiovascular health (Chiva-blanch & Badimon, 2020; Marmot & Brunner, 1991). A variety of animal models studies have supported the theory that moderate alcohol consumption has been shown to prevent the development and progression of AS, for example; C57BL/6 hyperlipidemic mice(E. E. Emeson et al., 1995; Eugene E. Emeson et al., 2000) and LDL receptor knockout ($LDLR^{-/-}$) mice (Dai et al., 1997). Research suggests this may be due to the increase in high-density lipoprotein (HDL) cholesterol and a decrease in low-density lipoprotein (LDL) cholesterol, total cholesterol and triglycerides (TG). The cardioprotective function of HDL may be enhanced by moderate alcohol consumption by upregulating the capacity of removing cholesterol through reverse cholesterol transport (RCT), esterification of cholesterol from its free form and the transfer of cholesteryl ester from HDL to the liver (Brien et al., 2011; Brinton, 2012). Evidence also shows that moderate consumption of alcohol reduces apoB and sdLDL-c levels (Y. Huang et al., 2017; Kovář & Zemánková, 2015; Vu et al., 2016). An increase in the transport rate of lipoproteins and hepatic production may be the cause of the elevation of HDL cholesterol (Chiva-blanch & Badimon, 2020). Lipoprotein lipase (LPL) is an essential enzyme that mediates hydrolysis and transports TG (He et al., 2018). Alcohol consumed in moderation has also been shown to have a cardioprotective effect on the cardiovascular system due to the increase in LPL activity which also leads to a decrease in TG concentration (Kovář & Zemánková, 2015). The correlation between HDL and the consumption of alcohol is mediated by the effect of alcohol on the activity of a plasma glycoprotein called on cholesteryl ester transfer protein (CETP). CETP drives the transfer of cholesteryl ester from HDLs to TG-rich lipoproteins in exchange for TG. An increase in the activity of CETP leads to a decrease in the level of HDL and leads to AS

(Shrestha et al., 2018; L. Zhang et al., 2012). Conversely, CETP activity is depleted due to alcohol consumption which leads to an increase in HDL levels (Gaubatz et al., 2020; B. J. Wu et al., 2015). While most studies support the cardioprotective effects of alcohol in moderation, it was reported that alcohol (red wine) did not reduce mature atherosclerotic plaques in ApoE −/− mice (Bentzon et al., 2001). Similarly, moderate consumption of beer does not alter the development of early or mature AS in mice (Goulet et al., 2004). These conflicting results may be due to differences in experimental variables, for example, dietary fat, cholate content, the strain of mouse and model type used (Deeg, 2003).

On the contrary, excessive alcohol consumption increases the risk of CVD, with an increase in cases of morbidity and mortality, as well as an increased risk of more than 50 diseases (Ding et al., 2021). Therefore, the effects of alcohol on the cardiovascular system are heterogeneous, various and vary based on dose (low to moderate vs. heavy) and pattern of intake (acute, binge or chronic) (Minzer et al., 2020). The Dietary Guidelines for Americans recommend that for the moderate consumption of alcohol, two standard drinks a day for males, and one standard drink for females should be consumed to reduce the risk of chronic disease. The relationship between CVD and alcohol consumption is complex, and the cell signalling mechanism involved in mediating the varying effects of alcohol is not fully elucidated. However, the heavy consumption of alcohol increases the risk of CVD. Higher doses of alcohol cause the induction of ED (Puddey et al., 2001). Many studies have shown excessive alcohol consumption can cause changes in the lipid blood profile, which causes hypertriglyceridemia, a condition in which TG levels are elevated, and a known risk factor for the onset of AS (Waśkiewicz & Sygnowska, 2013). This change is due to elevated levels of very-low-density lipoproteins (VLDL) and chylomicrons due to types of lipoproteins rich in TGs (Chait et al., 1972; J. Y. Cho et al., 2014). The alteration of OS and antioxidant defence is another mechanism in which alcohol consumption induces ED (Piano & Phillips, 2014). OS is a phenomenon caused by the imbalance between the oxidant and antioxidant system leading to an increase in ROS (Pizzino et al., 2017). Acetaldehyde, one of the most toxic metabolites of alcohol, modifies the structure of the mitochondria and impairs its function leading to high levels of ROS and a decrease in ATP generation (T. Yan & Zhao, 2020). OS is a well-researched component of AS pathogenesis, occurring alongside inflammation response (Peluso et al., 2012). Over-production of ROS plays a role in the oxidation of LDL. LDL consists of triglycerol, cholesterol ApoB protein molecule and phospholipids, due to alterations, LDL becomes pro-atherogenic and accumulates in the blood vessel walls. This LDL is oxidised by ROS, picked up by

macrophages scavenger receptors (SR), such as CD36 to give rise to foam cells (Dimitry A. Chistiakov et al., 2017).

Atherosclerotic plaques are complex structures that consist of multiple origins of cells such as vascular cells and immune cells. These various types of cells contribute to vascular inflammation and are key cell types involved in AS. Many signalling pathways are activated through inflammatory responses such as Wnt, Notch, toll-like receptors, NLRP3 inflammasome and proprotein convertase subtilisin/kexin type 9 (P. Kong et al., 2022). Research on EVs has exploded since it has become evident that any cell type can secrete them. They can be detected in most biofluids including saliva, blood, and conditioned media from cells in culture. EVs have become known potent mediators in cell-cell communication and signalling through the transport of cargo which can include DNA, RNA miRNA and enzymes (Colombo et al., 2014; Van Niel et al., 2018). EVs are present in atherosclerotic plaques and are involved in many pathologic processes, many of which underly the formation and progression of AS such as cell proliferation and migration, immune responses and endothelial inflammation (Charla et al., 2020). EC-derived EVs are the secretome of progenitor ECs and mature ECs. They account for a very low proportion (5%) of circulating EVs in physiological conditions, however, their levels become elevated in response to various CVD risk factors (Amabile et al., 2005; Arteaga et al., 2006). EC-derived EVs are known regulators of cardiac and vascular remodelling through cell-cell communication (Berezin et al., 2016). They have been shown to have anti-inflammatory effects, are atheroprotective and promote neovascularization (Hergenreider et al., 2012; Jansen et al., 2015; Sheldon et al., 2010) and therefore have the potential to act as therapeutic agents in vascular diseases. On the contrary, there have been reports of EC-derived EVs having adverse effects including the onset of vascular destabilisation (Jansen et al., 2013; Ju et al., 2014; Shan et al., 2015). Initiation of subclinical AS results from continuous endothelial injury, leading to ED and subsequent atherosclerotic lesion formation. This event triggers an inflammatory response initiating a cascade of events including the release of EVs (De Caterina R, 2007; Dignat-George & Boulanger, 2011). Levels of circulating EC-derived EVs are increased in patients with underlying cardiovascular risk factors such as high blood pressure (Nomura et al., 2004) and smoking (H. Liu et al., 2014). ED or activation induces the production of EVs from ECs. EVs could be used as a biomarker for the identification of patients with a high risk of developing CVDs. (Dignat-George & Boulanger, 2011)

This chapter investigated the effect of ethanol induced ED in the secretion of EC- derived EVs from HAECs and the modulation of EV size and phenotype.

3.2 Objective

The primary aim of this Chapter was to characterise, enumerate and phenotype extracellular vesicles (EVs) released from dysfunctional endothelial cells following exposure to alcohol using a human *in vitro* cell culture model. To achieve this goal, the following objectives were addressed:

- To develop an *in vitro* human cell model of HAEC and hSMC in culture and validate the expression of key lineage specific markers for ECs and SMCs
- To induce endothelial dysfunction of HAECs following ethanol treatment and characterise the number and phenotype of the EVs released using dynamic light scattering, immunoblot and Amnis ™ Cell Stream NanoFACS.

3.3 Strategy

The main objective was to develop an *in vitro* murine model and an *in vitro* human model to test our hypothesis that the induction of ED via ethanol can release HAEC-derived EVs.

Primary MAECs were purchased from Innoprot (C-12271) and cultured in a complete medium until reaching 70% confluency. ICC was carried out to characterise the cell population by staining for CD31, a membrane-bound protein that is also called Platelet endothelial adhesion molecule (PECAM1). Confirmatory qRT-PCR analysis was used to determine mRNA levels of *CD31*, *S100β* and *CNN1* compared to mouse smooth muscle cells. However, due to low levels of *CD31* expression and high levels of *S100β* and *CNN1* found in MAECs, led us to deem this cell line unsuitable to test our hypothesis. This led us to move on to our human model instead.

Primary HAECs were purchased from promocell (p10427) and cultured in a complete medium until reaching 70% confluency. ICC was carried out to characterise the cell population by staining for CD31. Confirmatory qRT-PCR analysis was used to determine mRNA levels of *CD31*, and *CNN1* compared to hSMCs.

hSMCs were purchased from and cultured in a complete medium until reaching 70% confluency. ICC was carried out to characterise the cell population by staining for *CNN1*. Transcript and protein expression of myogenic genes *CNN1* and *Myh11* were measured by qRT-PCR. Confirmatory qRT-PCR analysis was used to determine mRNA levels of *CD31*, and *CNN1* compared to HAECs.

To simulate conditions found *in vivo*, HAECs were treated with ethanol, with varying concentrations for 48 hours. Before ethanol treatments, HAECs were pre-treated with serum deprivation. The percentage of serum was decreased from 5% to 1% until reaching 0% serum. This allows us to eliminate FBS-derived contamination which can introduce unwanted exogenous FBS-derived EVs and other nanoparticles. It also allowed us to ensure cells were G0 quiescence and their cell cycles synchronised (Abramowicz et al., 2018). Once the cells reached 80-90% confluency, HAEC cell culture media was removed, the cells were washed three times with PBS, and were replaced with complete cell culture media with no treatment, 25mM ethanol and 50mM ethanol (which contains 0% serum). The cell culture media was collected following 48 hours of treatment, this was centrifuged for 2000g for 15 min at 4°C to remove any cellular debris and stored in the -80°C freezer. EVs were isolated from this HAEC conditioned (CM) media using ExoQuick™.This is a proprietary polymer that gently precipitates exosomes. EVs were then enumerated and characterised using dynamic light scattering (DLS), immunoblot and Amnis™ Cell Stream nano FACS analysis for EV biomarkers. DLS of isolated EC-derived EVs in normal/healthy and ethanol conditions was carried out to measure the size distribution of EVs. Amnis™ Cell Stream nano FACS analysis was used to characterise the EC-derived EVs. This method allows for high-throughput flow cytometry with increased fluorescence sensitivity for the ability to detect the smallest of particles, in particular exosomes. To detect and quantify these EC-derived EVs using this method, we labelled them with an ExoGlow-membrane EV labelling kit. Next, we carried out fluorescent labelling with tetraspanins to determine the percentage of CD9, CD63 and CD81 by using specific antibodies to stain the surface proteins of these EVs. Lastly, we used an antibody platform called Exo-Check antibody array to confirm the Exosomes were positive for known exosomal markers.

3.4 Results

3.4.1 Primary MAECs do not express distinct endothelial markers CD31 under normal conditions

MAECs were grown in cell culture and characterised for their expression of endothelial markers, *CD31*. MAECs displayed morphological features which included a distinct cobblestone monolayer which was observed by using phase-contrast microscopy at 4X and 10X magnification (Figure 11 A and B). The cells were not enriched for *CD31* mRNA levels compared to mSMCs in culture but were enriched for *S100β* (Figure 11 C and D). ICC was then carried out to characterise the cell population by probing for endothelial cell marker *CD31* and neural stem cell marker *S100β.* For ICC, cells were seeded onto sterile coverslips in a 6-well plate at a low density (5,000 cells/well) in MM1 and incubated at 37^{O}C for 48 hrs. Cells were fixed with 3.7% formaldehyde and stained with primary antibodies for Cd31 (1:100) and S100β (1:150). Fluorophore-conjugated secondary antibodies (Alexa Fluor 488-conjugated secondary antibody) were added to generate a fluorescent signal for staining and nuclei were stained using DAPI. A fluorescent microscope was used to visualise these stains and images were created and analysed to determine the expression of each protein. In figure 3.1 C-D the MAEC expressed the stem cell markers Sca1 but did not express the endothelial marker CD31. These results suggest that the MAEC are not terminally differentiated endothelial cells and may be contaminated as they have high expressions of S100β and Cnn1.

Relative quantification of mRNA expression was performed on MAEC using specific primers for the following transcripts, *S100β, Cnn1,* and *Cd31* to determine the levels of stem cell and EC and SMC specific markers. Unexpectedly, MAECs expressed low levels of Cd31 and high levels of Cnn1and S100β protein (Figure 12).

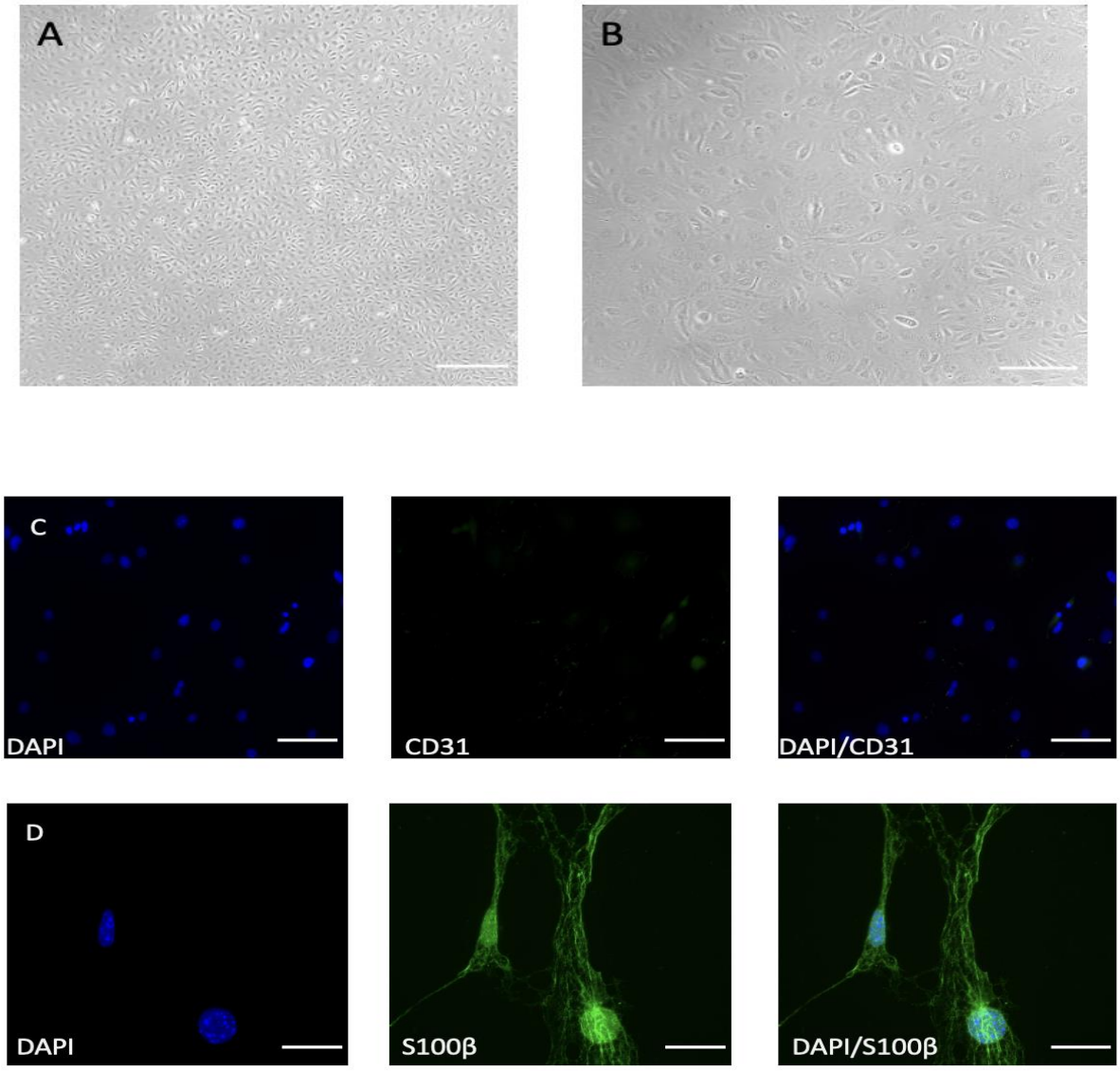


Figure 11 Immunocytochemical analysis of endothelial markers and Phase-contrast images of cultured MAECs for their characterisation. (A&B) Representative phase-contrast images of morphological features in MAECs in culture 4X and 10X magnification. Scale bar representative of 20μm and 50μm respectively. (C & D) Representative ICC data of the expression of an endothelial marker, CD31 and S100β in MAECs. AlexaFluor 488 secondary antibody used to visualise primary antibody binding. Images depict DAPI, CD31 Merge of DAPI/CD31and DAPI, S100β Merge of DAPI/ S100β. All images were taken on the Olympus BX51 Fluorescence Microscope and CellF Multi-fluorescence and Imaging Software. All images are representative of n > 6 images, scale bar is representative of 25μm

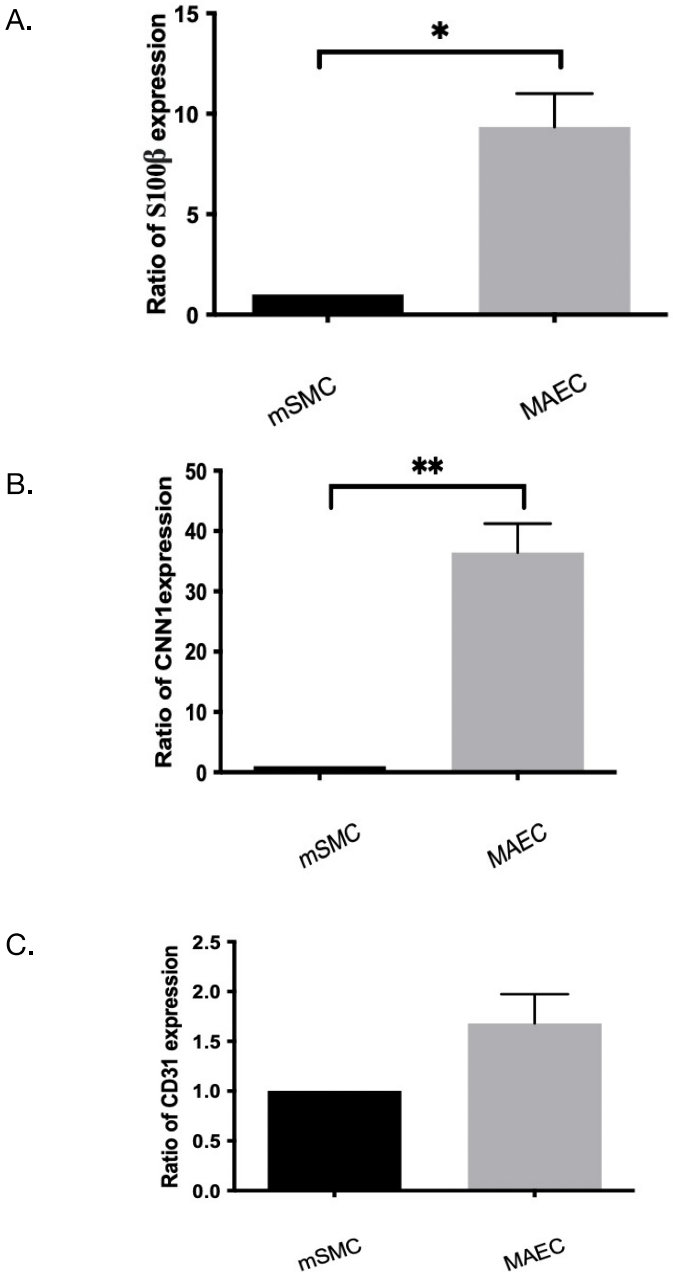


Figure 12 Gene expression analysis for the characterisation of MAEC using RT-qPCR.Relative mRNA levels of endothelial markers Cd31, Cnn1 and S100β in MAECs. Data are expressed as the ratio of mRNA expression relative to GAPDH and are the mean ± SEM (n= 6 samples * $p \leq 0.05$ versus mSMCs)

3.4.2 Primary HAECs express distinct endothelial markers CD31 and eNOS under normal conditions

HAECs were grown in cell culture and characterised for their expression of endothelial marker CD31. HAECs displayed morphological features which included a distinct cobblestone monolayer which was observed by using phase-contrast microscopy at 4X and 10X magnification (Figure 13 A-B). The cells were enriched for *CD31* mRNA levels when compared to mSMCs in culture and did not enrich for *CNN1* (Figure 13 C-D). ICC was then carried out to characterise the cell population by probing for endothelial cell marker CD31 and smooth muscle cell marker CNN1. For ICC, cells were seeded onto sterile coverslips in a 6-well plate at a low density (5,000 cells/well) in a complete endothelial medium and incubated at 37^{O}C for 48 hrs. Cells were fixed with 3.7% formaldehyde and stained with primary antibodies for Cd31 (1:100) and Cnn1 (1:200). Fluorophore-conjugated secondary antibodies (Alexa Fluor 488-conjugated secondary antibody) were added to generate a fluorescent signal for staining and nuclei were stained using DAPI. A fluorescent microscope was used to visualise these stains and images were created and analysed to determine the expression of each protein. HAEC expressed the endothelial cell marker CD31 but did not express the smooth muscle cell marker Cnn1 or stem cell marker S100β. The localisation of protein expression of CD31 was localised around the cell membrane (Figure 13 C-E)

Relative quantification of mRNA expression was performed on HAEC using specific primers for the following transcripts, eNOS and CD31 to determine the levels of EC. CD31 and eNOS proteins were abundantly expressed in HAECs (Figure 14 A-B).

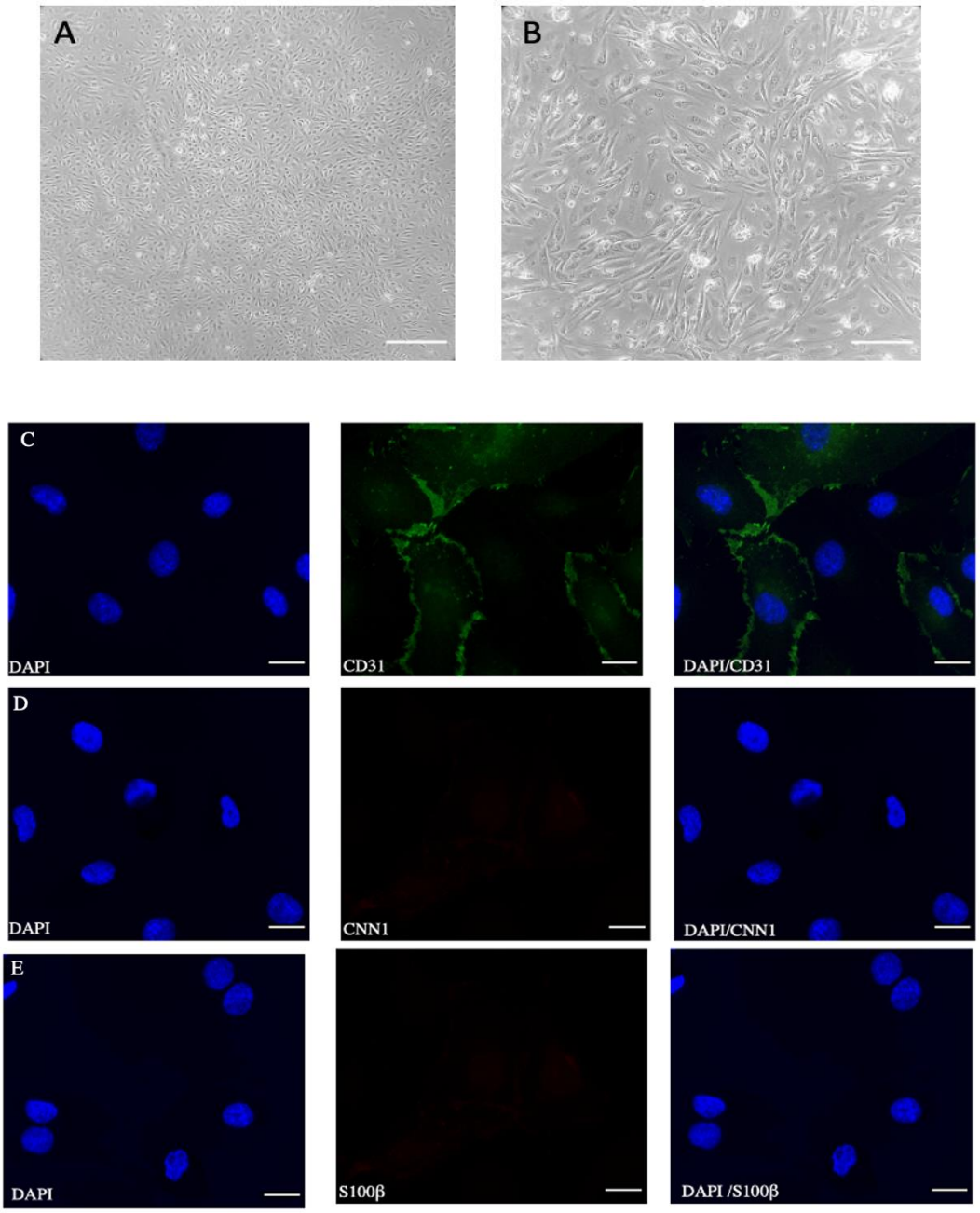
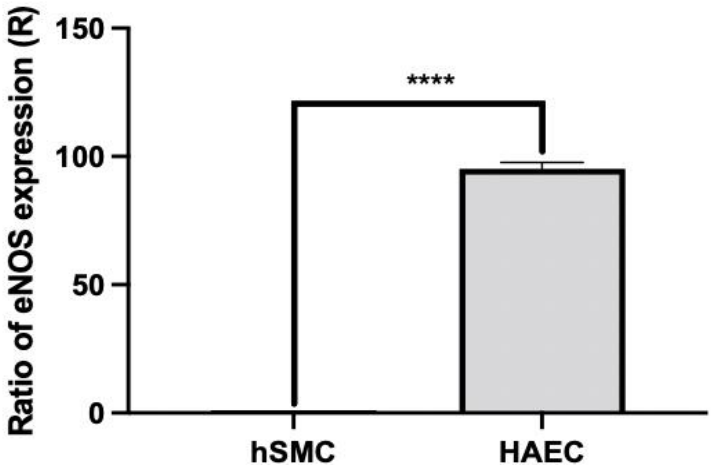


Figure 13. Immunocytochemical analysis of endothelial markers and Phase-contrast images of cultured HAECs for the characterisation of HAECs. (A&B) Representative phase-contrast images of morphological features in HAECs in culture 4X and 10X magnification. Scale bar representative of 20µm and 50µm respectively. (C-E) Representative ICC data of the expression of an endothelial marker, CD31, Cnn1 and S100**β** in HAECs. AlexaFluor 488 secondary antibody used to visualise primary antibody binding. Images depict DAPI, CD31, Merge of DAPI/CD31 and DAPI, Cnn1and Merge of DAPI/ Cnn1 and S100**β** and Merge of DAPI/ S100β. All images were taken on the Olympus BX51 Fluorescence Microscope and Cell^{F} Multi- fluorescence and Imaging Software. All images are representative of n > 6 images, scale bar is representative of 25µm

A.



B.

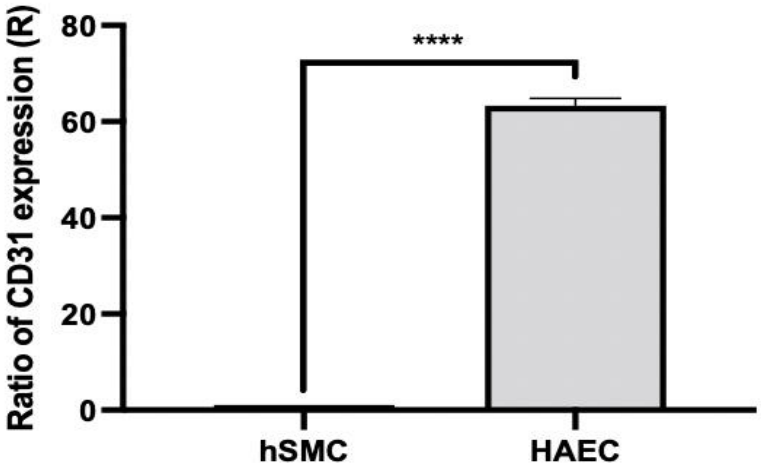


Figure 14Gene expression analysis for the characterisation of HAEC using RT-qPCR.Relative mRNA levels of endothelial markers CD31 and eNOS in HAECs. Data are expressed as the ratio of mRNA expression relative to GAPDH and are the mean ± SEM, n = 3 * $p \leq 0.05$ versus mSMCs.

3.4.3 Primary hSMCs express distinct smooth muscle markers CNN1 and Myh11 under normal conditions.

hSMCs were grown in cell culture and characterised for their expression of smooth muscle cell markers CNN1 and Myh11. hSMCs displayed morphological features which include elastic, not striated and spindle shape which was observed by using phase-contrast microscopy at 4X and 10X magnification (Figure 15 A and B). ICC was then carried out to characterise the cell population by probing for smooth muscle cell marker Cnn1 and endothelial cell marker CD31. For ICC, cells were seeded onto sterile coverslips in a 6-well plate at a low density (5,000 cells/well) in a complete endothelial medium and incubated at 37^{O}C for 48 hrs. Cells were fixed with 3.7% formaldehyde and stained with primary antibodies for Cd31 (1:100) and Cnn1 (1:200). Fluorophore-conjugated secondary antibodies (Alexa Fluor 488-conjugated secondary antibody) were added to generate a fluorescent signal for staining and nuclei were stained using DAPI. A fluorescent microscope was used to visualise these stains and images were created and analysed to determine the expression of each protein. hSMC expressed smooth muscle cell marker Cnn1 but did not express the endothelial marker CD31 (Figure 15 C-D).

