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Development of an Extracellular Vesicle-Based Biomarker of Hemodialysis Induced Vascular Injury

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Abstract

Hemodialysis (HD) is a life-saving treatment for severe chronic kidney disease but results in ischemic organ injury which contributes to an increased risk of cardiovascular (CV) disease, stroke, and death. HD-induced ischemic injury is monitored using complex imaging-based approaches which are not suitable for routine use. Currently there is no reliable blood-based biomarker that is comparable to findings seen from imaging-based studies and is also suitable for clinical practice. However, biomarkers of growing interest pertaining to HD-induced microcirculatory injury, are circulating endothelial and platelet derived Extracellular Vesicles (EVs), which are known to directly reflect cellular activation and apoptosis. Therefore, the purpose of this thesis was to optimize and validate nanoscale flow cytometry (nFC) for EV-based analysis to determine the utility of EVs as biomarkers of HD-induced vascular injury through using *in vitro*, *in vivo,* and observational patient-based studies. Our results showed that through refining pre-analytical guidelines, the nFC is an appropriate methodology for EV enumeration, characterization, and linear detection of EVs between approximately 100 and 1,000 nm. Utilizing this sensitive methodology, we found that the uremic milieu has minimal impact on EV levels, though inflammatory stress caused by lipopolysaccharide resulted in an increase in small sized (<500nm) endothelial EVs. To further understand the direct impact of HD and its potential influence on EVs (concentration and size), we used a rodent model of HD and applied intravital microscopy to observe muscle perfusion during HD. These experiments demonstrated that HD causes hypo-perfusion of muscle and hemodynamic instability, and an increase in endothelial and platelet EVs over HD. To translate to a clinical context, we used observational patient-based studies and observed that EV levels increased over HD. Additionally, we found that Pre-HD EV levels correlated with important measures of HD-induced injury such as ultrafiltration rate, hypotension, and cardiac contractile function. Altogether, my thesis describes a comprehensive approach which determines and evaluates an EV based assay for HD-induced vascular injury. Our overall findings are promising and warrant further refinement of an EV-based approach to monitor and risk assess patients receiving HD.

Keywords

Extracellular Vesicles, Hemodialysis, Biomarker, Vascular Injury, Nanoscale Flow Cytometry, Chronic Kidney Disease, Microparticles, Endothelial Dysfunction

Summary for Lay Audience

Hemodialysis (HD) is a lifesaving treatment for those suffering from kidney failure. HD replaces some functions of the kidneys by removing waste products and excess fluid from the blood. However, HD causes negative side effects, such as decreases in blood pressure, damage to blood vessels, and reduced blood flow to various organs (heart, brain, liver, and kidneys). Currently, only specialized, time-intensive, and costly imaging methods are used to monitor and understand this kind of HD based injury. Development of a blood test could provide similar results while also expanding access and reducing cost. Research has found that measuring components of blood called Extracellular Vesicles (EVs) may be valuable to study as markers of HD injury. EVs are tiny fragments of injured or activated cells that may be able to identify blood vessel injury and as a result organ injury. My work focuses on developing and refining an EV based blood test to determine whether it is a valuable tool to use among HD patients. To explore this, I first created and refined an EV based technique for a highly sensitive machine used to analyze EVs. This ensured that we would obtain reliable and consistent results. Next, I exposed human blood vessel cells to HD-related stress and saw that when inflammation conditions were present, smaller EVs are produced. Furthermore, to test the effect of HD we exposed rats to a scaled-down version of HD. Similar to humans, the rats presented with lowered blood pressure and reduced blood flow to their leg muscle during HD. They also had an increase in EV levels in their blood over the same HD treatment. Among HD patients, EV levels also increased over the treatment. We found that depending on the EV levels that patients had at the beginning of their HD treatments, there was a connection with the severity of complications experienced during their HD session. Higher EV levels before treatment were associated with greater decreases in blood pressure and heart injury during HD. Altogether my work has shown that an EV based blood test has the potential to monitor and assess HD induced injury.

Co-Authorship Statement

Chapters two, three, and four are adapted from original research studies performed by myself with contributions from other authors. These contributions are described below.

Chapter two was adapted from an original research manuscript titled, “Analytical Considerations in Nanoscale Flow Cytometry of Extracellular Vesicles to Achieve Data Linearity”, which was published in *Thrombosis and Haemostasis* in 2018. This manuscript was co-authored by myself, Dr. Fabrice Lucien, Dr. Tyler Cooper, Dr. Yohan Kim, Dr. Karla Williams, Xinyang Liao, Lauren Kaufman, Dr. Francois Lagugne-Labarthet, Oliver Kenyon, Justin Boysen, Neil E. Kay, Dr. Christopher McIntyre, and Dr. Hon Leong. This study was designed primarily by myself, Dr. Fabrice Lucien, Dr. Christopher McIntyre, and Dr. Hon Leong. I was responsible for carrying out experiments, collecting data, data analysis, figure creation, and manuscript preparation, all of which were completed with supervision by Dr. Fabrice Lucien, Dr. Christopher McIntyre, and Dr. Hon Leong. Dr. Tyler Cooper operated the conventional flow cytometer for the comparison experiment with beads. Lauren Kaufman and Dr. Francois Lagugne-Labarthet conducted electron microscopy to image the beads. Xinyang Liao, Dr. Karla Williams, Oliver Kenyon, Justin Boysen, and Neil Kay were involved in editing of the manuscript. The final manuscript was revised and approved by every co-author.

Chapter three and four are adapted from an original composite manuscript that is in the process of being submitted and titled “Endothelial and Platelet Extracellular Vesicles as a Measure of Hemodialysis-Induced Microcirculatory Stress”. This composite manuscript which encompasses two of my projects, is to be submitted in December 2022. This manuscript was co-authored by myself, Dr. Barry Janssen, Dr. Yanmin Zhang, Lisa Hur, Jarrin Penny, Dr. Thamara Dayarathna, Dr. Fabio Salerno, Dr. Stephen Pasternak, and Dr. Christopher McIntyre. The study was designed by myself, and Dr. Christopher McIntyre. I was involved in carrying out the majority of experiments, collecting data, data analysis, figure creation, and manuscript preparation which were completed with supervision by Dr. Stephen Pasternak and Dr. Christopher McIntyre. Dr. Barry Janssen and Dr. Yanmin Zhang facilitated the animal experiments and intravital microscopy data analysis. Lisa Hur performed and processed the echocardiography and CT imaging. Jarrin Penny acquired study

approval for human patients in this study. Dr. Thamara Dayarathna provided laboratory support and training. Dr. Fabio Salerno helped with data analysis. The final manuscript is to be revised and approved by every co-author before the manuscript is submitted.

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"What we know matters, but who we are matters more" – Dr. Brené Brown

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List of Abbreviations

HD	Hemodialysis
CV	Cardiovascular
EV	Extracellular Vesicle
nFC	Nanoscale Flow Cytometer
GFR	Glomerular Filtration Rate
mGFR	Measured Glomerular Filtration Rate
eGFR	Estimated Glomerular Filtration Rate
AKI	Acute Kidney Injury
CKD	Chronic Kidney Disease
ESRD	End Stage Renal Disease
RRT	Renal Replacement Therapy
PD	Peritoneal Dialysis
AV	Arteriovenous
ED	Endothelial Dysfunction
Echo	Echocardiography
PET	Positron Emission Tomography
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
VEGF	Vascular Endothelial Growth Factor

BNP	B-type Natriuretic Peptide
cTnT	Cardiac Troponin T
cTnI	Cardiac Troponin I
MVB	Multivesicular Bodies
MP	Microparticle
PS	Phosphatidylserine
TNF	Tumor Necrosis Factor
ISEV	International Society of Extracellular Vesicles
LPS	Lipopolysaccharide
ELISA	Enzyme-Linked Immunosorbent Assay
LFIA	Lateral Flow Immunoassay
DLS	Dynamic Light Scattering
NTA	Nanoparticle Tracking Analysis
RPS	Resistive Pulse Sensing
EM	Electron Microscopy
TEM	Transmission Electron Microscopy
AFM	Atomic Force Microscopy
FC	Flow Cytometry
FSC	Forward Angle Scatter
SSC	Side angle scatter

PMT	Photomultiplier Tube
ROI	Region of Interest
RI	Refractive Index
LALS	Large Angle Light Scatter
SALS	Small Angle Light Scatter
PMP	Platelet Microparticles
FITC	Fluorescein Isothiocyanate
MESF	Mean Equivalent Soluble Fluorochromes
PFP	Platelet Free Plasma
PRP	Platelet Rich Plasma
RT	Room Temperature
HUVEC	Human Umbilical Vein Endothelial Cells
IVM	Intravital Microscopy
SAD	Sum of Absolute Values of Light Intensity Changes
IDH	Intradialytic Hypotension
IDWG	Interdialytic Weight Gain
UFR	Ultrafiltration Rate
IQR	Inter Quartile Range

Chapter 1

1 Introduction

1.1 Introduction to the Renal System

In general, humans are born with two kidneys. They are located under the rib cage, behind the peritoneum, one on each side of the vertebral column. An adult kidney is approximately 12 cm long and weighs 150 g. On the indented surface of the kidney is a slit called the hilus, through which the renal artery and renal vein pass, as well as the lymphatics, the renal nerve, and the renal pelvis. Macroscopically the kidney is composed of an outer region, the cortex, and an inner region, the medulla. Within these regions are nephrons, the basic functional unit of the kidney (Figure 1.1). Each kidney contains about 1-1.5 million nephrons which filter blood to remove waste and fluid, excreted in the form of urine. The nephron is composed of a renal corpuscle and tubule. Within the renal corpuscle, blood is filtered first in the glomerulus, and then the resulting fluid is filtered further in a tubule made up of adjacent functional segments (proximal tubule, intermediate tubule, distal tubule, and collecting duct). The collecting ducts of each nephron eventually merge with each other to form a single ureter which transports the resulting fluid, urine, to the bladder. On average, the kidneys are responsible for filtering about 200 liters of blood daily.[1]

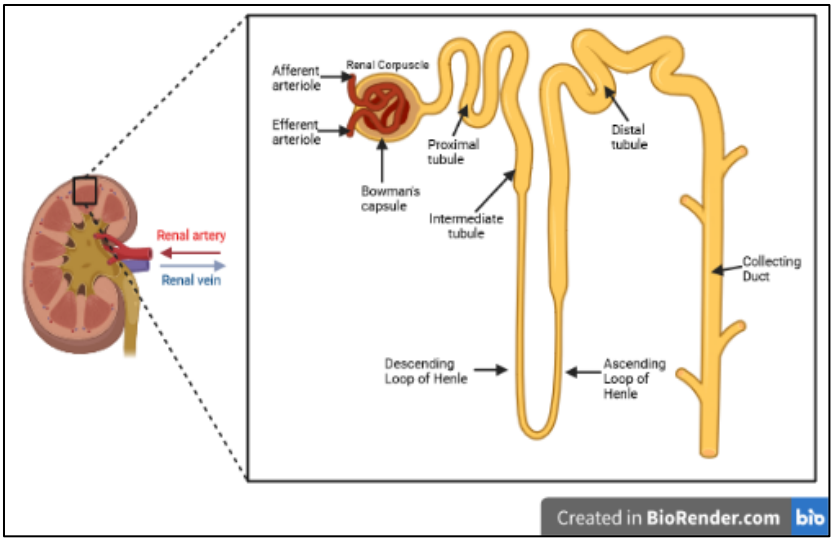

Figure 1.1. Structure of the nephron.

Blood is filtered in the renal corpuscle. The filtrate then travels to be further processed in the Proximal Tubule, the Intermediate Tubule (consisting of the Descending Loop of Henle and the Ascending Loop of Henle), the Distal Tubule, and the Collecting Duct. (Figure created with Biorender.com)

1.1.1 Normal Renal Physiology

The kidneys are responsible for maintaining homeostasis in the body. They are responsible for the excretion of waste, reabsorption of nutrients and electrolytes, maintenance of pH, regulation of osmolality, release hormones to regulate blood pressure, and control the production of red blood cells.[1]

A main responsibility of the kidney is filtering blood by removing excess fluid and waste-products at the level of the nephrons. This process begins when blood enters a nephron at the afferent arteriole into the glomerulus (renal corpuscle). The glomerulus is composed of capillaries enclosed in a pouch-like extension called the Bowman's capsule. The glomerulus functions to filter out small solutes and water from the blood, while preventing larger molecules from being filtered out from the blood. This forms the filtrate which will continue to be processed in the nephron. The resulting retained blood/plasma and blood cells exit through the efferent arteriole in the renal corpuscle. The filtrate eventually enters the renal tubule. The renal tubule consists of the proximal tubule, the

intermediate tubule, and the distal tubule. The proximal tubule functions to reabsorb most of the filtered water and solutes. The intermediate tubule consists of the thin descending and thin ascending limb of the loop of Henle. The descending limb is highly permeable to water, while the ascending limb is impermeable to water and further concentrates the filtrate. The filtrate eventually enters the distal tubule which encompasses the thick portion of the ascending limb of the loop of Henle and the convoluted portion. The distal tubule is water impermeable and is important for the reabsorption of sodium and chloride ions. The filtrate eventually moves into the collecting duct, which is the final regulator of fluid and electrolytes, and is responsible for handling sodium, chloride, potassium, and acid-base homeostasis. Altogether the passage of the filtrate through the nephron results in urine which is transported through the ureter to the bladder to be excreted.[1]

Additionally, the kidneys are responsible for gluconeogenesis, and regulating red blood cell and vitamin D production.[2] The kidneys produce erythropoietin, a peptide hormone that controls red blood cell production in the bone marrow. Erythropoietin production is stimulated by a reduction in the partial pressure of oxygen in the kidneys.[2] The kidneys are also involved in the *in vivo* synthesis of vitamin D. The kidney is the major site of the active hormonal form of vitamin D, 1α,25(OH)2D. The rate of synthesis is regulated by hormones that control calcium and phosphate balance.[3] Lastly, the kidneys have an important role in glucose homeostasis.[4] While the majority of gluconeogenesis occurs in the liver, a considerable fraction occurs in the kidney. Gluconeogenesis is the process where glucose is synthesized from non-hexose precursors during fasting and stress conditions.[5] The kidneys accounts for approximately 40% of endogenous gluconeogenesis, occurring in the proximal tubule. The whole process is regulated by insulin, cellular glucose levels, acidosis, and stress hormones.[5] Additionally, the kidneys are also responsible for insulin clearance. Insulin has a molecular weight of approximately 6000 Da, which makes it freely filtered. Of total renal insulin clearance, 60 percent will occur through glomerular filtration, and 40 percent through extraction by peritubular vessels.[6] Insulin is metabolized into amino acids by the lysosomes of proximal tubular cells.[7] Altogether the kidneys are involved in multiple important processes in the body.

1.1.2 Glomerular Filtration Rate (GFR)

The glomerular filtration rate (GFR) is an overall index of kidney function for health and disease. It is the average filtration rate of individual nephrons, multiplied by the average number of nephrons in both kidneys. GFR is determined by the sum of hydrostatic and oncotic pressure gradients from plasma in the Bowman space. The Starling equation is used to calculate the GFR of a single nephron:

$$K_f\left[\left(P_{gc} - P_{bs}\right) - \left(\pi_{gc} - \pi_{bs}\right)\right]$$

K_f is the ultrafiltration coefficient, P_{gc} is the glomerular capillary hydrostatic pressure (~45 mm Hg), P_{bs} is the bowman space hydrostatic pressure (~10 mm Hg), π_{gc} is the glomerular capillary oncotic pressure (~25 mm Hg), and π_{bs} is the bowman space oncotic pressure (0 mm Hg). The normal GFR for men and women, is 130ml/min/1.73 m^2 and 120ml/min/1.73 m^2, respectively. [8]

The GFR cannot be directly derived on the above basis, but instead is measured (mGFR) or estimated (eGFR) with renal clearance techniques. These are calculated through clearance measurements in urine or serum levels of different filtration markers. Filtration markers include solutes that are not bound to plasma proteins and are freely filtered by the glomeruli with molecular weight of less than 20,000 dalton. These markers can be exogenous (e.g. inulin, iohexol, and iothalamate) or endogenous (e.g., creatinine and urea).[8]

Urinary clearance, is defined as follows:

$$Cl_x = \frac{U_x \times V}{P_x}$$

Where clearance (Cl_x) of a substance (x) excreted into the urine can be calculated as a product of urinary concentration (U_x) and urinary flow rate (V) divided by the plasma concentration (P_x). The measurement of urinary clearance requires timed urine collection for measurement of volume, as well as urine and plasma concentrations of the selected filtration markers.[1]

Plasma clearance, is defined as follows:

$$Cl_x = \frac{A_x}{P_x}$$

Where plasma clearance (Cl_x) of a substance (x) after bolus of an exogenous filtration marker is calculated from original amount of the marker administered (A_x) divided by the average plasma concentration (P_x). Plasma clearance is usually estimated by using a two-compartment model which requires blood sampling early (2-3 time points over 60 minutes) and late (1-3 time points after 120 minutes).[1]

Overall, GFR is a useful measurement that serves as a criterion for staging of acute kidney injury and chronic kidney disease.[9]

1.1.3 Pathophysiology

1.1.3.1 Acute kidney injury (AKI)

Acute Kidney Injury (AKI) is a term that refers to an abrupt decrease in kidney function, encompassing kidney injury (structural damage) and impairment or loss of kidney function.[10] It is estimated that 1 in 5 adults and 1 in 3 children worldwide experience AKI.[11] Risk factors for AKI include environmental, socioeconomic, and patient-related sources such as inadequate drinking and waste water systems, insufficient health care systems, anemia, hypotension, hypoxia, use of nephrotoxic drugs, heart disease, liver or gastrointestinal disease, diabetes, sepsis, old age, acute organ failures, major surgeries, chemotherapy, and autoimmune disorders.[12] Moreover, some risk factors include genetic predispositions such as myoglobinuria, hemoglobinuria and urolithiasis.[12] The diagnosis for AKI is determined through increased serum creatinine levels (≥ 26.5 μmol/L or ≥ 1.5 times baseline levels within a 48 h period) and decreased urine output (< 0.5 ml/kg for ≥ 6 h).[13]

AKI can be grouped into three etiologies: prerenal, renal, and post renal. Prerenal causes result from hypovolemia or a decreased effective arterial volume. Renal causes are classified under the different anatomic components of the kidney such as the vascular

supply (e.g., intrarenal vessel damage), the glomeruli (e.g., glomerulonephritis), the tubules (e.g., acute tubular necrosis), and the interstitium (e.g., interstitial nephritis). Postrenal causes of AKI include urinary tract obstruction and obstruction to urinary flow as a result of cancer, fibrosis, and uretal stones.[14]

1.1.3.2 Chronic Kidney Disease (CKD)

More than 800 million individuals worldwide are currently affected by Chronic Kidney Disease (CKD).[15] From 1990 to 2017 the global prevalence of CKD has increased by 29.3 % with mortality increasing by 41.5 %.[16] CKD is defined as abnormalities in kidney function or structure for at least three months.[17] CKD is classified and based on eGFR and levels of proteinuria. Through utilization of eGFR, patients are classified into G1 to G5 (Table 1).[17] Based on proteinuria, or Albumin: Creatinine Ratio (ACR), patients can be classified by being in A1-A3 (Table 2).[17] CKD is diagnosed based on the following criteria: (1) GFR $\leq$ 60 ml/min/1.73m^2; (2) urine albumin is $\geq$ 30 mg/24 hours or the urine albumin-to-creatinine ratio is $\geq$ 30 mg/g; (3) abnormalities in urine sediment histology or imaging which suggests kidney damage; (4) renal tubular disorders; or (5) history of kidney transplant.[18]

There are several risk factors for the progression of CKD. Similarly, as AKI, the risk factors come from a variety of socioeconomic, environmental, and patient specific reasons. Susceptibility factors include older age, family history of CKD, reduction in kidney mass, low birthweight, and low income or education. Some initiation factors of CKD include diabetes, high blood pressure, urinary tract infections, urinary stones, lower urinary tract obstruction, drug toxicity, and autoimmune diseases. Progression factors include higher levels of proteinuria, higher blood pressure, poor glycemic control, and smoking.[19] The eventual cause of CKD can be categorized by the presence or absence of underlying systemic disease and the location of the abnormality. The types of CKD can be described as glomerular, tubulointerstitial, vascular, and cystic or congenital diseases. These are a consequence of (i) systemic diseases such as diabetes and hypertension, (ii) autoimmune reactions and renal transplant rejection, (iii) the action of drugs, toxins and metals, (iv) infections, (v) mechanical damage, (vi) ischemia, (vii) obstruction of the

urinary tract, (viii) primary genetic alterations, and (ix) undetermined causes (idiopathic).[20]

Table 1.1. GFR categories with description and range

Category	eGFR (ml/min/1.73m^2)	Classification
G1	≥ 90	Normal to high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	< 15	Kidney Failure