Relative quantification of mRNA expression was performed on hSMC using specific primers for the following transcripts, *CNN1*, and *Myh1* to determine the levels of SMC-specific markers. CNN1 and Myh11 proteins were abundantly expressed in hSMCs (Figure 16 A and B).

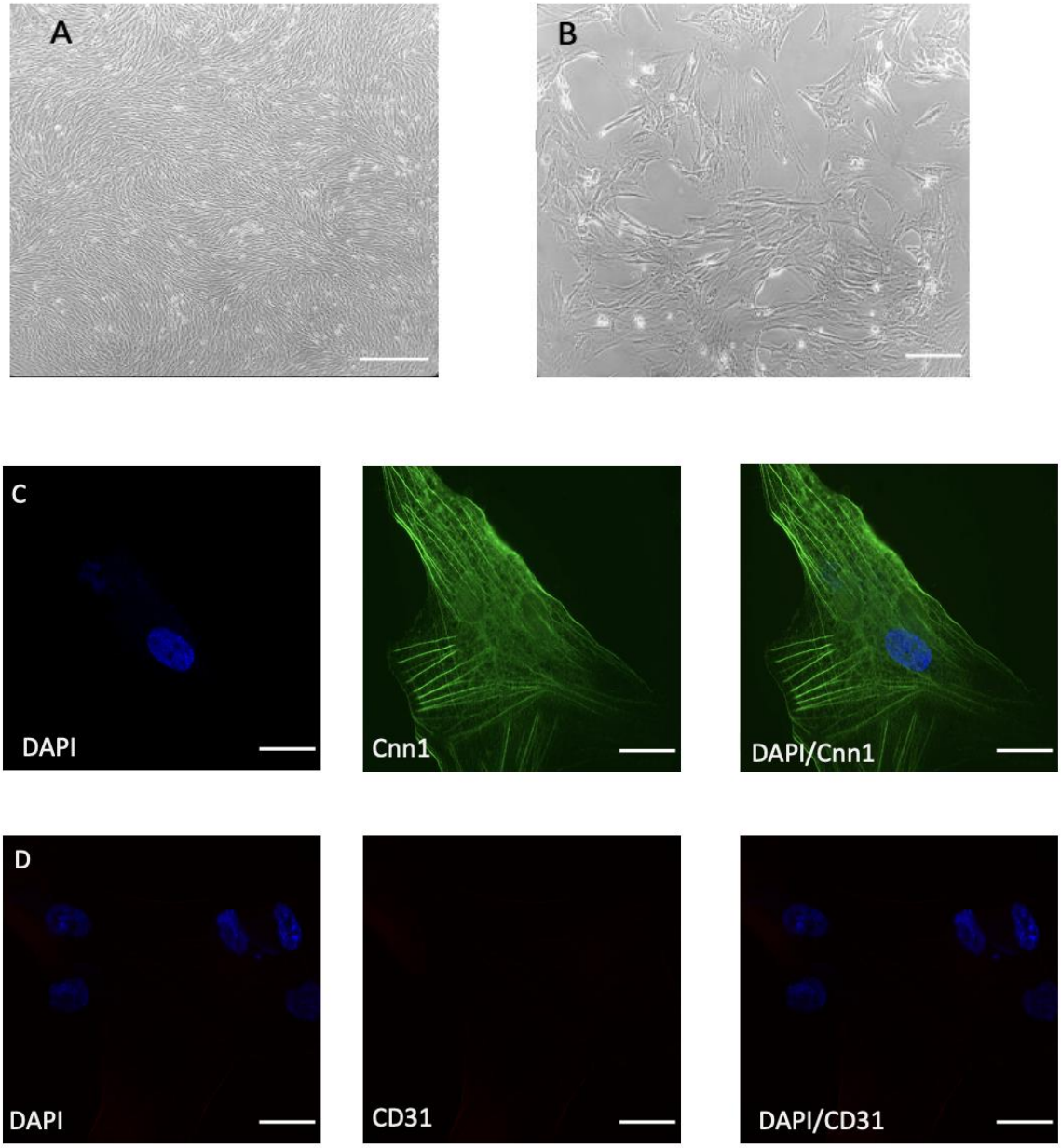
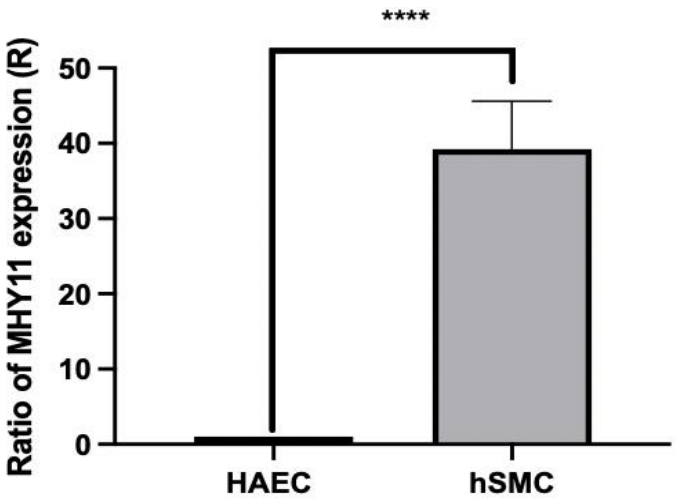


Figure 15. Immunocytochemical analysis of smooth muscle cell markers and Phase-contrast images of cultured hSMCs for their characterisation.(A&B) Representative phase-contrast images of morphological features in HAECs in culture 4X and 10X magnification. Scale bar representative of 20μm and 50μm respectively. (C & D) Representative ICC data of the expression of an endothelial marker, CD31 and Cnn1 in HAECs. AlexaFluor 488 secondary antibody used to visualise primary antibody binding. Images depict DAPI, CD31, Merge of DAPI/CD31 and DAPI, Cnn1and Merge of DAPI/ Cnn1. All images were taken on the Olympus BX51 Fluorescence Microscope and CellF Multi-fluorescence and Imaging Software. All images are representative of n > 6 images, scale bar is representative of 25μm

A.



B.

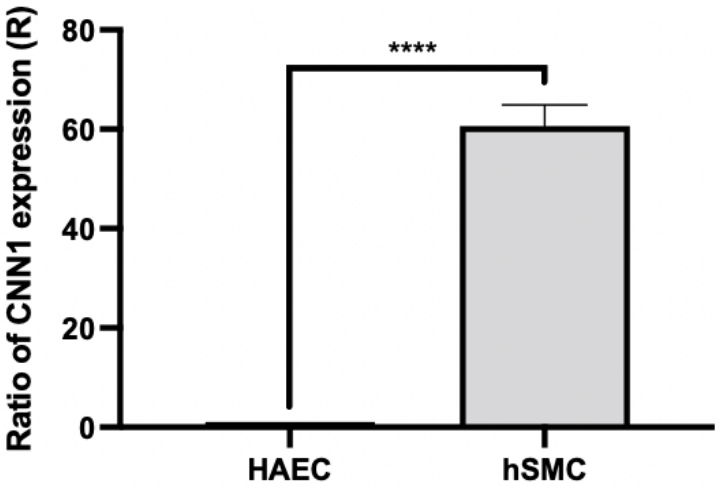


Figure 16 Gene expression analysis for the characterisation of hSMCs using RT-qPCR.mRNA levels of smooth muscle cell markers CNN1 and Myh11 in hSMCs. Data are expressed as the ratio of mRNA expression relative to GAPDH and are the mean ± SEM, n = 3 * $p \leq 0.05$ versus HAEC

3.4.4 Fluorescent labelling and detection of HAEC-derived EVs following exposure of cells to normal and ethanol conditions in vitro

As previously mentioned, EVs are small membrane-derived vesicles that are released by all types of cells, which secrete them into extracellular space. Through direct cell-to-cell contact or via paracrine signalling, this is a sophisticated form of intercellular communication. EVs have been reported to be located in intimal lesions of developing and advanced plaques, suggesting they play a role in both the initial and final steps of plaque formation. (Charla et al., 2020). EVs are now being recognised as attractive, a non-invasive circulating biomarker of CVDs. The need to find such a biomarker to detect the presence of ED and to predict subclinical AS is crucial. HAECs were cultured in both normal and ethanol conditions, which allowed these cells to secrete EVs into CM. As a control, this same experiment was set up in parallel, to which the same parameters and conditions were used, with the absence of HAECs, this will be described as 'non-conditioned' (NC). After 48hrs, the media was removed and harvested. . ExoQuick™ was then used to isolate these EVs. These EVs were then resuspended in deionised H_20.

To detect the particle size of EVs, DLS was employed to analyse the velocity distribution of particle motion caused by Brownian motion via measuring the fluctuations of scattered light intensity (Lyu et al., 2021). HAEC-derived EVs under control conditions (H-EXOs) ranged between 80nm-110nm with a small sub-population at 8nm-25nm. HAEC-derived EVs under 25mM ethanol conditions (25-EXO) ranged between 110nm-230nm with a sub-population between 30nm-50nm. HAEC-derived EVs under 50mM ethanol conditions (50-EXO) ranged between 110nm-500nm with a sub-population between 20nm-80nm (Figure 17 A-C).

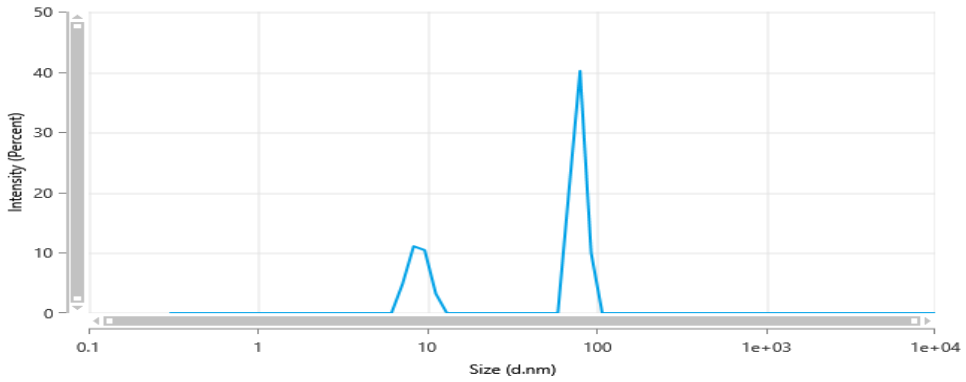
Amnis™ CellStream nanoFACs analyser was then used to carry out submicron particle detection and characterise the EVs that were secreted under normal and ethanol conditions. This analyser is a compact flow cytometer that uses time delay integration charge-coupled device TDI CCD) camera technology for detection. Fluorescent labelling (green) of all three EV types was achieved using ExoGlow-Membrane™ EV Labelling Kit which is commercially designed to specifically label EV protein cargo (465 nm excitation/635 nm emission). It can develop a robust signal specific to EV membrane, leading to low levels of backround. It can deliver a robust performance on EVs from different methods of isolation. It is a very quick and straightforward protocol and as little as 1 µg of EVs can be used.

Both treatments of ethanol increased the number of EVs released from HAECs when compared to control when enumerated using ExoGlow™. Control samples were collected for ExoGlow only and H-EXO, 25-EXO and 50-EXO only. To identify potential EVs, a gate was set up using an SSC vs FSC plot. Using this 'potential EVs' gated population, ExoGlow positive events were gated.

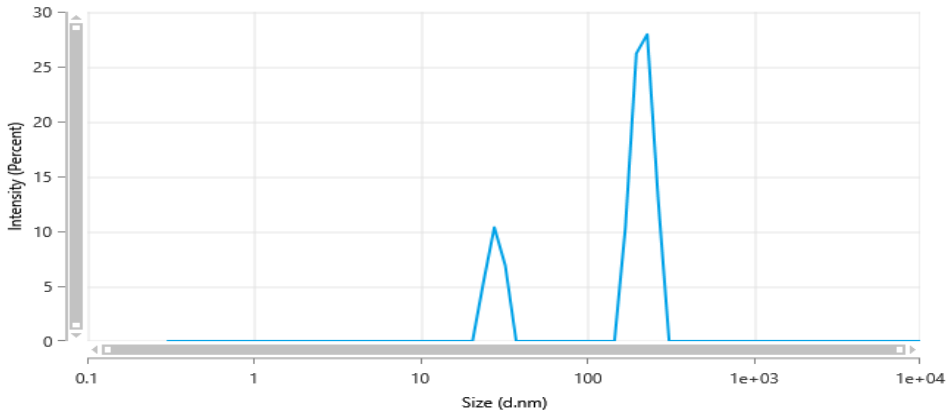
The existence of tetraspanins CD81 and CD63 were detected in EVs from both normal and ethanol conditions. There was little expression of CD9, however its expression increases in 25-EXO and 50-EXO when compared to the control. Control samples were examined for antibody only and IgG, respectively (Figure 19 A-C). The number of CD81 EVs slightly increased (Figure 20 A-C) and the number of CD63 EVs decreased when HAECs were under ethanol conditions respectively when compared to control (Figure 21 A-C).

Lastly, an Exo-Check™ antibody array was also performed on HAEC-derived EVs under control and EtOH conditions by analysing the expression of EV proteins. This is a membrane-based assay which comes with 12 preprinted spots which consist of known exosomal markers CD63, CD81, apoptosis-linked gene 2-interacting protein X (ALIX), flotillin 1(FLOT1), intercellular adhesion molecule 1(ICAM1), epithelial cell adhesion molecule (EpCam) annexin A5 (ANXA5) and tumour susceptibility gene 101 (TSG101). The GM130 cis-Golgi protein marker acts as a negative control to rule out cellular contamination. The positive control contains human serum exosome proteins. The expression of the ICAM1, ALIX and ANXA5 proteins was elevated in 25-EXO and 50-EXO respectively, when compared to the HAEC EV control (Figure 23 A-F).

A. H-EXO



B. 25-EXO



C. 50-EXO

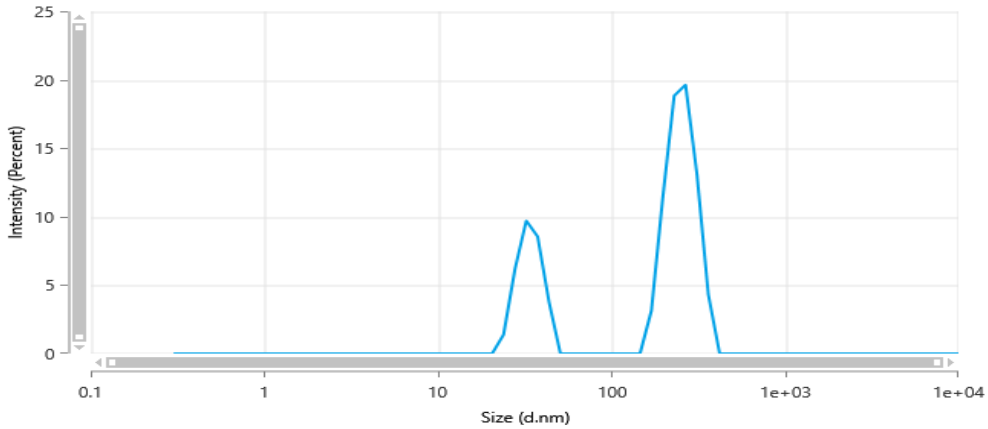


Figure 17 Characterisation of HAECs- derived EVs under normal, 25mM and 50mM ethanol conditions.HAECs were cultured in serum-free media under healthy conditions and in 25mM ethanol and 50mM ethanol conditions for 48hrs, the CM was collected from each treatment and used to isolate exosomes using ExoQuick TC, following this, the purified exosomes were then measured using DLS to display the size distribution profile of normal, vs 25mM and 50mM ethanol conditions.

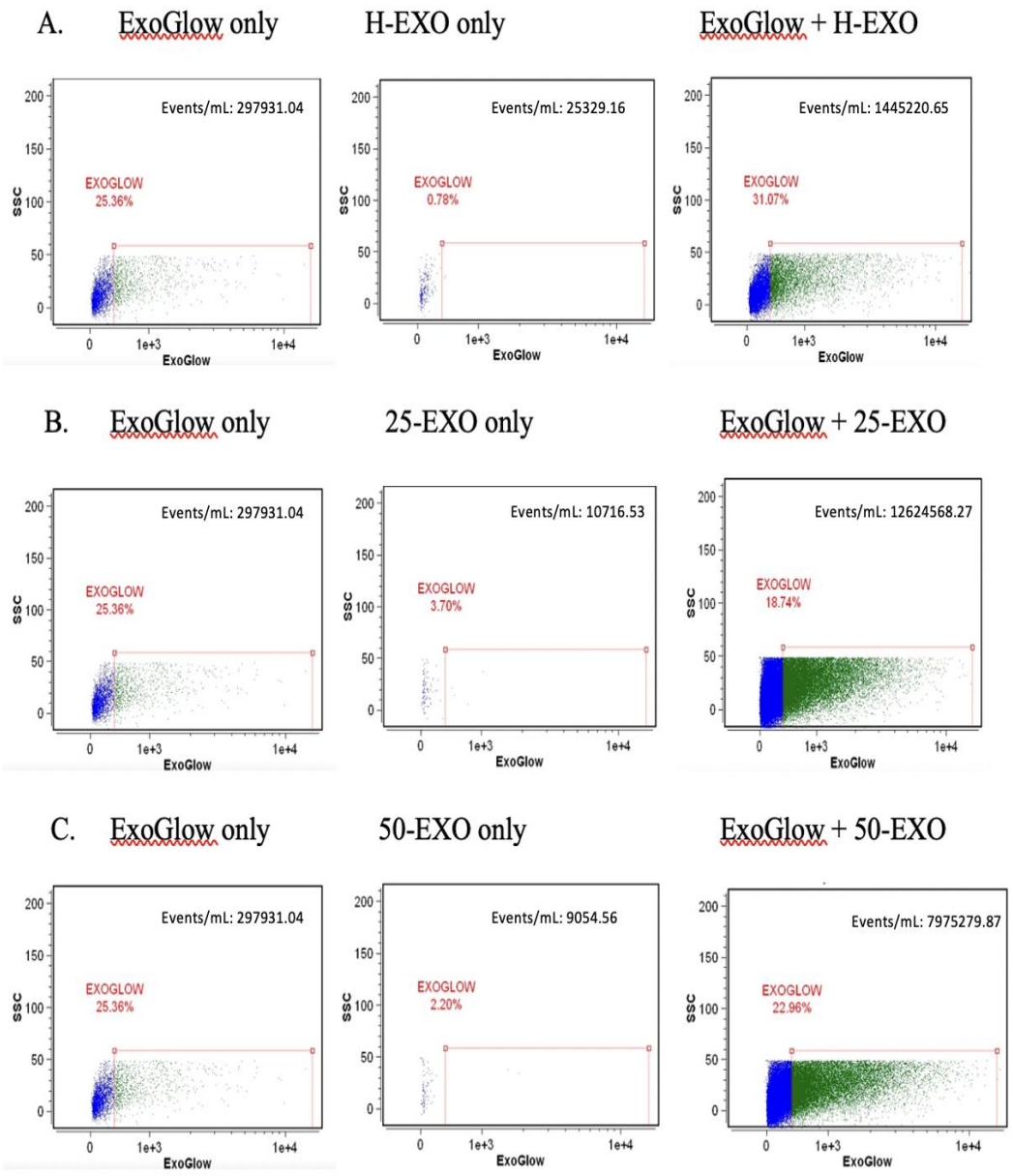


Figure 18 Detection of EV positive H-EXO, 25-EXO and 50-EXO following fluorescent labelling using ExoGlow.(A-C) Detection of ExoGlow positive healthy, 25mM ethanol and 50mM t EC-derived exosomes using Amnis™ CellStream nanoFACs. Representative images of control samples (A) ExoGlow dye only, H-EXO only, and ExoGlow+H-EXO. (B) ExoGlow dye only, 25-EXO only, and ExoGlow+25-EXO. (C) ExoGlow dye only, 50-EXO only, and ExoGlow+50-EXO. Data representative of n=3.

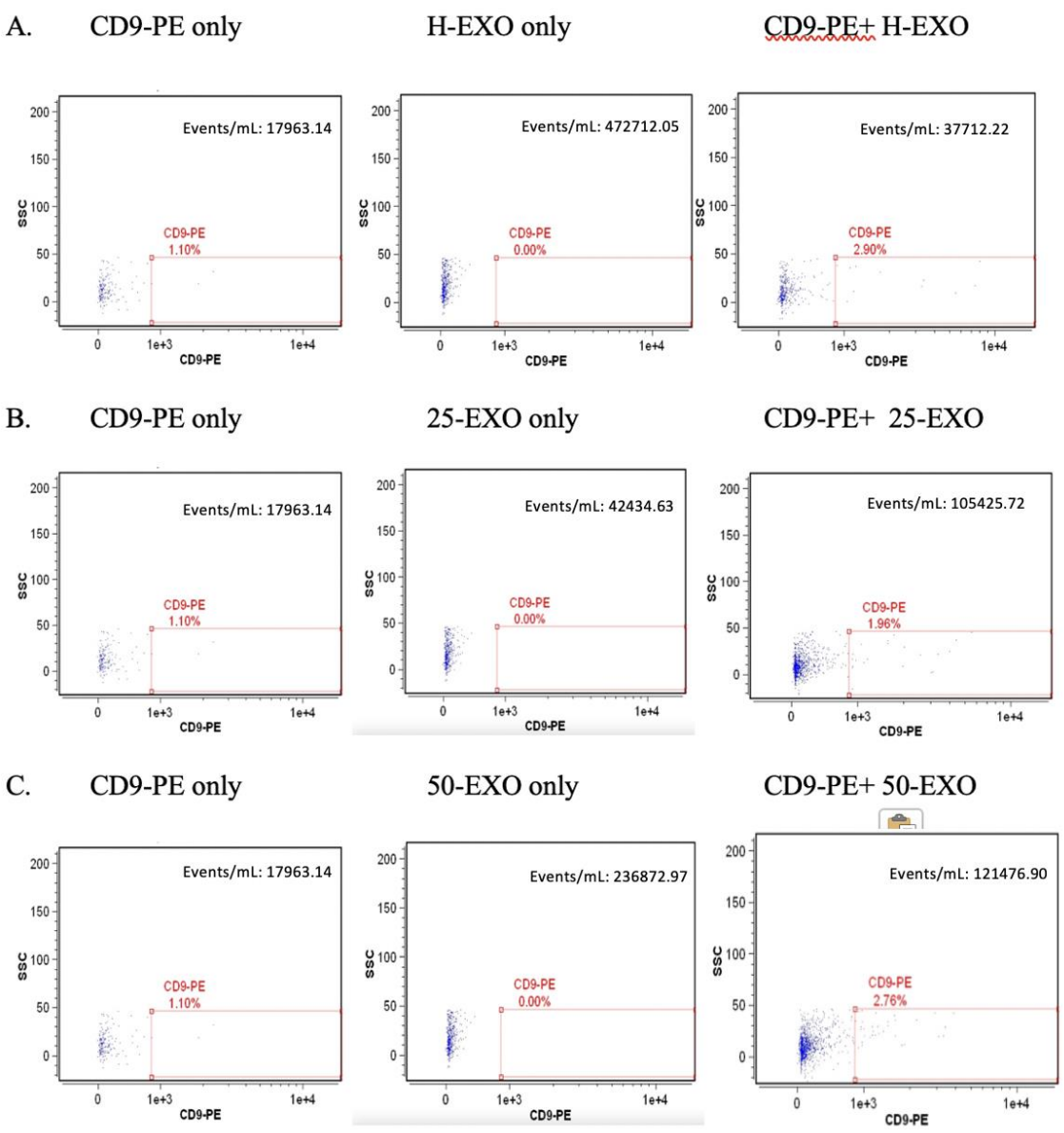


Figure 19 Particle quantification of EVs positive for tetraspanin protein CD9, determined by using Amnis™ CellStream flow cytometer (A-C) Detection of CD9-PE positive healthy, 25mM ethanol and 50mM EC-derived exosomes using Amnis™ CellStream nanoFACs Representative images of control samples (A) CD9-PE dye only, H-EXO only, and CD9-PE +H-EXO. (B) CD9-PE dye only, 25-EXO only, and CD9 +25-EXO. (C) CD9-PE dye only, 50-EXO only, and CD9-PE +50-EXO. Control comparisons include antibody only and IgG, n=3.

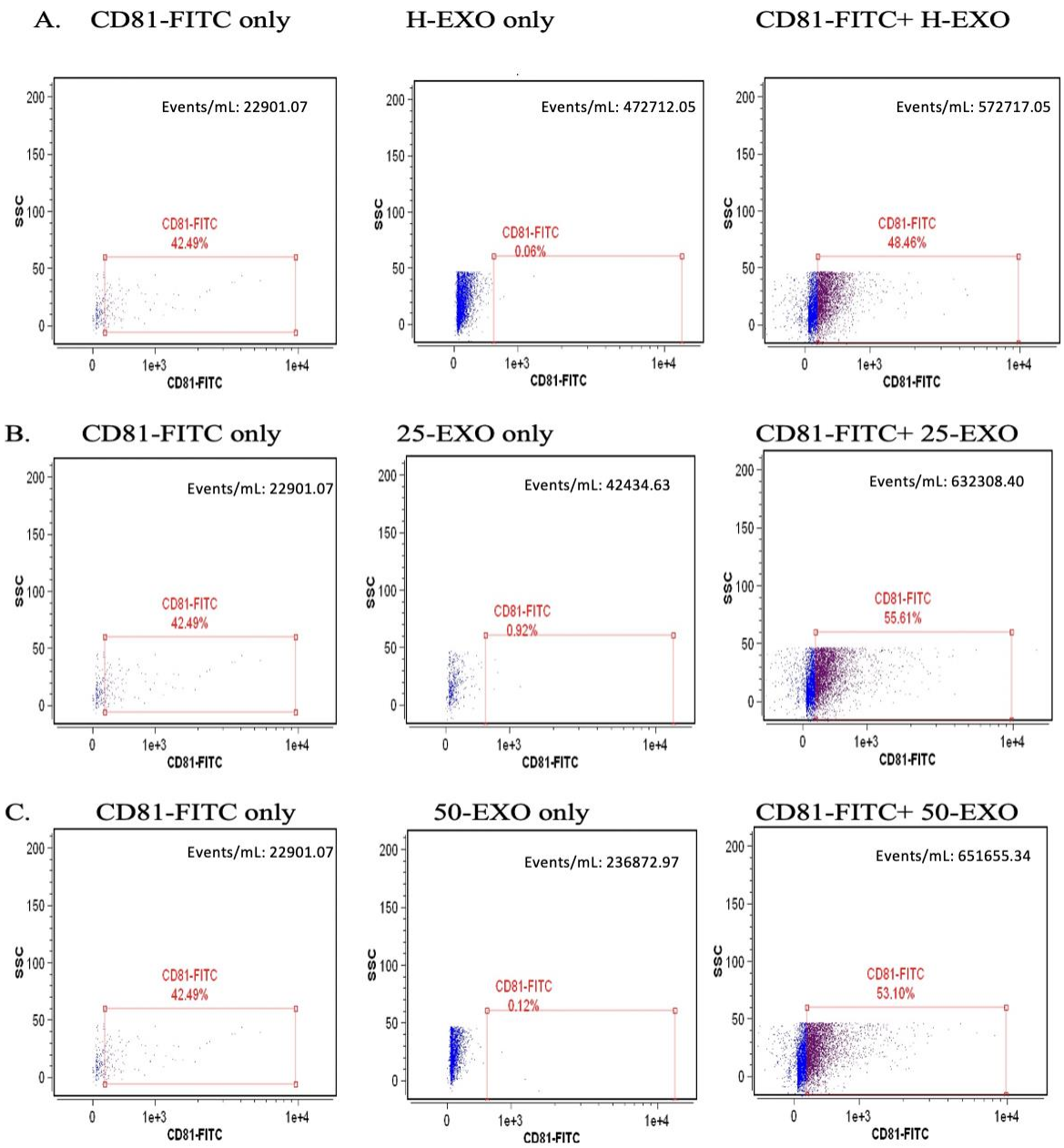


Figure 20 Particle quantification of EVs positive for tetraspanin protein CD81-FITC, determined by using Amnis™ CellStream nanoFACs.(A-C) Detection of CD81-FITC positive healthy, 25mM ethanol and 50mM EC-derived exosomes Amnis™ CellStream nanoFACs. Representative images of control samples (A) CD81-FITC dye only, H-EXO only, and CD81-FITC +H-EXO. (B) CD81-FITC dye only, 25-EXO only, and CD81-FITC -EXO. (C) CD81-FITC dye only, 50-EXO only, and CD81-FITC +50-EXO. Control comparisons include antibody only and IgG, n=3.