Table 1.2. Albuminuria categories with description and range

Category	ACR (mg/g)	Classification
A1	< 30	Normal to mildly increased
A2	30-300	Moderately increased
A3	≥ 300	Severely increased

1.1.3.3 End Stage Renal disease (ESRD)

ESRD or kidney failure is defined as a composite of GFR that is < 15 ml/min/1.73m^2 and increased levels of albuminuria.[17] This can be caused by AKI or by progression of a chronic nephropathy. ESRD results in the inability to excrete waste products, manage daily dietary and metabolic acid load, and maintain proper fluid balance and control serum electrolytes. For those with ESRD, they have options of renal replacement therapy (RRT) including kidney transplantation, peritoneal dialysis, and hemodialysis. However, RRT is usually not started until one's GFR in some cases is below <5-10 ml/min/1.73m^2. Modality choice of RRT depends on patient autonomy, medical and social factors, system-related issues, and patient outcomes.[21]

1.2 Renal Replacement Therapy

1.2.1 Kidney Transplant

Kidney transplantation is the most preferred mode of renal replacement in terms of outcomes and cost effectiveness.[22] Kidney transplantation for ESRD patients consists of receiving a kidney from either a living or deceased donor. Living donor transplantation is a preferred method when feasible, because it results in improved patient survival. Kidney transplants offer increased life expectancy, as well as an improved quality of life following transplantation. However, disadvantages of transplantation include the logistics of finding a suitable and compatible donor and a patient's compliance with immunosuppressive drugs.[23]

1.2.2 Peritoneal Dialysis (PD)

Peritoneal Dialysis is another treatment option for ESRD. The peritoneum membrane is a semipermeable lining of the abdominal cavity. PD treatment involves the transport of solutes and water across the peritoneum membrane in one's abdomen which separates two compartments: the blood in the peritoneal capillaries, and the dialysate solution in the peritoneal cavity. PD is performed through surgically inserting a catheter in the abdomen to the peritoneal cavity. Dialysate fluid is then inserted into the cavity. The dialysate fluid contains sodium, chloride, and lactate or bicarbonate, as well as a high concentration of glucose as an osmotic agent. The concentration of the solutes in the dialysate creates a high osmotic pressure gradient across the peritoneal space, which transports waste products and excessive water from the peritoneal capillaries across the peritoneum membrane by diffusion, ultrafiltration, and absorption into the dialysate solution. The degree of fluid and waste product removal depends on the volume of dialysate, how often the dialysate solution is exchanged, and the concentration of the osmotic and oncotic agents used.[23]

The benefit of using PD is that it offers patients a home-based therapy as it has simple equipment set-up and does not require special water systems. Patients who often favour PD include infants and children, patients with severe cardiovascular disease, patients with

difficult vascular access, and patients who desire a greater degree of freedom. However, there are contraindications that prompt patients to switch to another RRT, hemodialysis. Contraindications include unsuitable peritoneum due to adhesions, fibrosis, or malignancy. Additionally, some patients may experience an increased peritoneal membrane transport rate over time which results in inadequate ultrafiltration. Moreover, the abandonment of PD is also caused by the occurrence of episodes of peritonitis.[23]

1.2.3 Hemodialysis

Hemodialysis (HD) is the most used form of RRT worldwide.[24] It is a treatment which removes excessive fluid and waste products from the blood to restore the intracellular and extracellular fluid environment to that of one with relatively normal functioning kidneys. There are various HD treatment schedules such as short daily HD (1.5-2.5 hours, 5 or more days/week), daily nocturnal HD (6-10 hours, 5 or more days/week), long intermittent HD (8 hours, 3 days or nights/week), and conventional/maintenance HD (4 hours, 3-times per week), which is most commonly used.[21] The HD apparatus can be divided into three sections: blood circuit, dialysis solution circuit, and the dialyzer.

1.2.3.1 Blood circuit

To initiate the blood circuit, a vascular access site must be created in patients. This access is achieved by one of three options: arteriovenous (AV) fistula, AV graft, or venous catheter. An AV fistula involves the creation of an anastomosis (connection between two structures) of an artery and a vein. AV grafts are similar, however the distance between the artery and vein are bridged by a tube made of a prosthetic material. Venous catheters are more frequently used by patients whose AV creation cannot be readily created, this includes children, some diabetic patients, patients with severe vascular disease, and those who have undergone multiple AV access insertions and therefore do not have viable sites available for AV insertion.[23]

From the vascular access point, blood travels through extracorporeal tubing (inflow blood line), passing a blood pressure monitor, blood flow pump, anticoagulant (usually heparin) pump, and an inflow pressure monitor, eventually passing through the dialyzer (Figure

1.2). After the blood is filtered in the dialyzer, it then passes another blood pressure monitor, air trap, and air detector before returning back to the patient.

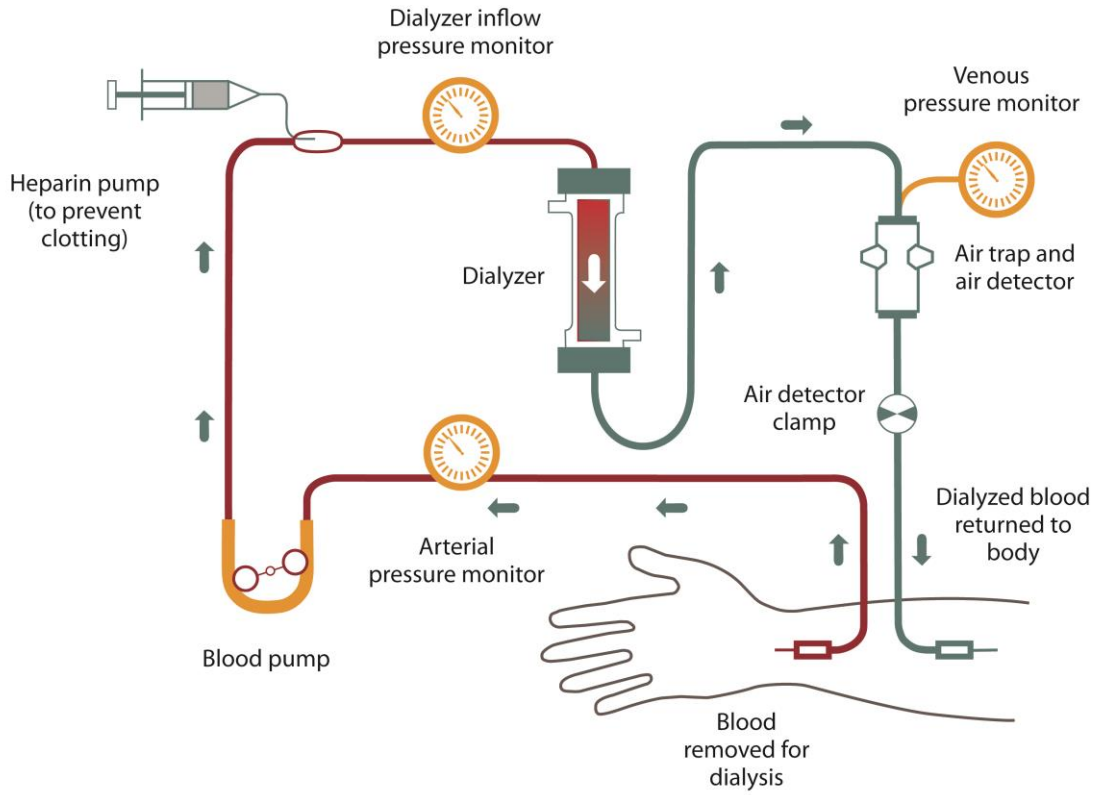

Figure 1.2. Schematic diagram of hemodialysis blood circuit.

Blood (red) travels from the AV access (AV fistula or AV graft) or a venous catheter through extracorporeal tubing past the arterial pressure monitor, blood pump, anticoagulation (heparin) pump, inflow pressure monitor, dialyzer, air detector, venous pressure monitor, and air trap and air detector returning filtered blood (blue) back to the patient. This figure was originally published online in the *National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.* [https://www.niddk.nih.gov/health-information/kidney-disease/kidney-failure/hemodialysis]. This figure is being reproduced for educational purposes only and not for any commercial use. Figure is included in the Ph.D. dissertation with attribution.

1.2.3.2 Dialysis Fluid circuit

The dialysis fluid circuit consists of components such as a stand-alone water purification system, a proportioning system (where concentrates and water are mixed and delivered to

the dialyzer), monitors and alarms, ultrafiltration control, as well as other controls. In this circuit, the water purification system and proportioning system create approximately 120-200 L of dialysate solution during the HD treatment. The solution flows into the dialyzer unit.[23]

1.2.3.3 Dialyzer

The blood circuit and the dialysis fluid circuit both interact at the dialyzer (Figure 1.3). The dialyzer shell consists of four ports, with two ports for blood (inflow and outflow), and two ports for dialysate (inflow and outflow). Within the dialyzer are thousands of narrow and hollow semipermeable fibers. Blood enters through the inflow of the dialyzer shell and is then channeled into the semipermeable hollow fibers, eventually exiting through the outflow port of the dialyzer. The dialysate solution flows around and outside the fibers in the opposite direction creating a countercurrent flow with the blood. It is within the dialyzer that through diffusion and ultrafiltration (convection) that excessive fluid and waste products are removed from the patient's blood across the semipermeable membrane and into the dialysate solution. Diffusion refers to the movement of solutes from random molecular motion, where molecules move from an area of high to low concentration. Ultrafiltration occurs when water is forced across the membrane by hydrostatic pressure and osmotic forces. Solutes that can easily pass through the membrane pores are also swept along with water (solvent drag).[23]

The dialyzer itself is crucial in the dialysis process. Dialyzers can differ by a variety of factors such as material composition, membrane thickness, surface area, pore size distribution, and pore density. These factors can influence diffusion, convection, adsorption, and ultrafiltration.[25] Dialyzer membranes have been traditionally classified by their material composition of cellulosic or synthetic membranes. While cellulosic membranes were widely used in the past, synthetic membranes are now more generally used because of their relative biocompatibility and higher permeability.[26] Other properties such as membrane thickness influences the distance that solutes travel between the blood and dialysate. The surface area of membranes can also influence the frequency of interactions of molecules with the dialyzer membrane impacting diffusion. Pore size distribution, and density can also influence diffusivity. Membranes with larger pores are

usually referred to as high flux because they can remove any molecules less than 25 kDa in size such as uremic toxins and middle molecules such as B2-microglobulin. Whereas low-flux dialyzers, usually referred to as having smaller pores, are only able to remove small molecules <500 Da. However, high flux and low flux does not necessarily always relate to pore size, as other properties can influence the dialyzers' ability to clear certain solutes. Therefore high-flux is also described as a B2-microglobulin clearance of greater than 20ml/min, whereas low-flux is clearance less than 10 ml/min.[27]

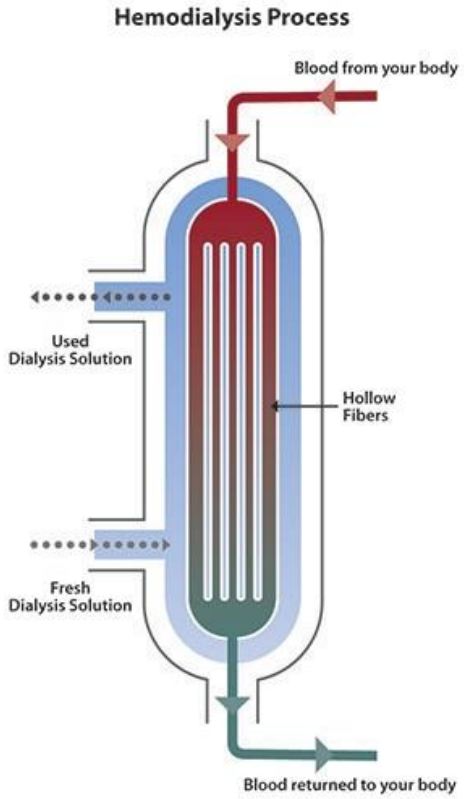

Figure 1.3. Schematic illustration of the dialyzer.

As blood enters the dialyzer, it is guided into thin hollow fibers. On the other side of the fibers, dialysate (dialysis solution) passes in the opposite direction of the blood. During this process, waste products and excess fluid will move from the patient's blood into the dialysate solution. This figure was originally published online in the *National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.* [https://www.niddk.nih.gov/health-information/kidney-disease/kidney-failure/hemodialysis]. This figure is being reproduced for educational purposes only and not for any commercial use. Figure is included in the Ph.D. dissertation with attribution.

1.3 Hemodialysis and Cardiovascular Injury

1.3.1 HD-Associated Microcirculatory Stress

While conventional/maintenance hemodialysis (3 times/week) is a life-saving treatment, it has many negative side effects and a range of complications. Hemodialysis is associated with large ultrafiltration volumes (fluid removed from patients), resulting in a decrease in the patients' intravascular volume. These decreases in plasma volume coupled with short treatment times (4 hours) usually exceeds the plasma refill rate (i.e. refilling rate of intravascular volume from the extravascular compartment) and results in intradialytic hypotension and decreased organ (heart, brain, kidney, and liver) perfusion.[28–30] Intradialytic hypotension (a decrease of 20 mmHg over treatment) has been shown to be an independent predictor of mortality among HD patients.[31,32] Moreover higher ultrafiltration rates (the rate at which fluid is removed per hour indexed to patient body weight) have also been shown to be associated with risk of mortality among HD patients.[33,34]

Patients are also less likely to tolerate their HD treatments and be further predisposed to hemodynamic stress because of a variety of factors such as endothelial dysfunction (ED), defective vasoregulation, and impaired cardiovascular compensation (redistribution in blood volume, and changes in heart rate, cardiac output, vascular total peripheral resistance).[35,36] The disruption of capillary blood flow and reduced tissue perfusion is referred to as microvascular ED.[37–40] ED refers to the change from a normal and healthy endothelium to a stressed/damaged phenotype characterized by the imbalance of endothelium derived vasodilating factors, and/or an increase in endothelial derived contracting factors, which results in an impairment of vasodilation, inflammation, coagulation, vascular tone, wall permeability, and impaired cell growth.[38,41,42] In a study comparing uremic patients (undergoing renal transplant or catheter insertion for dialysis) and healthy controls, they found that the blood vessels from uremic patients vasodilated less well than compared to controls.[39] Other than the influence of the uremic environment, other studies have found that endothelial dysfunction was worse among

hemodialysis patients compared to patients who had chronic kidney disease stage 3 or 4, or patients with a kidney transplant.[40]

1.3.2 HD-Induced Cardiac Injury

Hemodialysis patients display elevated rates of cardiovascular disease and cardiac mortality.[43] Cardiac injury has been observed through a variety of medical imaging-based studies which display indirect (Regional Wall Motion Abnormalities, RWMA) and direct (myocardial perfusion) forms of microcirculatory stress.

RWMAs provide a direct measurement of the contractile performance of the heart and indirectly reflect regional ischemia of the heart.[29] To observe intradialytic RWMA, speckle-tracking echocardiography (echo) which is a modality that uses a 2D ultrasound technique, tracks myocardial speckle signals over multiple temporal frames.[44] Through this modality, myocardial strain and RWMA can be determined among the 12 ventricular segments. It has been shown that patients experience RWMAs throughout HD.[45] Additionally, through using positron emission tomography, with radiolabeled water, ($H_2{}^{15}0$-PET), it has been shown that during HD treatments, patients experience a 30% reduction in myocardial perfusion, even in the absence of coronary stenosis.[29] Additionally, it was shown that at peak stress of HD (235 minutes into an HD session) compared to pre-HD, the ventricular regions with hypo-perfusion matched the regions with RWMA.[29] Overall, the repetitive nature of hemodialysis causes progressive injury which can lead to irreversible multi-organ injury including increased risk of cardiovascular disease, stroke, and death.[30,46–49]

1.3.3 HD-Induced Brain Injury

Hemodialysis patients are known to suffer cognitive impairment (predominantly sub-cortical defects in executive decision making), which are almost universal among HD patients, and appear early after starting hemodialysis treatment.[50–53] Similar to the studies on HD and heart injury, there are various studies which have used imaging-based methodologies to investigate brain hypo-perfusion and injury. For example, through using dynamic $H_2{}^{15}0$-PET-Computed Tomography (CT), it has been demonstrated that

HD associated circulatory stress and recurrent regional ischemia drives brain injury.[54] Through other imaging techniques such as magnetic resonance imaging (MRI), cerebral pathologies such as white matter hyperintensities have been revealed. These represent white matter ischemia, which is characterized by neuronal loss, demyelination, and gliosis. These overall characteristics constitute the appearance of Leukoaraiosis. Leukoaraiosis describes rarefaction of brain white matter, typically associated with vascular and cognitive impairment and is universally present in HD patients.[55],[56] Additionally the severity and reduction in cognitive function is proportional to the amount of white matter injury, with a predominant loss of subcortical functions (executive functioning).[57], [58]

1.4 Biomarkers of Endothelial and Cardiac Injury

Detection of microcirculatory stress among HD patients has been assessed through imaging modalities such as Echocardiography (Echo), Computed Tomography (CT), Positron Emission Tomography (PET), and Magnetic Resonance Imaging (MRI).[49],[59],[60] Medical imaging has provided informative observations, though are costly (ranging from $500-$2000/patient, Canadian Magnetic Imaging), time consuming, and generally require trained technicians. Therefore, identifying other ways, such as using blood-based biomarkers to understand vascular injury among HD patients may be a more practical method.

Biomarkers (short for biological markers) are objectively measured and can be evaluated as an indicator of either normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [61]. Biomarkers can have molecular, histologic, radiographic, or physiological characteristics. They are used for a wide variety of applications such as diagnosis, staging diseases, classifying the extent of a disease, indicating disease prognosis, and for predicting and monitoring clinical response to an intervention.[61] An ideal biomarker is non-invasive, easily measured, inexpensive, and produces rapid results. Additionally, they should be from readily available sources, have high sensitivity and specificity, levels should vary rapidly in response to treatment, and levels should aid in risk stratification and possess prognostic value in terms of real

outcomes. Lastly, biomarkers should be biologically plausible and provide insight into the underlying disease mechanism.[61]

Even though a variety of biomarkers exist for endothelial and cardiac injury, no biomarkers have had sufficient testing to qualify as being useful to identify HD associated injury or to guide the prescription of HD treatments.[62,63]

1.4.1 Blood-Based Biomarkers of Endothelial Injury

There are several blood-based biomarkers of vascular injury that are associated with endothelial biology, including growth factors, cell adhesion molecules, glycocalyx, and circulating endothelial cells.

1.4.1.1 Growth Factors

Angiopoietin (Ang) 1 and 2 are growth factors involved in angiogenesis and vascular permeability. Among sepsis, which is a disease associated with endothelial dysfunction, Ang 1 and 2 correlate with mortality and severity of disease.[64,65] Other growth factors include vascular endothelial growth factor (VEGF). VEGF stimulates endothelial cell migration, hyperpermeability, and angiogenesis. Moreover, VEGF has been shown to be elevated in diseases like sepsis and preeclampsia, and is associated with endothelial dysfunction.[66,67] While endothelial growth factors provide information regarding endothelial cell survival and angiogenesis, they are not used regularly in the clinical setting for HD patients. Moreover, some of these biomarkers have been found to be sex-dependent, and therefore might be limited for use among all HD patients. From a multicenter cohort study that investigated baseline serum Ang-2 concentrations among HD patients, they found that Ang-2 independently associated with all-cause mortality among male patients, but not female patients.[68]

1.4.1.2 Cell Adhesion Molecules

Selectins are a type of cell adhesion molecule expressed on activated endothelial cells and modulate leukocyte movement.[69] Endothelial cells may shed their selectins during cell injury.[70] Other than selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecules-1

(PECAM-1) are also involved in inflammation by facilitating leukocyte adhesion and movement. These cell adhesion molecules are found to be increased as a result of endothelial dysfunction, however, their ability to determine severity of disease is still uncertain.[71] Moreover, cell adhesion molecules such as ICAM-1 have not been demonstrated as useful biomarkers among hemodialysis patients. Comparing patients who did and did not experience HD-induced regional left ventricular systolic dysfunction, there were no significant differences in ICAM-1 levels.[63]

1.4.1.3 Glycocalyx

The endothelial glycocalyx is a carbohydrate rich layer that lines the endothelium. The glycocalyx is connected to the endothelium through several proteoglycan and glycoprotein molecules. A dynamic equilibrium exists between this layer and flowing blood, which impacts glycocalyx thickness.[72] The breakdown of the glycocalyx has been shown to correlate with endothelial dysfunction caused by malaria, dengue, sepsis, and acute respiratory syndrome.[73–76] Specific broken-down products of the endothelial glycocalyx such as syndecan-1, chondroitin sulfate, dermatan sulfate, serum hyaluronic acid and heparan sulfate can be measured. From these, syndecan-1 levels have been associated with severity of sepsis, acute kidney injury, and mortality.[77,78] Additionally, syndecan-1 levels are higher in patients receiving HD compared to healthy controls.[79] However, their potential as biomarkers of HD-associated injury is still to be determined.