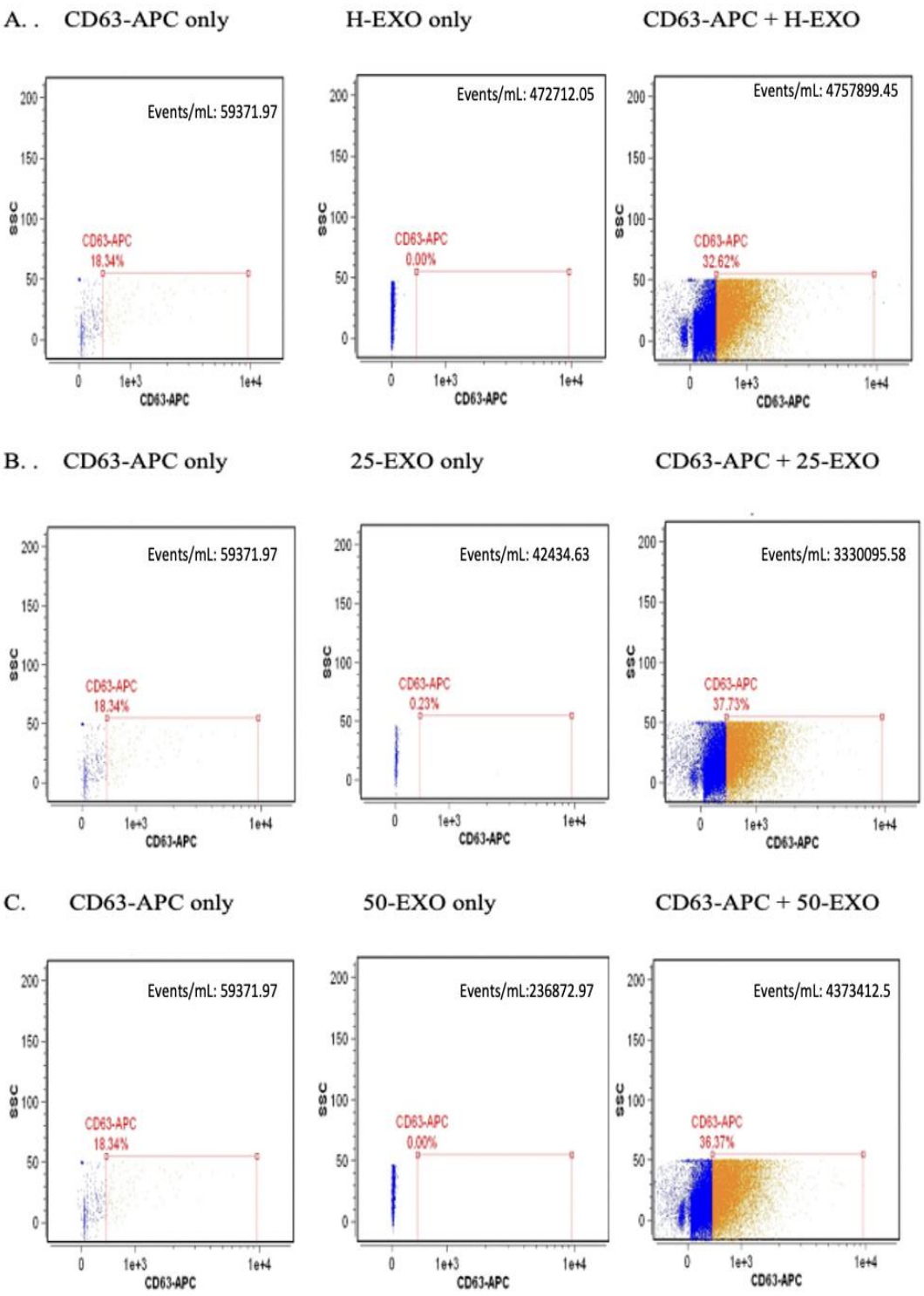
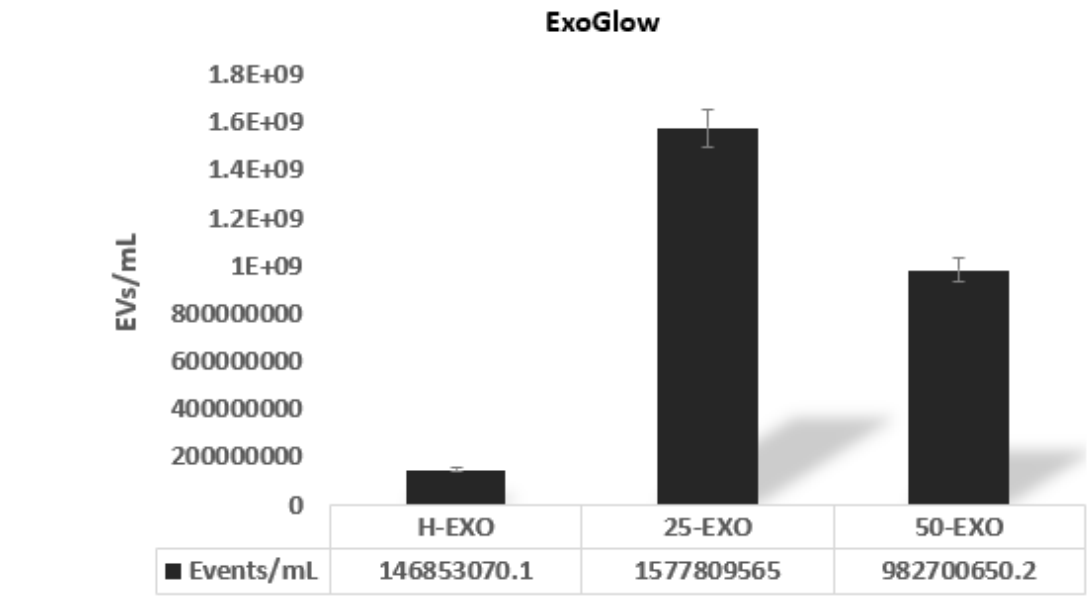


Figure 21. Particle quantification of EVs positive for tetraspanin protein CD63-APC, determined by using Amnis ™ CellStream nano FACs.(A-C) Detection of CD63-APC positive healthy, 25mM ethanol and 50mM EC-derived exosomes using Amnis™ CellStream flow cytometer. Representative images of control samples (A) CD63-APC dye only, H-EXO only, and CD63-APC +H-EXO. (B) CD63-APC dye only, 25-EXO only, and CD63-APC -EXO. (C) CD63-APC dye only, 50-EXO only, and CD63-APC +50-EXO. Control comparisons include antibody only and IgG, n=3.

A.



B.

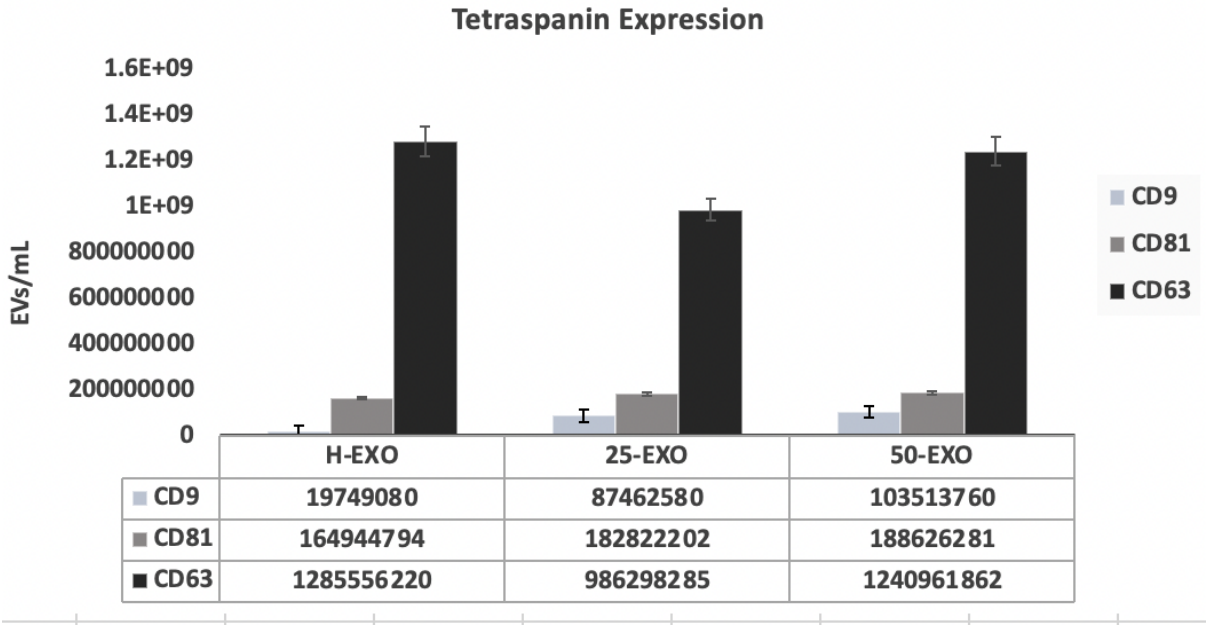


Figure 22 Graphical representation of particle quantification of EVs positive for Exoglow and tetraspanin proteins CD9, CD63, and CD81.The above graphs compare the events/ml detected by Amnis ™ CellStream nano FACs for particle quantification of EVs positive for (A) Exoglow and (B) tetraspanin proteins CD9, CD63, and CD81.

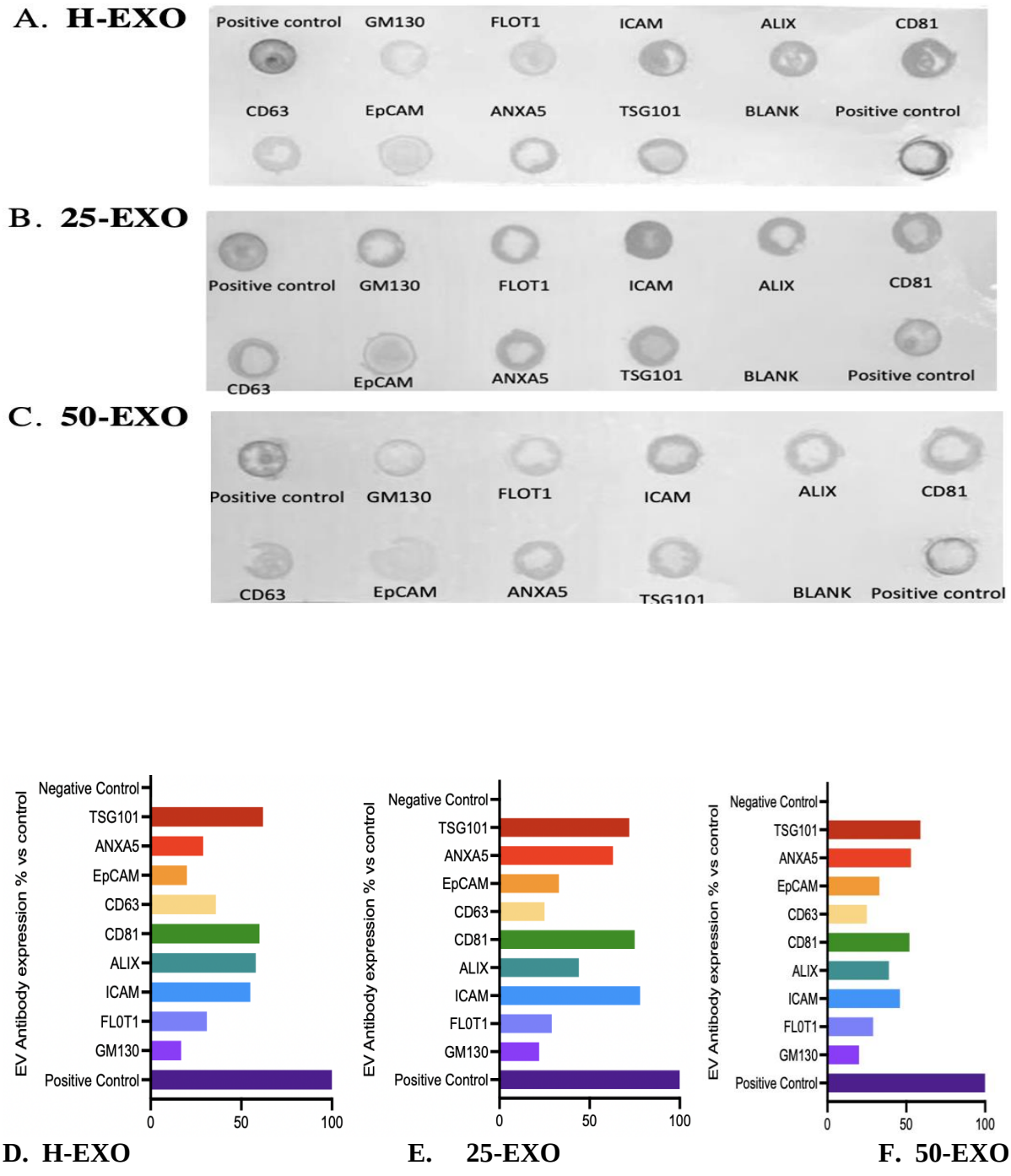


Figure 23 Characterisation of EVs using Exo-Check antibody array. (A-C) The quality and intensity of EV-associated protein expression in H-EXO, 25-EXO and 50-EXO using an antibody platform called Exo-Check antibody array. Exosomes were positive for known exosomal markers CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG101 were observed. the GM130 cis-Golgi protein marker acts as a negative control to rule out cellular contamination. There was a low expression of GM130 in all samples which indicated that exosome samples had good purity, although there may be a small amount of cellular contamination was minimal. (D-F) Densitometry analysis of the intensity of EV-associated protein expression in H-EXO, 25-EXO and 50-EXO were analysed using ImageJ. Data expressed as a percentage of the positive control, n=1.

3.5 Summary

- MAEC had distinct endothelial cobblestone morphology in culture. Confirmation analysis using ICC and transcriptional analysis showed there was very little expression of endothelial-associated markers/genes CD31 and eNOS. MAECs were contaminated with other cellular subpopulations, such as SMC and/or stem cell progenitors.
- Cultured HAEC display distinct endothelial cobblestone morphology in culture. Confirmation analysis using ICC and transcriptional analysis confirmed the expression of endothelial-associated markers/genes CD31 and eNOS.
- Cultured vSMC display distinct smooth muscle cell morphology in culture. Confirmation analysis using ICC and transcriptional analysis confirmed the expression of endothelial-associated markers/genes CNN1 and MYH11.
- HAECs under normal conditions or treated with varying concentrations of ethanol secretes EVs *in vitro*. Amnis™ Cell Stream nano FACs analysis using ExoGlow staining and tetraspanin staining (CD81,CD63) confirmed the presence of an EV population following isolation using the ExoQuick™. Their expression of EV-associated proteins are discretely different. An Exo-Check antibody array confirmed the exosomes were positive for known exosomal markers and also confirmed discrete changes in the cargo under different conditions.
- Using DLS, it was determined that HAECs generate and secrete EVs under different conditions that differ discretely in their range of size.

Chapter 4

Proteomic profile of endothelial cell-derived EVs following exposure of cells to alcohol and their role in dictating the phenotype of hSMCs.

4.1 Introduction

MS is a fundamental technique for identifying and characterizing the protein content of EVs, and it is applied to study EVs in several diseases, in particular CVD. As previously described, EVs can be secreted from almost all cell types and can be isolated from biological fluids (Konoshenko et al., 2021), cell culture medium (Purushothaman, 2019) and from dissociated tissues (Ishiguro et al., 2019). Proteomic studies of EVs using MS-based technology involve many steps, including isolation of EVs from specific biofluids, extraction of EV proteins using detergent sodium dodecyl sulfate (SDS) or non-detergent (8 M Urea) lysis buffer, separation of the extracted EV proteins and digestion before analysis using MS. This Bottoms-up MS approach is most commonly performed for proteomic studies of EVs (J. Li et al., 2019). Many methods can be used to isolate EVs such as differential ultracentrifugation (Jiao et al., 2019), density-gradient ultracentrifugation (Muraoka et al., 2020), size-based isolation (Osti et al., 2019) and immunoaffinity (Hildonen et al., 2016). The polymeric precipitation technique, which is based on the use of polyethylene glycol PEG, entraps EVs, which provides quick and easy isolation of EVs from biological fluids and cell culture medium (García-Romero et al., 2019). Many research groups have used this method of EV isolation to carry out in-depth proteome profiling (Brown & Yin, 2017; Y. Weng et al., 2016). Commercial isolation kits are now available for isolating exosomes, such as Exo-spin, ExoQuick ExoQuick Plus, exoEasy, ME kit, and Exo-Flow (Macías et al., 2019). Classical methods for preparing samples such as exosomes for proteomics can be time consuming and laborious and challenging. However, more recently in-StageTip (iST) methods have been introduced to address the challenges for reproducible, high-throughput and effective proteomic sample preparation (Ding et al., 2020). Preomic iST kit enables robust and reproducible sample preparation for proteomics. This method saved time as complex workflows that are usually carried out in other methods which require many different reagents and taking up to 48hrs is now replaced by a single kit containing all necessary reagents and materials.

Although exosomal protein contents depend on their origin and vary depending on cell type, all exosomes from various cell sources have a few common proteins that help characterise them (Boriachek et al., 2018). The most common proteins are the classes of membrane transport and fusion proteins and others such as carrier proteins, albumin, signalling, cytoskeleton and metabolic (Ibrahim & Marbán, 2016). Exosomal proteins which a most commonly used for exosome classification are tetraspanins, which include CD9, CD63, CD81, CD82, adhesion

molecules (CD45 and CD11b), lysosome-associated membrane glycoproteins (including LAMP-1 and 2B), MVB-associated protein (Alix-1), ESCRT-I associated protein (such as Tsg101), Rabs, and membrane-binding proteins (annexins) and major histocompatibility molecules (MHC-I and II) (C. Liu & Su, 2019). Exosomes also carry diverse enzymes within their cargo, including metabolic enzymes for example lipid kinase-1, peroxidase and pyruvate kinase and GTPases, in particular ones that are from the Rab family (Mathivanan et al., 2012). All protein content of exosomes that have been analysed to date has all been found in the plasma membrane, cytosol or in the membrane of endocytic origin. It is clear that the proteins found were not of Golgi-apparatus, mitochondrial, endoplasmic-reticulum or nuclear origin (Boriachek et al., 2018).

The table below lists some of the most common proteins that are found in exosomes (C. Liu & Su, 2019)

Table 9 Most common proteins found in exosomes.

Protein category	**Gene symbols**
MVB biogenesis	SNF8, CHMP, PDCD6IP, TSG101, VPS25, VPS36, VPS28,VPS37
Antigen-presentation	HLA
Signalling proteins	ARHGDIA, RHOC, RAP1B, YWHA, HRAS, RAP2B, RRAS2, IQGAP1, GNG, GNAS, GNA, GNAI, GNAO, GNAQ, GNB, RHOA
Cell adhesion	CLDN1, THBS1, MFGE8 ,ITG,
Heat shock proteins and chaperones	SP90AB1, HSPA8, CCT
Metabolic enzymes	PGK, PGAM1, PKM2, GAPDH, ALDOA, FASN
Cell structure and motility	ACT, ACTN, CFL1, VIL2, MSN, MYH, MYL, RDX, TUB
Trafficking and membrane fusion	GDI2, RAB, ANXA, ARF, AP2A1, SNAP23, STX3, AP2B1, CLTC,
Transcription and protein synthesis	EEF1A1, HIST, RPS, RPS27A,
Tetraspanins	CD9, CD63, CD81, CD82

ECs release cytokines and growth factors and can also release EVs to mediate their response to stress and communication with other cardiac cells (Bellin et al., 2019). A study carried out showed that ECs cultured and exposed to different types of cellular stress released EVs whose content reflected the cellular stress implicated and varied in relation to the stimuli received (de Jong et al., 2012). They found that the components of the ESCRT complex and many exosome marker proteins such as CD9, CD63, CD81, Flotillin-1, and HSP70 were present as expected. They identified over 1,354 proteins among which many were significantly altered following exposure to cellular stress. Suggesting the protein levels in ECs derived EVs depend on the type of stress and culture conditions suggesting a role of EC-derived EVs in the transfer of stress signals to other cells. This study demonstrated that exosomes had a higher content of proteins such as fibronectin and collagen which are involved in ECM remodelling and in miRNAs that are associated with apoptosis genes and response to stress when exposed to cellular stress. When ECs are treated with TNF-α to mimic inflammation, the cargo within these exosomes results in a high amount of factors concerning immune response, superoxide protection and nuclear factor κB (NF-κB) pathway. It is now well known that ECs release EVs to communicate with each other in particular for angiogenesis regulation. Label-free quantitative MS/MS analysis was used to show that Dll4, which is important for regulating and is normally overexpressed during angiogenesis, was transferred and incorporated into HUVECs derived exosomes from HUVECs overexpressing this Dll4 protein, these exosomes were transferred and taken up by neighbouring ECs, leading to an increase vessel density in vitro and increase branching *in vivo* via Notch signalling inhibition (Sheldon et al., 2010). Another study has shown that ECs secrete exosomes that contain factor hsa-miR214 which results in angiogenesis stimulation via silencing of ataxia-telangiectasia (ATM) mutated in neighbouring cells which prevented senescence and allow blood vessel formation (Balkom et al., 2013). For conditions like peripartum cardiomyopathy (PPCM) or postpartum cardiomyopathy, 16-kDa N-terminal prolactin fragment (16K PRL) induced the release of exosomes from EC that were loaded with miR-146a which have anti-angiogenic and anti-proliferative effects on ECs. These exosomes were taken up by cardiomyocytes, which elevated their mIR-146a levels which resulted in a decrease in the metabolic activity and downregulated Erbb4, Nras, Notch1, and Irak1 expression of proteins (Halkein et al., 2013). Exosomes may play a role in AS development and progression through intercellular communication. For example, exosomes released by ECs, via activation of CD137 inflammatory signalling, induced phenotypic switching of vSMC, which in turn promoted this SMC phenotype to proliferate and migrate leading to plaque formation as well as intimal hypoplasia after carotid injury (B. Li et

al., 2020). Exosomes derived from nicotine-treated macrophages accelerate AS by mediating vSMC migration and proliferation through a mechanism miR-21-3p/PTEN-related (Zhu et al., 2019). Moreover, exosomes isolated from the serum of patients with atherosclerosis as well as EC-derived exosomes induced by oxidized low-density lipoprotein (ox-LDL) induce neutrophil extracellular traps through the transfer of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). Exosomes derived from ox-LDL-treated ECs demonstrated to exaggerate inflammatory response, hyperlipidemia, and Neutrophil extracellular traps (NETs) release in a mouse model of AS (Gao et al., 2020). Interrogating the contents of exosomes and the proteins contained within is of significant interest as their cargo can give an insight and provide clues to exosome biogenesis, targets and cellular effects and may be a source of biomarkers for diseases such as CVD diagnosis and prognosis as well as treatment. Qualitative and quantitative characterisation of exosomes has been made possible through proteomic technology.

There are two mechanisms that inform cells about their required action and position. Through cell-cell interactions in which cells receive messages from neighbouring cells or via gradients of signalling proteins called morphogens that can specify cell fate in a graded manner (Bijlsma et al., 2006). EVs play an important role as cargo-carrying transporter of this secreted morphogen. Our main morphogen of interest is the sonic hedgehog (SHh). SHh, is a known regulator in blood vessel maturation, integrity and arterial differentiation, vascular wall remodelling, and myogenic differentiation (Alvarez et al., 2011; Yao et al., 2014). Hedgehog signalling pathway activation occurs via ligand-dependent interaction when SHh binds to the patched family receptors (ptch1, ptch2) at the cell membrane. In response to this binding, this causes Smoothened (SMO) a signal transducer to be released from ptch dependant suppression and activates signalling via Gli transcription factors (Carballo et al., 2018). SHh has been widely implicated in vascular disease and has been involved in atherosclerosis plaque angiogenesis. Research has shown evidence that Hh morphogen can be secreted by EVs (Callejo et al., 2011; Liégeois et al., 2006). Exosomes derived from insulin resistance adipocytes (IRADEs) transport SHh within their cargo which promotes a vulnerable plaque. This is due to elevated levels of VEGFa and Gli1 (F. Wang et al., 2018).

4.2 Objective

The main objective of this Chapter was to determine the proteomic profile of extracellular vesicles (EVs) released from dysfunctional endothelial cells following exposure to alcohol and assess whether endothelial-derived EVs dictate the differentiation state of hSMCs

To achieve this goal, the following objectives were addressed:

- To determine the proteomic cargo within EC-derived EVs following exposure of HAECs to alcohol using label-free LC/MS analysis
- To determine whether HAEC-derived EVs following exposure of HAECs to alcohol impact on the phenotype of hSMCs *in vitro.*
- To determine whether EC-derived EVs following exposure of HAECs to alcohol activates hedgehog signalling hSMCs *in vitro*

4.3 Strategy

The main objective was to explore the protein content of our EV isolates, by carrying out LC/MS analysis. To test our hypothesis that the induction of endothelial dysfunction via ethanol can release EC-derived EVs harbouring SHh ligands which may be involved in the communication between HAECs and hSMC to promote the activation of Hh signalling in hSMCs.

Due to the important role of the vascular endothelium, acting as the first line of defence against stress, inflammatory and hypoxic conditions, we hypothesize that endothelial cell-derived EVs, under normal and varying concentrations of EtOH, have differentially expressed proteins that reflect the ethanol induced endothelial dysfunction in the cell of origin. Exposure of endothelial cells to different concentrations modulates the protein content of exosomes derived from the cells.

To simulate conditions found *in vivo*, HAECs were treated with ethanol, with varying concentrations for 48 hours. Before ethanol treatments, HAECs were pre-treated with serum deprivation. The percentage of serum was decreased from 5% to 1% until reaching 0% serum. This allows us to eliminate FBS-derived contamination which can introduce unwanted exogenous FBS-derived EVs and other nanoparticles. It also allowed us to ensure cells were G0 quiescence and their cell cycles synchronised (Abramowicz et al., 2018). Once the cells

reached 80-90% confluency, HAEC cell culture media was removed, the cells were washed three times with PBS, and were replaced with complete cell culture media with no treatment, 25mM ethanol, and 50mM ethanol. The cell culture media was collected following 48 hours of treatment, this was centrifuged for 2000g for 15 min at 4°C to remove any cellular debris and stored in the -80°C freezer. EVs were isolated from this HAEC conditioned media using ExoQuick™.. This is a proprietary polymer that gently precipitates exosomes. PreOmics iST kit was used as per manual instructions, this allowed for proteomic sample preparation for mass spectrometry analysis. Each sample was analyzed in duplicate using a 60-minute peptide separation into an Orbitrap Fusion Lumos Tribid Mass Spectrometer. Qualitative LC MS analysis was carried out using the algorithm SEQUEST HT in Proteome Discoverer to identify peptides and proteins. Comparative Label Free Quantitation for differential expression analysis comparing the samples as healthy vs 25mM sample, healthy vs 50mM, and finally, 25mM vs 50mM using Progensis QI for proteomics software. Proteomic analysis was also carried out using PANTHER software and Funrich.

The role of HAEC-derived EVs under ethanol conditions (25mM and 50mM) in attenuating the contractile phenotype of hSMCs was determined. hSMCs were grown in complete medium to 70% confluency. The cell culture medium was replenished with a fresh complete medium and treated with the EVs derived from HAECs under normal and ethanol conditions. The hSMCs were harvested following 7 days of treatment and transcript and protein expression of myogenic genes *Cnn1* and *Myh11* were measured by qRT-PCR.

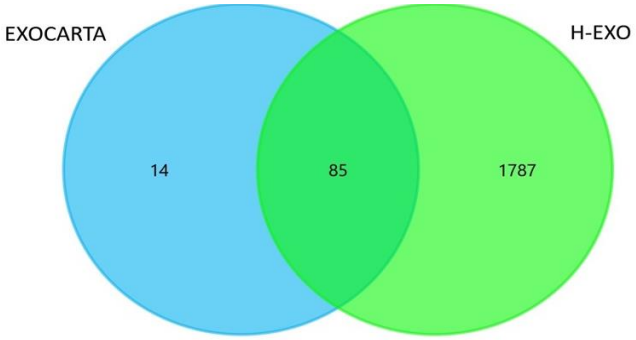
The role of HAEC-derived EVs under ethanol conditions (25mM and 50mM) in activating SHh signalling in hSMCs was determined. hSMCs were grown in complete medium to 70% confluency. The cell culture medium was replenished with a fresh complete medium and treated with the EVs derived from HAECs under normal and ethanol conditions. The hSMCs were harvested following 48hrs treatment and transcript and protein expression of myogenic genes *Gli1* and *Gli2* were measured by qRT-PCR.

4.4 Results

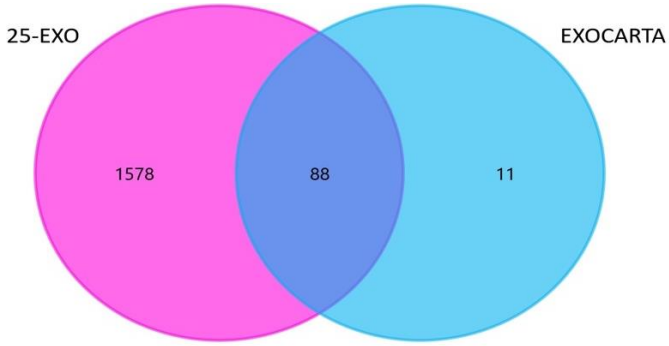
4.4.1 **Protein content confirms the cellular origin of exosomes**

To investigate whether the different ethanol conditions under which endothelial cells were cultured are reflected in exosome protein content, peptide separation and identification were achieved by combining reverse-phased capillary high-pressure liquid chromatography and the analytical capability of an Orbitrap Fusion Tribrid mass spectrometer. The data generated was searched using the algorithm SEQUEST to identify peptides and proteins, experimental design with biological repetition ($n \geq 3$). Using the ExoCarta database, we compared the top 100 proteins identified in exosomes to our own. In the H-EXO, 85 exosomal proteins were matched, leaving 14 exosomal proteins to be not present. In the 25-EXO, 88 exosomal proteins were matched, leaving 11 to be not present, and lastly, in 50-EXO, 84 exosomal proteins were matched, leaving 15 to be not present (Figure 24 A-C). The table below is a list of the top 100 proteins identified in exosomes which are represented by their gene name are compared to each of our exosomes, H-EXO, 25-EXO, and 50-EXO (Table 10). Given the fact that there is an 84-88% match with this ExoCarta list for all three of our exosomes, this further validates the successful isolation of our EVs through their protein Cargo.