1.4.1.4 Circulating Endothelial Cells

Circulating endothelial cells, which are thought to originate from sloughed off mature endothelium, are thought of as markers of endothelial injury.[80] Higher levels of circulating endothelial cells have been associated with cardiovascular risk factors and acute myocardial infarction.[81] Moreover, it has been shown that circulating endothelial cells are a strong predictor of long-term mortality in hemodialysis patients.[82] However, their association with direct HD-associated injury has not been thoroughly investigated.

1.4.2 Blood-Based Biomarkers of Cardiac Injury

1.4.2.1 B-type Natriuretic Peptide (BNP)

B-type natriuretic peptide (BNP) is a vasopeptide hormone. It is expressed and secreted by the ventricular myocardium. It is also expressed by other organs such as the brain, adrenal glands, kidney, and lungs.[83]

BNP is synthesized as an amino acid protein which undergoes modification to prohormone (proBNP). Once released into circulation, proBNP is cleaved into a C-terminal fragment and a N-terminal fragment (NT-pro-BNP). BNP and NT-Pro-BNP synthesis and secretion are influenced by left ventricular wall stress and may reflect left ventricular overload.[84] Population based studies have shown that plasma derived BNP and NT-pro-BNP are useful screening tests for heart failure and asymptomatic left ventricular dysfunction.[85]

Various studies have found that BNP and NT-pro-BNP levels are strongly associated with left ventricle hypertrophy and systolic dysfunction in patients who have ESRD and are on conventional HD.[86,87] However, a confounding factor is that HD itself may influence BNP and NT-pro-BNP, as it is shown to be eliminated by high flux dialysis membranes.[88]

1.4.2.2 Cardiac Troponin

Cardiac Troponin T (cTnT) and Troponin I (cTnI) are regulatory proteins that manage the calcium mediated interactions between actin and myosin molecules.[89] Cardiac troponins are released into the circulation in response to myocardial injury and can be used to diagnose acute myocardial necrosis.[90] The evaluation of serum concentrations to diagnose acute coronary syndromes is standard practice among non-renal populations.[91] Cardiac troponins have also been noted as being unreliable as they can be released from myocytes without necrosis and are observed at high levels among people without any clinical signs of myocardial injury.[92] Moreover, the interpretation of cardiac troponins is also controversial as there is a lack of understanding surrounding what specifically constitutes change in serial measurements, and there is disagreement as to whether

interpretation in chronic settings is best understood in a single or serial measurement. Within the dialysis patient population, majority of patients present with serum cardiac troponin levels that exceed the 99th percentile upper reference limit of troponin assays, despite being asymptomatic.[93] Additionally, cTnT and cTnI are partially cleared during HD with high flux dialyzer membranes compared to low flux dialyzer membranes.[93]

1.5 Extracellular Vesicles

1.5.1 Extracellular Vesicles: Exosomes, Apoptotic Bodies, Microparticles

Blood-based biomarkers of interest in my thesis are Extracellular Vesicles (EVs). First discovered in 1967, EVs were described as debris or "platelet dust" due to their prothrombotic functions.[94] However, research studies since then have shown that EVs are serological markers of cardiovascular disorders and are a means for intercellular communication.[95,96] Blood-based circulating EVs are an informative biomarker to evaluate endothelial function, as they are considered as endothelium-specific biomarkers.[97] The function and diversity of EVs have been further investigated leading to the traditional classification of EVs into three sub-groups: exosomes, microparticles, apoptotic bodies.[98]

1.5.1.1 Exosomes

Exosomes are classified as particles in the size range from 30-150 nm in diameter. Exosomes are referred to as intraluminal vesicles and are formed by the inward budding of the endosomal membrane. They are enclosed within a single outer membrane and are secreted by all cell types. Exosomes are found in plasma, urine, semen, saliva, bronchial fluid, cerebral spinal fluid, breast milk, serum, amniotic fluid, synovial fluid, tears, lymph, bile, and gastric acid.[99],[100] Exosomes form by the inward budding of the membrane of early endosomes, which eventually mature into multivesicular bodies (MVBs). MVBs are responsible for trafficking cell material and are involved in protein sorting, recycling, storage, transport, and release. MVBs are either sent to the lysosome to

be degraded, or fuse with the plasma membrane and release their contents into the extracellular space.[101,102] The regulation of exosome formation and release is through endosomal sorting complexes required for pathway transport.[103] Additionally, exosomes participate in a variety of processes, such as cell-cell communication, cell maintenance, tumor progression, and have been found to stimulate immune responses.[100] Exosomes have potential as candidate biomarkers for various diseases such as acute kidney injury, and pancreatic and lung cancer. [104–106] While various applications exist, more standardized methods for exosome isolation and analysis are needed for exosomes to be used as biomarkers, vaccines, drug delivery devices, and therapeutic tools.[107]

1.5.1.2 Apoptotic Bodies

Apoptotic bodies are vesicles released by dying cells as a result of programmed cell death and are considered a hallmark of apoptosis as they are released as a product of apoptotic cell disassembly.[108] Apoptosis is initiated in stages, beginning with condensation of nuclear chromatin, followed by membrane blebbing, then disintegration of the cellular contents into vesicles called apoptotic bodies.[109] After apoptotic bodies are released, they are phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes.[110] Their size is reported to be between 500 nm up to 5000 nm in diameter, with most occupying the larger end of the spectrum. Apoptotic bodies contain intact organelles, chromatin, and small amounts of glycosylated proteins.[111,112] Apoptotic bodies are involved in intercellular communication, as previous studies have reported horizontal transfer of oncogenes by uptake of apoptotic bodies.[113] However, compared to exosomes and microparticles, research on apoptotic bodies is limited, and more in-depth studies are needed to further understand their role and possible function.[114]

1.5.1.3 Microparticles

Microparticles (MPs), also referred to as microvesicles and ectosomes, are membrane-bound vesicles that are 100 nm-1000 nm in diameter and are released from various cell types under physiological and disease conditions.[108] They are secreted through outward blebbing and pinching of the plasma membrane and released in the extracellular

environment.[115] MPs have been found in peripheral blood, cord blood, saliva, urine, and cerebrospinal fluid.[116] The biogenesis of MPs is not fully understood, however it is thought to be the result of plasma membrane phospholipid redistribution and coordination of actomyosin contractile machinery (Figure 1.4).

In quiescent cells, there is an asymmetrical distribution of phospholipids. Negatively charged amino phospholipids, phosphatidylserine (PS), are located on the inner leaflet of the cell membrane, and neutral phospholipids are located on the outer membrane. The distribution of phospholipids is managed by three proteins: aminophospholipids translocase (or flippase), floppase, and scramblase. Flippase is an ATP-dependent protein which transports aminophospholipids from the outer to inner plasma membrane, and is inhibited by high levels of calcium.[117] Floppase is an ATP-dependent protein that transports phospholipids from the inner to outer plasma membrane.[118] Scramblase is a protein that induces random movement of phospholipids across the membrane and is activated by high calcium levels.[119] When there are normal intracellular calcium concentrations, flippase is the only protein of the three that is active, only moving phospholipids to the inner membrane. When cells are activated by stimuli, intracellular calcium levels rise, flippase is inhibited, and floppase and scramblase move phospholipids to the outer membrane. Stimuli include environmental and biochemical stimuli such as inflammatory cytokines (i.e. Tumor necrosis factor (TNF)[120], apoptosis inducers (i.e. staurosporin)[121], or cellular stress inducers (i.e. endotoxin, hydrogen peroxide, serum deprivation).[122,123] The movement of phospholipids and transmembrane protein clustering and changes in lipid composition lead to asymmetry of membrane and curvature of the cell membrane. This is followed by actomyosin contraction, where the outward budding of the membrane splits and the MP is released. In addition to modifying phospholipid symmetry, high calcium levels also activate proteases which trigger cytoskeleton reorganization and/or destruction. Caspases are also involved in MP release as it is has been shown that caspase-3 mediates the cleavage of Rho-kinase which leads to MP release.[124]

It is important to note that while external exposure of phosphatidylserine seems like an obvious way to identify MPs, some studies have reported vesicles the size of MPs, which

lack PS exposure.[125] As a result, PS is not considered the gold standard marker for MPs. MPs also contain material from their cell of origin and thus express antigens unique to those parent cells. Therefore, specific antibodies can be used to identify specific antigen markers present on the surface of EVs.

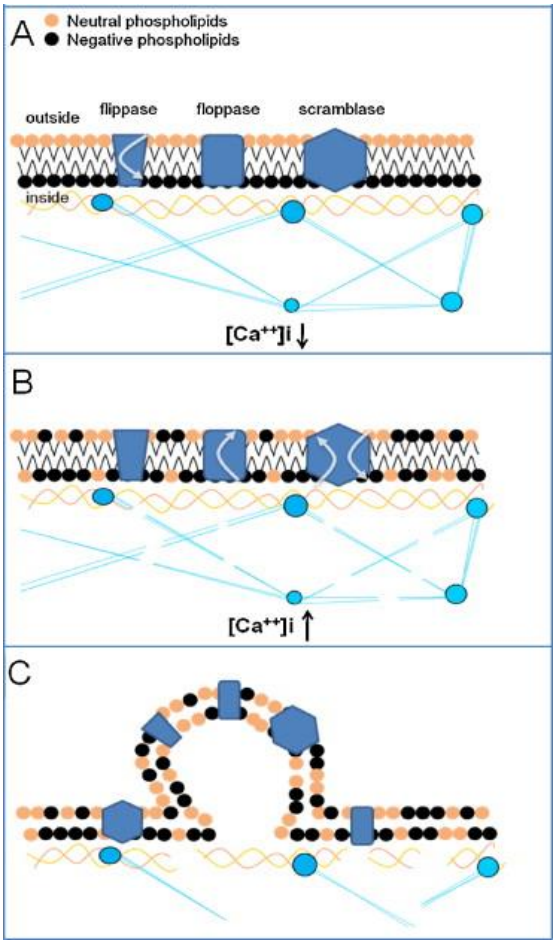

Figure 1.4. Schematic of Microparticle formation.

(A) There are three enzymes (flippase, floppase, and scramblase) involved in the initiation of MP formation. In quiescent cells, where cytoplasmic calcium concentration is low, only flippase is active, facilitating the localization of negatively charged phosphatidylserine (PS) onto the inner leaflet of the plasma membrane. (B) When cells are exposed to environmental or biochemical stimuli, there are increased intracellular calcium concentrations. Flippase is inactivated, while floppase is activated resulting in PS to be localized to the outer leaflet of the plasma membrane. Scramblase translocates phospholipids in a non-specific manner. As a result of the activated and inactivated

enzymes, there is phospholipid asymmetry, and thus activation of proteases and actomyosin contraction facilitates the outward blebbing of the cell membrane. (C) The cell membrane becomes less rigid and will bleb, resulting in a released MP which contains surface antigens and cargo (proteins and nucleic acids) of the cell of origin. Note: This figure was originally published in *Translational Proteomics. Tissot, J.D., Canellini, G., Rubin, Olivier, R., Angelillo-Scherrer, A., Delobel, J., Prudent, M., and Lion, N. Blood microvesicles: From proteomics to physiology. 2013;1(1), 38-52.* This figure is being reproduced for educational purposes only and not for any commercial use. This figure is included in the Ph.D. dissertation with attribution.[126]

1.5.1.4 EV Nomenclature used in this Thesis

The International Society of Extracellular Vesicles (ISEV) define EVs as particles released naturally from a cell, that are delimited by a lipid bilayer, and cannot replicate.[127] ISEV's Minimal Information for Studies of Extracellular Vesicles Guidelines from 2018, suggest that consensus has not yet been reached regarding specific markers of EV subtypes (exosome, microparticle, and apoptotic body), and it is therefore difficult to assign EVs to a particular sub-group, that is unless live imaging techniques are used to confirm the pathway of release. To be transparent among the scientific community and to appropriately discuss EVs, ISEV recommends that subtypes should be avoided, and instead operational terms for EV subtypes should be used, those being physical characteristics (size), biochemical composition (external markers of interest), or description of conditions of the cells of origin. Therefore, these guidelines will be followed through the following chapters of my thesis. MPs will be described as EVs which are 100-1000 nm in diameter, and I will discuss the relevant surface antigen markers on the EVs, or conditions involved.

Note: A version of the content in Chapter 2, was published prior to the release of the 2018 guidelines by ISEV, which encourages all researchers to use the term EVs rather than specific EV subcategories (such as MPs). Therefore, it is important to note that in chapter 2 specifically, I have retained the term MP, rather than using the term EV (100-1000 nm). This is to maintain transparency of my work that was already published, prior

to ISEV's guidelines. For the remainder of this introduction chapter, chapter 3, chapter 4, and chapter 5, the term EV (100-1000 nm) will be used instead of MP to reflect the 2018 guidelines established by ISEV.

1.6 Extracellular Vesicles as Biomarkers of Microcirculatory Stress

Currently, many existing studies support the use of EVs (100-1000 nm) as a biomarker for cardiovascular disease.[128],[129] Circulating EVs from human plasma have been identified as biomarkers of vascular injury and inflammation in severe cardiovascular pathologies including acute myocardial infarction, diabetes, atherothrombosis, preeclampsia, hypertension, and metabolic syndrome.[130,131] Existing literature supports that EVs of endothelial and platelet origin may reflect endothelial dysfunction as well as contribute to endothelial dysfunction.[132–135] Also, endothelial and platelet EVs have been shown to correlate with functional assessments of endothelial function, such as flow mediated vasodilation.[136]

1.6.1 Endothelial EVs

Endothelial EVs (100-1000 nm in diameter) are potential candidate biomarkers of endothelial dysfunction, as well as endothelial inflammation, angiogenesis, and thrombosis.[132] Past studies have shown that they are directly indicative of endothelial stress and damage, and may reflect endothelial inflammation, increased coagulation, and vascular tone.[135] Endothelial EVs (100-1000 nm) are released through shedding of the endothelial plasma membrane in response to stimuli such as TNF-alpha, lipopolysaccharide (LPS), reactive oxygen species (ROS), thrombin, C-reactive protein, and uremic toxins.[134,137,138] Shear stress has also been implicated as a stimulus for endothelial EV release.[139,140]

When endothelial EVs are released, they retain various markers. These markers include vascular endothelial cadherin (CD144), platelet endothelial cell adhesion molecule-1 (CD31), intercellular cell adhesion molecule (CD54), endoglin (CD105), E-selectin (CD62e), S endo 1 endothelial junction protein (CD146), and vascular endothelial growth

factor receptor (VEGF-R2).[132] While there are a variety of endothelial markers, some have different clinical implications. Apoptotic endothelial cells shed EVs that are known to have constitutive markers such as CD31+/Annexin V+, and activated endothelial cells shed EVs with inducible markers such as CD62e+.[122] Among pulmonary hypertension patients, elevated levels of CD62e+ EVs, rather than EVs with constitutive markers (CD144 and CD31) were associated with worse outcomes such as death and re-admission for heart failure or worsening heart failure.[141] Moreover CD62e+ EVs are known to be associated with ischemic injury, as studies have found that high levels of these EVs are associated with extracranial carotid atherosclerosis.[142] Additionally, these endothelial EV levels were associated with endothelial dysfunction, and are suggested as being a robust predictor of severe cardiovascular outcomes.[143–145]

1.6.2 Platelet EVs

Platelet EVs (100-1000 nm) are generated by platelets and megakaryocytes with expression markers such as glycoproteins (CD41,CD42, CD61), P-selectin (CD62p), and platelet endothelial cell adhesion molecule-1 (CD31).[146] Of all these markers of platelet origin, CD41 is the most commonly used among studies.[146] Platelet EVs, known for their strong procoagulant activity are the most abundant fraction of EVs in the blood, making up 70-90% of circulating EVs.[147] Platelet EVs are continuously shed under normal physiological conditions and after platelet activation.[148] Stimuli such as calcium ionophore, thrombin, collagen, and endotoxin can activate platelets and lead to increased intracellular calcium and influence EV release. Platelet EVs are also formed in response to physical stimuli such as shear stress, hypoxia, or blood storage conditions which can create artifact EVs.[149]

Platelet EVs also demonstrate an important role in venous and arterial thrombosis. They are known to bind to the subendothelial matrix, and act as the catalytic center for progressive platelet binding in areas of endothelial injury. They are also involved in the formation of fibrin fibrils.[150,151] Elevated concentrations of platelet EVs are found in patients with coronary syndrome, transient ischemic attacks and strokes, and diabetes with atherothrombotic disease.[152–154] Therefore, platelet derived EVs are thought to

indirectly reveal endothelial dysfunction by identifying increased coagulation and inflammation. [146,155]

1.7 Methods for Extracellular Vesicle detection

There are a variety of methods that have been used to characterize and enumerate EVs. These methods include Enzyme-Linked Immunosorbent Assay (ELISA), Lateral Flow Immunoassay (LFIA), Nanoparticle Tracking Analysis (NTA), Resistive Pulse Sensing (RPS), Electron Microscopy (EM), Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS), and Flow Cytometry (FC).

1.7.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs are used to detect and quantify peptides, proteins, or antibodies. This method relies on an antigen and antibody reaction. In an ELISA assay, antibodies are used to target specific molecules of interest. The antibodies are linked to enzymes which will produce a detectable signal (usually a colour change) after the addition of a substrate. By measuring the signal in reference to appropriate standards, levels of EVs can be quantified. This method is beneficial for detecting and quantifying EVs with a specific antigen marker, however cannot distinguish EVs based on size.[156]

1.7.2 Lateral Flow Immunoassay (LFIA)

LFIA uses similar logic to that of ELISAs, where an antibody or immobilized antigen is bound to a membrane. However, unlike ELISA, this assay can be performed within a few minutes and in a single step, avoiding the tedious steps involved in ELISA. LFIA is conducted with various components (sample pad, conjugate pad, and absorbent pad). To conduct this assay, sample volume is added to the sample pad. The sample will migrate to the conjugate pad, which has a detection reagent that is conjugated to a specific biological component. The sample will interact with the conjugate, eventually migrating into the membrane. Moreover, like ELISA, the antigens on the EV surface facilitate the detection of EVs. However LFIA is known to have a lack of sensitivity.[157] This is another reason that LFIA must usually be performed with another detection methodology.[156]

1.7.3 Dynamic Light Scattering (DLS)

This method is also referred to as photon correlation spectroscopy. To conduct this method, a monochromatic laser beam passes through a suspension of the sample with EVs. Vesicles in the pathway of the beam of light cause the light to scatter in various directions. By recording the intensity of scattered light as a function of time, the fluctuations can be observed due to Brownian motion of the suspended particles within the sample. To determine particle size, an autocorrelation of the intensity spectra is created to further determine size. The advantages of using DLS include the ability to measure particles from 1 nm to 6 µm. However, the reliability of this method is negatively affected when there is a heterogenous population of particles. For example, when larger particles are present within samples, the detection of smaller particles is inconsistent.[158] Therefore, DLS can provide some information regarding a diameter range of vesicles within a sample, though is unable to determine information about antigens present on EV surfaces.

1.7.4 Nanoparticle Tracking Analysis (NTA)

NTA is a common technique used to determine the size and concentration of EVs within samples.[159] NTA involves the use of dynamic light scattering and Brownian motion to determine the size and concentration of particles. During NTA, a laser beam illuminates the sample cell by which the scattered light travels through the objective lens of a microscope, where the information is eventually analyzed by a charged-couple device (CCD) camera. The Brownian motion of the captured particles is recorded and analyzed by using the Stokes-Einstein equation to determine the diameter of EVs. Through this method, the concentration of particles can also be acquired.

NTA is capable of capturing EVs from 10-2000 nm in size. In addition to having size sensitivity, NTA also has the capability to use fluorescent labelling to detect particles with specific antigen markers.[160] While there are some benefits to using NTA, there are some limitations. Some of the limitations include difficulty determining the correct dilution factor of samples due to an overlaying effect of larger vesicles masking smaller vesicles.[161] Another disadvantage is that while NTA is capable of detecting fluorescence,

its practical use for EV phenotyping is limited, as past studies indicate that fluorescent signal must be very bright to be detected by NTA.[162] Therefore, using NTA for EV phenotyping by antigen markers is treated with caution.