A. H-EXO



B. 25-EXO



C. 50-EXO

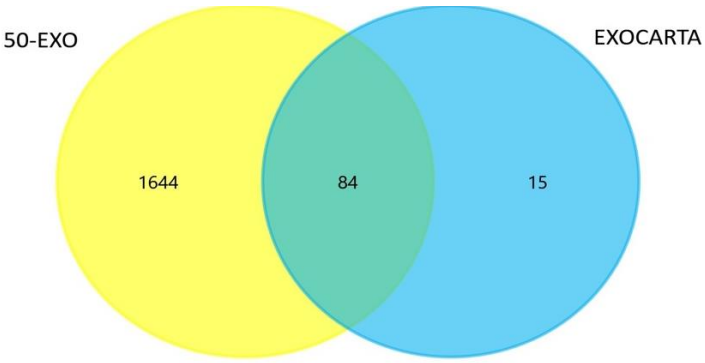


Figure 24 Venn diagram showing the distribution of identified exosomal proteins in each fraction when compared to the top 100 exosomal proteins on ExoCarta. Each Venn diagram represents the unique and common protein between (A) H-EXO vs ExoCarta, (B) 25-EXO vs ExoCarta and (C) 50-EXO vs ExoCarta.

Table 10 List of top 100 exosomal proteins in Exocarta compared to H-EXO, 25-EXO and 50-EXO.

Protein name	H-EXO	25-EX0	50-EXO
CD9 molecule	CD9	CD9	CD9
programmed cell death 6 interacting protein	PDCD6IP	PDCD6IP	PDCD6IP
heat shock protein family A (Hsp70) member 8	HSPA8	HSPA8	HSPA8
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GAPDH	GAPDH
actin beta	ACTB	ACTB	ACTB
annexin A2	ANXA2	ANXA2	ANXA2
CD63 molecule	CD63	CD63	CD63
syndecan binding protein	SDCBP	SDCBP	SDCBP
enolase 1	ENO1	ENO1	ENO1
heat shock protein 90 alpha family class A member 1	HSP90AA1	HSP90AA1	HSP90AA1
tumor susceptibility 101	TSG101	TSG101	TSG101
pyruvate kinase M1/2	PKM	PKM	PKM
lactate dehydrogenase A	LDHA	LDHA	LDHA
eukaryotic translation elongation factor 1 alpha 1	EEF1A1	EEF1A1	EEF1A1
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	YWHAZ	YWHAZ	YWHAZ
phosphoglycerate kinase 1	PGK1	PGK1	PGK1
eukaryotic translation elongation factor 2	EEF2	EEF2	EEF2
aldolase, fructose-bisphosphate A	ALDOA	ALDOA	ALDOA
heat shock protein 90 alpha family class B member 1	HSP90AB1	HSP90AB1	HSP90AB1
annexin A5	ANXA5	ANXA5	ANXA5
fatty acid synthase	FASN	FASN	FASN
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon	YWHAE	YWHAE	YWHAE

clathrin heavy chain	CLTC	CLTC	CLTC
CD81 molecule	CD81	CD81	CD81
Fas binding factor 1	ALB	ALB	ALB
valosin containing protein	VCP	VCP	VCP
triosephosphate isomerase 1	TPI1	TPI1	TPI1
peptidylprolyl isomerase A	PPIA	PPIA	PPIA
moesin	MSN	MSN	MSN
vacuolar protein sorting 72 homolog	CFL1	CFL1	CFL1
peroxiredoxin 1	PRDX1	PRDX1	PRDX1
profilin 1	PFN1	PFN1	PFN1
RAP1B, member of RAS oncogene family	RAP1B	RAP1B	RAP1B
integrin subunit beta 1	ITGB1	ITGB1	ITGB1
heat shock protein family A (Hsp70) member 5	HSPA5	HSPA5	HSPA5
solute carrier family 3 member 2	SLC3A2	SLC3A2	SLC3A2
H4 clustered histone 1	HIST1H4A	HIST1H4A	HIST1H4A
G protein subunit beta 2	GNB2	GNB2	GNB2
ATPase Na+/K+ transporting subunit alpha 3	ATP1A1	ATP1A1	ATP1A1
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta	YWHAQ	YWHAQ	YWHAQ
flotillin 1	FLOT1	FLOT1	FLOT1
filamin A	FLNA	FLNA	FLNA
chloride intracellular channel 1	CLIC1	CLIC1	CLIC1
chaperonin containing TCP1 subunit 2	CCT2	CCT2	CCT2
cell division cycle 42	CDC42	CDC42	CDC42
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma	YWHAG	YWHAG	YWHAG

alpha-2-macroglobulin	A2M	A2M	A2M
tubulin alpha 1b	TUBA1B	TUBA1B	TUBA1B
ribonuclease A family member 1, pancreatic	RAC1	RAC1	RAC1
galectin 3 binding protein	LGALS3BP	LGALS3BP	LGALS3BP
heat shock protein family A (Hsp70) member 1A	HSPA1A	HSPA1A	HSPA1A
G protein subunit alpha i2	GNAI2	GNAI2	GNAI2
annexin A1	ANXA1	ANXA1	ANXA1
ras homolog family member A	RHOA	RHOA	RHOA
milk fat globule EGF and factor V/VIII domain containing	MFGE8	MFGE8	MFGE8
peroxiredoxin 2	PRDX2	PRDX2	PRDX2
GDP dissociation inhibitor 2	GDI2	GDI2	GDI2
EH domain containing 4	EHD4	EHD4	EHD4
actinin alpha 4	ACTN4	ACTN4	ACTN4
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	YWHAB	YWHAB	YWHAB
RAB7A, member RAS oncogene family	RAB7A	RAB7A	RAB7A
lactate dehydrogenase B	LDHB	LDHB	LDHB
GNAS complex locus	GNAS	GNAS	GNAS
RAB5C, member RAS oncogene family	RAB5C	RAB5C	RAB5C
ADP ribosylation factor 1	ARF1	ARF1	ARF1
annexin A6	ANXA6	ANXA6	ANXA6
annexin 11	ANXA11	ANXA11	ANXA11
actin gamma 1	ACTG1	ACTG1	ACTG1
karyopherin subunit beta 1	KPNB1	KPNB1	KPNB1
ezrin	EZR	EZR	EZR

annexin A4	ANXA4	ANXA4	ANXA4
ATP citrate lyase	ACLY	ACLY	ACLY
tubulin alpha 1c	TUBA1C	TUBA1C	TUBA1C
transferrin receptor	TFRC	TFRC	TFRC
RAB14, member RAS oncogene family	RAB14	RAB14	RAB14
H4 clustered histone 14	HIST2H4A	HIST2H4A	HIST2H4A
G protein subunit beta 1	GNB1	GNB1	GNB1
thrombospondin 1	THBS1	THBS1	THBS1
RAN, member RAS oncogene family	RAN	RAN	RAN
RAB5A, member RAS oncogene family	RAB5A	RAB5A	RAB5A
	PTGFRN	PTGFRN	PTGFRN
chaperonin containing TCP1 subunit 5	CCT5	CCT5	CCT5
chaperonin containing TCP1 subunit 3	CCT3	CCT3	CCT3
adenosylhomocysteinase	AHCY	AHCY	AHCY
ubiquitin like modifier activating enzyme 1	UBA1	UBA1	UBA1
RAB5B, member RAS oncogene family	RAB5B	RAB5B	RAB5B
RAB1A, member RAS oncogene family	RAB1A	RAB1A	RAB1A
lysosomal associated membrane protein 2	LAMP2	LAMP2	LAMP2
integrin subunit alpha 6	ITGA6	ITGA6	ITGA6
H4 clustered histone 2	HIST1H4B	HIST1H4B	HIST1H4B
basigin (Ok blood group)	BSG	BSG	BSG
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta	YWHAH	YWHAH	YWHAH
tubulin alpha 1a	TUBA1A	TUBA1A	TUBA1A
discoidin domain receptor tyrosine kinase 2	TKT	TKT	TKT
t-complex 1	TCP1	TCP1	TCP1

stomatin	STOM	STOM	STOM
solute carrier family 16 member 1	SLC16A1	SLC16A1	SLC16A1
RAB8A, member RAS oncogene family	RAB8A	RAB8A	RAB8A
myosin heavy chain 9	MYH9	MYH9	MYH9
major vault protein	MVP	MVP	MVP

The exosomal proteins written in red represent the proteins that are absent from the list of top 100 exosomal proteins in Exocarta. The proteins that are absent in all three fractions of EXOs, are HIST1H4A, GNB2, HSPA1A, ARF1, ACTG1, TUBA1C, HIST2H4A, PTGFRN, HIST1H4B, and TUBA1A.

4.1.2 Comparative cargo proteome analysis of HAEC- derived EVs under normal, 25mM, and 50mM ethanol conditions.

The proteomic signatures of the EVs isolated from normal and both EtOH groups were examined by mass spectrometry. FunRich, a functional enrichment analysis tool, which is used mainly for functional enrichment and interaction network analysis of genes and proteins was used as a bioinformatics tool. Bioinformatics analyses were applied, identifying the 1967 total amount of proteins in H-EXO, 1749 total amount of proteins in 25-EXO, and 1819 total amount of proteins in 50-EXO. A total of 1421 proteins were shared by all three groups, H-EXO, 25-EXO, and 50-EXO. Whereas 241 proteins were exclusive to H-EXO, 82 proteins were exclusive to 25-EXO and 116 were exclusive to 50-EXO. The results of the analyses are depicted graphically in the form of a Venn diagram (Figure 25).

Next, we carried out a differential expression (DE) analysis to compare the significant differences in protein expression levels between the EVs. The following comparisons were made H-EXO vs 25-EXO, H-EXO vs 50-EXO, and 50-EXO vs 25-EXO revealing proteins were differentially expressed between the two EV groups. The results were visualized on volcano plots, (Figure 4.1B-C), highlighting differentially expressed proteins with adjusted p-value <0.05 and absolute LogFC>±1.3 as upregulated (red) and downregulated (blue) (Figure 26).

For H-EXO vs 25-EXO, among these proteins, nine proteins were significantly upregulated; RANBP6, UBE2NL, UBE2L3, PIGR, ALYREF, CD2AP, PRPF40A, ROCK2, USP39, and five proteins PSMB10, PSMB3, CLPTM1, PRPSAP2, and TFAM were significantly downregulated when 25-EXO was compared to the control group H-EXO. For H-EXO vs 50-EXO, among these two proteins, GALNS and MRPS36 were significantly upregulated, and seven proteins, CASP14, ROCK2, BCLAF1, PRPF40A, LDLR, KRT2, and SRRM1 were significantly downregulated in 50-EXO compared to the control group H-EXO. For 25-EXO vs 50-EXO, among these proteins, five proteins were significantly upregulated; KRT2, PRPSAP2, KRT14, KRT9, and SKIV2L, and twenty-four proteins; SKP1, UBE2L3, CNN3, ALYREF, PIGR, ME1, S100A6, USP39, RANBP6, AP15, DAZAP1, TCEA1, ZNF622, CFL2, NT5C2, PIEZO2, IGHA1, RAB14, AFP, EIF4H, EZR, GLRX3, ITM2B, MICU2 were significantly downregulated in 25-EXO compared to 50-EXO.

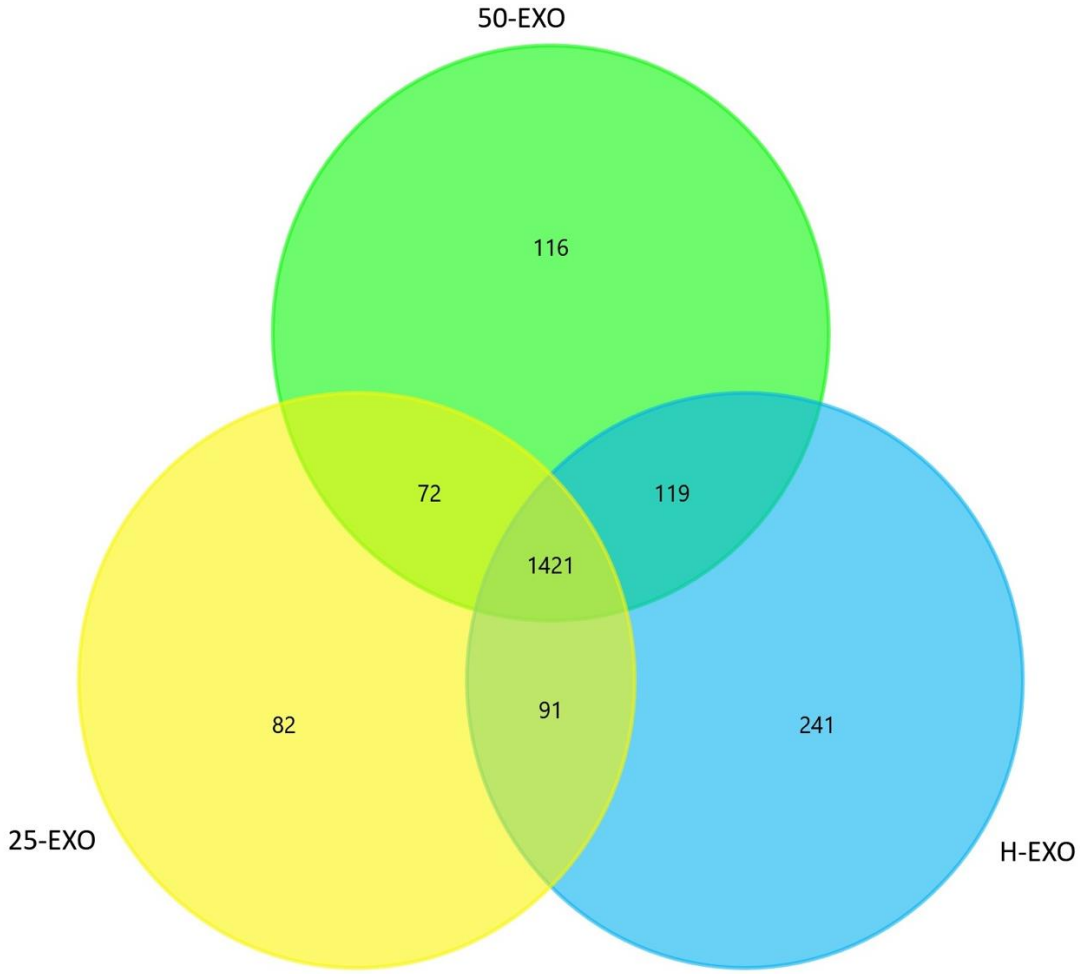
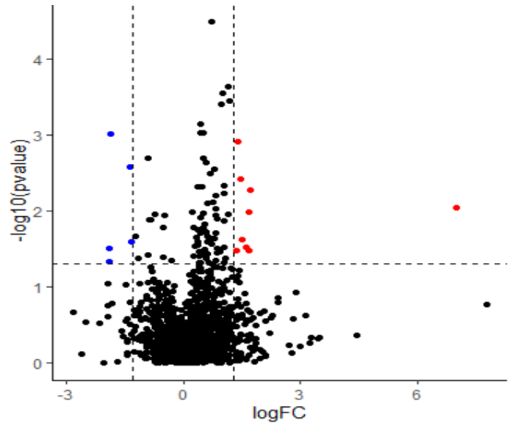
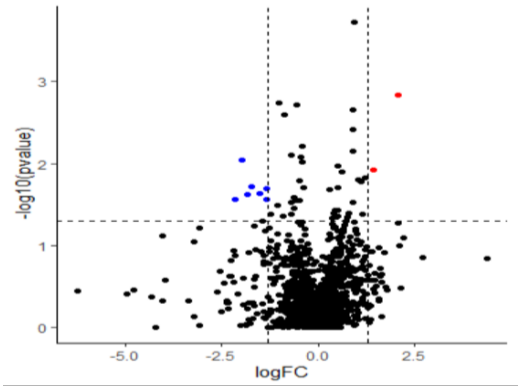


Figure 25Venn diagram showing the distribution of identified proteins in each fraction. Each Venn diagram represents the unique and common protein between H-EXO (blue) and 25-EXO (yellow), and 50-EXO (blue).

A. H-EXO vs 25-EXO



B. H-EXO vs. 50-EXO



C. 25-EXO vs. 50-EXO

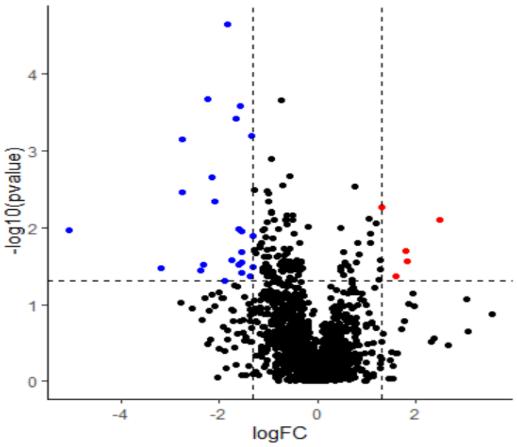


Figure 26 Volcano plots of differentially regulated proteins. Volcano plots of differentially expressed proteins with adjusted p-value <0.05 and absolute LogFC>±1.3 as upregulated (red) and downregulated (blue), (A) H-EXO vs 25-EXO, (B) H-EXO vs 50-EXO, (C) 25-EXO vs 50-EXO

4.1.3 Functional Annotation of H-EXO, 25-EXO and 50-EXO and their unique proteins Based on Gene Ontology (GO)

The proteomic data sets from the comparative analysis of HAEC- derived EVs under normal vs 25mM and 50mM ethanol conditions were analysed by standard bioinformatics software programs such as FunRich, which stands for "Functional enrichment analysis tool" which provides functional enrichment and network analysis integrated with gene ontology, biological pathways, protein interactions, or associated diseases (Benito-Martin & Peinado, 2015; Fonseka et al., 2021). Each protein was functionally annotated, firstly by its cellular component (Figure 27), proteins from H-EXO, 25-EXO, and 50-EXO were located mainly in the cytoplasm, nucleus, and exosomes based on GO. In addition, all three exosomes shared a series of common proteins involved in signal transduction, protein metabolism and cell communication (Figure 28). According to biological pathways (Figure 29), integrin, and TRAIL VEGF/VEGFR signalling, were the dominant pathways in all three exosomes. Next, we used the bioinformatics software PANTHER (Protein ANalysis Through Evolutionary Relationships) classification system which is a large database of protein families and their functionally related subfamilies that can be used to classify and identify the function of genes (Thomas et al., 2003). Functional Annotation based on Gene Ontology was also carried out on the unique proteins that were present within each of the EVs, to determine whether these proteins are involved in different pathways, molecular functions and biological processes (Figures 28-32.).

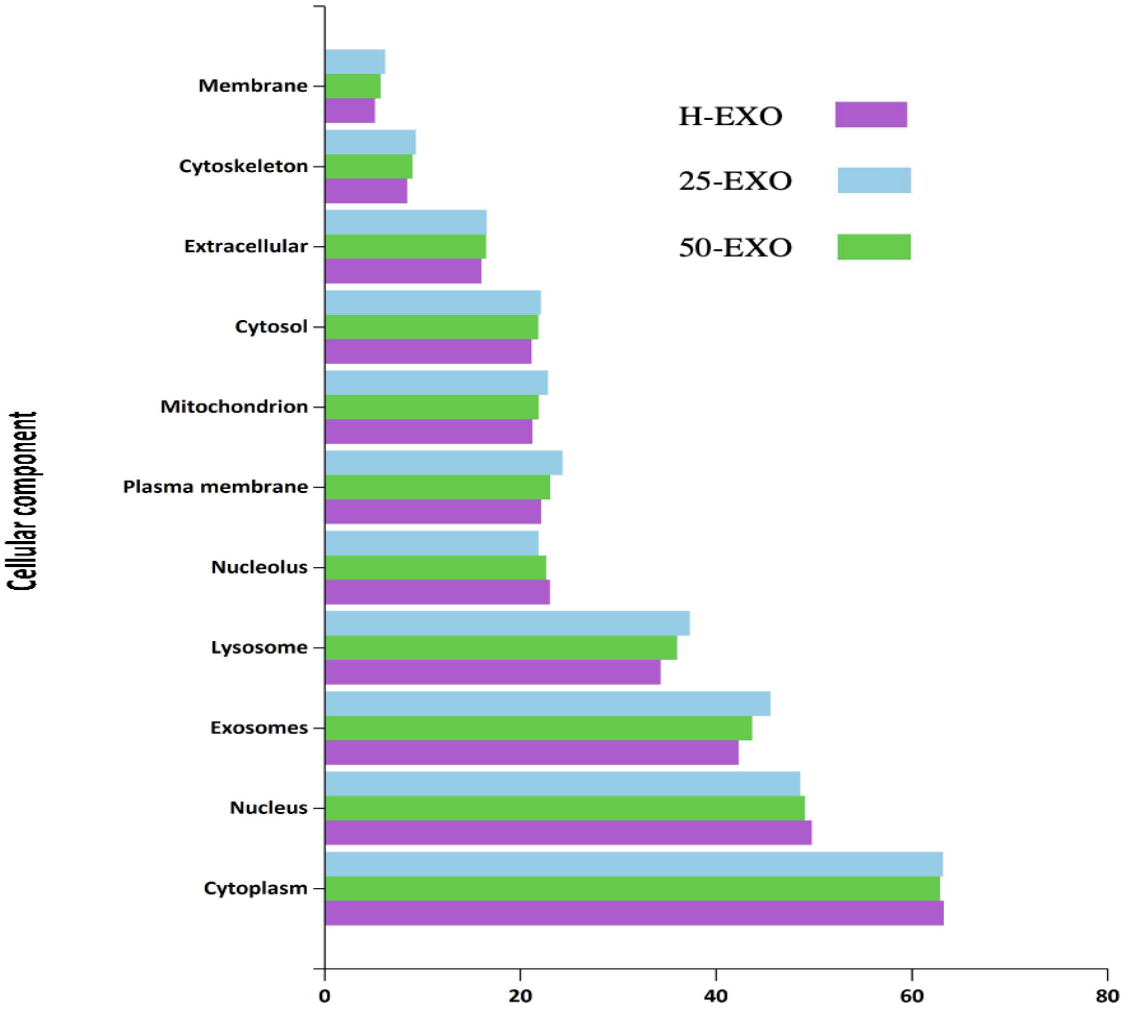


Figure 27 Cellular component comparisons. GO comparative analysis of cellular components in H-EXO, 25-EXO and 50-EXO depicted by a bar chart using the FunRich analysis tool

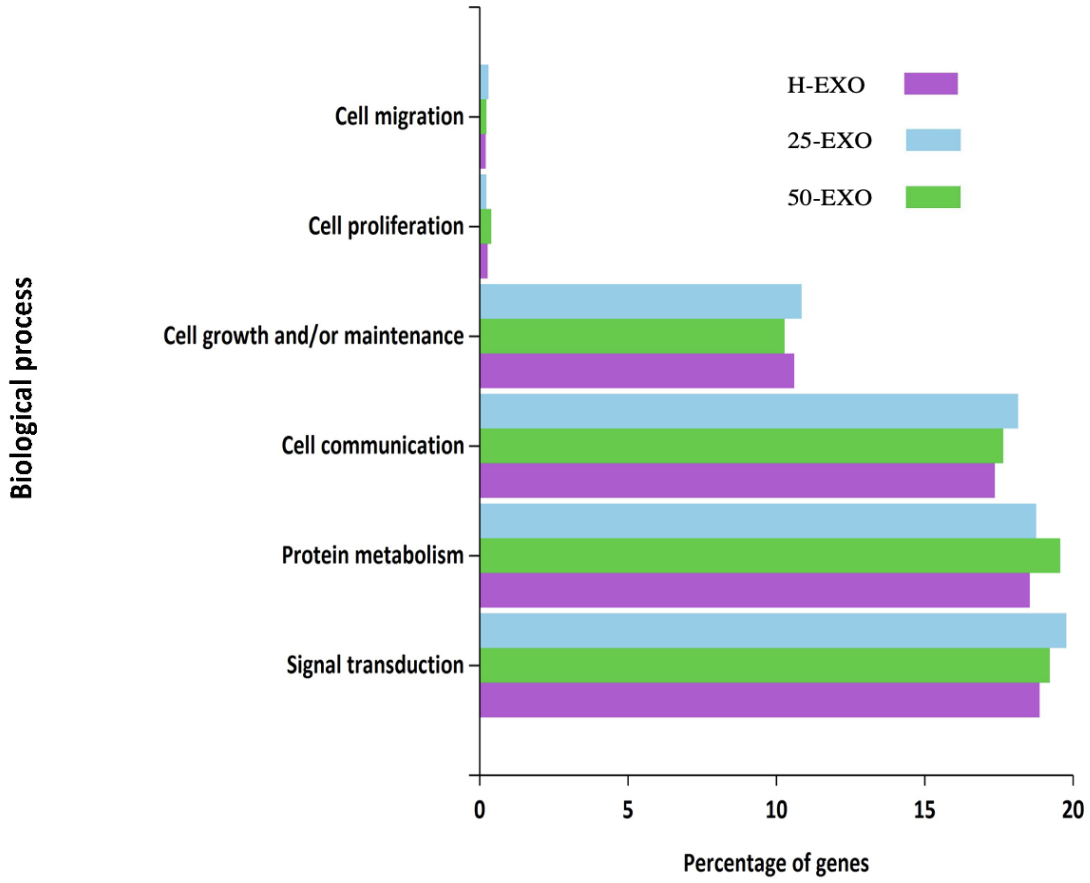


Figure 28 Biological process comparisons.GO comparative analysis of biological processes in H-EXO, 25-EXO and 50-EXO depicted by a bar chart using the FunRich analysis tool

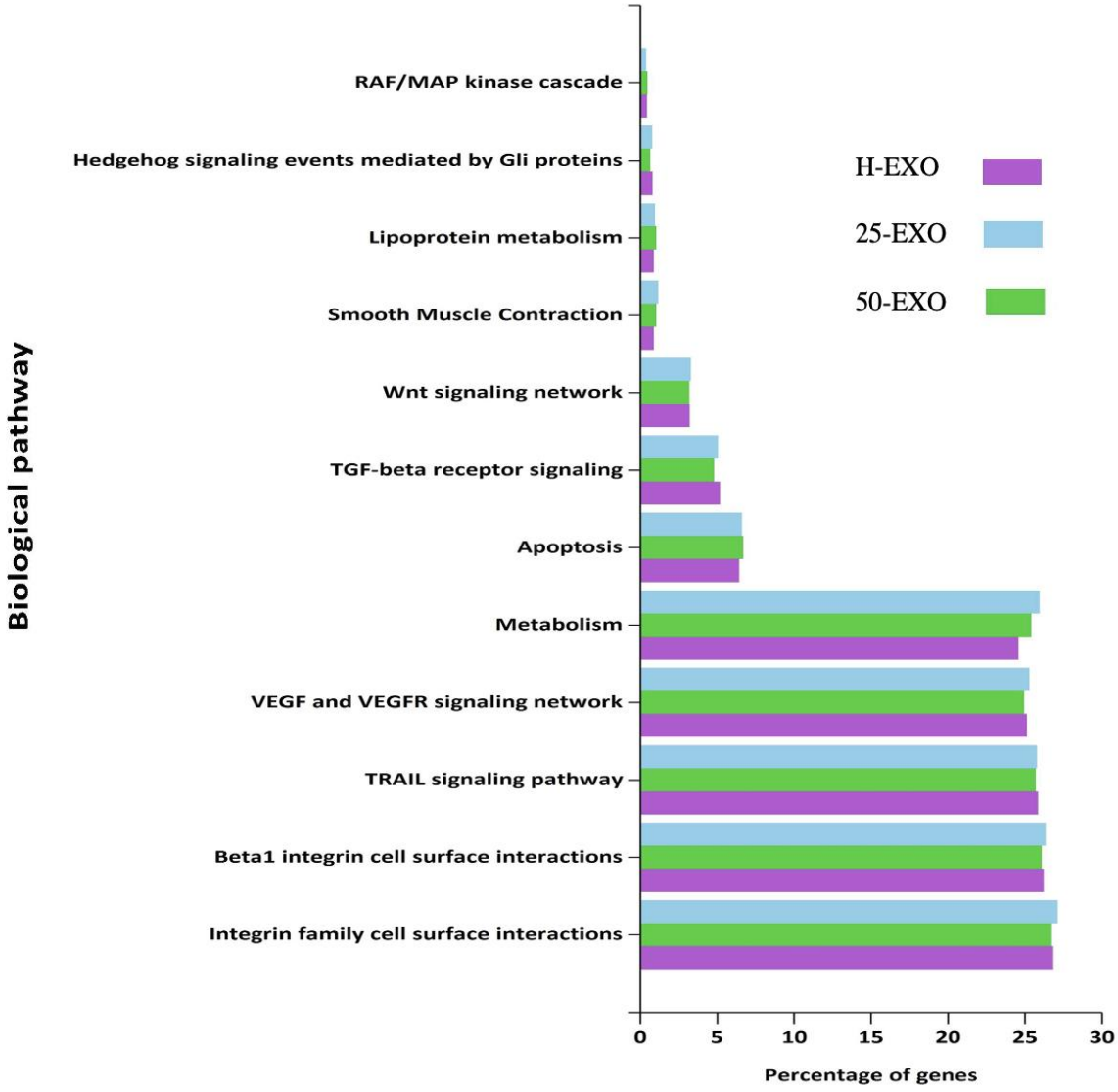


Figure 29 Biological pathway comparisons. GO comparative analysis of biological processes in H-EXO, 25-EXO and 50-EXO depicted by a bar chart using the FunRich analysis tool

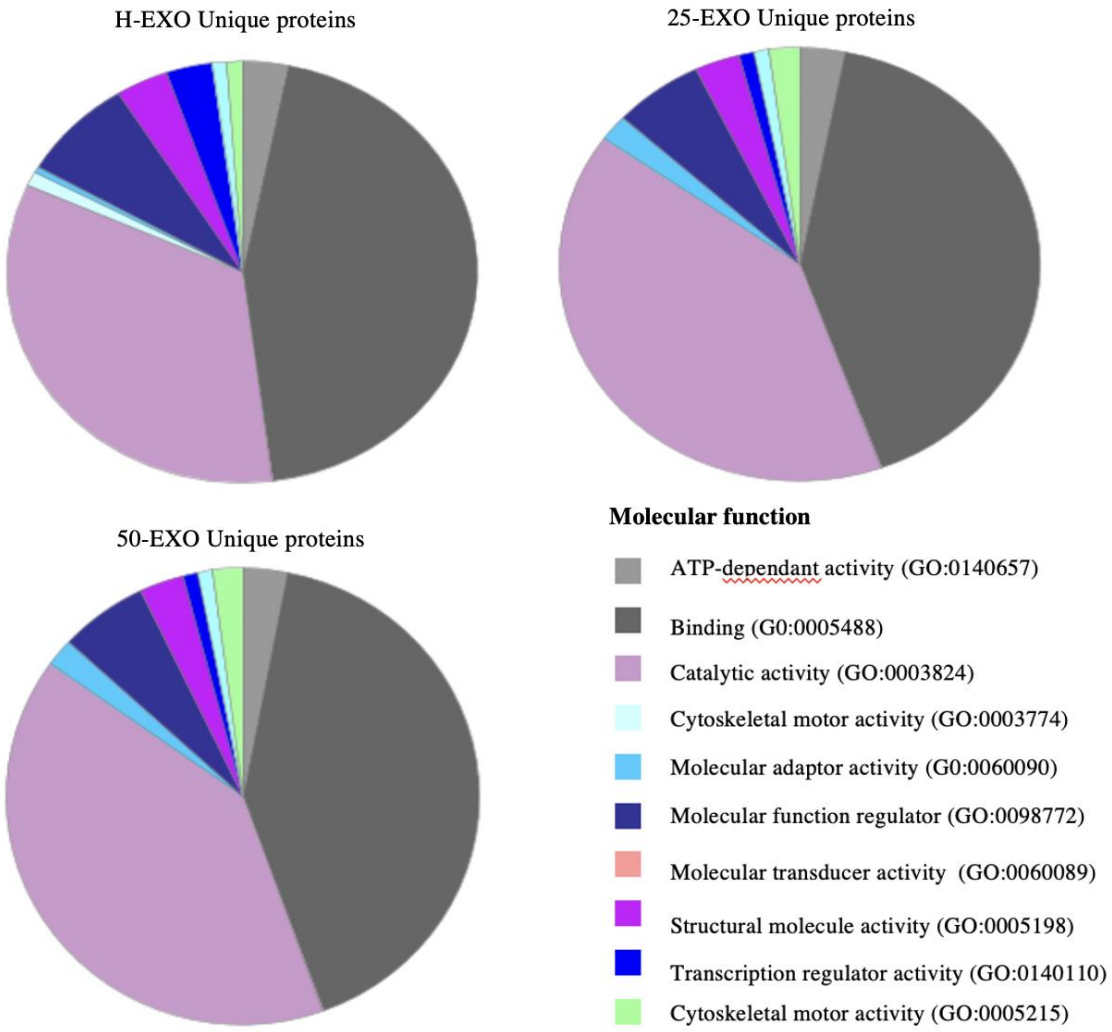


Figure 30 Molecular function comparisons of Unique proteins in EVs.GO comparative analysis of Molecular function of proteins unique to H-EXO, 25-EXO and 50-EXO depicted by a pie chart using Panther software.