1.7.5 Resistive Pulse Sensing (RPS)

This method is similar to NTA as it can determine the concentration and size of EVs. It consists of a membrane with nanosized pores that separates two fluid cells. A voltage is applied across the nanopore resulting in an ionic current. While particles move through the nanopore, the current is disrupted resulting in pulses proportionate to particle volume. The flow rate is proportional to concentration.[163] RPS can also be used to determine information regarding particle shape and movement profile.[164] While RPS has its benefits, it also has some limitations. Due to the nanostructure, the sample efficiency and detection speed may be small with most of the sample processing being performed in nanoliter to picolitre volumes.[164]

1.7.6 Electron Microscopy (EM)

This methodology utilizes a beam of electrons to create an image of the sample. There are two types of EM: transmission electron microscopy (TEM), and cryo-electron microscopy (cryo-EM). During TEM, an image is created by electron interference when the electron beam passes through the sample, creating a resolution of 1 nm. While TEM provides high resolution, there are disadvantages that make it difficult for EV analysis. For example, specimens examined by TEM must be fixed and dehydrated before assessment and must be processed under vacuum conditions. Moreover, the multi-step preparations required for EM can induce morphological changes to EVs.[165] To avoid some of these issues, cryo-EM can be applied as it omits invasive steps such as dehydration or sample fixation.[161] Additionally, two dimensional and three-dimensional images can be created through using this process.

1.7.7 Atomic Force Microscopy (AFM)

AFM or scanning probe microscopy is a surface analysis technique. This method detects and records interactions between a probing tip and sample surface. The sample surface is

probed by a cantilever (delicate flat spring) with a sharp tip mounted at the other end. AFM produces three-dimensional topography imaging by utilizing the probe over the surface of samples and can determine EV size. Sample analysis can be completed with minimal sample preparation. EVs can be bound to an observational surface using specific antibodies in order to gather information about specific EV concentrations by antigen markers of interest.[166] A disadvantage of using this method is that because EVs have no internal structural support, the vesicles are easily deformed during sample preparation and imaging by the scanning cantilever.[164]

1.7.8 Flow cytometry (FC)

Flow cytometry is the most used method for EV analysis as it can identify EV size and antigen markers of interest. Flow cytometry enables high throughput and fast measurements of EVs. This method uses three systems: fluidics, optics, and electronics. First, samples are aspirated into the machine where samples are suspended within a pressurized buffered saline solution (sheath fluid). This is to align EVs in single file through the flow cell, where they pass by a laser beam. The place where EVs interact with the laser is called the interrogation point. In this area, as each EV passes through the laser beam, light scatters in multiple directions. Light scatter is analyzed by detectors, in a forward-facing direction (forward angle scatter or FSC) which converts the light scattered into a voltage pulse, which is proportional to the amount of light scattered, helping determine EV size. Moreover, another detector is placed perpendicularly to the laser beam and is referred to as the Side Angle Scatter (SSC), and this is proportional to complexity or granularity. Therefore, by analyzing FSC and SSC together, size and complexity can be understood. Flow cytometers are also able to analyze fluorescent light from excited fluorophores such as fluorescently conjugated antibodies, dyes, or stains used within samples. To analyze fluorescence, fluorophores are excited by their corresponding wavelengths from the laser beam. Then dichroic filters are able to focus the emitted fluorescent light to specific sensors and bandpass filters to determine the wavelength of light that is read by each fluorophore used.[167] The sensors are called photomultiplier tubes (PMTs), and convert photon energy into a voltage pulse. These pulses are known as events and can be correlated with fluorescent intensity.

While conventional flow cytometry is widely used, it has some limitations. To detect small particles on the basis of FSC light scatter is challenging, which is why most conventional flow cytometers are not able to detect EVs smaller than ~500 nm.[168] Conventional flow cytometers also have difficulty determining the size of EVs in relation to standards (specific sized beads). To determine EV size, a set of standards, which are specific sized polystyrene beads are used to calibrate the flow cytometer and create specific representative regions of interest (ROI). Therefore, criteria such as the bead's refractive index (RI) is crucial because if RI differs from the RI of cell membrane, the size of EVs is then incorrectly described. For example, polystyrene beads have an RI of 1.59, while cell membrane has a RI of 1.35-1.4.[169] Due to the difference in RI, specific EV size remains uncertain because it cannot be accurately measured in relation to standards. Moreover, if samples have EVs with high concentrations, conventional flow cytometers may identify multiple vesicles as a single vesicle, which is referred to as swarm effect.[170] Swarm effect is a significant problem as it can impact the enumeration of EVs detected and thus linearity of data.

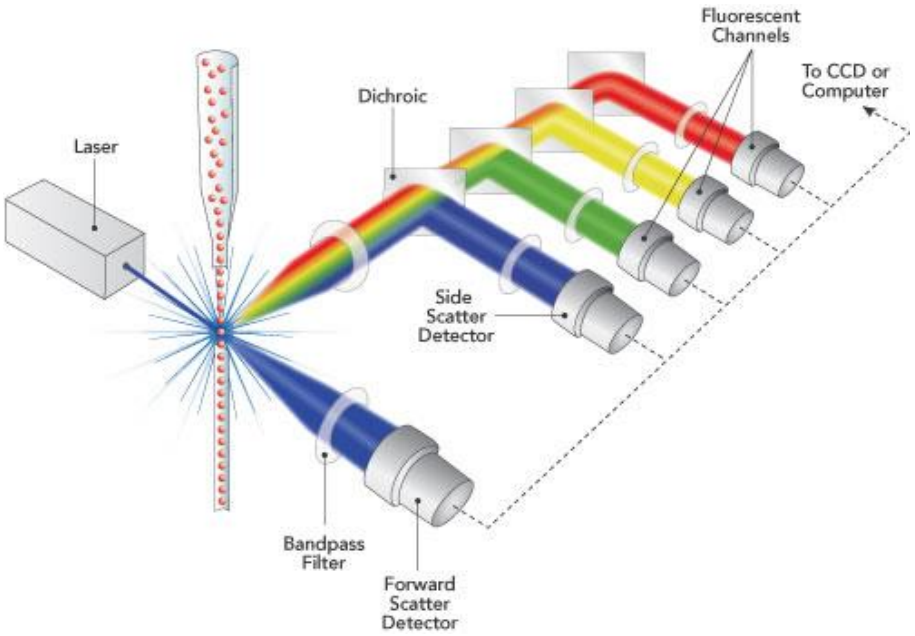

Figure 1.5. Flow cytometry

Flow Cytometry schematic which displays the laser beam, as well as the various detectors (forward scatter, side scatter, and fluorescent channel detectors). This figure was originally published online in *IDEX Health and Science.*

[https://www.semrock.com/flow-cytometry.aspx]. This figure is being reproduced for educational purposes only and not for any commercial use. Figure is included in the Ph.D. dissertation with attribution.

1.7.8.1 Nanoscale Flow cytometry (nFC)

While conventional FC has some limitations pertaining to the acquisition of EV size and sensitivity, FCs such as the Apogee A50-Micro Plus nanoscale flow cytometer (nFC) have been able to overcome these limitations. nFC uses two light scatter detectors instead of a single forward-facing light scatter detector for size, which therefore results in better resolution of particle size as low as 80 nm. The Apogee machine has enhanced optics including large angle light scatter (LALS) and small angle light scatter (SALS) and uses more sensitive photomultiplier tubes than conventional FCs. Therefore, rather than using FSC and SSC detectors like conventional FC, the Apogee nFC uses LALS and SALS detectors to determine size. Additionally, sensitive photomultiplier tubes can multiply the signals with higher quantum efficiency, allowing for better detection of dim light signals. When comparing the Apogee nFC with a conventional FC it was found that the Apogee nFC detected approximately 18-fold more vesicles than conventional flow cytometry, which further suggests that nFC may be a more appropriate method for EV analysis.[168] Moreover in a study comparing 14 different types of FCs, they found that the Apogee A50-Micro Plus had the best resolution.[171] However, standard protocols and pre-analytical guideline for using the Apogee nFC do not exist, and further investigation is needed to understand whether this machine is limited by issues (such as swarm effect) which are common for conventional FCs.

1.8 Motivation, Hypothesis, and Aims

1.8.1 Motivation

Chronic kidney disease (CKD) is a debilitating disease, affecting ~2.6 million Canadians.[172] Of the several stages of CKD, Stage 5 (kidney failure) is the most detrimental stage and requires renal replacement therapy in the form of a kidney transplant or dialysis (hemodialysis or peritoneal dialysis). Hemodialysis (HD) is the

most common form of dialysis. While HD is a life-saving treatment, it has many negative side effects. HD causes episodic hypotension, myocardial ischemia, abnormal perfusion to various organs, and damage to vulnerable vascular beds.[28–30] The disruption of capillary blood flow suggests that vascular injury is being worsened by microvascular endothelial dysfunction.[37]

Currently, detection of vascular injury among HD patients has been assessed through advanced forms of imaging such as echocardiography (echo), computed tomography (CT), and magnetic resonance imaging (MRI).[49,59,60] These imaging modalities have provided visual observations of microcirculatory stress during HD treatments. While these imaging modalities provide informative observations, they are costly ($500-$2000/patient, Canadian Magnetic Imaging), time consuming, and generally require skilled technicians. Therefore, there is a need to identify alternative methods of detecting vascular injury that are more cost-effective and efficient, such as the use of blood-based biomarkers.

Biomarkers of interest are endothelial and platelet EVs, as they have both been suggested to indicate vascular injury.[132–135] Many studies have demonstrated that HD leads to higher circulating endothelial and platelet EV (100-100 nm) levels, indicative of higher hemodynamic stress.[138,143,173–176] There are contradictory studies that state HD has no effect or that HD results in decreased endothelial and platelet EV (100-1000 nm) levels.[177–181] These discrepancies may be the result of EV based methodologies used, as well as study design.

Methodology is an important factor when enumerating EVs. In the last two decades, the methodology used to report EVs in plasma from patients with CKD has mostly been conventional flow cytometry (Figure 1.6).[182] Unfortunately, conventional flow cytometry cannot detect EVs less than ~300 nm –500 nm. EVs less than 300-500 nm represent an important size range as previous studies have demonstrated that the majority of EVs in human blood are below this range.[183,184] Moreover, previous studies which have used more sensitive methodologies such as sensitive flow cytometers have shown that small EVs (~100-500 nm) are indicative of vascular injury, and are even markers of

responsiveness to interventions such as chemotherapy.[185–187] While non-flow cytometry methods such as NTA provide valuable insight regarding the size of EVs, they do not reliably allow for detection of both EV size and characterization by antigen markers, and are unable to distinguish EVs from proteins.[188] Therefore, using sensitive flow cytometry instrumentation, such as the Apogee A50-Micro Plus nanoscale flow cytometer, is valuable because it has the ability to observe EVs by antigen markers and detect size as small as ~80-180 nm.[189] While nFC may be a reasonable method to use to enumerate EVs in the clinical setting, it lacks analysis protocols, standard operating procedures regarding plasma preparation and storage, and investigation of parameters that may impact massive coincidence (swarm effect).[190–193]

Study design is also crucial. It is still unclear what aspects of HD might cause changes to EV levels and how these levels may directly impact patients during their HD sessions. To investigate this, *in vitro* experiments are required to understand the biological utility of EVs. This includes exposing endothelial cells to the uremic milieu and analyzing the impact on EVs. Additionally, pre-clinical *in viv*o animal models are valuable as they provide an opportunity to understand the impact of HD, independent of underlying renal pathology. Even though pre-existing animal models (goats, pigs, sheep, cats, and dogs) of HD exist [194–198], to our knowledge none have investigated the impact of the HD procedure on EV levels within a healthy animal, to eliminate any confounding factors related to renal failure. A recently created rodent model of HD incorporates intravital microscopy which allows for tandem analysis of muscle perfusion through HD, and would further improve the understanding of biological plausibility of EVs as biomarkers of vascular injury.[199]

Lastly, while there are various studies which have investigated the use of EVs as biomarkers among HD patients, the clinical utility of EVs has still not been thoroughly understood. To our knowledge, no other studies have demonstrated how EV levels may be related to important measures such as ultrafiltration rate, intra-HD hypotension, and other measures of cardiac injury such as intradialytic RWMAs and intradialytic myocardial hypo-perfusion.

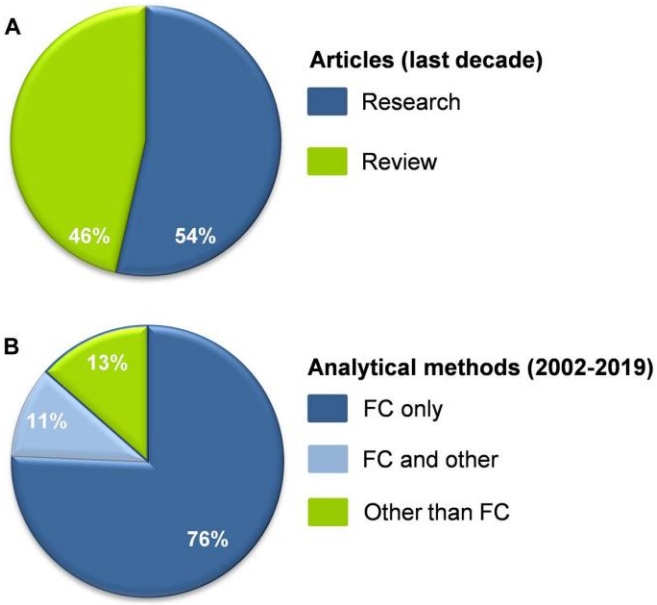

Figure 1.6. Overview of publications and EV methods used (2002-2020).

(A) There is a high ratio of reviews to research articles. This pie chart represents the percentage of research and review articles dealing with plasma derived EVs in CKD published in the last decade (*N* = 56), research (N=37), review (N=26). **(B)** The analytical methods that have been used primarily lack the sensitivity to identify both size and antigen marker of EVs together. Most research articles published on plasma derived EVs in CKD from 2002 until 2020 were based exclusively on conventional flow cytometry (FC) data. Fewer studies combined conventional FC with other methodologies such as immunoblotting, nanoparticle tracking analysis, proteomics, ELISA, and electron microscopy. Note: This figure was originally published in *Frontiers in Cell and Developmental Biology. Georgatzakou, H.T., Pavlou, E.G., Papageorgiou, E.G., Papassideri, I.S., Kriebardis, A.G., and Antonelou, M.H. The Multi-Faced Extracellular Vesicles in the Plasma of Chronic Kidney Disease Patients. 2020; 8, 1-9.* Copyright © 2020 Georgatzakou, Pavlou, Papageorgiou, Papassideri, Kriebardis and Antonelou. This figure is being reproduced for educational purposes only and not for any commercial use. This figure is included in the Ph.D. dissertation with attribution[182].

1.8.2 Hypothesis

I hypothesize that circulating endothelial and platelet EVs (concentration and size) have utility as markers of vascular injury and end organ dysfunction among HD patients.

1.8.3 Aims

Project 1: To establish a standard operating procedure for EV enumeration with nanoscale flow cytometry

- To assess for massive coincidence (swarm effect) and linearity by validating flow rate and dilution effect among standards and plasma samples.
- To determine the effect of plasma centrifugation, plasma storage, and freezing and thawing of samples for EV analysis.

Project 2: To determine the biological plausibility of endothelial and platelet EVs (concentration and size) *in vitro* and *in vivo*

- To assess the impact of the uremic milieu as a factor leading to endothelial EV release and EV size distribution.
- To assess the impact of HD on endothelial and platelet EVs (concentration and size) within a rodent model of HD.

Project 3: To determine the clinical utility of endothelial and platelet EVs as candidate biomarkers among observational HD patient studies

- To determine the impact of HD on endothelial and platelet EV (concentration and size) levels among HD patients.
- To investigate the association between endothelial and platelet EV (concentration and size) levels and HD-induced intradialytic myocardial injury.

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Chapter 2

2 Analytical Considerations in Nanoscale Flow Cytometry of Extracellular Vesicles

A modified version of this chapter has been published.

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2.1 Introduction

Extracellular Vesicles (EVs) that are 100-1000nm in diameter, traditionally referred to as microparticles (MPs) are cell-derived membrane vesicles that are released into bodily fluids following cell activation or apoptosis.[1–3] Their budding from the cell is propagated by cytoskeletal rearrangement and membrane asymmetry in a process called membrane budding.[4] MPs, also known as microvesicles and ectosomes, can be distinguished from other MPs such as exosomes and apoptotic bodies by their size (100–1,000 nm) and retention of surface antigens specific to donor cells.[5,6] Originally, MPs were thought to be 'cell garbage' or 'platelet dust'.[7,8] However, these submicron particles have gained more interest as an important intercellular communication mediator involved in normal and pathological processes such as Alzheimer's disease, cancer, and infectious diseases.[9–11] Like the cells that released them, MPs are composed of nucleic acids, lipids and proteins, which in some cases makes them an abundant source of biomarkers with potential functional impact.

Platelet microparticles (PMPs) are distinct from other MPs because they are a significant fraction of MPs in the blood of healthy individuals.[12] Rises in PMP levels have been associated with various pathological conditions such as atherosclerosis, rheumatoid arthritis, thrombosis, endothelial dysfunction, inflammation, and cancer.[13–16] Therefore, PMP levels could contribute to multivariable analyses for non-invasive liquid biopsies to

guide clinicians in pathological classification, diagnoses, and potential treatments. While MPs (100-1000nm sized EVs) have the potential to be used in the clinical setting, methods of isolation, characterization, and enumeration remain contentious as there is a lack of gold standard procedure(s). A rigorous standardization of an MP based protocol is required to assess the potential of MPs as biomarker in disease.

Currently, microscopy, dynamic light scattering, nanoparticle tracking analysis (NTA), tunable resistive pulse sensing, and flow cytometry (FC) are some physical methods that have been used to characterize MPs by cellular origin, size, population, number, and structure.[17–22] Of these methods, FC is advantageous because it has the ability to analyze individual MPs and detect two or more antigens simultaneously on their surface through the use of fluorophore conjugated antibodies in a high-throughput and multiplexed manner.[22] While conventional FC allows for rapid detection of antigen-specific MPs and size, it has limited sensitivity and can only detect particles as small as 300 nm based on light scattering.[23] This limitation in MP detection may result in the underestimation of MP levels. More recently, few groups have studied MPs by using nanoscale flow cytometry (nFC).[24–27] Although nFC is similar to conventional FC in terms of light scattering and high-throughput quantification, nFC is able to detect MPs as small as ~80 nm with multiplex fluorescent detection.[28,29] While the use of nFC is emerging as a suitable tool to enumerate MPs in a clinical setting, it still lacks analysis protocols that are supported by reproducibility and cross-validation studies. Furthermore, there is a need for standardization in terms of sizing parameters with the use of size-calibrated fluorescent beads, intra-instrument reproducibility, detection of massive coincidence (swarm effect) and whole blood and plasma preparation and storage.[21,23,30,31] While these are common areas of regulation among users of conventional FC, this level of standardization is also needed for FC instruments that analyze within the nanoscale realm (100–1000 nm). Here, we tested different conditions of plasma preparation and storage to optimize analysis of PMPs. Additionally, we developed a detailed methodology to accurately quantify sub-populations of PMPs from patient plasmas for future use in a clinical setting.

2.2 Methods

2.2.1 Nanoscale Flow Cytometry Settings

All samples were analyzed using the A50-Micro Plus Nanoscale Flow Cytometer (Apogee Flow Systems Inc., England) equipped with 70 mW 405 nm (violet), 53 mW 488 nm (blue) and 73 mW 639 nm (red) lasers. Parameters in the control panel were set to sheath pressure of 150 mbar and number of flush cycles to 3. Sample flow rate of 1.5 µL/min (or as indicated) was used for all measurements (or as indicated) and the time of acquisition was held constant for all samples at 60 seconds to yield enough events. An illumination wavelength of 405 nm (70 mW) was used to detect scattered light by MPs. Before sample analysis, calibration of flow cytometer was performed using a reference bead mix (Apogee Mix, Apogee Flow Systems Inc.) composed of a mixture of silica nanoparticles with diameters of 180, 240, 300, 590, 880 and 1,300 nm with a refractive index (RI) of 1.42; and 110 and 500 nm green fluorescent (excited by blue laser) polystyrene nanoparticles with an RI of 1.59 (latex) were used. These beads were used to assess the FC's light scattering detection of MPs and fluorescence detection resolution. Light scatter triggering thresholds were determined with the smallest particles distinguishable from noise (110 nm polystyrene beads). Thresholds were set at 20 a.u. (small angle light scatter [SALS]) and 25 a.u. (large angle light scatter [LALS]) to eliminate optical and electronic background noise without losing particles of interest. Photomultiplier tube (PMT) voltages were set as follows: LALS (320 V), SALS (300 V) and L488-Grn (425 V). All measurements were performed in log mode. Noise levels in PMT panel were kept below 0.6. Fluorescent latex nanoparticles (Tetraspek nanoparticles, Thermo Fisher Scientific) of 200 and 1,000 nm sizes were used in experiments to validate the effect of mass coincidence and verify accurate enumeration. Massive coincidence ('swarm effect') was tested by analyzing various dilutions of plasma samples that were run at a slow (1.5 µL/min), moderate (6 µL/min) and fast (10.5 µL/min) rates. All dilutions were also enumerated by confocal microscopy (Nikon Fast A1R ,60x objective) and counted using the Fiji software. nFC performance was also compared with a conventional flow cytometer BD FACS Canto (BD Biosciences Inc.) by using 880 nm silica beads and 1,000 nm latex beads.

2.2.2 Nanoscale Flow Cytometry Fluorescence Sensitivity

Fluorescence calibration was performed using commercial spectral matching fluorescein isothiocyanate (FITC) fluorescent particle beads (Cat No. ECFP-F1–5K, lot AJ01, Spherotech Inc.) in which the fluorescence intensity had been calibrated in units of mean equivalent soluble fluorochromes (MESFs). Standard curve was created to assign MESF values to the fluorescent bead mix under the same instrument settings used for experiments. A 488-nm (50mW) laser with a 530/40 filter was used to detect FITC fluorescence. Flow rate was set at 1.5 µL/min and PMT set at 475 V.

2.2.3 Plasma Samples

Blood was collected from three healthy donors (25, 32 and 45 years of age) using a 21-gauge needle. The first 3 ml of blood was discarded and approximately 8 ml was transferred within sodium citrate Vacutainers (BD Biosciences Inc.). Platelet-free plasma (PFP) was isolated within 1-hour post-collection by double centrifugation at 2,500 x g for 15 minutes (room temperature (RT), no brake) according to the International Society on Thrombosis and Haemostasis (ISTH) guidelines.[32] Plasmas were aliquoted and stored at -80°C.

2.2.4 Variation of Handling and Processing of Whole Blood

Various storage experiments were conducted to analyze MP integrity with varying temperature, length of storage, and thawing conditions. Platelet Rich Plasma (PRP) was generated through centrifugation of whole blood at 200×g for 15 min. To show the impact of freeze/thaw of platelet rich plasma, PRP sample aliquots were stored at -20°C and -80°C and were analyzed following seven Freeze/Thaw (on ice) cycles.

PFP was stored at RT, 4°C, –20°C and –80°C and was analyzed in terms of total MPs and PMPs every week for 4 weeks. Additionally, PFP aliquots of –80°C plasma samples were analyzed following six freeze/thaw cycles with thawing at different temperatures (ice, 4°C, RT and 37°C).

2.2.5 Immunostaining of Platelet EVs

Titration of all detecting antibodies was performed with pre- conjugated clones and dilutions were determined from the original concentration (µg/ml) as provided by the manufacturer. Dilutions of Tetraspek nanoparticles/beads (5,000x, 10,000x, 20,000x, 40,000x, and 80,000x) were performed using 0.20 µm filtered phosphate-buffered saline (PBS). Dilutions of pre-conjugated antibodies (0.0125, 0.0250, 0.050, 0.100 µg/reaction) were also performed using 0.20 µm filtered PBS. To enumerate PMPs in each of the experiments, 10 µL of plasma was taken and incubated with 25 ng of CD41a-FITC (HIP8 clone, BD Biosciences Inc.) for 30 minutes at RT in the dark. Samples were also incubated with isotype-matched antibody IgG1k-FITC (MOPC-31C clone, BD Biosciences Inc.) to determine the nonspecific binding and autofluorescence within each sample. The sample was then further diluted with PBS and run using the nFC. All experiments were completed in triplicate. In experiments where Triton X-100 was added to patient plasma, 10 µL of plasma stained with CD41a-FITC antibody was incubated with 0.5% of Triton X-100 for 30 seconds prior to analysis.

2.2.6 Confocal Microscopy of Calibration Beads

A Fast A1R confocal microscope (Nikon Fast A1R+, 60X objective) equipped with 405/491/565/ 643 nm solid state lasers was used to perform imaging of calibration beads. In summary, 0.5 µL of each diluted calibration bead mixture was placed onto a slide and coverslip. A 60-x oil immersion objective lens was used to visualize the beads and the entire coverslip was imaged by using the NIS Elements software.

2.2.7 Electron Microscopy of Calibration Beads

Scanning electron micrographs were acquired for a 1 in 80,000 dilution (v/v) of 1 µm diameter latex beads (Thermo Fisher Scientific). Images were obtained using a LEO Zeiss 1530 (Zeiss, Oberkochen, Germany). 20 µL of the sample was pipetted onto a silicon wafer and allowed to dry by heating to 45^0C for 10 minutes. The sample was subsequently coated with a 5 nm thick layer of osmium using an osmium plasma coater (Filgen OPC80T, Nagoya, Japan) to reduce sample charging.

2.2.8 Statistical Analyses

Parameters recorded were as follows: total MPs within sample, CD41a + ve MP events/μL. The number of MP events/μL in the isotype sample was subtracted from the MP events/μL in the antibody sample. GraphPad Prism 7.0 was used to run statistical analysis. Ordinary one-way ANOVA was used for analyses with multiple comparison, with ad-hoc Bonferroni correction. Two-way ANOVA was used to evaluate statistical significance across the groups. The confidence interval was set at 95% and a p-value of <0.05 was considered significant.

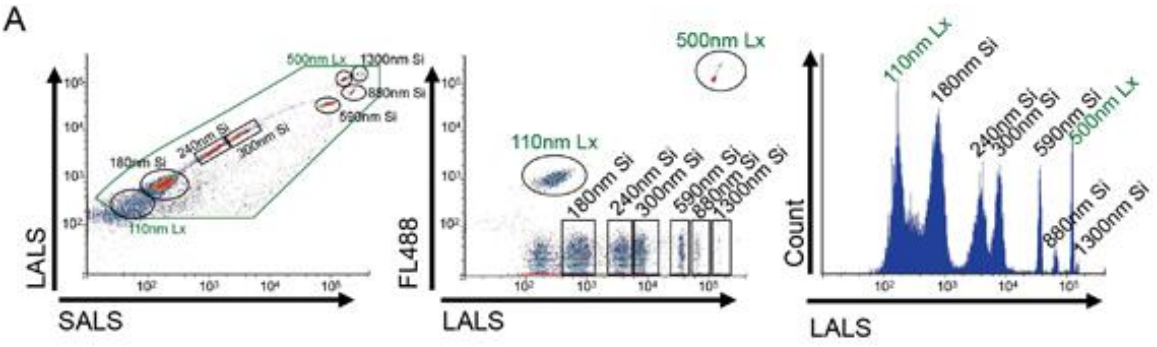
2.3 Results

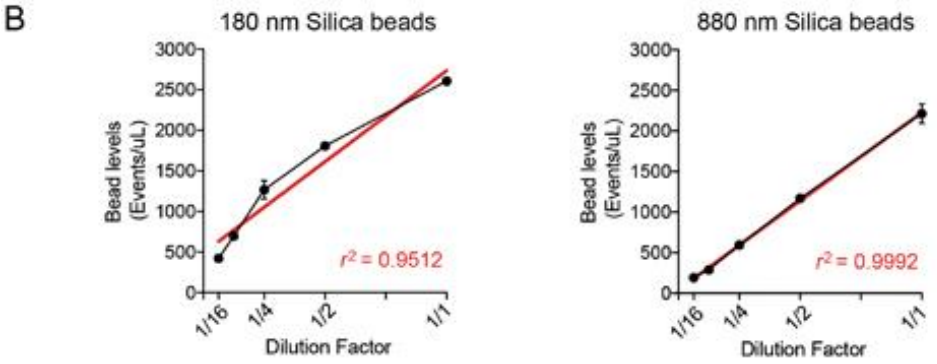
2.3.1 Linear Light Scatter Detection of Nanoscale Events Regardless of Dilution Factor

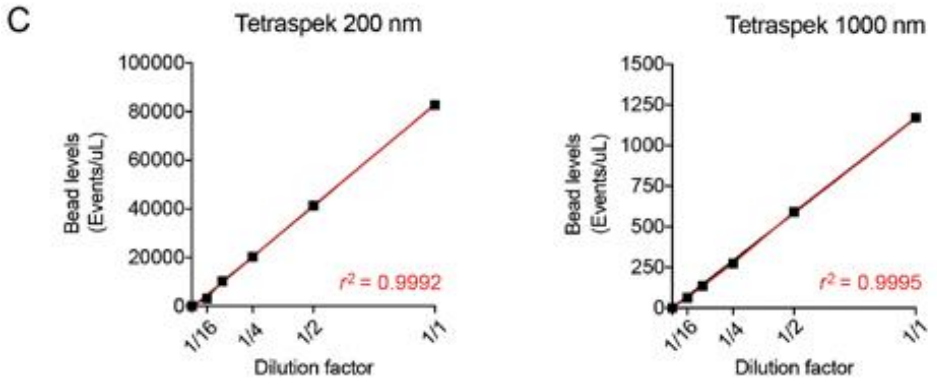
The A50-Micro Plus nFC relies on any three parameter-based triggers to analyze EVs: SALS, LALS and/or the fluorescence channel(s) of interest. Sensitivity of the A50-Micro Plus for sub-microparticles is based on the incident wavelength used to detect scattered light. The A50-Micro Plus uses a 405-nm laser instead of a 488-nm laser present on conventional flow cytometers.[33,34] A mix of fluorescent and non- fluorescent calibration beads was used to create various gates corresponding to different calibration size beads, and when presented on the LALS versus SALS plot provides a sizing gradient for each bead (Figure 2.1 A). The fluorescent 110 and 500 nm beads are visibly distinct sub-populations (FL488, Figure 2.1A, middle plot) indicating that there is negligible 'swarm effect', that is, other FL488 events that are not 110 or 500 nm in diameter. The triggering threshold for LALS and SALS was optimized to maximize sensitivity of all true EVs while minimizing dark current noise (LALS 25 a.u, SALS 20 a. u). Calibration beads from known concentrations were enumerated and for every single size the particle count did not differ significantly with a coefficient of variation below 10% (Table 2.1). Serial dilutions of 180 nm (RI, 1.42) did not produce a linear correlation between dilution factor and event levels beyond a dilution factor of 1/4 (Figure 2.1 B, left panel). Note that 880 nm silica beads produced a linear correlation between dilution factor and event levels (Figure 2.1 B, right panel). Hence, analytical performance of the A50-Micro Plus is

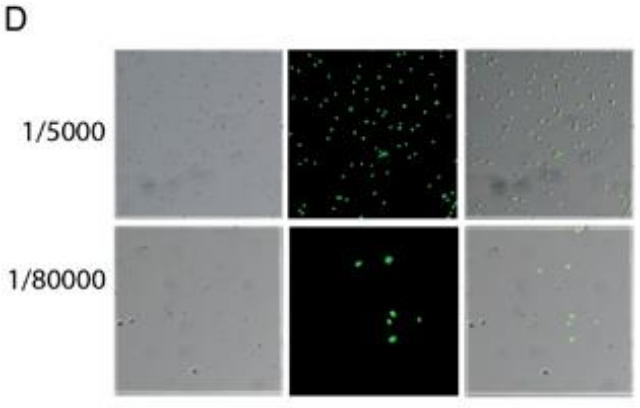
affected by smaller particles ($r^2 = 0.9512$) that are close to the lower detection limit of the instrument. In contrast, light scatter detection of 880 nm silica beads was not achievable with a conventional flow cytometer (BD FACS Canto) (Figure 2.2).

Latex beads are also used for calibration and to assess light scatter performance of flow cytometers albeit having a higher RI (1.59) than silica beads and biological vesicles. Serial dilutions of latex bead (200 and 1000 nm) concentrations were found to generate linear correlations between dilution and event rates (Figure 2.1 C). Additionally, the count of beads that was determined with nFC was confirmed via confocal microscopy of the various diluted bead preparations (Figure 2.1 D, E). Additional SEM image of 1um latex bead is also included for reference (Figure 2.3).


A
LALS
SALS
500nm Lx
1300nm Si
880nm Si
590nm Si
240nm Si
300nm Si
180nm Si
110nm Lx
FL488
LALS
Count
LALS


B
180 nm Silica beads
880 nm Silica beads
Bead levels (Events/uL)
Dilution Factor
r² = 0.9512
r² = 0.9992
1/16
1/4
1/2
1/1


C
Tetraspek 200 nm
Tetraspek 1000 nm
Bead levels (Events/uL)
Dilution factor
r² = 0.9992
r² = 0.9995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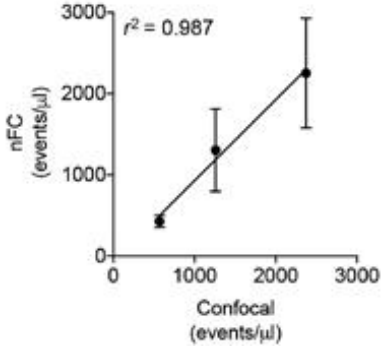
E
r² = 0.987
nFC (events/µl)
Confocal (events/µl)

Figure 2.1 Linearized detection of microparticles by using calibration sized beads and nanoscale flow cytometry and confocal microscopy

(A) Calibration of the flow cytometer with a bead mix of fluorescent latex and non-fluorescent silica beads. Cytograms and histograms show bead populations detected with light-scatter and fluorescence. The green box indicates the sort of gate for microparticle-related events based on light-scatter detection of calibration beads. **(B)** Enumeration of 110 and 880 nm silica beads at various serial dilutions. **(C)** Enumeration of 0.2 and 1 μm Tetraspek bead dilutions. Linear regression was performed on serial dilution graphs representing technical triplicates. **(D)** Representative confocal microscopy images of the fluorescent 1 μm latex beads deposited onto a hemocytometer. Panel are images presented at higher power. **(E)** Correlation curve of bead counts with nanoscale flow cytometer and confocal microscope. Each experiment represents three technical replicates.

Table 2.1 Accurate Enumeration of Calibration Beads with Light Scatter Threshold

Size	Count (EV/μL)	Fluorescent	Counted	%CV
110	8,000	Green	8,386	4.80
180	23,000	None	21,749	5.40
240	10,000	None	10,732	7.30
300	9,000	None	9,304	3.30
500	3,600	Green	3,794	5.30
590	2,700	None	2,767	2.40
880	3,900	None	4,214	8
1,300	3,400	None	3,122	9.80

Abbreviations: CV, coefficient of variation; EV, extracellular vesicle.

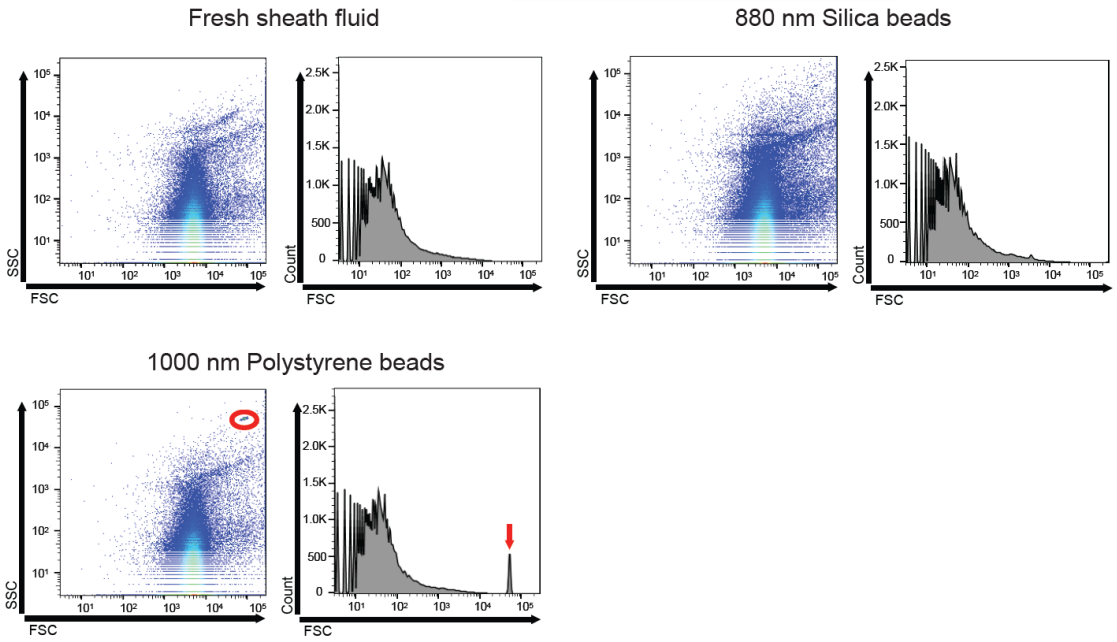

Figure 2.2 Detection of calibration beads by conventional flow cytometry

Dot plots and histograms showing analysis of 880 nm Silica beads and 1000nm polystyrene bead with a conventional flow cytometer (BD FACS Canto).

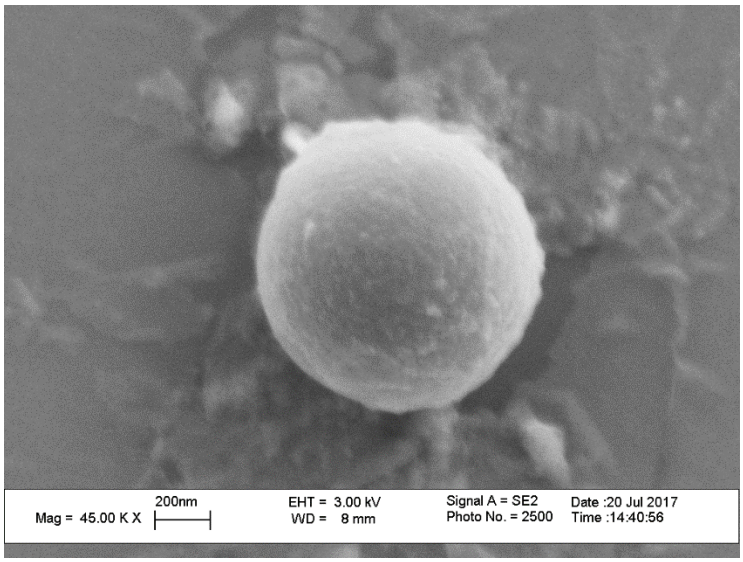

Figure 2.3 SEM micrograph of 1 µm latex beads

2.3.2 Impact of Flow Rate on EV Enumeration by nFC

Flow rate in flow cytometry is a critical parameter for linear detection and concentration measurements of MPs.[36] An excessively high flow rate may result in swarm effect when multiple small vesicles below the detection limit appear as a single event during acquisition. A lower flow rate provides suitable separation between events during acquisition, thereby increasing reproducibility of measurements.[36] The Apogee A50-Micro Plus allows for acquisition at flow rates as low as 0.75 μL/min because it is equipped with a Hamilton syringe-driven delivery system to control flow rate. Serial dilutions of 180 nm silica beads were analyzed at three different flow rates (1.5, 6.01 and 10.5 μL/min) (Figure 2.4 A) with no differences observed in event rates compared with theoretical bead concentrations. However, analysis of patient plasma samples did not consistently generate linear correlations when dilution was varied (Figure 2.4 B). Two patient samples (#1 and #3) revealed discrepancy in particle number at lower dilutions, likely due to the larger proportion of events < 180 nm (5x and 2x more events in the 180-nm gate in plasmas #1 and #3) in those two plasma samples (Figure 2.4). These patient samples also exhibited a higher concentration of total MPs compared with sample #2. At higher flow rates, particle number exceeds nFC acquisition limit resulting in an underestimation of MP concentrations. At 10.5 μL/min, the number of events per second recorded was 38,640 (plasma #1), 11,585 (plasma #2) and 16,218 (plasma #3) at dilution of 1/20. The Apogee A50-Micro Plus is only reliable for light scatter-based enumeration of MPs when the event rate does not exceed approximately 12,000 events per second. This suggests that in addition to dilution factor, flow rate is also critical for linear and accurate enumeration of MPs. Altogether serial twofold dilutions of plasmas from three healthy donors at appropriate dilution and flow rate reveal linear relationships and demonstrates that ‘true’ events are recorded, and enumeration is predominantly caused by single vesicles and not by massive coincidence when using light scatter detection (Figure 2.5).