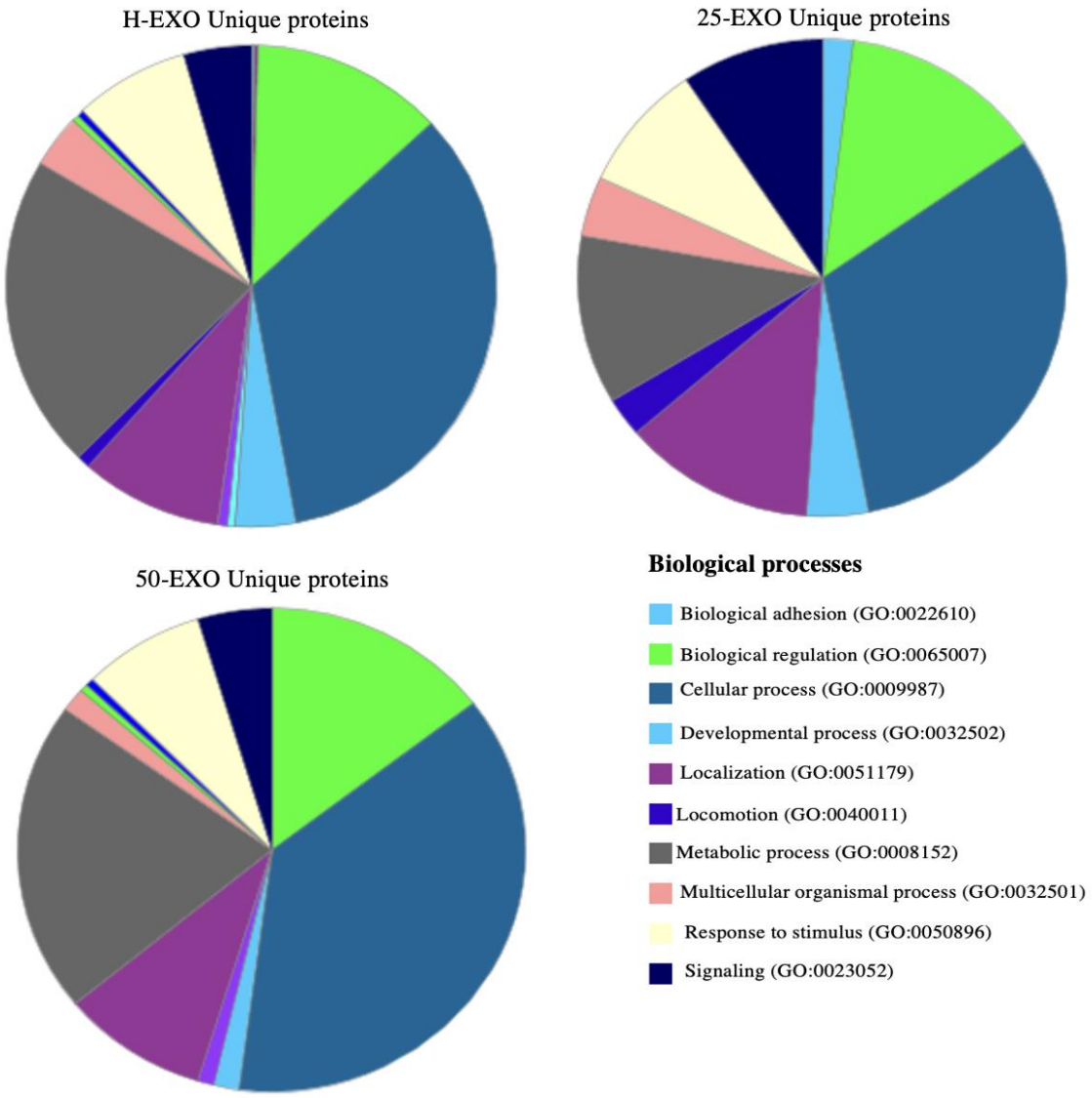


Figure 31 Biological process comparisons in unique proteins in EVs.. GO comparative analysis of Biological processes of proteins unique to H-EXO, 25-EXO and 50-EXO depicted by a pie chart using Panther software.

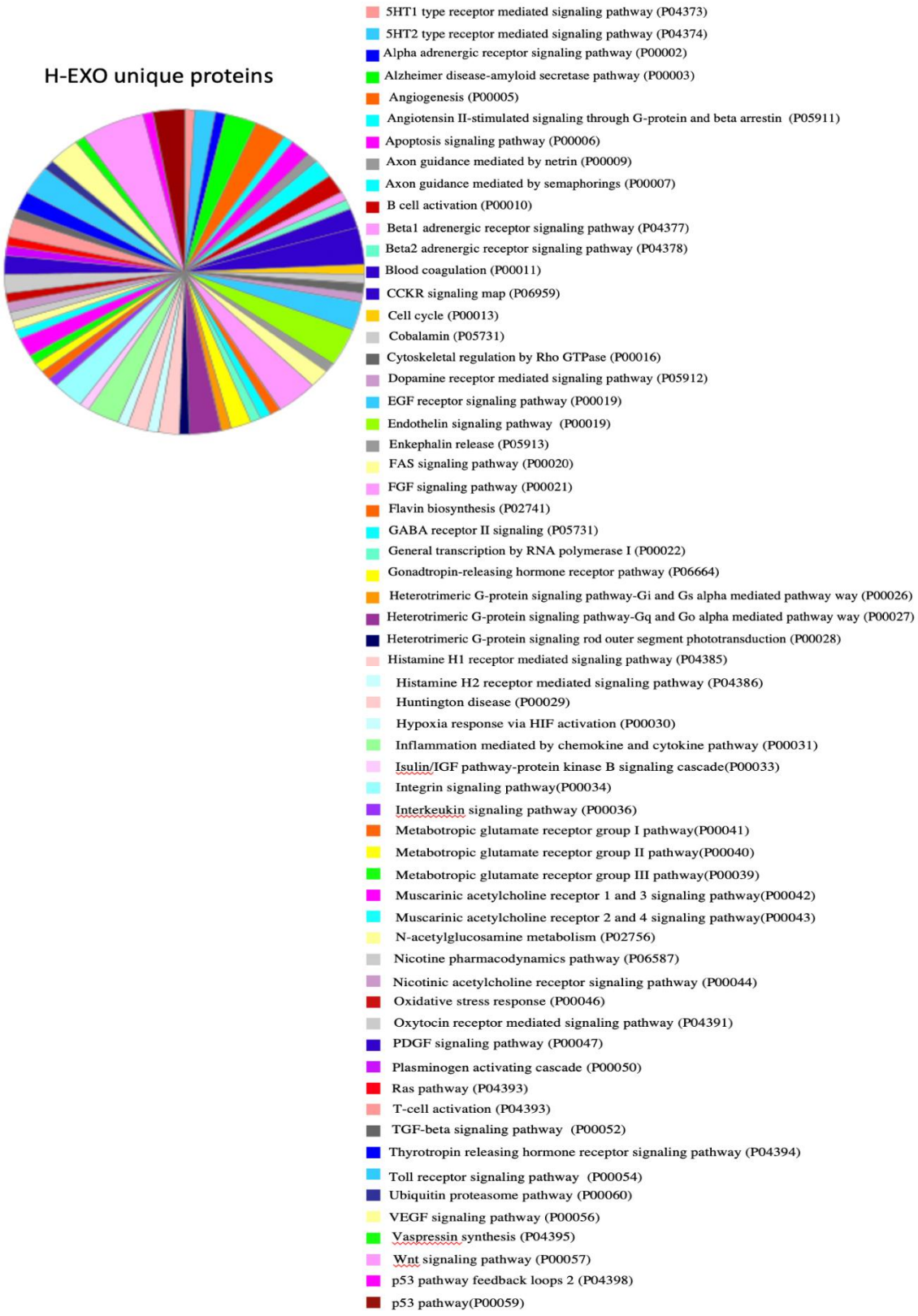


Figure 32 Signalling pathways in proteins unique to H-EXO. GO analysis of signalling pathways involved in proteins unique to H-EXO depicted by a pie chart using Panther software.

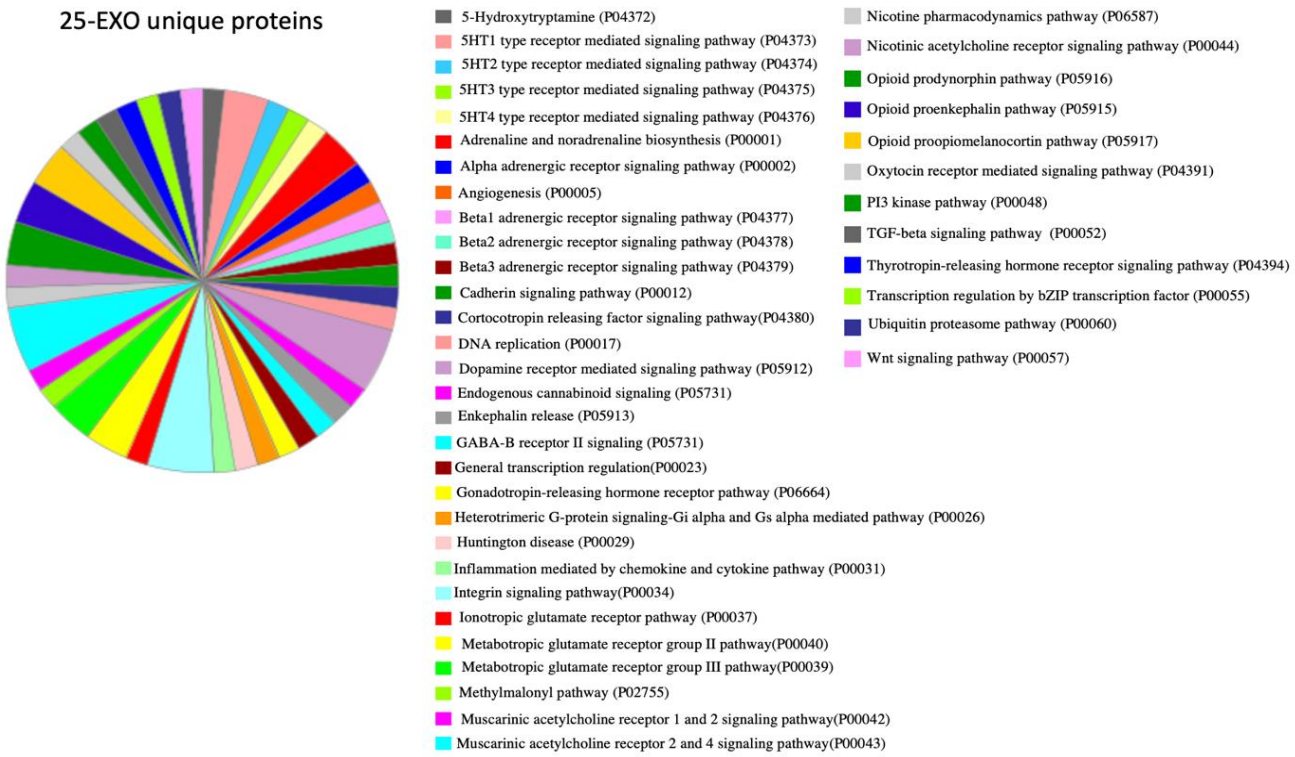


Figure 33 Signalling pathways in proteins unique to 25-EXO. GO analysis of signalling pathways involved in proteins unique to 25-EXO depicted by a pie chart using Panther software.

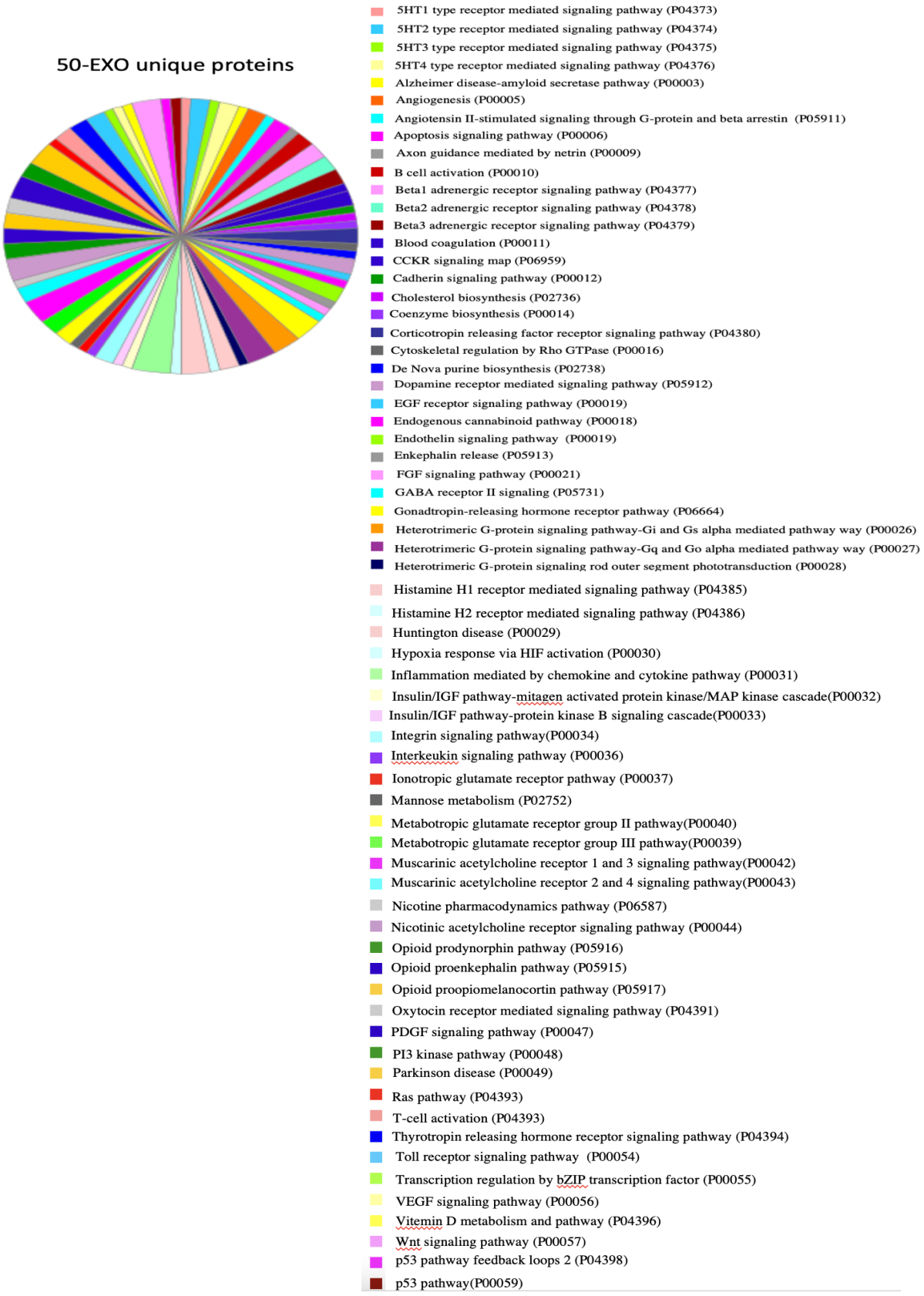


Figure 34Signalling pathways in proteins unique to 50-EXO. GO analysis of signalling pathways involved in proteins unique to 50-EXO depicted by a pie chart using Panther software.

4.1.4 Direct effect of ethanol and HAEC-derived EVs under normal and ethanol conditions attenuates the hSMC contractile phenotype

vSMCs are a major component of a healthy artery and play a major role in atherogenesis. The de-differentiation theory led to the idea that both the contractile and synthetic smooth muscle cell types exist. Contractile vSMCs can alter their phenotype to a synthetic state, which allows them to upregulate important genes involved in the remodelling of the vasculature. This includes proteases, adhesion proteins, cytokines, and chemokines. vSMC can also give rise to foam cells, osteochondrocytes, macrophages, or mesenchymal stem cells. Synthetic VSMCs can also regain many characteristics of the contractile phenotype, suggesting a reversible phenotypic switch is possible (Harman & Jørgensen, 2019). Contractile SMCs are classically spindle-shaped and elongated morphologically and are in a mature state. They generate proteins associated with the extracellular matrix such as elastin and they express smooth muscle cell markers associated with early (marker smooth muscle alpha-actin), intermediate (Calponin1) stage and late (major histocompatibility complex class II) stage SMCs. These contractile SMCs are in a state of quiescence in the vessel wall, therefore their proliferation and migration rates are reduced (Cunningham & Gotlieb, 2005). On the other hand, synthetic SMCs are shorter and less elongated and tend to have a "cobblestone" morphology. They contribute to vessel development and play a role in protein synthesis, thus containing a higher number of organelles whereas these are mostly replaced by contractile filaments in contractile SMCs. In comparison to contractile SMCs, synthetic SMCs exhibit a higher rate of growth and migration (Rensen et al., 2007). The vSMCs are a major source of many proliferative synthetic cells that contribute to forming the extracellular matrix to form the fibrous cap and therefore stabilise plaques (Basatemur et al., 2019). It is essential to reduce pathological arterial remodelling and its associated aberrant blood flow, therefore it is important to gain better insight into identifying the origin of SMC-like cells that accumulate in the intima. vSMCs present in the vessel wall may be triggered in response to injury to undergo de-differentiation and contribute to vascular disease development.

The first reports of SMCs grown in culture were by Champy, he described them as "elongated elements with fibrillae that lost these characteristics with repeated mitosis". Laqueur, who noted contractions were evidently spontaneous in SMC explants and Lewis and Lewis, who stained mitochondria of SMCs (Chamley-Campbell et al., 1979). Cultured vSMCs grown *in vitro* are a powerful tool for understanding their functionality and their contribution to vessel wall contraction as well as vascular diseases. However, there have been limitations with the

growth and proliferation of vSMCs. It is well known that vSMC can undergo the process of phenotypic switching, causing a loss of expression in vSMC markers (G. K. Owens et al., 2004). vSMCs from mature vessels are difficult to access due to the complexity of the structure and the presence of many other cellular components within the vessel wall, which can increase the possibility of cell contamination such as EVs and fibroblast. They can also have a limited expansion ability *in vitro*.

Our aim was to determine the effect of ethanol (EtOH) exposure on hSMCs and their contribution to pathologic vessel remodelling. To investigate the role of EV facilitated dedifferentiation of hSMCs via transport of vesicular cargo, it was first important to determine whether the direct treatment of ethanol causes myogenic de-differentiation of hSMC. Various concentrations of Ethanol (0mM, 5mM, 25mM, 50mM and 100mM) were used to treat the cells for dose-response experiments. hSMCs were incubated for 24 hours, cells were then harvested, and RT-qPCR was carried out. No significant changes in expression of *Myh11* were observed after treatment with 5mM and 50mM, however, after treatment with 25mM and 100mM, there was significant attenuation in *Myh11* (Figure 35). Similar observations were detected in *CNN1*, whereby no significant changes in expression of *CNN1* were observed after treatment with 5mM and 50mM. However, after treatment with 25mM and100mM there was significant attenuation in *CNN1* (Figure 36). These effects suggest an inhibitory effect of EtOH on smooth muscle proliferation. The expression levels of these contractile marker proteins decrease when SMCs are cultured with 25mM and 100 concentrations of ethanol. The reduction in the expression of these proteins that are associated with the contractile phenotype is generally taken as characteristic of the synthetic phenotype. SMCs with different phenotypes express varying levels of the marker proteins as opposed to entirely different marker proteins. Although the SMC phenotype appears to be genetically programmed, vascular injury cues can still modulate the characteristics of the SMCs.

Having developed a human endothelial cell model, HAEC, allowed for the generation of EVs using ethanol to mimic moderate and chronic alcohol consumption to induce endothelial cell dysfunction and confirm the presence of these purified EVs from normal and ethanol (25mM and 50mM) treated cell culture media. Following on from our experiments on the effect of exposure to ethanol on vSMCs, we test our hypothesis, if secreted HAEC-derived EVs under normal and ethanol conditions impact the myogenic de-differentiation of vSMCs *in vitro.* A panel of controls were run in parallel which included EVs derived from normal HAEC culture,

as well as normal, and ethanol conditions from NC media in the absence of cells. To determine the role of HAEC-EVs in promoting de-differentiation of hSMCS, hSMCs were cultured in the absence or presence of EVs purified from normal (H-EXO) and ethanol-treated (25-EXO,50-EXO) CM and NC NC medium before the relative mRNA levels myogenic differentiation marker gene *CNN1* and *Myh11* were assessed after 7 days.

The relative levels of *Myh11* mRNA were significantly decreased in hSMCs following treatment with 25-EXO-CM when compared to H-EXO-NC and no treatment (NT) control after 7 days (Figure 37). The relative levels of *Myh11* mRNA did not decrease in hSMCs treated with 50-EXO-CM as seen with *CNN1* mRNA levels (Figure 38). The relative levels of *CNN1* mRNA were significantly decreased in hSMCs following treatment with 25-EXO-CM when compared to H-EXO-NC and H-EXO-CM controls after 7 days. The relative levels of *CNN1* mRNA were also significantly decreased in hSMCs treated with 50-EXO-CM when compared to H-EXO-CM controls, respectively (Figure 38).

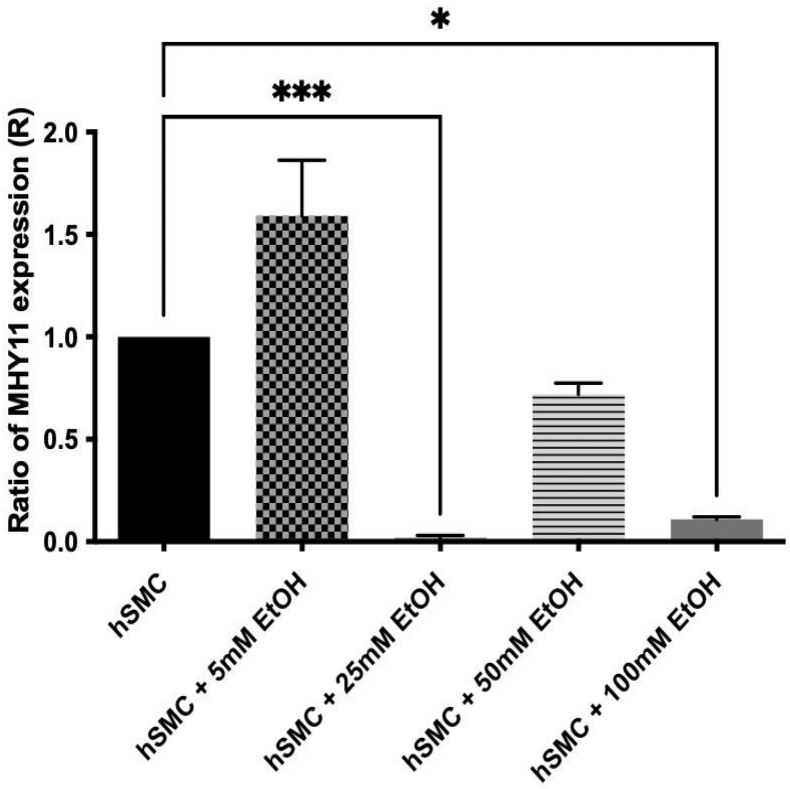


Figure 35Expression of Myh11 in hSMCs treated with varying concentrations of ethanol (EtOH) for 24 hours. qRT-PCR was used to measure the expression of Myh11 in hSMCs cultured in DMEM, treated with different concentrations of EtOH (5mM, 25 mM, 50 mM AND 100 mM) for 24 hours. Data are given as ratio expression relative to the negative control sample with no ethanol treatment. Error bars represent the standard error of the mean (SEM) *P≤ 0.05. Data representative of n=3.

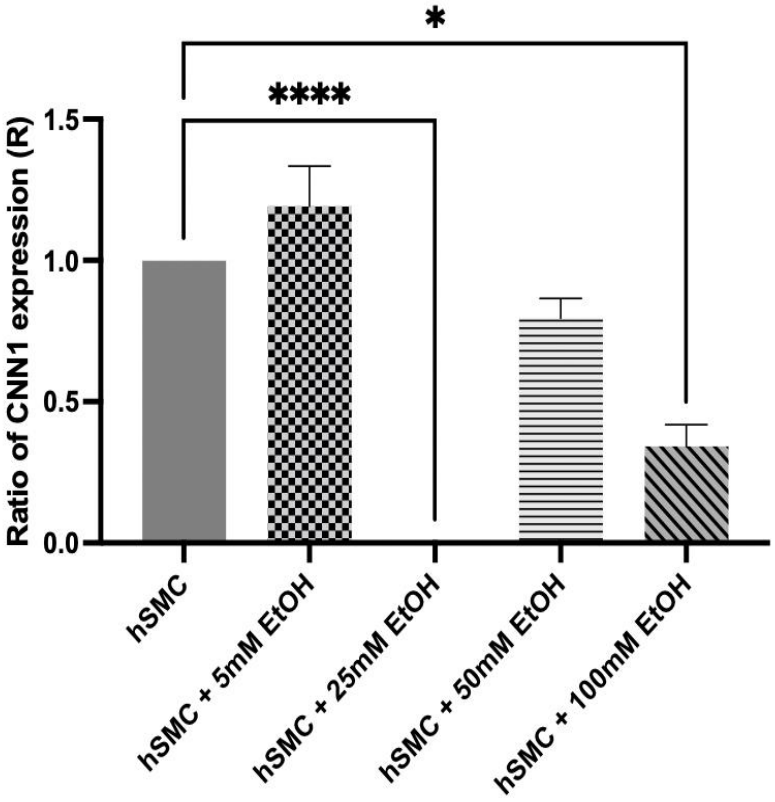


Figure 36 Expression of Myh11 in hSMCs treated with varying concentrations of ethanol (EtOH) for 24 hours. qRT-PCR was used to measure the expression of CNN1 in hSMCs cultured in DMEM, treated with different concentrations of EtOH (5mM, 25 mM, 50 mM AND 100 mM) for 24 hours. Data are given as ratio expression relative to the negative control sample with no ethanol treatment. Error bars represent the standard error of the mean (SEM) *P≤ 0.05. Data representative of n=3.

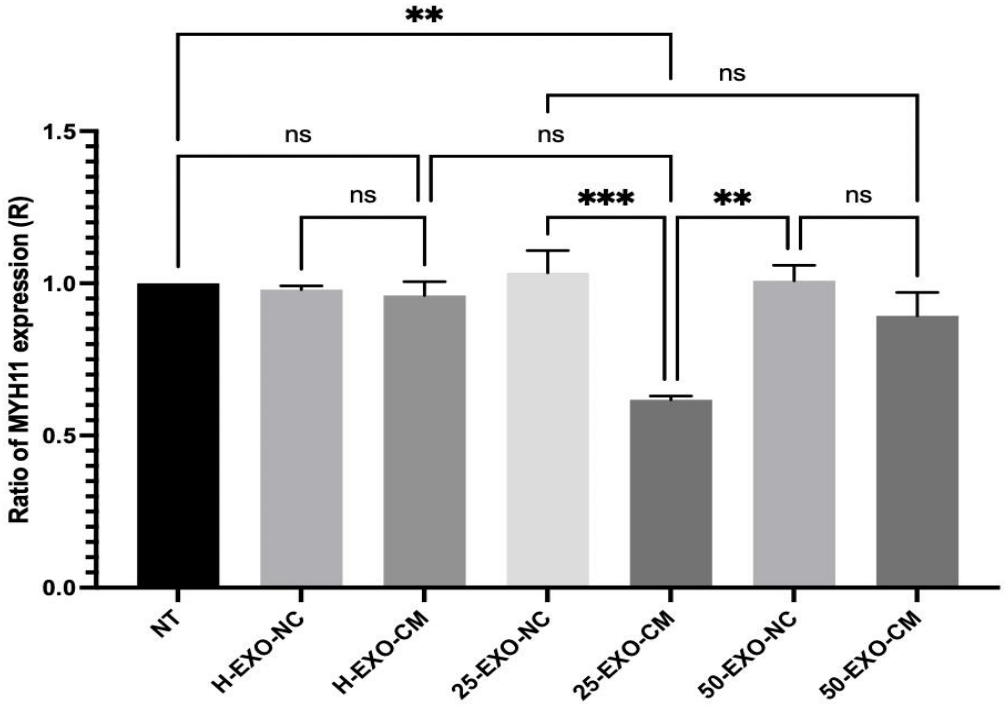


Figure 37 Expression of Myh11 in hSMCs treated with HAEC-derived EVs under normal and ethanol conditions from both condition and NC medium. qRT-PCR was used to measure the expression of Myh11 in hSMCs cultured in DMEM, treated with EVs derived from NC and CM media treated in normal and 25 and 50mM ethanol conditions for 7 days. Data are given as ratio expression relative to the negative control sample with no treatment. Error bars represent the standard error of the mean (SEM). *P≤ 0.05. Data representative of n=3.

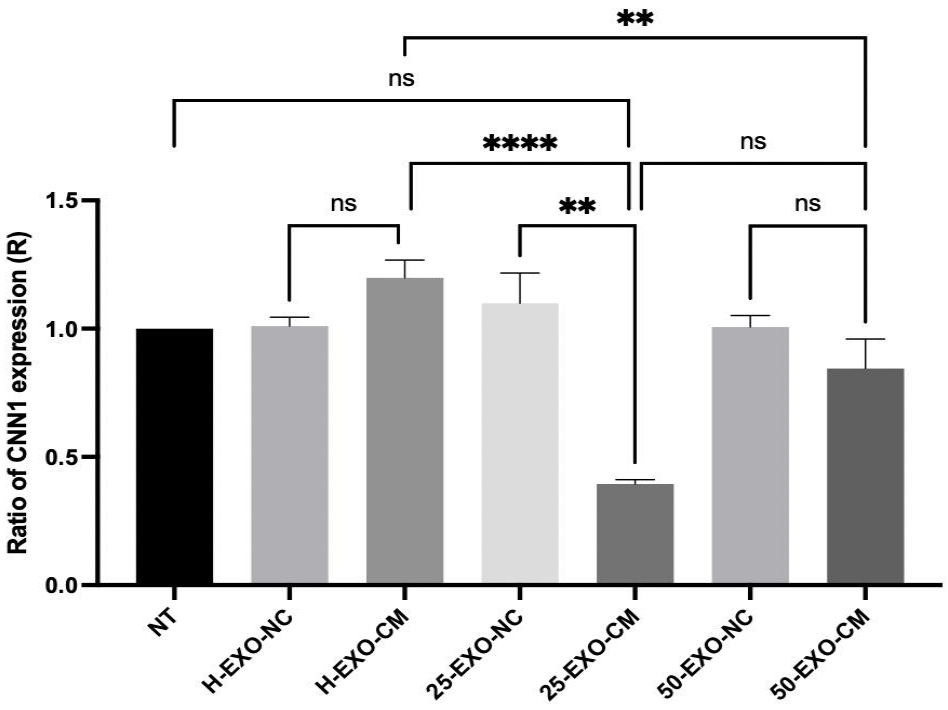


Figure 38. Expression of CNN1 in hSMCs treated with HAEC-derived EVs under normal and ethanol conditions from both condition and NC medium. qRT-PCR was used to measure the expression of CNN1 in hSMCs cultured in DMEM, treated with EVs derived from NC and CM media treated in normal and 25 and 50mM ethanol conditions for 7 days. Data are given as ratio expression relative to the negative control sample with no treatment. Error bars represent the standard error of the mean (SEM). *P≤ 0.05. Data representative of n=3.

4.1.5 The effect of HAEC-derived EVs under normal and ethanol conditions on Hedgehog signalling in hSMC

Previous research in our lab has shown that HAECs exposed to hyperglycaemic conditions resulted in an increase in SHh expression *in vitro.* When HiPSC-derived NE SNEPs were treated with these EVs, this led to an increase in Hh signalling, growth and myogenic differentiation of these progenitor cells. To determine whether EVs are harbouring SHh ligands in dictating the phenotypic changes in hSMCs, the levels of SHh target genes were evaluated in vSMCs after no treatment and treatment with EVs derived from normal and ethanol (25mM and 50mM) conditions from HAECs in CM for 48 h. As previously mentioned, a panel of controls were run in parallel which included EVs derived from normal HAEC culture, as well as EVs derived from normal, and ethanol conditions from NC media in the absence of HAEC cells. To determine the role of HAEC-EVs in promoting the activation of SHh target genes in hSMCS, HAEC-derived EVs were isolated and purified from normal and ethanol-treated CM and NC medium using ExoQuick™.. Isolated EVs were then resuspended in hSMC complete medium (DMEM). Cultured hSMCs were seeded at a density of 200,000/well for 24 h at 37°C. Following removal of DMEM media, hSMCs were treated with fresh DMEM media supplemented with the isolated EVs from different conditions for 48hrs. hSMCs were then harvested before the relative mRNA expression of SHh target genes Gli1 and Gli2 were assessed using RT-qPCR. There was no significant change in the ratio of Gli1 or Gli2 across all EVs treatments, H-EX0, 25-EXO and 50-EXO (CM) when compared to no treatment and their control groups (Figure 39-40). This suggests that EVs derived from HAECs under ethanol conditions do not activate SHh target genes

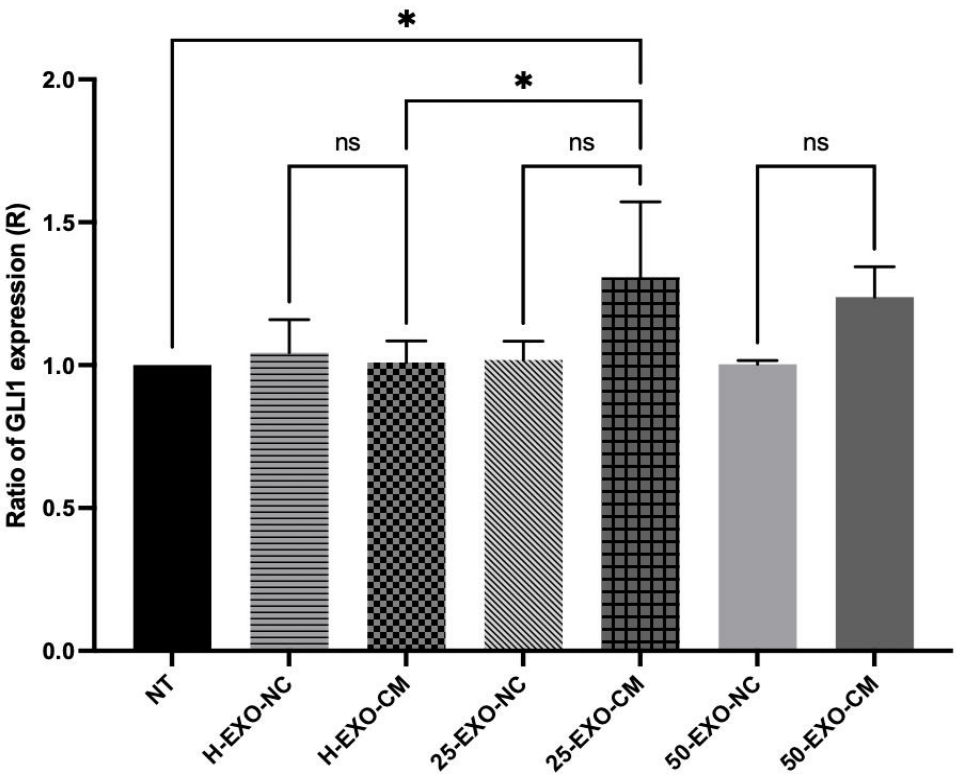


Figure 39 Expression of Gli1 in hSMCs treated with H-EXO, 25-EXO and 50-EXO. RT-qPCR was used to measure the expression of Gli1 in hSMCs cultured in DMEM, treated with exosomes from NC and CM in normal, and 25mM and 50mM ethanol treatments for 48hrs. Data are given as ratio expressions relative to the negative control sample with no treatment. Error bars represent the standard error of the mean (SEM). *P≤ 0.05. Data representative of n=3.

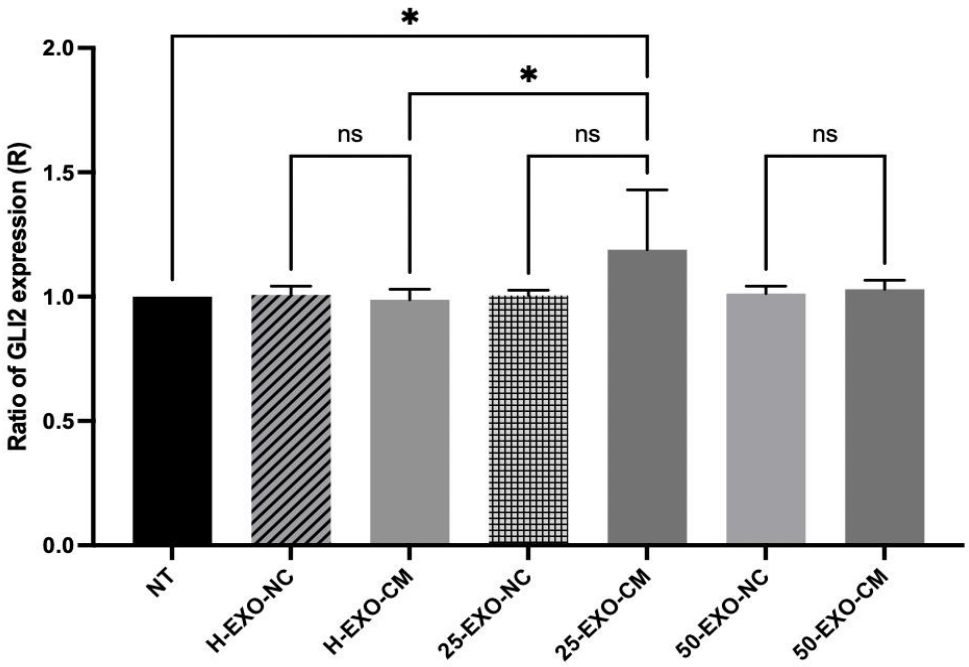


Figure 40 Expression of Gli1 in hSMCs treated with H-EXO, 25-EXO and 50-EXO.RT-qPCR was used to measure the expression of Gli1 in hSMCs cultured in DMEM, treated with exosomes from NC and CM in normal, and 25mM and 50mM ethanol treatments for 48hrs. Data are given as ratio expression relative to the negative control sample with no treatment. Error bars represent the standard error of the mean (SEM). *P≤ 0.05. Data representative of n=3.

4.5 Summary

- HAEC-derived EVs under normal and ethanol conditions carry exosomal proteins within their cargo.
- Proteins are differentially expressed under 25mM and 50mM ethanol conditions when compared to the control, or compared to each other, such as up-regulation of PIGR in H-EXO vs 25-EXO and 25-EXO vs. 50-EXO and downregulation of proteins MICU2, GLRX1 and TFAM in 25-EXO vs 50-EXO.
- GO analyses revealed the proteins involved in pathways, biological processes and molecular functions that are associated with proteins within the whole EV and the unique proteins found within each EV. Bioinformatic pathway analyses yielded protein hit counts against Endothelin signalling pathway, FGF signalling pathway and wnt signalling pathways unique to H-EXO, dopamine receptor-mediated signalling pathway, integrin signalling pathway and muscarinic acetylcholine receptor 2 and 4 signalling pathway unique to 25-EXO and inflammation mediated by chemokine and cytokine pathway and wnt signalling pathway unique to 50-EXO. Indicating that proteins within the ethanol derived EVs are involved in different pathways when compared to the control.
- hSMC attenuate their contractile phenotype when treated with concentrations of 25mM and 100mM ethanol.
- HAEC-derived EVs from 25 mM conditions that were treated on vSMC induce de-differentiation of hSMCs *in vitro.*
- EVs derived from HAEC CM under ethanol conditions did not promote SHh target genes (*Gli1* and *2*).

Chapter 5

General Discussion

5.1 Alcohol consumption and it's impact at a vascular cellular level.

Vascular complications due to excessive alcohol consumption can account for a significant proportion of morbidity and increased mortality in patients with chronic and binge drinking patterns. Numerous studies have investigated the various effects of alcohol consumption and its relationship with the incidence and severity of the outcome of CVD which can result in a heart attack or stroke. With respect to CVD, meta-analysis indicated that compared to abstinence, low-moderate consumption (1-3 drinks/day) which gives rise to blood alcohol levels of a range between 2-25mM, appears to be protective, and has the lowest risk of CVD progression (Bagnardi et al., 2008; Corrao et al., 2000). On the contrary, chronic alcohol or binge drinking, by which 5 or more drinks are consumed in less than two hours, leads to elevated blood alcohol levels as high as 50mM which can cause an increased risk of CVD and greater mortality (Ruidavets et al., 2010; Thun et al., 1997). The mechanisms by which consumption of moderate alcohol decreases the risk of CVD are complex. The main favourable and beneficial effect of alcohol on AS is its effect on plasma lipids. Plasma HDL has been shown to increase and plasma LDL has been shown to decrease because of moderate alcohol consumption. LDL is said to be pro-atherogenic leading to an increase in the risk of heart disease, whereas HDL is said to be anti-atherogenic, lowering the risk of disease (Wood et al., 1998). A review of past studies has shown that LDL levels are elevated by binge drinking episodes (Mckee & Britton, 1998). These findings were also supported by other studies carried out that showed there was an increase in LDL-C levels in rats when they ingested heavy concentrations of EtOH (Z. Wang et al., 2010). Another study showed that both patterns of alcohol consumption (moderated and binge drinking) resulted in increased plasma HDL levels. Despite this, they found that daily-moderate alcohol consumption lowered LDL levels by 40%, contrary to the elevated LDL levels by 20%, as a result of weekend-binge alcohol consumption (W. Liu et al., 2011). It is now well known that when plasma LDL levels elevate by 10% this results in a 20% increase in AS risk (Wood et al., 1998). Atherosclerotic plaque develops under certain conditions following interactions between cells (ECs, SMCs, fibroblasts), lipids and inflammatory cells that exist within the vessel wall. Although the precise population of cells and the signalling mechanisms that are targeted by alcohol to mediate these effects is still unknown, they continue to be investigated.

vSMC accumulation is a hallmark of AS and vascular injury. The hyperplasia of vSMCs in the media and accumulation of these cells in the tunica intima plays a key role in the process of

intimal medial thickening (IMT) due to de-differentiating SMCs (Chappell et al., 2016). The occurrence of IMT and restenosis has become a major issue in the treatment of CVD such as stenting and balloon angioplasty (Doran et al., 2008). The most common hypothesis is the phenotypic switching of contractile vSMCs to a synthetic SMC phenotype that is capable of proliferation and migration. The concept of phenotypic switching, re-programming and de-differentiation of SMCs was first theorised to show that contractile SMCs that were isolated and expanded underwent phenotypic changes (Campbell & Campbell, 2012; Chamley-Campbell & Campbell, 1981). The main mediator in SMC de-differentiation *in vitro* is PDGF-BB (Yoshida Tadashi, 2007). SMC that has been sub-cultured have shown a distinct loss in the expression of SMC-specific markers such as CNN1 and Myh11. They also acquire synthetic and proliferative cellular machinery, for example, the cell increases its abundance of endoplasmic reticulum, ribosomes and Golgi apparatus (J. H. Chamley-Campbell & Campbell, 1981). A reduction in microRNA levels can also result in a less contractile and more synthetic phenotype (Cordes et al., 2009). Myocardin levels are decreased in parallel with the attenuation of SMC marker expression when compared to the fresh aorta (J. Chen et al., 2002). Epigenetic mechanisms play a critical role in the control of SMC differentiation and phenotypic plasticity. PDGF-bb and other stimulators of SMC de differentiation have been used to treat SMCs in culture and characterise them epigenetically (M. R. Alexander & Owens, 2012). The epigenetic modification, histone acetylation is depleted during the de- differentiation process, however, the H3K4Ddime of the Myh11 locus is restricted to SMC lineage in humans and mice, and this mark persists through phenotypic modulation within the atherosclerotic lesion which expresses no SMC markers (Gomez et al., 2013). Transcription factor KLF4 is another important factor that modulates SMC phenotypic switching. Studies have shown that KLF4 represses the expression of many SMC genes (Yan Liu 2005). This factor is not expressed in SMCs that are differentiated *in vivo*, however in response to vascular injury KLF4 is rapidly upregulated *in vivo.* In response to vessel injury, the adventitia undergoes significant remodelling, this leads to adventitial myofibroblasts proliferating and migrating into the neo-intima, these cells resemble medial SMCs and are believed to be associated with the development of the neointima (Scott et al., 1996; Shi et al., 1996). The origin of neointimal SMC-like cells is controversial but lineage tracing and scRNA-seq have provided compelling evidence in various animal models for the involvement of a rare population of differentiated Myh11 medial SMC that are Sca1+, that de-differentiate and undergo phenotypic switching (Chappell et al., 2016; Dobnikar et al., 2018). It is accepted that data retrieved from lineage tracing shows that differentiated

SMCs undergo phenotypic switching when induced by injury which causes the onset of SMC proliferation (Herring et al., 2014; Nemenoff et al., 2011).

Alcohol has an effect on lipoproteins (G. Hao et al., 2015). Light to moderate alcohol intake has been shown to decrease lipid accumulation product (LAP) and reduce its relation to hypertension. LAP is an index calculated by waist circumference and TG levels, and has been used to predict CVD (Wakabayashi, 2014). It is speculated that OS plays a key role in the pathogenesis of many EtOH-related disorders. OS and the generation of ROS are a result of EtOH metabolism in the vascular system (J et al., 2005). EtOH has also been shown to cause aortic vSMC proliferation. This is due to an increase in Ox-LDL-mediated OS and elevated levels of homocysteine which leads to changes in the aortic wall that is proatherogenic (Shirpoor et al., 2013). This study also supported evidence that the level of serum lipids such as LDL, TG and cholesterol increased after EtOH egestion, whilst a simultaneous reduction in apoA levels was observed. They show an association between Ox-LDL and amino acid homocysteine and vSMC proliferation. An increase in OS in various EtOH-induced organ disorders has been reported in animal and human studies (Shirpoor et al., 2008; D. Wu & Cederbaum, 2006). Hyperhomocysteinemia (high levels of homocysteine in the blood) is associated with a higher risk of CVD. Studies have shown that homocysteine can initiate SMC growth that can lead to the progression of AS(Desai et al., 2001; Halkos et al., 2000). EtOH is known to control the growth and migration of vSMCs. It has been shown that EtOH has an inhibitory effect on SMC proliferation and on the MAPK signalling pathway *in vitro* (Hendrickson et al., 1998). Data from another study has also demonstrated that EtOH inhibits vSMCs by a mechanism that modulates the expression and activity of cell cycle regulatory molecules in rat aortic SMCs (Sayeed et al., 2002). Reports have also shown that daily moderate EtOH consumption inhibits SMC proliferation through inhibition of the Notch signalling pathway (Morrow et al., 2010) and inhibits carotid artery remodelling and causes an increase in plaque in mice (W. Liu et al., 2011). The need for diagnostic biomarkers that predict the presence of subclinical AS at an early stage is of fundamental importance however is still an unmet clinical need. Further research is required to understand the role of cell-cell communication as the primary instigator of neointimal VSMC-like cell hypoplasia leading to IMT and neointima formation.

5.2 EVs and their cues driving cargo make up and release.

EVs have been described as lipid membrane-enclosed vesicles that are secreted from most cell types into the extracellular space and play a role in cell-cell communication. EVs are separated into three distinct types, microparticles, exosomes and apoptotic bodies (Noble et al., 2020). Several studies reported that EVs secreted from an EC play an important role in endothelial function and contribute to angiogenesis-related diseases such as CVD. EVs can play a critical role in vascular homeostasis and pathological processes such as inflammation (Fujimoto et al., 2021). EVs derived from ECs were shown to contribute to the SMC contractile phenotype (X. Lin et al., 2016). EVs released by ECs have been shown to be associated with vascular dysfunction. A study reported that vSMC proliferation and migration are regulated by EVs derived from ECs. Idoxyl-Sulfate induced EVs released by ECs stimulated these cellular processes in a concentration dependant manner. The EVs contained TGF-β and promoted the production of TGF-β in SMC by phosphorylating p38 MAPK, Smad3, ERK1/2 and Akt, which was inhibited by an anti-TGF-β antibody. EVs under uremic conditions are suggested to be involved in the development of stenosis by controlling TGF-β signalling in vSMCs (Ryu et al., 2019). EVs derived from other sources such as plasma, fibroblasts and macrophages have also been reported to regulate SMC proliferation and migration (Otani et al., 2020; Ren et al., 2020; Sharma et al., 2018). EVs are known to encapsulate various molecules such as lipids, proteins and nucleic acids (Berezin & Berezin, 2020; Xing et al., 2020). These bioactive molecules are well documented to be involved in implementing the effects of EVs on EC to vSMC regulation. EVs derived from HUVECs can control the vSMC phenotype through EV cargo containing miRNAs (Hergenreider et al., 2012). Exposure of ECs to hyperglycaemic conditions stimulated the release of exosomes which transports a circular RNA to vSMC causing senescence in the vSMCs (S. Wang et al., 2020). Hyperglycaemic conditions stimulated the release of HUVEC-derived EVs containing exosomal protein Notch3 which modulated vSMC calcification and ageing (X. Lin et al., 2019). Studies have also reported that in response to smoking, a CVD risk factor, there has been a significant increase in EC-EV levels (C. Gordon et al., 2011; H. Liu et al., 2014). EVs have also been shown to be upregulated in patients with AS and ED (Chironi et al., 2006). ED is an independent predictor of vascular disease. CD31+/Annexin V+ EVs increased in patients with CVD risk factors and impaired endothelial function (Sinning et al., 2011). One study showed that EtOH increases EC-derived EV vascularization bioactivity which could initiate alcohol-induced tumour angiogenesis (Lamichhane et al., 2017). Several proteins in EtOH-derived exosomes were found to be of mitochondrial origin (Rahman et al.,

2020). Mitochondrial malfunction is known to play a role in OS, and chronic alcohol consumption contributes to cardiomyopathy through OS (Lazarević et al., 2000). EVs derived from cells under EtOH conditions are likely to deliver mitochondrial apparatus which contributes to OS-induced intercellular signalling (Rahman et al., 2020). EtOH increases EV levels generated from cardiac myocytes via OS (Malik et al., 2013)

EC-derived EVs mediate stress signals, and the cargo within these EVs reflects the cellular stress that occurred in the cell of origin (de Jong et al., 2012). Proteomic analysis of EC-derived EVs under high glucose conditions and normal conditions has been shown to have different protein compositions, indicating cargo within these EVs varies, even though the EVs originated from the same cell source (Burger et al., 2017). This suggests that risk factors such as Diabetes could indeed modulate EV biogenesis and their cargo within. The effects of alcohol exposure on the contents of EVs, furthermore the transfer of these alcohol modified EVs to a recipient cell can play a critical role in the inflammatory pathways in the transfer of these alcohol modified EVs to nearby or distant cells can play a vital role in inflammatory pathways in the pathogenesis of alcoholism (Rahman et al., 2020).

To explore our hypothesis, our first aim was to generate a reliable *in vitro* cell culture mouse model for ECs to determine whether EVs are secreted under normal and EtOH conditions. EVs are secreted by most cell types under stable conditions in various body media and can pass through many barriers due to their minuscule size. The use of a cell culture conditioned medium allows us to mimic this setting in a controlled environment *in vitro* to allow cells to secrete EVs. MAECs were characterised using ICC and RT-qPCR to detect the expression of EC markers; CD31 and eNOS (Figure 11 C-D). However, due to unexpected data results, it was deemed that this commercially available cell line of MAECs had displayed stem-cell-like and SMC-like markers. Although the morphology of these cells suggests they were of a distinct EC population through their 'cobblestone' structure, their lack of expression of endothelial markers suggested otherwise (Figure 11 A-B). Although this cell line was extremely proliferative, it is possible there are two causes of this problem. Firstly, there may be contamination of other cells within the culture. This is due to the proximity of other cell types within the blood vessel wall. There is a possibility that during the isolation of MAECs, an SMC and/or stem cell population may also have been present. Another theory is that the MAECs may have transdifferentiated into these smooth muscle-like or stem cell-like cells during culture. Due to this issue, this cell line was deemed unreliable, as EVs would be collected from an incorrect cell source. This led

us to move on to a human model. HAECs generated were a reliable cell source to carry out this study. HAECs morphology was defined by their distinct cobblestone structure that is typical of ECs (Figure 12 A-B). They were characterised using ICC (Figure 12 C-D) and RT-qPCR (Figure 13 A-B) to detect the expression of EC markers; CD31 and eNOS, which were abundantly available, classifying this cell type as ECs. There was no detection of Cnn1 or S100β which confirms there is no contamination of any other cell types within the culture. To investigate whether EVs could be secreted by HAECs, they were cultured under normal and EtOH conditions for 48hrs. We used a commercially sold exosome isolation kit called ExoQuick™,, which allows for high exosome purity. Although there are several other EV isolation techniques such as size exclusion chromatography and ultracentrifugation, this method has been proven to be reliable and least time-consuming and laborious (Y. Cheng et al., 2020). There are disadvantages to exosome isolating methods, such as contamination of non-EV components such as proteins and lipoproteins (Van Deun et al., 2014). Characterisation and verification of EC-derived EVs were carried out to demonstrate the validity of the isolation of EVs from HAECs. The isolated EVs from normal and EtOH-treated HAEC culture was assessed using DLS (Figure 17 A-C). This method is widely used to characterise these membrane vesicles, which have a cell diameter of approx. 20-150nm (although this range tends to vary across literature). As EVs exist on the submicron (nm) scale, this laser-based technique allows us to determine the size of these isolated EVs by sending a beam through a polarised sample and simultaneously measuring the scatter light to determine the particle size. HAEC-derived EVs under control conditions (H-EXO) ranged between 80nm-110nm with a small sub-population at 8nm-25nm. HAEC-derived EVs under 25mM EtOH conditions (25-EXO) ranged between 110nm-230nm with a sub-population between 30nm-50nm. HAEC-derived EVs under 50mM EtOH conditions (50-EXO) ranged between 110nm-500nm with a sub-population between 20nm-80nm. The sub-population is a homogenous population of EVs, which we determine to be exosomes. We observed a significant increase in vesicle size following EtOH treatments, interestingly, in particular the EtOH treatment of 50mM. We hypothesise that this size and zeta potential increase is corresponding to larger-sized MVs, which span a significant and heterogenous size range (Clancy et al., 2021). Unlike exosomes, which are more specific and regular in size, microvesicles span between 100-1000nm. There is the possibility that this heterogeneous MV populations could in fact contain a sub-population of exosomes. This may be caused by alcohol effecting plasma membrane integrity and potentially stimulating membrane blebbing. The mechanism behind the difference in EV sizes due to alcohol needs to be explored, and may show the way for assessing the nature and degree

of cell injury. MVs are involved in cell-cell communication and the size might be related to different protein interaction with MVs to regulate many different cellular functions. Interestingly MVs are typicall released from healthy activated cells by being secreted from the endosomal cell membrane or by proteolytic cleavage of the cytoskeleton (Janina Ratajczak et al., 2013). They are mostly present when under steady state conditions in most bodily fluids that have been investigated so far. In pathological conditions the number of EVs released increases, and most likely to be enriched in malignant proteins (Rak, 2015) causing their cargo to be different in molecular composition. MVs are released from all cell types such as ECs. MVs can also be internalised by target cells through the process of phagocytosis and fuse with the cell membrane of the target, this allows the cell membrane of the host cell to incorporate fragments of the cell membrane and delivers the cargo to the cytosol (Deregibus et al., 2007; J. Ratajczak et al., 2006). These effects can make changes the target cell phenotype and function. MVs are derived from cell surface membrane blebbing whereas exosomes are derived by MVB pathway or via exocytosis. Ethanol has been shown to modulate the production of both exosomes and MVS (Awoyemi et al., 2022; Zou et al., 2022). Both EVs are capable modulating responses in the target cells, therefore perhaps both MVs and exosomes could be involved in our obeservations.