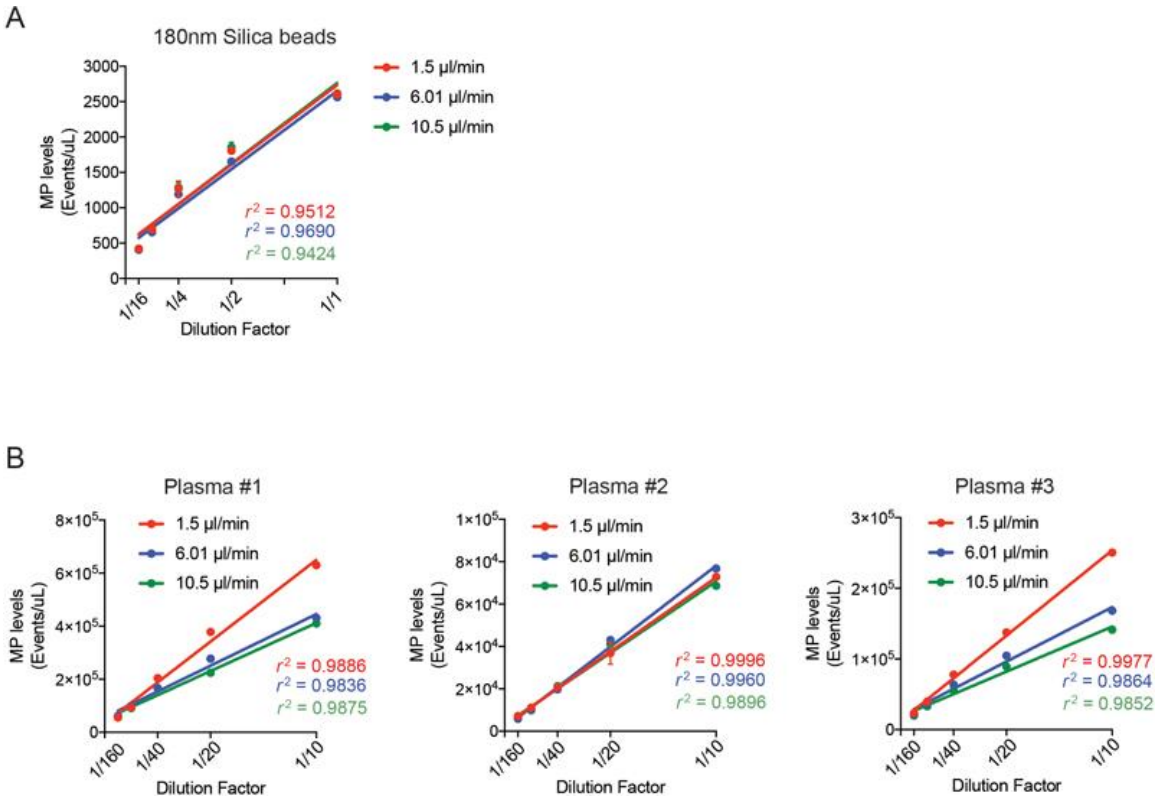

Figure 2.4 Impact of flow rate on extracellular vesicle (EV) enumeration by nanoscale flow cytometry

Serial dilutions of 180 nm silica beads **(A)** and three human plasmas **(B)** were analyzed at different flow rates: low (1.5 µL/min), medium (6.0 µL/min) and high (10.5 µL/min).

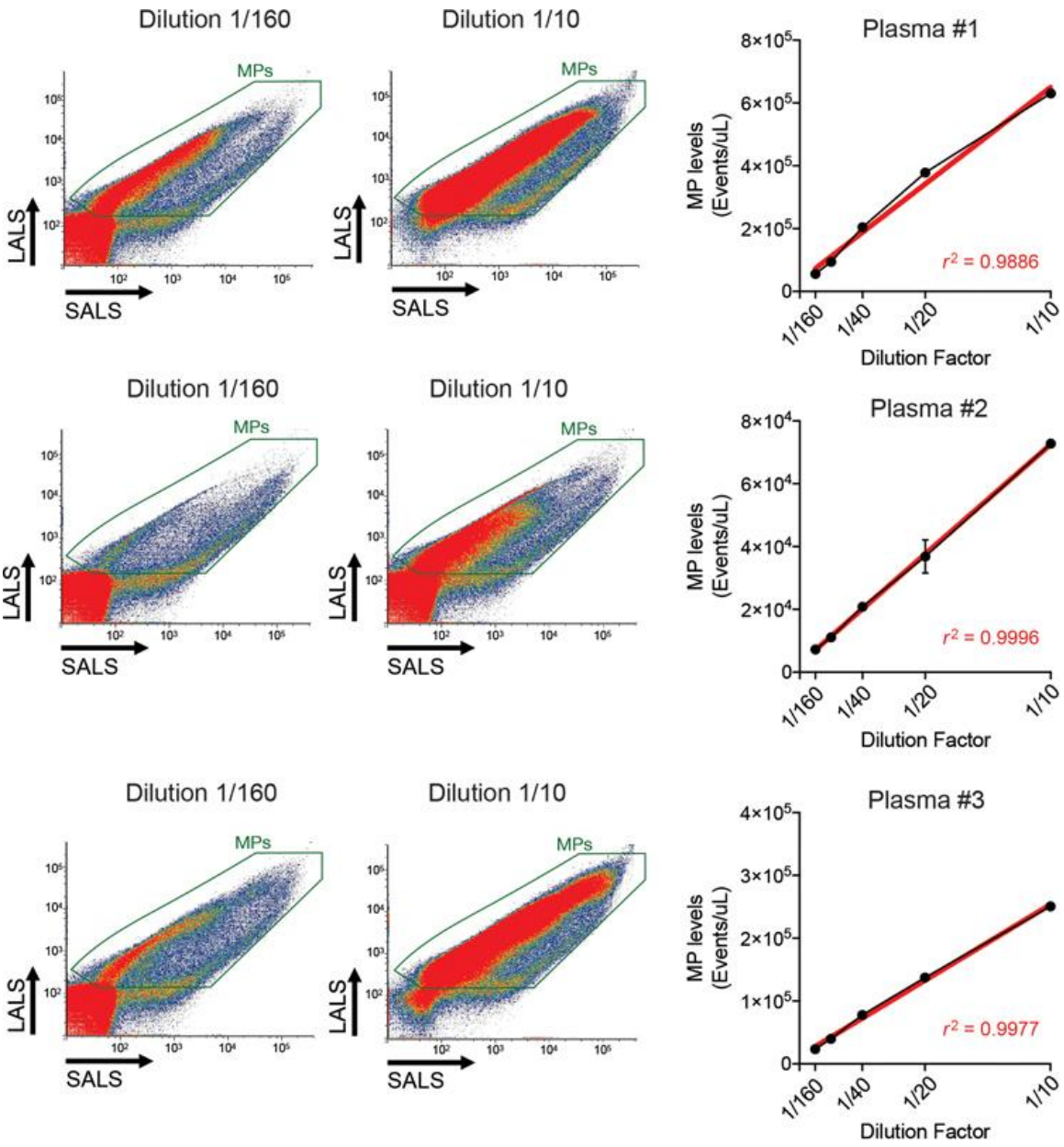

Figure 2.5 Linearized detection of microparticles from healthy donor plasmas by nanoscale flow cytometry (nFC)

Cytograms show light scatter microparticle (MP) detection (large angle light scatter/small angle light scatter [LALS/SALS]) in plasmas of three healthy donors at two different dilutions. Graphs represent enumeration of MPs in plasma serial dilutions. Trend lines for each enumeration were recorded to confirm linearity of dilutions.

2.3.3 Detection of Platelet EVs by nFC from human plasma

Accurate and reproducible enumeration of MP or EV populations of interest requires detectors with high sensitivity on both light scatter and fluorescence detection. Most conventional flow cytometers used for MP analysis prioritize fluorescence sensitivity over light scatter detection performance suggesting that fluorescence-based triggering is a more accepted alternative to detect fluorescent-labelled MP sub-sets.[37,38] A recent study indicated that fluorescence resolution limit of the A50-Micro Plus is 304 MESF-FITC which is similar to high-sensitive flow cytometers.[34,38] We assessed the A50-Micro Plus performance to enumerate CD41a-positive PMPs using light scatter and fluorescence triggering. Fluorescence calibration of the A50-Micro Plus was performed by using Sphero FITC polystyrene beads of known intensities (Figure 2.6 A, B). The smallest particles distinguishable over the noise (110 nm polystyrene beads) revealed a value of 12,500 MESF-FITC. Mean fluorescence intensities produced by CD41a-positive PMPs in human PFP do not change regardless of dilution which confirms the lack of massive coincidence during PMP enumeration by nFC (Table 2.2). PMPs were quantified from human PFP by using immunolabelling with an antibody against CD41a.[20] Antibody titration curves were generated to identify the appropriate antibody concentration for immunostaining (Figure 2.6 C, D). This population was abolished when Triton X-100 was added to permeabilize PMPs, resulting in more events with lower LALS and SALS being abundant. This observation is consistent with previous reports of Triton X-100 inducing lysis of microvesicles leading to smaller events being generated.[39] Interestingly, the number of CD41a-positive MPs (PMPs) when detecting with a light scatter trigger or a fluorescence trigger were similar in three different healthy volunteer PFP samples (Figure 2.6 E).

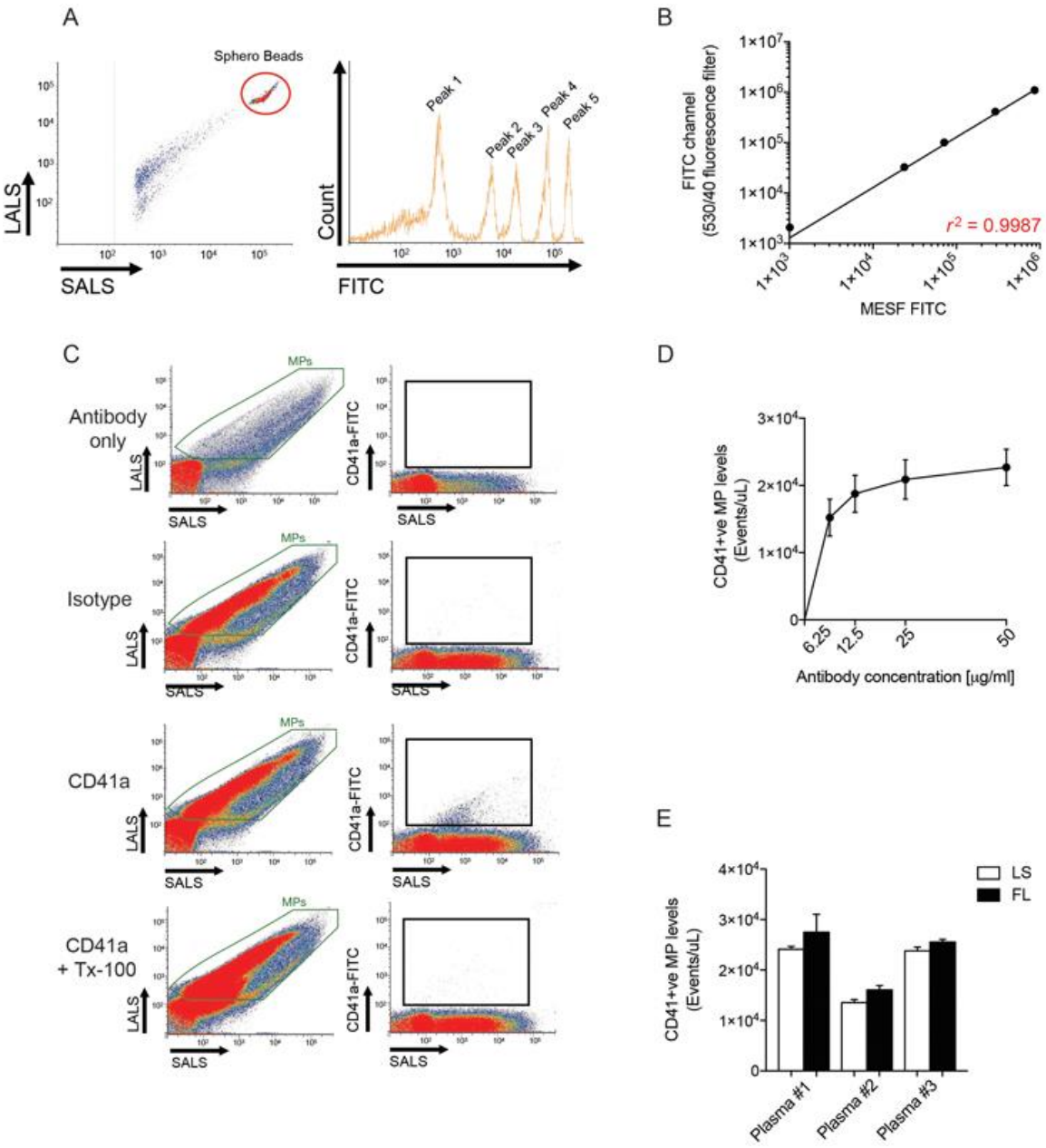

Figure 2.6 Detection of platelet microparticles (PMPs) by nanoscale flow cytometry (nFC)

(A) Representative cytograms showing Spherotech beads used for mean equivalent soluble fluorochrome (MESF) calibration. Five peaks of different MESF intensities were accordingly detected by nFC. **(B)** Linear regression of MESF versus green channel indicating intensities of every peak detected by the fluorescein isothiocyanate (FITC) detector. **(C)** CD41a-positive platelet microparticles in human plasmas. Note that 0.025 μg of CD41a-FITC antibody or isotype-matched control were used. Plasma

immunostained with anti-CD41a-FITC antibody and then treated with Triton X-100 (final concentration of 0.5%) (bottom panel). **(D)** Antibody titration curve was performed with human platelet-free plasma. **(E)** CD41a-positive PMPs levels in three human plasmas analyzed with light scatter triggering threshold or fluorescence threshold. Bars represent mean ± standard error of the mean (SEM) for n = 3 independent experiments.

Table 2.2 Mean Fluorescence Intensities of CD41a +ve EVs does not change with plasma dilution

	Plasma #1	Plasma #2	Plasma #3
Dilution 1/10	2,056.1	2,361	2,603
Dilution 1/20	1,937.1	2,497	2,578
Dilution 1/40	2,045.9	2,520	2,575
Dilution 1/80	2,101.8	2,579	2,659
Mean ± SD	2,583 ± 89	2,489 ± 92	2,603 ± 39

Abbreviations: MESF, mean equivalent soluble fluorochrome; SD, standard deviation.
Note: Values are expressed in MESF units.

2.3.4 The Impact of Sample Storage and Freeze/Thaw of Samples

To assess how MP integrity is affected by thawing of plasma, we enumerated total MPs, and PMPs from PRP following multiple freeze/thaw cycles (Figure 2.7). As more freeze thaw cycles are performed, total MP levels and CD41a + ve MPs increase (Figure 2.7 A, B). This observation was more pronounced when -80°C was used to freeze samples (Figure 2.7 A, B). However, when PPP was submitted to the same experimental protocol, PMP levels did not change after repeated freeze thaws, regardless of thawing conditions (37°C, RT, 4°C, on ice) (Figure 2.8).

PFP stored at different temperatures (RT, 4°C, –20°C, –80°C) for up to 4 weeks was analyzed to determine total MP counts and CD41a +ve MPs levels. Storage at RT induced a significant decrease of total MPs after a 1-week period (Figure 2.9 A), whereas other storage conditions did not affect total MP count (Figure 2.9 B–D). Storage at–80°C led to the lowest amount of PMP loss regardless of storage timeframe (Figure 2.9 D). Storage at RT or 4°C led to a complete loss of PMPs after 1 week and a partial loss of approximately 40% of PMPs was observed at–20°C after 4 weeks (Figure 2.9 A–C).

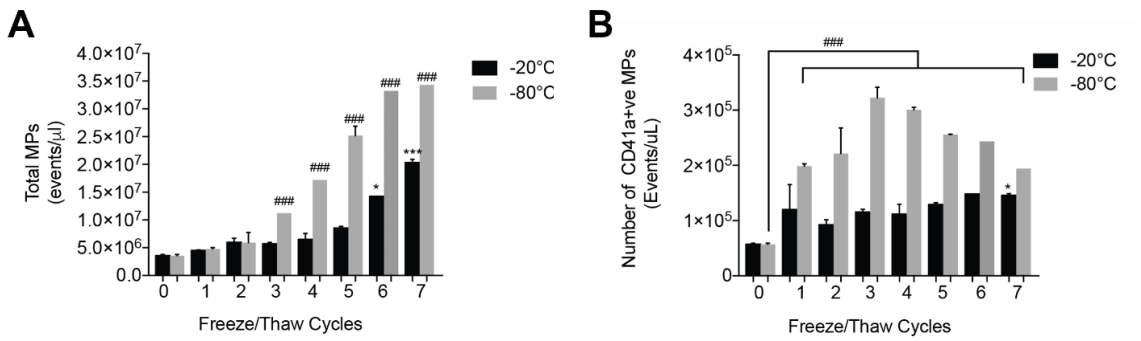

Figure 2.7 Impact of freeze thaw cycles of human platelet rich plasmas on MP integrity and enumeration

(A) Enumeration of total MPs, **(B)** CD41a+ve MPs in a healthy control platelet-rich plasma sample that frozen at -20°C (black bars) and -80°C (grey bars) and subsequently thawed on ice. Bars represent mean +/- SEM for n=3 independent experiments. Two-way ANOVA test, *p<0.05, ***/###p<0.001.

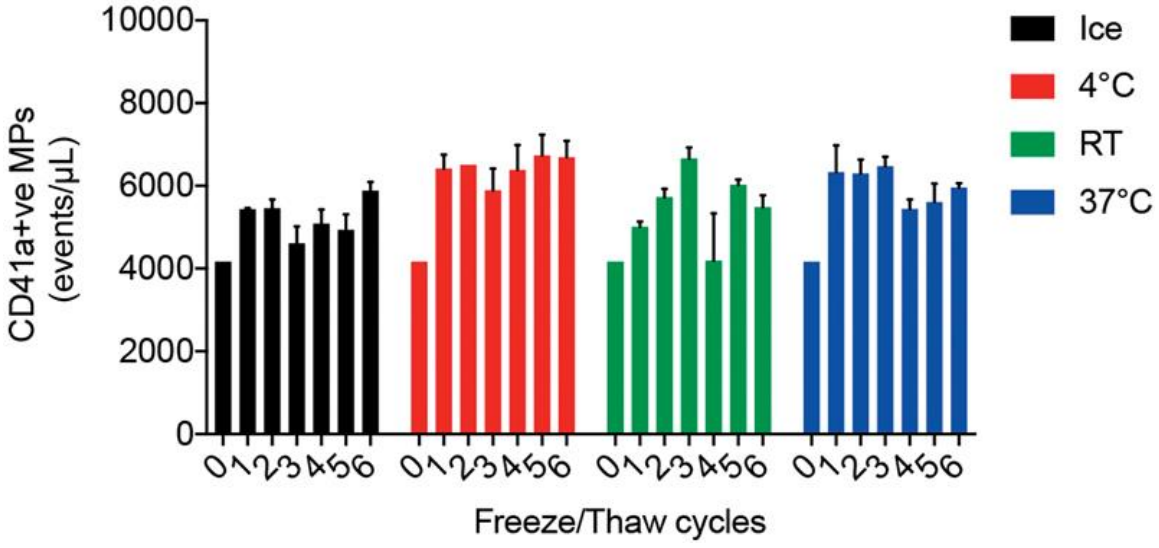

Figure 2.8 Impact of freeze thaw cycles of human plasmas on microparticle (MP) integrity and enumeration

Enumeration of CD41a + ve MPs of a healthy control platelet-free plasma (PFP) sample that was frozen at –80°C and differentially thawed gently on ice (black), at 4°C (red), at room temperature (RT) (green) and at 37°C. Bars represent mean ± standard error of the mean (SEM) for n = 3 independent experiments.

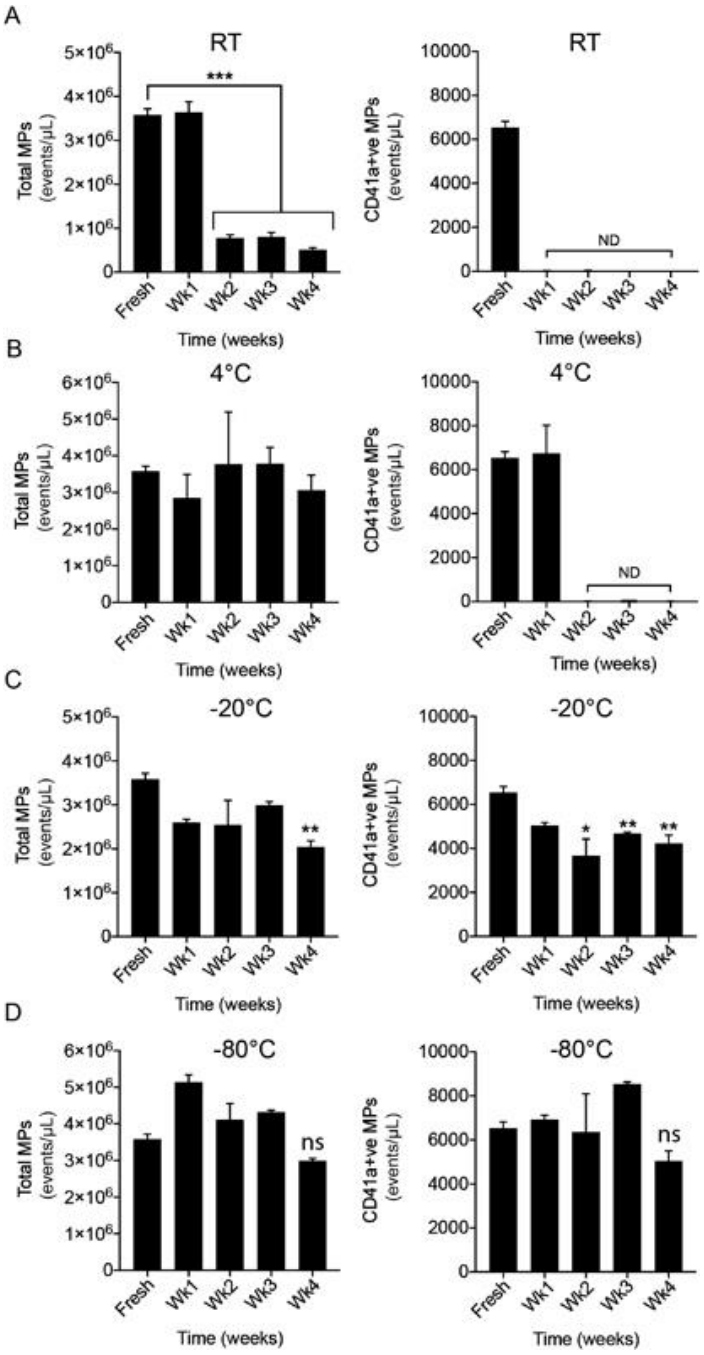

Figure 2.9 Effect of storage of plasma samples over 4 weeks at various temperatures

(A) Enumeration of total microparticles (MPs) and CD41a + ve MPs from plasma stored for 4 weeks at room temperature (RT), **(B)** at 4°C, **(C)** at –20°C and **(D)** at –80°C for 4 weeks. Bars represent mean ± standard error of the mean (SEM) for n = 3 independent experiments. One-way ANOVA test, *p < 0.05, **p < 0.01 and ***p < 0.001; ns = not significant, nd = non-detectable.