To further characterise EC-derived EVs to confirm these results we used the AmnisTM CellStream nano FACs to analyse isolated EVs, through labelling fluorescent techniques and FACS analysis. This nanoscale FACs technology has been used by many other research groups to detect EVs (Morales-Kastresana et al., 2017; Thane et al., 2019). HAEC-derived EVs under normal and EtOH conditions were labelled using exogenous EV-labelling dye ExoGlow, an exosome-specific dye (Figure 18 A-C). These EVs both demonstrated a positive expression of ExoGlow confirming the detection of EVs. We observed an increase in the number of EVs released from HAECs under EtOH conditions when compared to control when enumerated using ExoGlow™. This suggests that EtOH conditioning of HAECs increases EV production.

Secondly, we investigated whether these EVs were enriched with tetraspanins CD9, CD81, and CD63. Due to an exponential increase in exosomal studies, ExoCarta was created which is a free web-based resource that catalogues proteins and RNA identified in exosomes (Simpson et al., 2012). EVpedia was also created, which is an integrated database of high-throughput datasets from EVs (D. K. Kim et al., 2013). Both platforms provide information on proteins, mRNAs, miRNAs, and lipids enclosed in prokaryotic, non-mammalian eukaryotic, and

mammalian EVs. Tetraspanins are often used as exosome biomarkers, for example tetraspanins CD9, CD63, and CD81. These are especially enriched in the membrane of exosomes and are classical markers of exosomes. Using the AmnisTM CellStream nanoFACs, antibody staining was carried out for these exosomal markers. HAEC-derived EVs were positive for the expression of CD81(Figure 20 A-C) and CD63 (Figure 21 A-C) in both normal and EtOH conditions. Many other research groups have also confirmed the expression of tetraspanins in EC-derived exosomes (Banizs et al., 2014; Wong et al., 2019). There was a minimal expression of CD9 (Figure 19 A-C). Although other research groups have detected CD9 within the cargo of their endothelial-derived EVs (Boyer et al., 2020; Fontaine et al., 2021), it is clear that this particular tetraspanin is not highly expressed in EVs derived from HAECs under normal or EtOH conditions. It was observed that there was a slight increase in the number of CD81 and the number of CD63 EVs decreased, respectively, in HAEC-derived EVs under both 25mM EtOH conditions when compared to control, using Amnis™ Cell Stream analysis.

Lastly, an ExoCheck™ exosome antibody array for the detection of exosomes. Each array has 12 pre-printed spots and features 8 antibodies for known exosome markers. These markers include FL0T1, ICAM, EpCAM, and ANXA5 as well as EV biogenesis ESCRT proteins ALIX and TSG101. An EV-purity marker GM130 is also present to detect any cellular contamination in our exosome isolations. It is evident that CD63 and CD81 expression was observed in normal and EtOH conditions which confirms our results obtained using the Amnis™ CellStream flow cytometer (Figure 23 A-F). An increase in CD81 and a decrease in CD63 tetraspanin proteins were observed when compared to the control. The expression of the ICAM1 and ANXA5 proteins was also elevated in 25-EXO when compared to the HAEC EV control. Amalgamating the data obtained using ExoGlow and tetraspanin staining as well as ExoCheck™ exosome antibody array, confirms exosomes have been successfully isolated. It also suggests that when HAECs are conditioned with EtOH, this results in an increase in EV production. Lastly, EVs released in response to EtOH conditions cause differential expression of EV-associated proteins in comparison to EVs derived under normal conditions.

5.3 A proteomic analysis of Endothelial derived EVs under normal and ethanol conditions.

Several research groups have used proteomic approaches in characterising and interrogating EVs by applying gel electrophoresis (2-DE) and LC-MS/MS or MALDI-TOF/TOF MS (Mallia et al., 2020). Over a thousand different proteins have been identified and with the help of Exocarta and EVpedia databases, this allowed researchers to compile the most common exosomal proteins. The most common are multivesicular body synthesis proteins (ALIX and TSG101), heat shock proteins, membrane transporters, fusion proteins (annexins, GTPases, and flotillin), ESCRT proteins and tetraspanins (CD9, CD63 and CD81) (D. K. Kim et al., 2013; Simpson et al., 2012). In this present study, we have already demonstrated that cultured ECs release EVs under normal and ethanol conditions. EVs isolated using ExoQuick ™ from each condition displayed physical characteristics (size) consistent with microvesicles and exosomes We were able to detect these EVs using ExoGLOW and detect certain "canonical" transmembrane markers of EVs and exosomes: the tetraspanins CD63 and CD81 using Amnis™ Cell Stream analysis. Lastly using an ExoQuick™ to confirm EVs by detecting EV-associated proteins FL0T1, ICAM, EpCAM, and ANXA5. ALIX and TSG101. To further characterise these EVs, we performed mass spectroscopy and proteomic analysis of EVs, H-EXO, 25-EX0 and 50-EXO. Using the ExoCarta database, we compared the top 100 proteins identified (Table 10) in exosomes to our own. In the H-EXO, 85 exosomal proteins were matched, leaving 15 exosomal proteins to be not present. In the H-EXO, 88 exosomal proteins were matched, leaving 12 to be not present and lastly in 50-EXO, 84 exosomal proteins were matched, leaving 16 to be not present (Figure 24 A-C). This data supports the previous data obtained, confirming that all H-EXO, H-25 and H-50 are exosomes. CD81 and CD63 tetraspanins were present in all three fractions. Although there was very low expression detected from our cell stream analysis, CD9 was present in H-EXO and 25-EXO, however, was absent in 50-EXO suggesting this is not a major phenotype in HAEC-derived EVs. Alix-1 (PDCD6IP), TSG101, ANXA5 were also present. Although EpCAM and ICAM are not listed in the top 100 exosomal proteins on ExoCarta,(however are included in the ExoCarta database) they are considered to be exosomal proteins and were also present in all three EVs. Contradictory to our ExoCheck™ exosome antibody array results where we detected FLOT1 in all three EVs, our proteomic results revealed it was only present in 25-EXO and absent in H-EXO and 50-EXO. The exosomal proteins that are absent in all three fractions of EXOs are Histones; HIST1H4A, HIST1H4B, and HIST2H4A which are core components of the

nucleosome (Sundar et al., 2019). TUBA1A and TUBA1C, are major constituents of microtubules (Aiken et al., 2017). Extracellular heat-shock protein 72, HSPA1A plays a role in the release of proinflammatory cytokines (Périard et al., 2012). GNB2, a G-protein which is involved in various transmembrane signalling systems (Stallmeyer et al., 2017). ARF1 is a GTP-binding protein involved in protein trafficking among different compartments (Cevher-Keskin, 2013). ACTG1 is a part of the actin family which are highly conserved proteins (Sundby et al., 2022). PTGFRN is a cell surface transmembrane protein in the immunoglobulin superfamily (Aguila et al., 2019). All three EVs also carried endothelial-associated proteins within their cargo which signifies their cell origin. The proteins identified were VEGFC, FLT1, VWF, BMP1, BMP6, S100A9, ANGPTL4, RAI14, PECAM1, ESAM and SRPX2 supporting their endothelial origin. These proteins were also present in primary ECs derived from patients with AMI (Nukala et al., 2019). Overall, the EVs secreted by HAECs under normal and ethanol conditions are determined to be exosomes based on the amalgamation of the results generated.

Using LC/MS and bioinformatic analyses, we identified 1967 total amount of proteins in H-EXO, 1749 total amount of proteins in 25-EXO and 1819 total amount of proteins in 50-EXO. A total of 1421 proteins were shared by all three groups, H-EXO, 25-EXO and 50-EXO. The results of the analyses are depicted graphically in the form of a Venn diagram (Figure 25). As these EVs originate from the same cell source, it is not surprising that they share 1421 proteins in common. However, there are unique proteins to each of the EVs. There were 241 proteins exclusive to H-EXO, 82 proteins exclusive to 25-EXO and 116 exclusive to 50-EXO. This suggests that EVs secreted under normal and ethanol conditions, resulted in minor differences in protein profiles. Differential expression (DE) analysis was carried out to compare the significant differences in protein expression levels between the EVs. The following comparisons were made; H-EXO vs 25-EXO, H-EXO vs 50-EXO, 50-EXO vs 25-EXO revealing proteins were differentially expressed between the two EV groups compared. The results were visualized on volcano plots, (Figure 26), highlighting differentially expressed proteins with adjusted p-value <0.05 and absolute LogFC>±1.3 as upregulated (red) and downregulated (blue). For H-EXO vs 25-EXO, nine proteins were significantly upregulated; RANBP6, UBE2NL, UBE2L3, PIGR, ALYREF, CD2AP, PRPF40A, ROCK2, USP39 and five proteins PSMB10, PSMB3, CLPTM1, PRPSAP2 and TFAM were significantly downregulated when 25-EXO was compared to the control group H-EXO. UBE2NL gene name is ubiquitin-conjugating enzyme E2N-like, this gene encodes a member of the ubiquitin-

conjugating enzyme family and is listed as an exosomal protein on ExoCarta. It has been found in exosomes derived from melanoma cells and platelets (Lazar et al., 2015; Pienimaeki-Roemer et al., 2015). Interestingly, it has been shown that IL-1b- and IL-3-stimulated EC secretomes secrete proteins, one of which is UBE2NL (Bal et al., 2013). UBE2L3, another one of the many E2 ubiquitin-conjugating enzymes that are involved in the ubiquitination of numerous substrate proteins and regulate signalling pathways, such as the glycogen synthase kinase 3β (GSK3β/p65) pathway and the transcription factor-kappa B, NF-κB pathway. Studies have shown this protein has an abnormal expression in many diseases, particularly inflammatory diseases (Chiaroni-Clarke et al., 2014; Orozco et al., 2011; Yu Wang et al., 2016). During the cell cycle and differentiation, levels and activation states of UBE2L3 change. The proportion of cells in the G_1 phase relative to the S phase is increased when UBE2L3 is elevated and while its depletion extends the S phase (Guo et al., 2004; Whitcomb et al., 2009). Levels of ubiquitin charging of UBE2L3 are increased in differentiated tissue (Caceres et al., 2010). ALYREF is an RNA-binding protein which is important for the export of miRNAs. These miRNAs can possess sorting sequences that determine their secretion in EVs. ALYREF has been shown to be involved in the export of miRNAs carrying the strongest EXOmotifs, CGGGAG (Garcia-Martin et al., 2021). ALYREF is upregulated in many malignancies and is associated with poor prognosis including hepatocellular carcinoma, head and neck squamous cell carcinoma and bladder cancer (Han et al., 2021; J. Z. Wang et al., 2021). Endothelial actin-binding protein CD2-associated-protein CD2AP is an adopter protein. This protein has been shown to bind the receptor of ICAM-1 to control mechanosignaling, and leukocyte adhesion and drive excessive transmigration of leukocytes from the blood to the tissues via ECs that line the blood vessels. This is often associated with chronic inflammatory diseases such as AS (Schaefer et al., 2017). Exosomal CD2AP mRNA in urinary exosomes has been used as a biomarker for kidney disease. It was shown to be decreased in patients with kidney diseases when compared to healthy controls (Lv et al., 2014). PRPF40A is a pre-mRNA processing factor (Lin et al., 2004). It has been shown to be upregulated in malignant tissues (Ayyasamy et al., 2011; Ramaswamy et al., 2003). PRPF40A leads to hypoxia in lung cancer and has been shown to exhibit a strong hypoxia association pattern which correlates with hypoxic markers (Oleksiewicz et al., 2017). Rho-associated protein kinase 2 (ROCK2) has been shown to be involved in immune defence, inflammation and rheumatoid arthritis (C. H. Weng et al., 2016; Zanin-Zhorov et al., 2014). This protein has major roles in various biological processes via Wnt/β-catenin signalling (Luo et al., 2019). USP39, Ubiquitin-specific peptidase 39 plays a major role in mRNA processing and can act as a pro-tumour factor in many types of malignant tumours such as breast cancer

(Wang et al., 2013). Polymeric immunoglobulin receptor (PIGR) plays a role in mediating selective transcytosis of polymeric IgA and IgM across mucosal epithelial cells. PIGR among other proteins have a correlation with plaque thickness and has an independent association with subclinical AS. PIGR levels were significantly associated with an increased probability of subclinical AS (Fuster et al., 2022). All the proteins are upregulated in HAEC-derived EVs under moderate ethanol conditions (25-EXO) when compared to the control (H-EXO), suggesting that ethanol-induced ED led to an upregulation of these particular proteins when compared to the normal conditions. Many of these proteins are implicated in various diseases, in particular PIGR which is associated with AS.

Proteasome subunit beta-10 (PSMB10), is a protein-coding gene. PSMB10 expression has been shown to be upregulated in arterial fibrillation. Proteasome subunit beta type-3 (PSMB3) plays various important roles within the cell by associating with different regulatory particles. Palate associated transmembrane protein 1, CLPTM1 is a regulator of GABA type A receptor forward trafficking. Down-regulation of CLPTM1 has been associated with Alzheimer's disease (Ge et al., 2018).TFAM, mitochondrial transcription factor A, plays a major role in the maintenance of mitochondrial homeostasis and mtDNA (Campbell et al., 2012; Kanki et al., 2004). Cardiac-specific TFAM knockout mice display a critical decrease in mtDNA copy number, respiratory chain deficiency and fatality due to chronic cardiac dysfunction (Li et al., 2000; Wang et al., 1999). In contrast, an increase in mtDNA copy number is observed with TFAM overexpression, improves cardiac remodelling and ameliorates the survival in mice post-MI. In contrast, TFAM overexpression increases mtDNA copy number (Ikeuchi et al., 2005).

For H-EXO vs 50-EXO, among these two proteins, GALNS and MRPS36 were significantly upregulated and seven proteins, CASP14, ROCK2, BCLAF1, PRPF40A, LDLR, KRT2 and SRRM1 were significantly downregulated in 50-EXO compared to the control group H-EXO. N-acetylgalactosamine-6-sulfatase (GALNS), which plays a role in oligosaccharide sulfation is an enzymatic reaction involved in homing by the endothelium. Morquio A syndrome is an autosomal recessive disease, caused by mutations in the *GALNS* gene (Víctor Álvarez et al., 2019). GALNS levels in patients with cancer and pneumonia were significantly higher than those of the healthy controls (Ho et al., 2019).MRPS36 is a mitochondrial ribosomal protein. Overexpression of this protein has been shown to impede cell proliferation through the phosphorylation of p53 and inducing the expression of p21 which affects mitochondrial function (Chen et al., 2007).

CASP14 is an apoptosis-related cysteine peptidase. It has been identified in exosomes derived from liver cancer cells (Zhao et al., 2014) and platelets (Pienimaeki-Roemer et al., 2015). CASP14 is a promising diagnostic marker for bladder cancer, as there was a significantly higher expression of this protein in bladder cancer patients when compared to healthy controls (H. Huang et al., 2021). BCLAF1, Bcl-2-associated transcription factor 1 is a death-promoting transcriptional repressor and plays a major role in maintaining DNA genomic stability. BCLAF1 was shown to be a regulator of SMC lipid transdifferentiation in AS. This protein is essential for the transdifferentiation of SMCs into a macrophage-like phenotype. The downregulation of BCLAF1 was associated with plaque vulnerability and cardiovascular risk in patients with carotid AS. Moreover, decreased BCLAF1 plaque levels were shown to marginally predispose to a higher amount of adverse cardiovascular events following surgery (Rykaczewska et al., 2022). Low-density lipoprotein receptor (LDLR) is a cell surface glycoprotein that is highly expressed in hepatocytes. It is involved in the binding and cellular uptake of apolipoprotein B- and E-containing lipoproteins. Mutations in LDLR expression lead to myocardial infarctions in young patients (Defesche, 2004). Studies using animal models have shown that genetic deletion of LDLR causes plasma LDL cholesterol levels to elevated when mice are fed normal chow. However, animals had highly increased plasma LDL cholesterol levels associated with AS when fed a high-fat diet (Ishibashi et al., 1994). Keratin complex-2 (KRT2) is associated with keratinocyte proliferation (Bloor et al., 2003). KRT2 has been shown to be significantly enriched (EVs) from rheumatoid arthritis synovial fluid (Foers et al., 2020).

For 25-EXO vs 50-EXO, among these proteins, five proteins were significantly upregulated; KRT2, PRPSAP2, KRT14, KRT9 and SKIV2L and twenty-four proteins; SKP1, UBE2L3, CNN3, ALYREF, PIGR, ME1, S100A6, USP39, RANBP6, AP15, DAZAP1, TCEA1, ZNF622, CFL2, NT5C2, PIEZO2, IGHA1, RAB14, AFP, EIF4H, EZR, GLRX3, ITM2B, MICU2 were significantly downregulated in 25-EXO compared to 50-EXO. Of the five upregulated proteins, three (KRT2, KRT14, KRT9) belong to tissue-specific cytoskeletal components known as keratin intermediate filaments (Kononikhin et al., 2019). Post-translational modifications of keratins can play an important role in apoptosis and differentiation, as well as disease states. Overexpressed keratins in RA patients have also been reported (Chang et al., 2009). Disruption of keratin filaments of hepatocytes in response to EtOH is characteristic of alcoholic liver disease (Osna et al., 2017). Ski-complex factor SKIV2l is an RNA helicase that can unwind RNA substrates and pass them into the RNA exosomes for

their degradation (Yang et al., 2022). SKP1 is an adopter protein that is involved in the ubiquitination and proteasomal degradation of target proteins. Together with CUL1 and F-box protein, this forms a SCF E3 ubiquitin complex which is responsible for the protein turnover regulation. This SCF E3 ubiquitin complex has been termed FBXO3 which has been shown to modulate inflammatory response in AS. Patients develop less AS with a hypo functioning genetic variant of FBOX3 .Monocytes within the carotid plaques contain this FBOX03 protein, and its levels elevate in patients with characteristics of AS when compared to those with asymptomatic AS. Inhibition of FBX03 reduced the inflammatory response to OxLDL by macrophages without affecting the uptake of OxLDL. This data shows that FBX03 regulates inflammation in AS (Chandra et al., 2019). A research group screened 69 groups of the atherosclerotic carotid artery and used STRING analysis to show the protein-protein interaction network of downregulated differentially regulated genes and identification of hub genes of AS. SKP1, UBE2N and UBE2H were among those genes differentially regulated (Youwei Lu et al., 2021). Calponin is expressed in SMCs and is an actin filament-associated regulatory protein. It affects many cellular activities such as differentiation, proliferation and migration. There are three homologous genes, one of which is an acidic calponin called CNN3, which plays a role in actin cytoskeleton-based activities in myogenesis (R. Liu & Jin, 2016). It has been shown to be a pro-invasive protein in trophoblast cells that is induced in hypoxia conditions (Appel et al., 2014). During wound healing, CNN3 has been observed to be involved in the proliferative stage. Primary fibroblast with CNN3 knockdown has been shown to impair contractility and fibre formation and reduces cell motility (Kwon et al., 2022). S100A6 is a member of the EF-hand Ca^{2+} binding protein superfamily that is involved in regulating various molecular and cellular functions, including differentiation and proliferation as well as hypertrophy, Ca^{2+} dynamics, apoptosis and contractility (Nowotny et al., 2000). Post MI, S100A6 expression levels are increased in the heart (X. Y. Cai et al., 2011), and is a negative regulator of the induction of cardiac fetal gene promotor in rat cardiac myocytes *in vitro* which suggest this protein is important in modulating cardiac hypertrophy (Tsoporis et al., 2005). Overexpression of S100A6 in cardiac myocyte-specific transgenic mice attenuates cardiac hypertrophy and apoptosis following MI compared to control animals (Tsoporis et al., 2014). S100A6 has been shown to regulate the EC cycle activity, and the progression through the cell cycle can be inhibited by transcription factors known as signal transducers and activators of transcription (STAT).

Overexpression of S100A6 has been shown to reduce apoptosis, elevate Ca^{2+} cycling and attenuate hypertrophy (Mofid et al., 2017). Enhanced intimal S100A6 protein expression in

proliferating ECs is involved in remodelling arteries, a study was able to show that S100A6 could suppress antiproliferative STAT1/IFITM1 signalling to facilitate reendothelialization in injured vessels by promoting the expression of protein inhibitors of activated STAT1 (Lerchenmüller et al., 2016). Peizo, comes from the Greek word for piezi, meaning pressure (Coste et al., 2010). PIEZO1 and PIEZO2 are two genes known to encode piezo ion channels in humans. The encoded PIEZO2 protein has a large transmembrane structure. There is growing evidence showing PIEZO channels are significant in vascular tone maintenance, AS and NO generation. These PIEZO channels play a role in cardiovascular development and its associated disorders (Coste et al., 2010). PIEZO2 has been shown to have an emerging role CVDs such as varicose veins, hypertensive heart disease and distal blood pressure (Shah et al., 2022). Immunoglobulin heavy constant alpha 1 (IGHA1) contributes to immunoglobulin receptor binding activity. Total AS burden consists of calcified or non-calcified plaque. A study carried out HDL proteome analyses using mass spectrometry in 126 patients who had undergone coronary computed tomography angiography. IGHA1 has been shown to be a positively associated protein in non-calcified plaque burden (Gordon et al., 2018). IGHA1 has been identified in exosomes derived from urine (Saraswat et al., 2015) and human plasma-derived EVs (Repetto et al., 2021). Alpha-fetoprotein (AFP) is a protein made in the liver of a developing baby (Adigun et al., 2021). It is also classified as a liver cancer dedifferentiation marker (Li et al., 2019). Ezrin (EZR) is said to be involved in the connection of major cytoskeletal structures to the plasma membrane. Long non-coding RNA ANRIL is recognised for playing a role in CAD and regulates EC functions associated with CAD. It regulates these EC activities by upregulating genes such as EZR (H. Cho et al., 2019). Glutaredoxin-3 (GLRX3) is one of 4 members part of the glutaredoxin family in mammals and is primarily contained within the cytoplasm. Glrxs play a major role in CVDs such as cardiac hypertrophy, myocardial ischemia and AS. In particular, Glrx3 plays an important role in cardiac hypertrophy. Glrx3 CKO in mice leads to the development of cardiac hypertrophy and HF (Donelson et al., 2019). A mitochondria Ca^{2+} uptake protein 2 (MICU2) has been observed to increase with CVDs in humans and animals. $Micu2^{-/-}$ mice exhibited diastolic dysfunction and 30% lethality from abdominal aortic rupture. There was an increased expression in genes associated with remodelling of the extracellular matrix and single-cell RNA-seq showed that expression of genes related to ROS, inflammation and proliferation in SMCs were elevated (Bick et al., 2017). The results reported here suggest ethanol conditioning plays a role in up and down-regulation of proteins within HAEC-derived EVs when compared to the control. It also suggests that varying ethanol conditions play a role in up and down-regulation of proteins

within HAEC-derived EVs when chronic and moderate conditions are compared. Table 5.1 shows the differentially expressed proteins associated with CVDs such as HF, AS and hypertrophy.

Table 11. Differentially expressed proteins associated with CVD

Protein name	Comparison	Upregulated	Downregulated
MICU2	25-EXO vs. 50-EXO	-	✓
GLRX1	25-EXO vs. 50-EXO	-	✓
EZR	25-EXO vs. 50-EXO	-	✓
PIEZO2	25-EXO vs. 50-EXO	-	✓
S100A6	25-EXO vs. 50-EXO	-	✓
SKP1	25-EXO vs. 50-EXO	-	✓
BCLAF1	H-EXO vs. 50-EXO	-	✓
LDLR	H-EXO vs. 50-EXO	-	✓
TFAM	H-EXO vs. 25-EXO	-	✓
CD2AP	H-EXO vs. 25-EXO	✓	-
PIGR	H-EXO vs. 25-EXO	✓	-
PIGR	25-EXO vs. 50-EXO	✓	

The proteomic analysis performed in this study, by comparing EV samples under normal and different ethanol conditions provides a comprehensive dataset to be used for further integrative studies in the future. Some of the observed protein changes are associated with CVD-related diseases. Ethanol has been demonstrated to alter exosome cargo, as well as regulate proteins within their membranes.

A Gene Ontology (GO) analysis was conducted to gain insight into the potential physiological relevance of EV proteins. Functional enrichment analysis using FunRich revealed all three EVs, (H-EXI, 25-EXO and 50-EXO) contain the highest proportion of percent protein hits for cytoplasm, nucleus, exosomes, lysosomes, plasma membrane, mitochondrion and extracellular (Figure 27) which is consistent with the extracellular and vesicular nature of EV proteins. While the cellular component is the same across all three EVs, there seems to be a slight

increase in the percentage of exosome cellular components in 25-EXOs. It was also revealed that all three EVs were enriched in proteins associated with biological processes such as signal transduction, protein metabolism, cell communication and cell growth and/or maintenance (Figure 28). Moreover, the panther GO pathway classification revealed “integrin family cell surface interactions”, “Beta1 integrin cell surface interactions”, TRAIL signalling” “VEGF and VEGR signalling network” and “metabolism”, were among the more abundant pathways in all three EVs (Figure 29).

Vessel intima remodelling during AS involves the modulation of vSMC phenotype, modifications of cell migration and proliferation and extracellular matrix remodelling. Cell adhesion molecules integrins represent the targets of all these responses. Many aspects of AS are affected because of alterations in integrin signalling. Integrin signalling has been shown to regulate endothelial phenotype and drive SMC proliferation and migration (Li et al., 2010; Moiseeva et al., 2003; Schaller, 2001). Medial vSMCs are surrounded by a thin basement membrane that is rich in laminin and collagen IV (Voss & Rauterberg, 1986). SMCs express integrin α1β1 and α2β1 which bind to collagen (Moiseeva, 2001), α1β1 has been observed to bind more strongly to collagen IV and α2β1 more to collagen I (Heino, 2000). Basement membrane proteins play a role in limiting endothelial activation, α1β1 shows a decrease in expression during SMC phenotypic modulation (Obata et al., 1997) and deletion of α1β1 Integrin reduces AS and induces a stable plaque phenotype in ApoE−/− Mice (Schapira et al., 2005). Atherosclerotic plaque formation is associated with an impairment in endothelial NO production and vasodilation, which is termed ED (Funk et al., 2012; Pober & Min, 2006). eNOS is an enzyme that is involved in the conversion of amino acid L-arginine to NO and is the main source of endothelial NO generation. During ED, reduced NO production is a result of decreased eNOS activity, elevated NO scavenging by ROS and increased eNOS uncoupling (Funk et al., 2012; Pober & Min, 2006). Endothelial-derived NO vasodilatory characteristics don’t seem to contribute to its association with AS, NO also reduced integrin activation on platelets and inhibits NF- *K*b activation (De Caterina R, 2007; B. V. Khan et al., 1996). Induction of intracellular calcium and phosphorylation of eNOS is a result of shear stress which promotes eNOS activation (Fleming, 2010). Integrin signalling has been shown to regulate kinases involved in eNOS phosphorylation, such as Akt and PKA (Dimmeler et al., 1999; Fleming, 2010). Integrin family cell surface interaction are activated in all three EVs suggesting that they could be implicated in AS. Focus has been placed on components of the Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) axis as proteins involved in the

progression and development of CVDs. vSMCs and cardiomyocytes express TRAIL receptors within the cardiovascular system (Secchiero et al., 2004). Studies have shown evidence of TRAIL contributing to the pathophysiology of AS (Di Bartolo et al., 2013; Watt et al., 2011), pulmonary hypertension (Hameed et al., 2012) and cardiomyopathy (Tanner et al., 2019).
An increase in VEGF-A and IL-18 has been detected in the serum and plasma levels of patients with coronary artery disease (Vm et al., 2016). VEGF-A has been identified as a marker of AS and has positive and negative effects on functions related to AS (Yu et al., 2018). One study demonstrated that VEGF-A protects ECs by inducing NO synthesis and expression of anti-apoptotic proteins (M. Kong et al., 2019). On the contrary, VEGF-A has increased adhesion protein expression and endothelium permeability thus promoting trans endothelial migration and activation (Camaré et al., 2017) suggesting that VEGF-A has a dual role in AS. All three of the pathways discussed are implicated in AS and are activated in our EVs.