2.4 Discussion

nFC is a powerful means of performing multi-parametric analysis of EVs, carrying the same concepts of ‘fluorescence-activated cell sorting (FACS)’ for cell immunophenotyping over to EVs. This has enabled investigators to use many cytometry compatible antibodies for use in this emerging area of EV analysis. Our main findings in this work are that the conditions for MP/EV analysis by nFC are highly dependent on flow rates and the dilution factors used when processing and preparing plasma samples for analysis. What was not previously appreciated was the fact that plasma contains a much higher concentration of EVs than previously thought and is at a level that could exceed analysis rates if not accounted for. For our study, we focused on PMPs because of their relative abundance and variation in certain disease phenotypes such as sepsis,[40] thrombosis[41] and cancer.[42] The scientific committees for the ISTH have instituted several recommendations for standardization of PMPs enumeration by FC and inter-laboratory reproducibility.[31,43] Overall, by using nanoscale or high-resolution FC instrumentation for EV research, we have confirmed findings from previous reports and offer additional recommendations for plasma processing and handling, as well as MP enumeration that would make MP analysis feasible in a clinical laboratory environment.

Blood collection and sample preparation are critical to maintaining MP integrity and concentration. Following blood draw, samples must be consistently processed in a short time and should undergo two centrifugations at 2,500 × g for 15 minutes to obtain clinical quality PFP. In addition, cryopreservation has an effect on MP levels and integrity. In all situations, –80°C appears to be ideal for maintaining the original EV profile of the plasma sample. Repeated freeze/thaw cycles did not appear deleterious, although minimizing freeze/thaw cycles is still recommended. Interest for plasma analysis in MP/EV research is growing since large institutional biobanks store patient plasmas in anticipation for their use in retrospective biomarker-based studies. Unfortunately, blood collection and plasma isolation are not consistently controlled in such clinical studies. While prospective clinical studies are required to include the latest recommendations in terms of sample handling and processing, retrospective studies must also provide these details if similar studies are to be completed and results to be compared.

The opportunities afforded by nFC are numerous; only 10 to 20 µL of plasma sample is required for analysis which is not processed and is subsequently diluted 10 to 60× fold for final analysis. Moreover, 20 to 50 ng of pre-conjugated antibody is needed for analysis at this scale, whereas approximately 1 µg is often used to immunostain cells prior to 'FACS' analysis. Moreover, the antibody titration experiments show that there is no need to wash EVs even if feasible for the investigator because there is a lack of non-specific signal/events when antibody alone is analyzed. This is an important benefit and avoids the need for time-consuming ultra-centrifugation steps. Exploiting the full capacity offered by nFC does require many technical considerations to ensure accurate enumeration of MPs beyond optimal sample preparation and handling. For instance, antibody titration experiments along with isotypes are pre-requisite to determining the optimal antibody concentration to be used. On that note, antibody working concentration may differ depending on sample type (culture media, plasma, urine, and so on) where MP/EV concentrations are not identical in body fluids. Sample dilution must be tested to determine the full range of dilutions responding to linearity and to avoid saturation of acquisition capacity of nFC as well as too dilute of a concentration for each sample. Use of isotype-matched controls is also required for gating and control of background to minimize inter-experimental batch-to-batch variability. Differences in non-specific binding present between plasma samples justify the inclusion of isotype controls. Interestingly, Andersen et al raise some concerns regarding the use of isotype-matched controls and recommend incubation of plasma samples with a blocking reagent prior to FC analysis.[44] Therefore, it would be relevant to compare blocking reagent versus isotype control in terms of background levels for EV analysis by nFC.

EVs have demonstrated great potential as translational biomarkers in several disease conditions, but biomarker validation for future clinical use requires large number of samples to be analyzed. The ideal technology for EV-based biomarker validation will allow for accurate particle enumeration, multi-parameter phenotyping in a high-throughput manner. Even though no such technology is commercially available, FC is the most suitable and fulfils these criteria. Advanced optical methods including NTA, electron microscopy, atomic force microscopy and high-resolution microscopy have revolutionized the EV research field offering an unprecedented closer look to EV shape,

composition, and concentration. However, these technologies are very expensive and time-consuming, hindering their use in the clinical setting. FC is a well-established methodology but lack of sensitivity of conventional flow cytometers for sub-micron particle detection is an on-going limitation in EV research in terms of absolute quantification of EVs and reproducibility. Interestingly, Arraud et al[38] presented a strategy to refine EV enumeration by using FC and fluorescence triggering wherein EV levels are generally higher as determined by fluorescence-based detection compared with light scatter-based detection. We observed that PMP levels from human plasmas were similar when using both light scatter or fluorescence triggering. These results align with a recent study showing superiority of light scatter detection of EVs over fluorescence triggering.[34] Our values are also consistent with those from Brisson and colleagues[38] when we enumerated 20,484 CD41a + ve PMPs per microliter (± 5,250) compared with 21,986 CD41a + ve PMPs per microliter (± 5,977). This cross-validation result confirms accurate and reliable quantification of CD41a + ve PMP sub-populations by nFC and light scatter detection only.

Superiority of nanoscale flow cytometers over conventional flow cytometers resides within significant improvements made for optical parameters and fluidic systems. For instance, unlike the majority of conventional flow cytometers using a high-powered blue laser (488 nm) to illuminate sub-micron particles, the A50-Micro Plus utilizes a 405-nm laser and a slow flow rate resulting in better sensitivity for particles below 200 nm.[36] To our knowledge, only Beckman Coulter also offers a flow cytometer (Cytoflex S) that relies on light scatter from EVs with a violet laser (405 nm). Calibration of FC instruments and standardization are required to attain inter-laboratory reproducibility and the validation of EVs as clinical biomarkers. In our study, the Apogee A50-Micro was calibrated for light-scatter resolution with silica-based calibration beads, whereas numerous studies erroneously encourage the use of latex/polystyrene beads. The latter exhibit high refractive indices that do not reflect those of EVs leading to underestimation of EV concentrations and their sizes. In contrast, use of silica beads appears more relevant, exhibiting a RI closer to EVs.

2.5 Limitations

There are some limitations with this study. This study primarily focused on platelet derived EVs. We acknowledge that limiting our study to platelet EVs may infer that these results are cell-type specific. However, our choice for examining platelet EVs are because they are the most abundant EV in the plasma, and thus are a great candidate for study in terms of sample processing and enumeration. Due to their abundance, refining pre-analytical guidelines with focus on platelet EVs limits their potential of massive platelet EV-artifact creation, and thus makes samples purer to analyze other cell-derived EVs in the plasma. Moreover, our study focused on solely using a single nFC machine. We believe that inter-laboratory comparisons with other nFC machines would have provided additional validation of the machine's robustness for EV enumeration.

2.6 Conclusion

nFC, or in some cases, 'microflow' cytometry, will remain a cornerstone for EV analysis because the majority of experiments currently performed are of the immunophenotyping variety. This technology allows for high-throughput detection of specific MP populations and association with prognosis and outcome in various diseases. Proper calibration of nanoscale flow cytometers, standardization of sample processing and analytical conditions may allow for accurate determination of MP concentrations in hundreds of patient samples within a week. Guidelines of this nature will have significant bearing as EVs continue to have diagnostic and prognostic potential in various pathophysiology states.

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Chapter 3

3 Utilizing *In Vitro* and *In Vivo* Methodology to Investigate the Biological Plausibility of Endothelial and Platelet EVs as Biomarkers of HD-Induced Vascular Injury

A modified version of this chapter is in preparation for publication.

Gomes et al., Endothelial and Platelet Extracellular Vesicles as a Measure of HD-Induced Microcirculatory Stress. *In preparation*. December 2022

3.1 Introduction

Hemodialysis (HD) is a life-saving treatment. It does however have many negative side effects such as episodic hypotension, myocardial ischemia, abnormal perfusion to various organs (heart, brain, liver, and kidneys), and damage to vulnerable vascular beds.[1–3] HD is a repetitive treatment which exposes patients to multiple episodes of circulatory stress, resulting in an increased risk of cardiovascular (CV) disease, stroke, and death.[3–7] This particular type of microvascular injury has been assessed through advanced forms of imaging.[7–9] While imaging modalities have provided valuable observations regarding HD associated microcirculatory stress, they are costly and time-consuming procedures. Therefore, identifying alternatives, such as blood-based biomarkers, to better understand and monitor vascular injury among HD patients, may be a more cost-effective and efficient method. Currently, there are no reliable blood-based biomarkers for HD patients. Of those examined, most were identified through extrapolation from observational association with outcomes, without examination of the role they play in the pathophysiology of end organ damage. To date they have not been successfully translated to clinical use.[10]

A current biomarker of interest is circulating extracellular vesicles (EVs). EVs that are 100-1000nm in diameter are traditionally referred to as microparticles, and are considered to be biomarkers of vascular injury and inflammation in pathologies including acute

myocardial infarction, diabetes, atherothrombosis, preeclampsia, hypertension, and metabolic syndrome.[11,12] Specifically, 100-1000nm EVs of endothelial and platelet origin have been shown to reflect vascular injury (endothelial dysfunction, increased inflammation, and increased coagulation).[13–19] Conventional flow cytometry is the most common methodology used to enumerate EVs; however, this technique is unable to identity EVs below ~300-500nm in size. This represents a challenge, as the majority of EVs in human blood including those that may further reflect vascular injury, are below this limit of ~300-500nm.[20–22] Additionally, while past studies have found that Tumor Necrosis Factor (TNF-α), interlukin-1 (IL-1), interferon-γ (IFN- γ), bacterial lipopolysaccharide (LPS), and uremic toxins (oxalate, indoxyl sulfate, p-cresylsulfate, and homocysteine), produce an increase in endothelial EV levels[23–25], without the use of specialized methodology, the biological usefulness of EVs below 500nm cannot be ascertained.

Moreover, past studies have investigated the impact of HD on EV levels, with some reporting an increase in EV levels over HD,[25–28] and others reporting a reduction or no change in EV levels.[29–32] No existing studies have compared EV levels with direct visualization of microcirculatory stress experienced through HD and with the use of a sensitive flow cytometer for EV enumeration and characterization. This may further demonstrate the biological likelihood that EVs are linked to HD-associated injury.

Therefore, to assess the significance of EV size, as well as biological plausibility of an EV based assay, we used nanoscale flow cytometry (nFC), which is optimized for data linearity and sensitivity, to assess the impact of the uremic milieu, as well as the HD procedure itself. We hypothesize that the uremic milieu and the HD procedure are factors impacting circulating EV levels and their size distribution.

To investigate the utility of EVs as biomarkers of HD associated vascular injury, we exposed endothelial cells *in vitro* to HD serum, used appropriate positive control (LPS), negative controls, and analyzed endothelial (CD62e+) EV levels and size distribution using a sensitive nanoscale flow cytometer. To further understand the impact of HD, we used a previously described rat model of HD[33] combined with intravital microscopy for

direct visualization of tissue perfusion during treatment. We collected animal blood samples throughout the HD procedure and determined the change in endothelial (CD62e+) and platelet (CD41a+) EV levels and EV size distribution over HD. We also recorded the change in tissue perfusion and hemodynamic stability of the animals. To enumerate endothelial EVs, we used an antibody against CD62e (e-selectin), as previous studies have demonstrated that it is a marker of endothelial activation, and is located solely on endothelial cells.[15,34] To enumerate platelet EVs, we used an antibody against CD41a (platelet glycoprotein) as we have used it previously.[20]

3.2 Methods

3.2.1 *In vitro* cell culture to investigate the impact of the uremic milieu

To investigate the impact of the uremic milieu on EV levels *in vitro*, we exposed endothelial cells to four conditions: pooled serum from HD patients, pooled serum from healthy individuals, a positive control (LPS), and a negative control (normal cell culture media). We chose to use pooled HD serum, because even though past studies[23–25] provide information regarding the independent effects of specific stressors, they might not reflect the complex collective uremic milieu. LPS, endotoxin, was chosen as a relevant positive control as previous studies have demonstrated that HD-induced circulatory stress causes recurrent regional ischemia in the gut, which may lead to an increased translocation of endotoxin.[35–37] HD is also known to redistribute liver perfusion, and reduces hepatic clearance function, which may result in endotoxemia among patients.[38] Cell culture media from each condition was collected, further processed, and enumerated using the nFC.

3.2.1.1 Cell culture

Normal Primary Human Umbilical Vein Endothelial Cells, Pooled cells (Pooled HUVEC, PCS-100-013) were obtained from American type Culture Collection (ATCC; VA, USA). The cells were maintained in Endothelial Cell Growth Basal Medium-2 (EGM-2) supplemented with BulletKit and 2% FBS (Lonza, CC-3162), and maintained in a humidified 37°C incubator with 5% CO_2. The cells were seeded at 0.4 x 10^6 cells/well in

6-well plates. When the cells were at 70-80% confluency, different conditions were administered to 3 wells per condition.

For *in vitro* experiments, HUVEC cells were exposed to media (+2%FBS), media (+2%FBS) with LPS (500ng/ml), media (-2%FBS) supplemented with pooled HD serum (2%), and media (-2% FBS) supplemented with pooled HC serum (2%). After 24 hours, media from each condition was collected, and centrifuged for 10 minutes at 500g (4°C) to remove any dead cells or large debris. The media from each condition was further spun and concentrated using a 100x kDA filter for 10 minutes at 3500g to remove excess fluid and to further concentrate the samples. The samples were collected and stored at -80°C for later analysis by nFC. The experiments were performed in triplicate.

3.2.1.2 Serum sample collection and preparation

To obtain serum samples for *in vitro* experiments, samples were obtained from 10 healthy volunteers, and 10 ESRD patients treated by HD (renal diagnoses: Hypertension/Hypertensive nephrosclerosis, 2; Diabetic Kidney Disease (DKD),2; Autosomal Dominant Polycystic Kidney Disease (ADPKD), 1; Glomerular Focal and Segmental Glomerulosclerosis (FSGS), 1; Glomerular Membranous Glomerulonephritis (MN), 1; Membranoproliferative Glomerulonephritis (MPGN), 1; Reflux Nephropathy, 1; Glomerular (Unknown), 1). Patients had been on HD for a minimum of 6 months (mean 56, range 9-192 months) and had a mean age of 66 years (range 40-79). All patients underwent three, 3-4 hour, HD sessions per week. Blood was collected at the mid-week HD session from the arterial access port prior to passing through the dialyzer at the start of a HD treatment session. Serum samples were centrifuged for 10 minutes at 1,300 x g and stored in a -80°C freezer. Prior to cell culture use, serum samples were centrifuged by 1000g for 5 minutes to remove large cell debris. Samples were further processed using a 100x kDA filter to remove any residual EVs before adding to cell culture media.

3.2.1.3 Quantification of EVs in cell culture media with nFC

EV analysis was accomplished using the Apogee A50-Micro Plus nanoscale flow cytometer (nFC) with autosampler, capable of EV resolution between ~80-180nm to

1000nm. The set-up and specific machine settings have been described in Chapter 2 (section 2.2.1) and were the same parameters that were followed for this chapter and chapter 4 in regard to nFC. Before sample analysis, silica calibration beads (Apogee Flow Systems Inc.) with diameter of 180nm, 240nm, 300nm, 580nm, 880nm, and 1300nm, as well as 100nm and 500nm fluorescent latex beads were used. The machine was set-up as previously described.[20] Titrations of all detecting antibodies were performed, and dilutions were ascertained from the original concentration (µg/ml) as provided by the manufacturers. To determine endothelial EVs, CD62e (e-selectin), was the EV antigen of interest as previous studies highlight the importance of CD62e as a marker of endothelial activation, and because it is solely located on endothelial cells, unlike most endothelial markers.[15,34] To enumerate endothelial EVs from cell culture media supernatant, 50 µL of media was incubated with 0.1 µg anti-CD62e-PE (P2H3 clone, monoclonal, catalog #12-0627-42, eBioscience). All samples were incubated with respective isotype matched antibody IgG1k-PE (P3.6.2.8.1 Clone, catalog #12-4714-82, eBioscience) to determine any non-specific binding and autofluorescence within each sample. All samples were incubated at RT in the dark for 30 minutes. The samples were further diluted with PBS and run using the nFC with settings as previously published.[20] All experiments were completed in triplicate.

3.2.2 Rat model of HD to assess the impact of the HD procedure on EV levels

To elucidate the impact of HD on EV levels, we used a previously described small animal model of HD combined with intravital microscopy for the direct visualization of tissue perfusion during HD.[33] By utilizing healthy animals, we avoided any confounding factors that may result from the initiation of CKD in animals or underlying kidney pathology. A healthy animal model is justified as past research indicates that HD induces ischemic injury among patients without CKD. Patients who had Acute Kidney Injury (AKI) experienced ischemic injury, independent of severity of uremia.[39] Moreover, the current animal CKD models to induce kidney failure include major surgical procedure (bilateral or 5/6 nephrectomy).[40] This model has a prolonged phase to develop kidney failure, and a recovery phase that may result in animals being too weak to endure the HD procedure.

Other models which include an adenine diet result in the deposition of 2,8-dihydroxyadenine in the renal tubules, and results in interstitial fibrosis.[41] However this model is known to results in heterogeneity of CKD and would thus make it difficult to compare animals. Therefore, we chose to use our existing model with a healthy rat, and measured EV levels at different time-points and compared them with direct observation of microcirculatory perfusion, and blood pressure throughout HD.

3.2.2.1 Animals

To conduct these experiments, 10 healthy male Wistar Kyoto rats (approximate weight 250 - 300 g) were obtained from Charles River, Wilmington, MA, USA. The animals were housed under standard conditions (water and food ad libitum, 12/12 light-dark cycle at room temperature). Animals had approximate blood volume between 17.5 and 21 ml. Of the 10 animals that were used, only 7 animals had blood collected for all timepoints.

3.2.2.2 Blood pressure measurement

Hemodialysis-induced hypotension is a significant and independent risk factors for mortality among HD patients.[42] Therefore, we measured the animal's blood pressure throughout the procedure and correlated this with circulating endothelial and platelet EV levels. To measure blood pressure, a catheter was placed in the carotid artery and connected to a blood pressure transducer to measure mean arterial blood pressure (MAP) using a rodent blood pressure analyzer (DMSI-400, Micro-Med Inc, Louisville KY USA). Out of the 7 animals that had blood collected for EV levels, 5 of these animals had blood pressure recordings throughout the experiment, while 2 animals had issues with access for blood pressure recording throughout HD.

3.2.2.3 *In vivo* extracorporeal circuit

To set up the extracorporeal circuit for the HD procedure, the animals were first anesthetized with a 4% isoflurane oxygen mixture. The body temperature of animals was maintained at 36.5 °C using an infrared lamp connected to an automated temperature monitor (TCAT-2 Temperature Controller, Physitemp Instruments Clifton NJ). Extracorporeal blood flow for animals was established using two indwelling catheters

that were placed in the left femoral artery to enable blood supply towards the dialyzer, and the left femoral vein to enable blood return to the systemic blood circulation. Once the catheters were connected to the dialyzer unit, the HD procedure was initiated using an extra-corporeal peristaltic pump (P720, Instech Lab., Plymouth Meeting, PA, USA) with single-lumen tubing (FL-093S-LL, Instech Lab., Plymouth Meeting, PA, USA)

3.2.2.4 *In vivo* hemodialysis procedure

To understand the impact of HD procedure on animals, the animals underwent a two-hour hemodialysis procedure. To conduct the experiments, microdialyzers were assembled as previously described using polysulphone fibers collected from new dialyzers (FX 600 Helixone, Fresenius, Canada) and placed in a polycarbonate tubular housing.[33] Prior to the start of the experiment, all connecting fluid lines and dialyzer were thoroughly flushed with fresh dialysate fluid. To simulate countercurrent dialysate flow, peristaltic infusion pumps (Sigma Spectrum V8 Infusion System, Baxter, Deerfield, Ill, USA) were used with pump rate set to 1ml/min.