GO analysis was also carried out on the unique proteins found in H-EXO, 25-EXO and 50-EXO. The molecular function (Figure 30) involved in each unique subset of proteins was very similar. Catalytic activity and binding were the main functions identified. However, in H-EXO, it appears that proteins involved in the cytoskeletal motor activity are present, which seem to be absent in both 25-EXO and 50-EXO, there also seems to be a decrease in proteins involved in cytoskeletal motor activity when compared to the ethanol-treated EVs. The biological process (Figure 31) involved in each unique subset of proteins was similar between all three EVs, such as cellular process, biological regulation, and localisation.
Bioinformatic pathway analyses (Figure 32) also yielded protein hit counts against the endothelin signalling pathway, FGF signalling pathway and wnt signalling pathway in unique H-EXO, dopamine receptor-mediated signalling pathway, integrin signalling pathway and muscarinic acetylcholine receptor 2 and 4 signalling pathway in 25-EXO (Figure 33) and inflammation mediated by chemokine and cytokine pathway and wnt signalling pathway in 50-EXO (Figure 34). Wnt /β-catenin signalling pathway is a signalling pathway that has been conclusively demonstrated to be involved in dedifferentiation. β-catenin plays a role in E-cadherin-mediated cell-cell adhesion and is an essential intermediate for the wnt signalling pathway (Cai et al., 2007). When wnt signalling is suppressed or absent, glycogen synthase kinase 3 beta functionally interacts with β-catenin via phosphorylation and becomes a target for the degradation ubiquitin-proteasome system. (Polakis, 1997). Glycogen synthase kinase 3 beta is inhibited via activation of the wnt pathway, which stabilises and translocates β-catenin

to the nuclei, where it associates with TCF/LEF HMG domain family leading to target genes being transcriptionally activated (Clevers & Van De Wetering, 1997).

An important finding was the presence of hedgehog interacting protein (HHIP) protein in H-EXO and 25-EXO. *Hedgehog interacting protein-like 1* (HHIPL1) is a secreted pro-atherogenic protein that increases hedgehog signalling which regulates SMC proliferation and migration and promotes AS . (Aravani et al., 2019). Hedgehog pathway components such as PTCH and GLI have been detected in atherosclerotic plaques, and disrupting Hh signalling in *Apoe*$^{-/-}$ mice increased AS due to increased lipid uptake by macrophages (Beckers et al., 2007). Studies from our research group have demonstrated that RAEC-derived EVs under hyperglycaemic conditions promote Hh target gene expression in murine S100β resident vascular stem cells *in vitro.* It was also reported that HAECs exposed to hyperglycaemic conditions resulted in an increase in SHh expression *in vitro.* When HiPSC-derived NE SNEPs were treated with these EVs, this lead to an increase in Hh signalling, growth and myogenic differentiation of these progenitor cells indicating that these EVs could be used as an early diagnostic marker for AS (Burtenshaw, n.d.).

EVs show diagnostic and therapeutic value to serve as a biomarker and serve as a treatment delivery tool for CVD due to their ability to deliver their encapsulated contents to various targets cells and tissues within the body (Zamani et al., 2019). Various cell types can release EVs, and the cargo of these EVs varies based on the cell source and the conditions these cells are under. The cargo of these EVs and the type of reporter cell that uptakes these EVs are what determine their functional effects (Yáñez-Mó et al., 2015). ED caused by injury stimuli is a key element in the complex pathophysiology of atherogenesis and initiates the secretion of EC-derived EVs such as exosomes and microvesicles (Dignat-George & Boulanger, 2011). AS, which is a vascular disease associated with systemic ED, shows significantly increased levels of circulating EVs (Jansen et al., 2017; Koga et al., 2005). EVs can transfer cytokines, proteins, mRNA, or non-coding RNA such as microRNA (miRNA) to target cells to influence their phenotype as well as their function. EVs have come a long way from being considered just ‘cellular dust’. EVs have now become relevant effectors in intercellular signalling in the vascular system. EVs have been shown to contribute to the development and progression of CVD by promoting the formation of the initial lesion, plaque progression, intravascular calcifications and thrombus formation in atherosclerotic lesions following plaque rupture (Zifkos et al., 2021). In contrast, many studies have demonstrated that EVs can play a role in

endothelial regeneration and mediate vascular protection *in vitro* and *in vivo* (Boulanger et al., 2017; Oggero et al., 2019). EVs released from other cell sources such as MSCs and progenitors have also been shown to improve cardiac function following cardiac infarction which highlights their therapeutic potential in the pathogenesis of CVD (Barile et al., 2017). Recent studies have demonstrated that EC and SMC-derived EVs can contribute to processes that control vascular homeostasis and the pathophysiology of vascular diseases. EC-derived EVs have been shown to increase the release of EVs in response to CVD risk factors such as angiotensin II and tumour necrosis factor- α (Andrews & Rizzo, 2016; Burger et al., 2011). The RNA contents of these EVs are different in unchallenged cells to those that are under stress conditions. For example, shear stress or exposure to KLF-2 enhances the release of EC-EVs which contain MiRs 143 and 145. These EC-EVs containing these miRs were incubated with vSMCs cultures or injected into *ApoE*$^{-/-}$ mice, these EVs were protected against plaque formations through enhanced repression of miR-143/145 target genes and de-differentiation-associated gene expression (Hergenreider et al., 2012).

5.4 EVs from dysfunctional endothelial cells following exposure to alcohol facilitates the attenuation of vascular Smooth muscle cells

To investigate our hypothesis on the effect of ECs on SMCs phenotype using exosomes released under normal and EtOH conditions, we first determined the direct effect of EtOH on hSMCs. Alcohol has been widely identified as an effective vasodilator. It has been reported that it gives rise to SMC vasodilation/relaxation but also vasoconstriction/ contraction, depending on vessel type, species and whether it is assessed in preserved vessels or endothelial denuded vessels (Alleyne & Dopico, 2021). The response of vessels to alcohol can be caused by EtOH itself and its metabolites, for example, acetaldehyde. Dilation of arteries by toxicologically relevant levels of EtOH has been reported by many research groups (Piano et al., 1991; Tabrizchi & Pang, 1993). Many *In vitro* (North et al., 2018; Simakova et al., 2017; Werber et al., 1997) and *In vivo* (Acevedo et al., 1997; R. Yang et al., 2018) studies have reported evidence of the effect of toxicologically relevant levels of alcohol on SMCs which leads to their contraction and in turn their vasoconstriction. A study also showed that EtOH attenuates the contraction of rat airway SMCs. They found that EtOH significantly inhibits methacholine-stimulated changes in RASM cell length and area (Oldenburg et al., 2010).

Another study examined the direct effect of alcohol on intimal proliferation following balloon injury in the rabbit iliac artery. This reduced phenotype conversion of SMCs which reduced SMC proliferation in the neointima (M. W. Liu et al., 1996). In overstretched porcine coronary arteries, intrapericardial delivery of a single dose EtOH reduced neointima proliferation. (Hou et al., 2000). Many research groups have isolated vSMCs in culture and confirm that alcohol has an anti-proliferative effect. One study showed the inhibitory effect of EtOH on rat aortic SMC proliferation and MAPK signalling *in vitro* (Hendrickson et al., 1998). Modulation of the activity and expression of key cell cycle regulatory molecules by EtOH, has be shown to be a possible mechanism by which EtOH inhibits vSMC proliferation (Sayeed et al., 2002). Other findings have shown that EtOH inhibits FGF-induced proliferation of human aortic SMCs (Ghiselli et al., 2003).

hSMCs were cultured in a complete medium until they reached 70% confluency. These cells were then treated with EtOH in a dose-dependent manner for 24 hours to determine the direct effect of EtOH on hSMCs in comparison to the control of hSMCs with no treatment. There was a significant decrease in expression in SMC markers, MHY11 and CNN1 in hSMCs treated with 25mM and 50mM respectively (Figures 33 and 34). During phenotypic modulation, vSMCs decrease the expression of specific markers of contractile phenotype. Thus changes in contractile proteins such as MYH11 and CNN1 in response to EtOH may be associated with the phenotypic switch of vSMCs. MYH11 and CNN1 are widely considered specific markers for SMC lineage, they are highly expressed in fully differentiated SMCs and show high specificity for the contractile phenotype SMCs (Gomez & Owens, 2012; Iwata et al., 2010). Phenotypic switching or de-differentiation is associated with the notable decrease in SMC-selective marker gene expression. Our results suggest that vSMC under specific EtOH concentrations (25mM and 50mM) attenuate contractile capacity of hSMCs.

Following this, HAEC-derived EVs were isolated from normal, and EtOH CM and NC media followed by subsequent incubation with hSMCs in culture for 7 days to determine the effect of these EVs on hSMCs. A significant decrease in MYH11 in hSMC treated with HAEC-derived EVs from 25mM EtOH conditions in comparison to their relevant controls (Figure 37). This decrease is observed when compared to the no treatment control and EVs isolated from NC media under 25mM conditions. A significant decrease was also observed in CNN1 was shown in hSMC treated with HAEC-derived EVs from 25mM EtOH conditions in comparison to their relevant controls (Figure 38). Although there was no significance when compared to the no

treatment control, there was a significant decrease when compared to the hSMCs treated with normal HAEC-derived EVs and EVs derived from 25mM conditions in NC media. This notable decrease in SMC-selective marker gene expression suggests that HAEC-derived EVs under 25mM EtOH conditions attenuate the contractile phenotype of hSMCs. Our results suggest that EC-derived EVs under 25mM induced vSMC phenotype transition upon this exposure. Although previous research indicates that compared to abstinence, low-moderate consumption (1-3 drinks/day) which gives rise to blood alcohol levels of a range between 2-25mM, appears to be protective, and has the lowest risk of CVD progression (Bagnardi et al., 2008; Corrao et al., 2000). However, this data suggests that at a moderate concentration, HAEC-derived EVs under 25mM ethanol cause dedifferentiated hSMCs which play a major pathophysiologic role in the development of AS. Our data seems to illustrate a biphasic effect of Myh11 and CNN1 expression of hSMCs treated with EC-derived EVs under 25mM when compared to treatment with EC-derived EVs under 50mM. As shown in figure 22, we demonstrate there is an increase in EV production when HAEC cells are treated with 25mM EtOH. Although there is little difference in numeration of tetratspanin positive exosomes when these two treatments are compared . Perhaps the increase in EVs available allows the reporter cell to uptake proteins from these EVs which leads to dedifferentiation. Futher proteomic studies on hSMCs following uptake of EC-derived EVs under these two different EtOH conditions will expose more information on the effect of the EVs on this reporter cell.

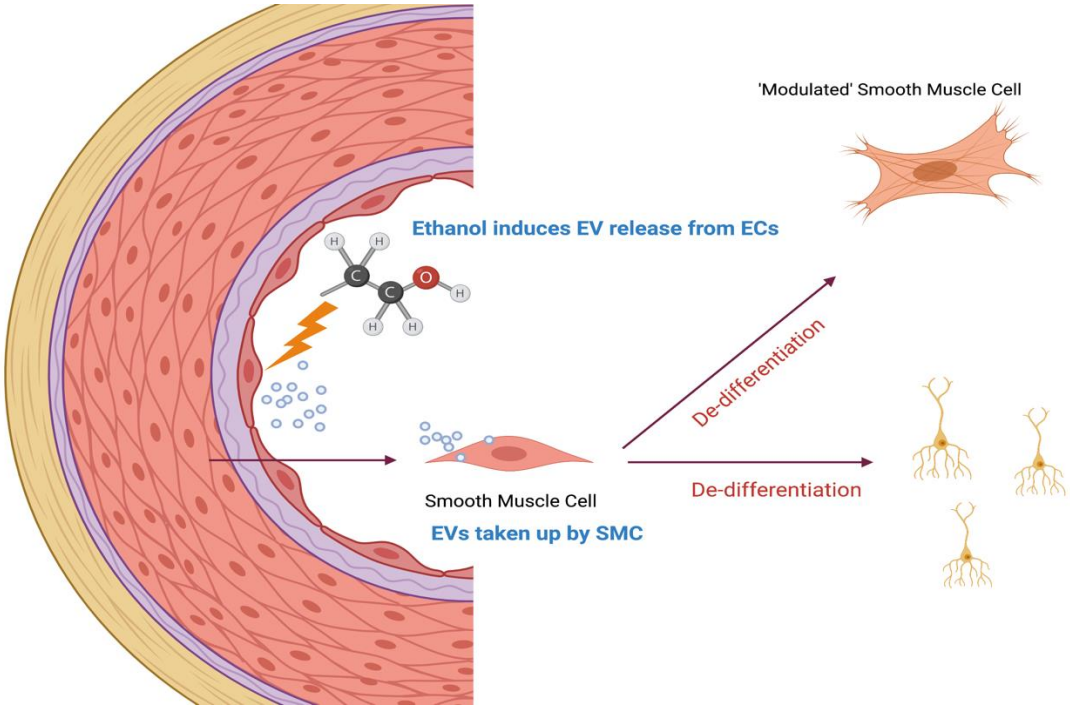


Figure 41 Ethanol induced EV release from ECs. Ethanol causes ED causing the release of EVs from ECs, which then causes SMC attenuation.

There is limited data on EtOH-induced EVs and their effects on SMCs. However, a recent study observed that ECs under atherogenic stimulation increased secretion of EVs that contained inflammasome product IL-1β which promoted synthetic phenotype transition of vSMCs, which led to their proliferation and migration (X. Yuan et al., 2020). Another recent paper observed that EC-EVs reduced the proliferation and migration and lipid storage of HASMCs. However, lipopolysaccharide (LSP) induced EVs derived from ECs had the opposite effect and did not inhibit migration or lipid accumulation, but promoted HASMC proliferation (Xiang et al., 2021). Another study also showed that EVs release microparticles or apoptotic bodies to promote endothelial repair and inhibit AS.EVs derived from HAECs exposed to EtOH could play a crucial role in disease progression by modulating biological pathways via delivery of cargo to reporter cell line hSMCs. The impact of alcohol on EV levels and their cargo content in alcohol-related diseases is an understudied area, even though its widespread use and implicated in cellular signalling processes in AS development.

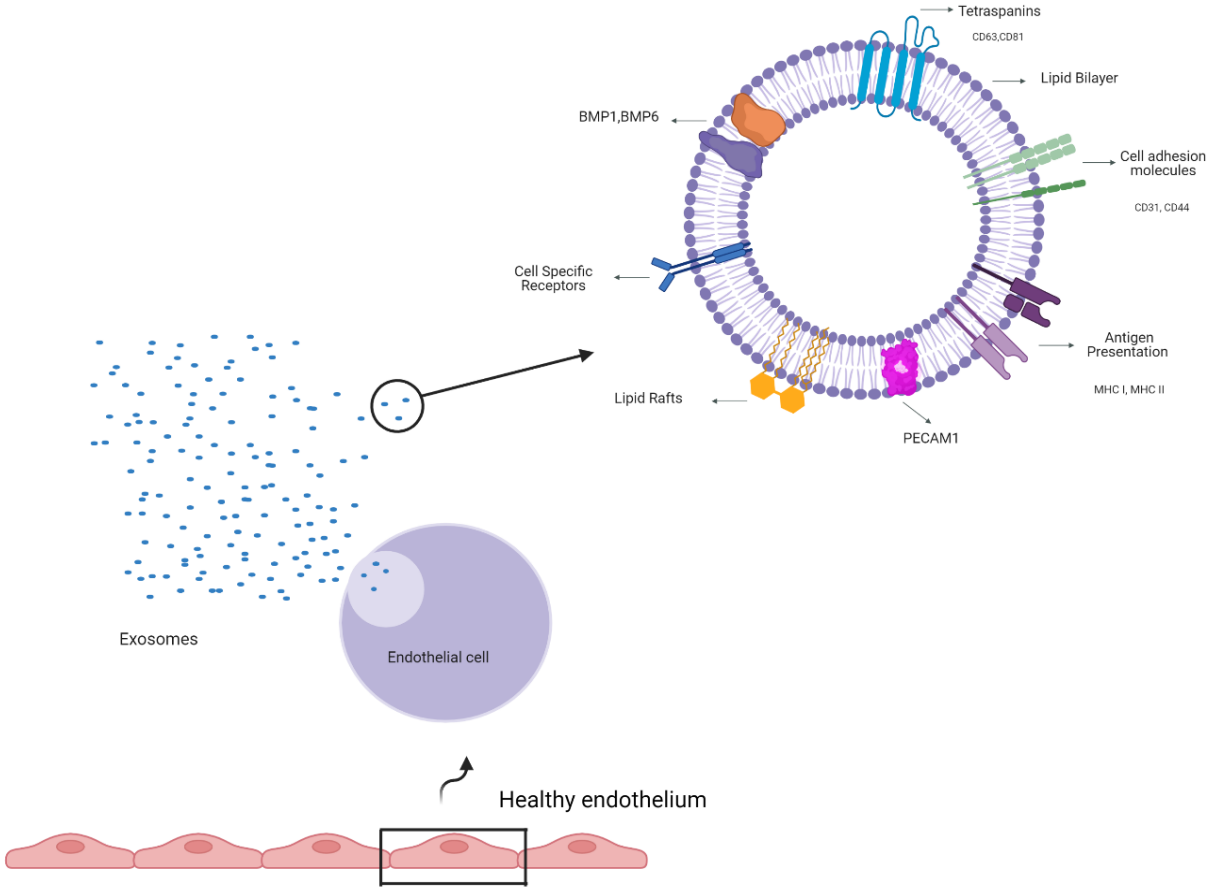
SHh has been shown to be important in embryonic and adult blood vessel development in vascular development. It regulates blood vessel maturation (Yao et al., 2014), integrity (Alvarez et al., 2011), arterial differentiation (Lawson et al., 2002) and vascular homeostasis (Hollier et al., 2020). Impaired Shh signalling can lead to cardiac dysfunction (Lavine et al., 2008; Qing Xiao et al., 2012). It is believed to be involved in AS. It has been made evident that hedgehog components such as Gli1 and SHh are downregulated and contribute to atherosclerotic lesions (Beckers et al., 2007; Dunaeva et al., 2015; Queiroz et al., 2012). Several groups have demonstrated that SHh signalling plays a pivotal role in the control of phenotypic switching and growth in vSMCs (Morrow et al., 2007; Redmond et al., 2013). *Paired box 9* regulated vSMC phenotypic switch, proliferation, and migration via the Shh signalling pathway, which may represent a novel target for the treatment of AS (J. Xu et al., 2020). Shh is also involved in PDGF-induced VSMC phenotypic switching and proliferation (F. Li et al., 2010; Q. Zeng et al., 2016). Microparticles that are harbouring exogenous SHh have been shown to have therapeutic potential for repairing OS-induced ED by increasing eNOS expression, inducing the release of nitric oxide and improving EC function (AgouTii et al., 2007). As a result, SHh signalling agonists have become a new therapeutic target for vascular disease (Cristofaro & Emanueli, 2009; Lavine & Ornitz, 2007; Y. Wang et al., 2017). The

cellular and molecular mechanisms that mediate the role of SHh signalling in vascular biology are not fully elucidated.

The next objective of this study was to investigate if activation of the Hh signalling pathway in hSMCs could be facilitated by HAEC-derived EVs from ethanol-conditioned cells. SHh function in a paracrine manner, where it binds to cells in proximity or initiates changes in cells further away. Paracrine signalling allows the secreted morphogen SHh to influence signalling in recipient cells by binding to its receptor Ptch. This binding revokes its inhibition on Smo and activates signalling via Gli transcription factors (Vyas et al., 2014). The mechanism by which SHh is secreted into extracellular space required two solubilising factors, DISP1(Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2005) and a member from the SCUBE family for example SCUBE2 (Hollway et al., 2006). During this release from cells, the SHh interacts in a lipid-dependant manner with DISP1 and SCUBE1 respectively (Tukachinsky et al., 2012). Although the full mechanism of SHh secretion is still not fully understood, studies have reported many different secretion mechanisms including lipoprotein particles (Panáková et al., 2005) EVs (Matusek et al., 2014; Vyas et al., 2014)and oligomeric complexes (X. Zeng et al., 2001). How SHh morphogen is secreted into extracellular space, particularly EV-mediated Hh in vertebrates still requires an extensive amount of research. As previously mentioned, data obtained from our research group has demonstrated that RAEC-derived EVs under hyperglycaemic conditions promote Hh target gene expression in murine S100β resident vascular stem cells *in vitro.* It was also reported that HAECs exposed to hyperglycaemic conditions resulted in an increase in SHh expression *in vitro.* When HiPSC-derived NE SNEPs were treated with these EVs, this led to an increase in Hh signalling, growth and myogenic differentiation of these progenitor cells indicating that these EVs could be used as an early diagnostic marker for AS.

HAEC-derived EVs exposed to ethanol conditions were isolated from normal and two ethanol concentrations; 25mM and 50mM CM and NC, followed by subsequent incubation with hSMCs in culture for 48 hours. There was no significant change in Gli1 and Gli2 demonstrated between hSMCs treated with HAEC-derived EVs from normal and ethanol conditions in both NC and CM (Figure 39- 40). It seems that the cargo within these vesicles does not harbour SHh ligands which contribute to the phenotypic switching that was seen in vSMCs treated with HAEC-derived EVs under 25mM EtOH conditions.

A


Tetraspanins
CD63,CD81
Lipid Bilayer
BMP1,BMP6
Cell adhesion molecules
CD31, CD44
Cell Specific Receptors
Antigen Presentation
MHC I, MHC II
Lipid Rafts
PECAM1
Exosomes
Endothelial cell
Healthy endothelium

B

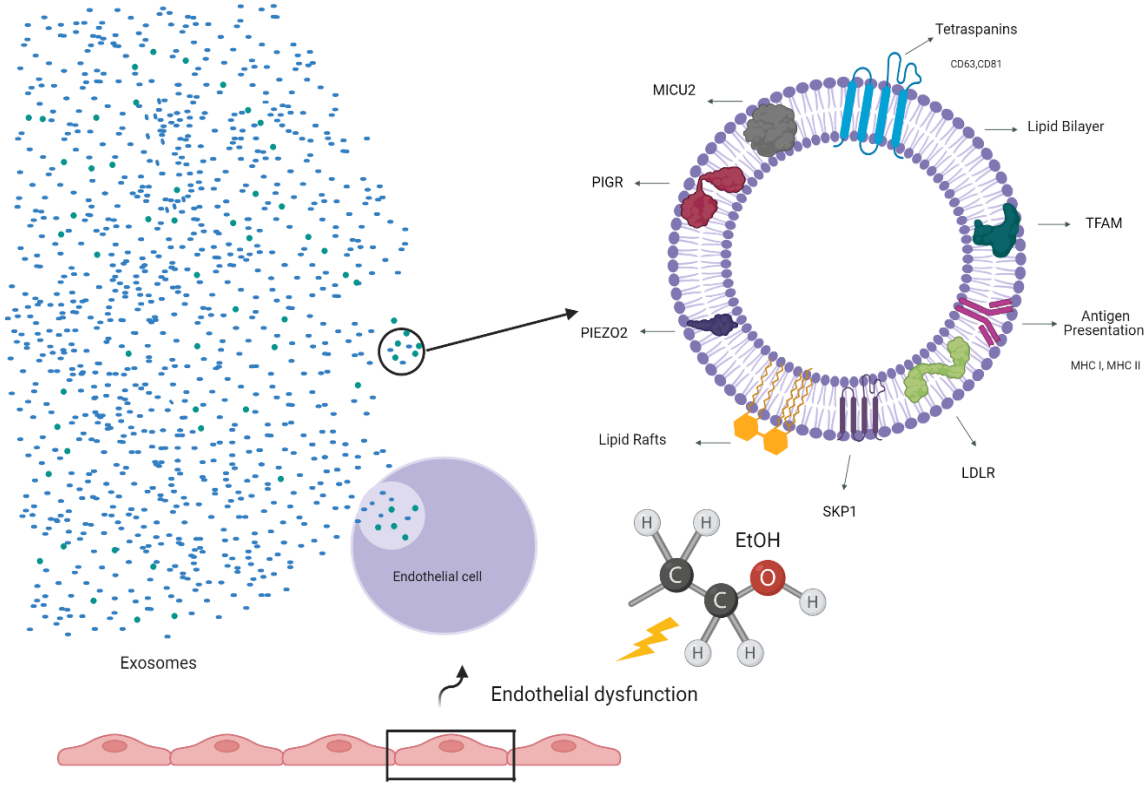


Figure 42 HAEC-derived EVs under normal and ethanol conditions. HAEC derived EVs under (A) normal and (B) ethanol conditions cause the release of EVs. The amount of EVs released from ECs under ethanol conditions increases when compared to normal conditions. The size of EV increases, and phenotypic characteristics differ. The cargo within EVs also change, such as unique proteins exclusive to each EV, or up or down regulation of certain proteins.

In conclusion, the development of a human cell culture model in vitro was validated to investigate the role of HAEC-derived EVs on vSMCs. Using this model it was shown that HAECs under both normal and ethanol conditions secrete EVs. Induction of HAEC dysfunction following EtOH treatment resulted in an elevated increase in EVs and a change in their size and expression of exosome proteins when compared to normal EC conditions. We were able to interrogate the cargo of EVs to further characterise them as exosomes. The data presented here demonstrate that protein expression and levels in EVs secreted by ECs vary depending on culture conditions, suggesting that ECs employ EV-mediated cell communication to cope with cellular stress such as ED. Many differentially expressed proteins were identified to be associated with inflammatory diseases such as AS and the pathways

involved are also implicated in AS. It still remains to be determined whether EVs from ethanol-induced HAECs also serve different functional goals, our data revealing the cargo within these EVs could be used to assess the physiological condition of their producing cells. This approach has highlighted exosomes as a source of disease biomarkers for diseases such as AS. Exposure of vSMCs to moderate and high concentrations of EtOH leads to attenuation of the vSMC phenotype. Treatment of vSMCs with HAEC-derived EVs under 25mM EtOH conditions leads to attenuation of vSMC phenotype. SHh signalling was not activated in vSMCs when treated with HAEC-derived EVs, suggesting that Hh morphogen is not involved in the onset of sub-clinical AS in an alcohol environment. Our data suggest that ethanol induces ED leading to the release of phenotypic specific HAECs-derived EVs. These EVs display a functional role leading to the dedifferentiation of vSMC in vitro.

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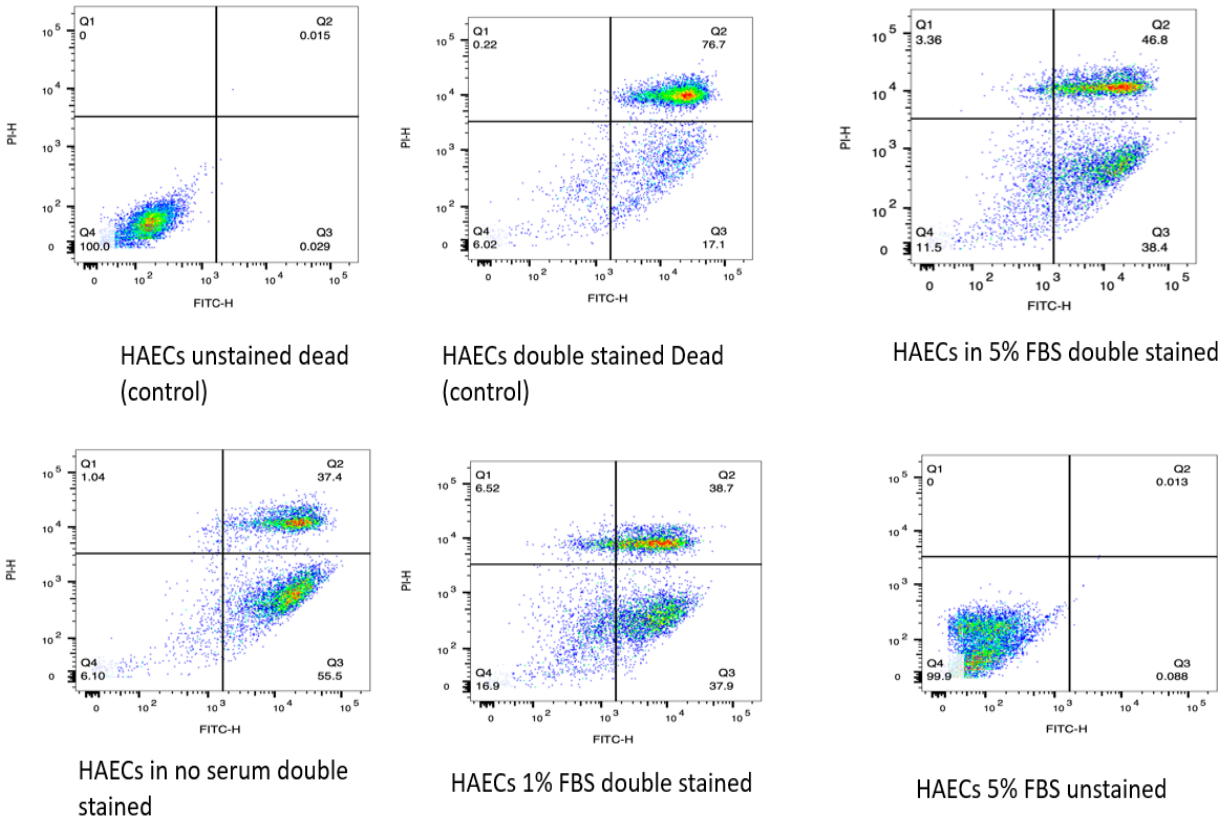
Appendices

Appendix A



Supplementary Figure 1. Apoptosis assay of HAECs treated with normal serum (5%) serum and serum deprivation conditions. Fluorescence staining of Annexin V-FITC in HAECs grown in either normal conditions,5% FBS and serum deprivation conditions, 1% and 0% FBS for 48 hours. Q4 are live cells, q3 are early apoptotic cells and Q2 are necrotic cells. n=1