Prior to the two-hour HD treatment, the animals also underwent a one-hour SHAM extracorporeal circulation (SHAM ECC) by-pass procedure to allow for physiological adaptation of the animals to the additional 2.3 ml extracorporeal priming volume in the HD circuit. The SHAM ECC procedure consisted of a bypass with the same internal volume as the mini-dialyzer (290uL). Previous study by our group which elucidates the pre-clinical model used in our study shows that this procedure is well tolerated by animals as it only creates a transient decrease in blood pressure and no changes to heart rate.[33] After the one-hour SHAM ECC procedure, HD was initiated, where the animal's blood was pumped through the dialyzer for two hours at a pump rate of 2ml/kg/h. To ensure a euvolemic HD procedure, the dialysate pumps were adjusted based on the baseline hematocrit levels as previously described.[33]

3.2.2.5 Blood collection

To determine circulating endothelial and platelet EV levels, blood samples were collected throughout the experiment at five different time points. Blood was collected at: **Pre-SHAM ECC (0 Hr**), sample taken immediately before start of SHAM ECC; **Pre-HD (1**

Hr), sample taken before the start of HD; **1-Hr HD (2 Hr),** 1-hour post-HD procedure; **2-Hr HD (3 Hr),** 2-hours post-HD procedure; and **Post-HD (3.05 Hr),** blood collected immediately after HD procedure is stopped (approximately 5 minutes post-experiment) as the animal is sacrificed. Figure 3.1 shows a schematic of when blood was collected.

At each time-point, 250 µL blood samples were collected from the dialyzer inflow, and a replacement 250 µL volume of sterile dialysate fluid was supplied back into the animal to compensate for the change in volume. Blood samples were collected in microtubes with EDTA and centrifuged twice for 15 minutes at 2500g to obtain platelet-free-plasma. The plasma was collected and stored at -80°C for later analysis by nFC.

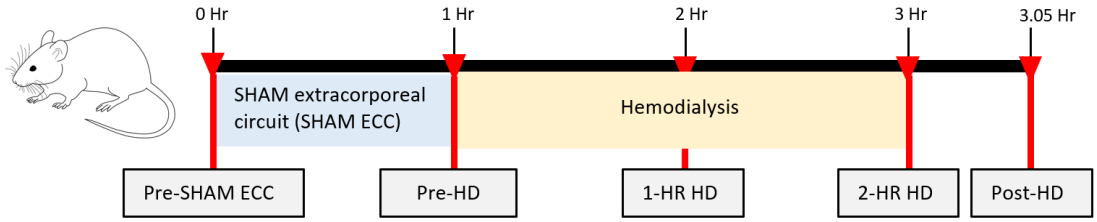

Figure 3.1 Schematic of when blood was collected throughout experiment

3.2.2.6 Dialyzer elution

To determine whether EVs are adhering to the dialyzer membrane, dialyzer membranes were eluted at the end of experiments. To elute membranes, sterile dialysate fluid was flushed through the dialyzer and extracorporeal tubing at the fastest pump speed to elute EVs from the dialyzer. Samples were centrifuged twice for 15 minutes at 2500g to remove large cell debris. Subsequently, the samples were further concentrated using a 100x kDA filter for 10 minutes at 3500g to remove excessive fluid and concentrate samples. The samples were collected and stored at -80°C for later analysis by nFC.

3.2.2.7 Quantification of EVs in rat plasma by nFC

EV analysis was completed using the Apogee A50-Micro Plus. The machine was calibrated as described (in section 3.2.1.3). To enumerate endothelial EVs from rat plasma, 10 µL of plasma was incubated with 0.15 µg anti-CD62e (20894-1-AP, polyclonal, catalog #20894-1-AP, Proteintech), which was conjugated to AF647 using

Alexa Fluor 647 Antibody Labelling kit (Invitrogen). To enumerate platelet EVs from rat plasma, 10 µL of plasma was incubated with 0.1 µg anti-CD41a-FITC (96-2C1 clone, monoclonal, catalog #sc-365938 Santa Cruz). All samples were also incubated with respective isotype matched antibodies IgG-AF647 (RbNP15 clone, catalog #14-4616-82, eBioscience), and IgG1k-FITC (MOPC-21 clone, catalog #551954, BD Pharmingen) to determine any non-specific binding and autofluorescence within each sample. All samples were incubated at RT in the dark for 30 minutes. The samples were further diluted with PBS and run using the nFC with settings as previously published.[20] Experiments were run in duplicate.

3.2.2.8 Intravital microscopy set-up and quantification of perfusion

To determine the change in perfusion through HD, intravital microscopy of the right Extensor Digitorum Longus (EDL) muscle was performed as previously described.[33,43] Through using an inverted microscope (Nikon Eclipse-Ti, Nikon Instruments, Melville, New York, USA), the EDL muscle was trans-illuminated (100 W xenon light-source, PTI LPS 220, Horiba Scientific, Piscataway NJ, USA) with optical light guide (Thorlabs, Newton, NJ, USA,) and with an additional filter (400-550nm bandpass) to prevent xenon light-source tissue damage. To enhance visualization of red blood cell movement, an additional filter (450 nm/20 nm band-pass filter; 450BP20; Omega Optical, Brattleboro, VT, USA) was used. Through using this set-up, intravital microscopy images (1200 × 1920 pixels; 16 bit) depicting EDL microcirculation were acquired using a multispectral multi-camera imaging system (MSMC-23-1-A, Spectral Devices Inc., London, Canada). At Pre-SHAM ECC, a selection was made of several adjacent fields of view of the muscle. These pre-selected views were imaged again at time-points of Pre-HD, 1-HR HD, and 2-HR HD to observe the change in perfusion index (i.e., number of perfused micro-vessels). IVM was conducted at only four time-points: Pre-SHAM ECC, Pre-HD, 1-HR HD, and 2-HR HD. The IVM images consisted of 60 second sequences (30 frames per second). This was subsequently stored on a hard drive for later off-line analysis which utilized an in-house written MATLAB based software, (MATLAB 2020a, the MathWorks Inc, Natrick, MA USA; https:// www. mathworks. com). To identify vessel perfusion, an algorithm is used which identifies vessels that are actively perfused by red

blood cells through registering the absolute value of light intensity changes. Through adding the sum of absolute values of light intensity changes (SAD), we determined which vessels were perfused.

Furthermore, an additional two-step machine learning algorithm was also applied. To quantify vessel perfusion, SAD images from subsequent time points were superimposed in a 10x10 grid. Intersections between the grid and vascular geometric structure that is associated with vascular blood flow were recorded. The number of points of intersection on the grid represented a perfusion index. Additional details regarding this analysis are described by Janssen et al.[33] Out of the 7 animals that had blood samples collected at each time-point, only 6 animals had tissue perfusion recordings.

3.2.2.9 Study approval

The animal experiments described in this manuscript were performed in accordance with the legal guidelines and regulations set by the Canadian Council of Animal Care (CCAC). Study approval was granted by the Animal Care and Use Committee (ACUC) of Western University, London, Ontario, Canada.

3.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Continuous variables were assessed with parametric or non-parametric tests. To assess normal distribution, the Shapiro-Wilk test was used. Any continuous variables were analyzed using t-tests for normally distributed variables, and Mann-Whitney U test or Wilcoxon matched-pairs signed rank test was used for non-normally distributed variables. Ordinary one-way ANOVA was used for analyses with multiple comparison, with ad-hoc Bonferroni correction. Friedman Test was used for multiple comparisons when data was not normally distributed, with Dunn's multiple comparisons test. Correlations were assessed utilizing the Pearson Correlation coefficient (r) for normally distributed variables and the Spearman correlation coefficient (ρ) for nonnormally distributed variables, with 95% confidence intervals, and P-value <0.05 considered as statistically significant.

3.3 Results

3.3.1 Small sized endothelial EVs may be indicative of endotoxic stress

I first used an Apogee A50-Micro Plus nanoscale flow cytometer to examine a mix of fluorescent and non-fluorescent calibration beads to identify regions of interest (ROI) based on size (Figure 3.2, A, B). These gates provided representative ROIs for EVs within specific size ranges (Figure 3.2 C). I then exposed endothelial cells in cultured monolayers to pooled serum from 10 HD patients, pooled serum from 10 healthy participants, LPS (positive control), and regular cell culture media (negative control). The media was collected from each condition, processed, and enumerated with the nFC to determine CD62e+ EVs. I observed an increase in 240nm and 300nm sized endothelial (CD62e+) EVs in the LPS condition compared to control (Figure 3.2, E, F).

I then examined EVs bearing the endothelial marker, CD62e, examining total endothelial EVs (CD62e+, 180-1300nm), small endothelial EVs (CD62e+, between 180 nm and up to 580 nm), and large endothelial EVs (CD62e+, between 580nm and up to 1300nm) (Figure 3.3 A). This creation of endothelial EV sub-population labels was guided by MISEV 2018 guidelines[44], which advises that EVs be described by size and markers of interest rather than broad categories such as microparticles. Through quantifying EVs within these sub-populations, I observed an approximately three-fold increase in total endothelial EV concentration when cells were exposed to LPS (1294 ± 295.5 events/uL) compared to control (447.8 ± 288.5 events/uL). There was also a difference in small sized endothelial EVs between control (mean: 270.8 ± 183.7 events/uL) and the LPS condition (1012 ± 252.8 events/uL) that was statistically significant (P=0.01) (Figure 3.3 A-C). There were no differences between control and the HD-serum condition among total, small, or large endothelial EV levels (Figure 3.3 A-C).

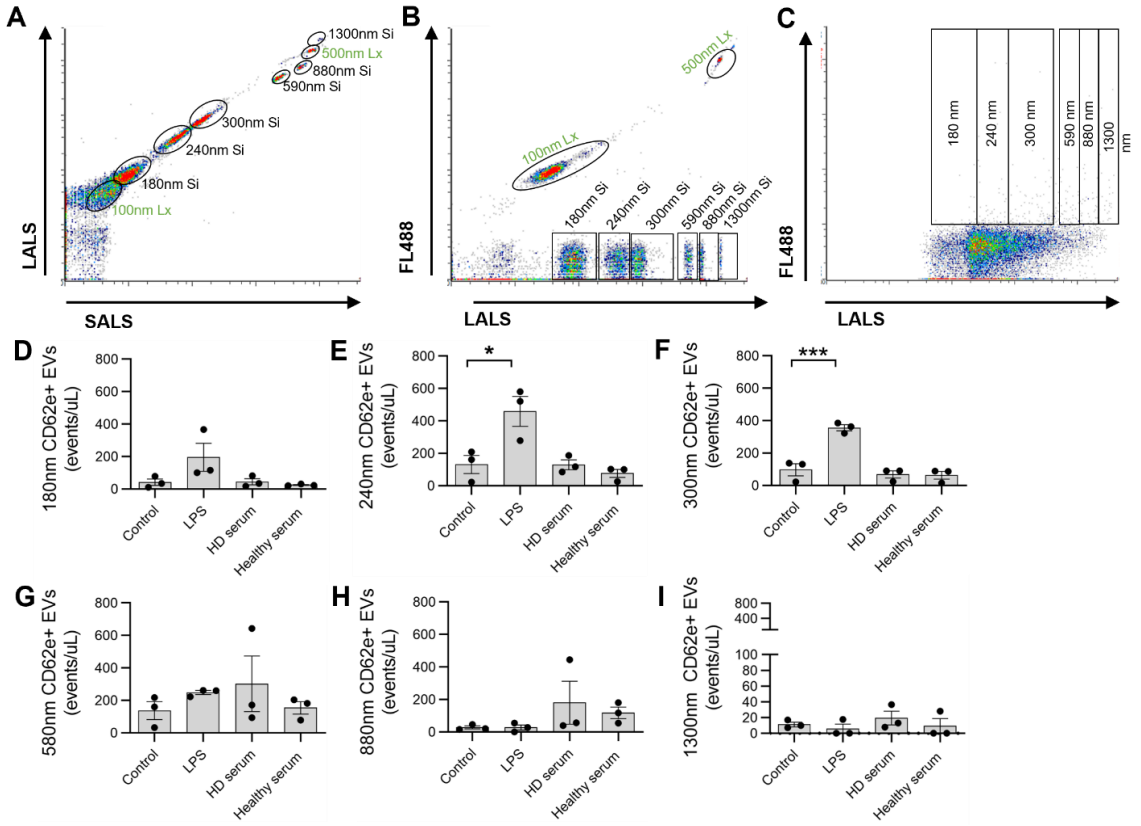

Figure 3.2 Endothelial EVs can be quantified by size and concentration using nanoscale flow cytometry

(A) Cytogram of the calibration of the nanoscale flow cytometer with bead mix (fluorescent latex and non-fluorescent silica beads) detected with short angle light scatter (SALS, x-axis) and long angle light scatter (LALS, y-axis). **(B)** Cytogram of the calibration of the nanoscale flow cytometer with bead mix (fluorescent latex and non-fluorescent silica beads) detected with long angle light scatter (x-axis) and fluorescent detector (y-axis). **(C)** An example of a cytogram depicting gates for EVs in various size ranges based on calibration beads**. (D)** HUVEC-derived endothelial (CD62e+) EVs from cell culture media collected 24 hours after incubation with cell culture media and LPS (2% FBS and 500ng/ml lipopolysaccharide), HD serum (pooled 2% hemodialysis patients' serum), and healthy control serum (pooled 2% healthy volunteers' serum) within size range of 180nm, **(E)** 240nm, **(F)** 300nm, **(G)** 590nm, **(H)** 880nm, **(I)** 1300nm. (n=3; the error bars represent the mean ± SEM, **P* < 0.05, ****P* < 0.001, one-way ANOVA)

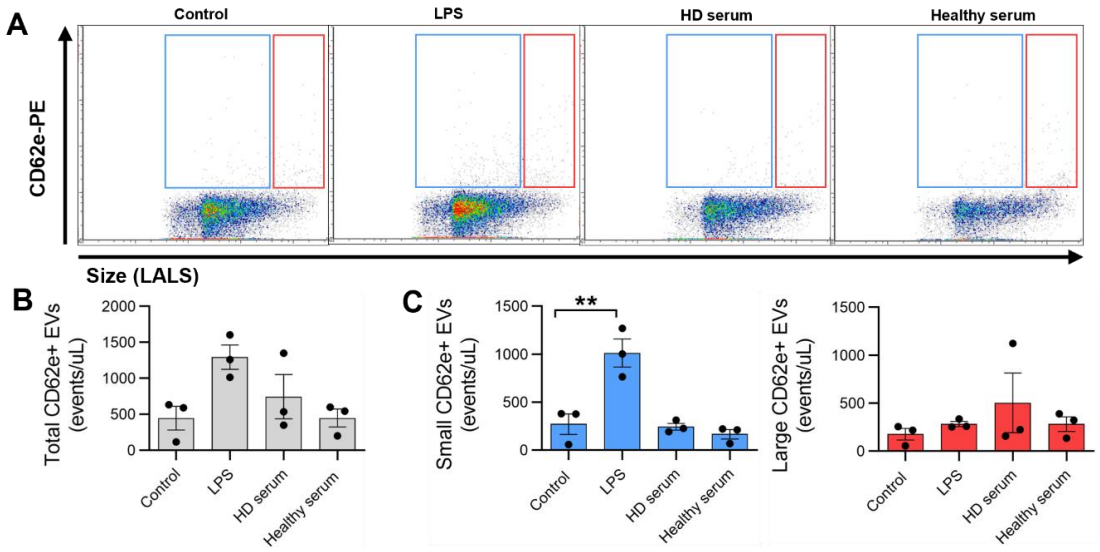

Figure 3.3 Small CD62e+ EVs may be indicative of LPS-induced endothelial stress

(A) Representative nanoflow cytograms of CD62e+ HUVEC-derived EVs from cell culture media collected 24 hours after cells were incubated with LPS (2% FBS and 500ng/ml Lipopolysaccharide), HD serum (pooled 2% hemodialysis patients' serum), and healthy control serum (pooled 2% healthy volunteers' serum). Blue box represents small EVs (between 180 nm and up to 580 nm), and red box represents large EVs (between 580nm and up to 1300nm). **(B)** Quantification of total (small and large) CD62e+ EVs from cell media conditions. **(C)** Left graph is quantification of small CD62e+ EVs and right graph is quantification of large CD62e+ EVs from cell media conditions. (n=3; the error bars represent the mean ± SEM, $^{**}P < 0.01$, one-way ANOVA)

3.3.2 Rat plasma-derived endothelial (total and small) and platelet (total) EV levels increase over HD

We then examined plasma for endothelial (CD62e+) and platelet (CD41a+) EVs from rats undergoing hemodialysis. We focused on quantifying both endothelial and platelet derived EVs because in the literature both types of EVs have been shown to reflect vascular injury (endothelial dysfunction, increased inflammation, and increased coagulation).[13–19] We observed an increase in total endothelial EVs from pre-HD (681.3 ± 65.14 events/uL) to post-HD (956.3±85.42 events/uL) (P=0.018) in our experiments (Figure 3.4 A). There was also a significant increase in small endothelial EV levels from pre-HD (492.0 ± 37.7 events/uL) to post-HD (684.6 ± 53.9 events/uL) (P=0.003), but no increase in large endothelial EVs (P=0.161) (Figure 3.4 C, D). There was an increase in total platelet (CD41a+) EVs from pre-HD (862.9 ± 283.0 events/uL) to post-HD (1578.0 ± 334.6 events/uL) (P=0.049), but no changes in small (P=0.093) and large (P=0.1945) platelet EV levels that were statistically significant (Figure 3.4 E-G). Additionally, through comparing endothelial and platelet EVs in plasma collected at all time points (Pre-SHAM ECC, Pre-HD, 1-HR HD, 2-HR HD, and Post HD), we observed no substantial fluctuations over all time-points that were statistically significant (Figure 3.5 A-G).

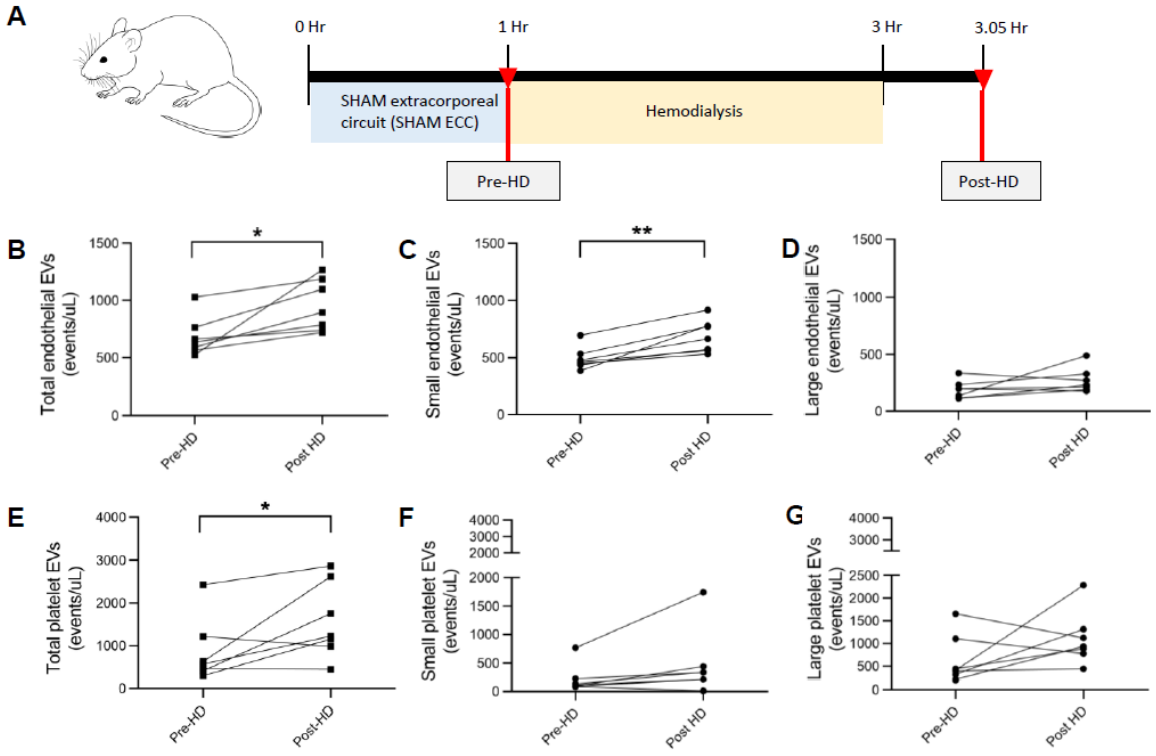

Figure 3.4 Rat plasma-derived endothelial (total and small) and platelet (total) EV concentration increases over HD

(A) Schematic of when blood was collected. Comparison of EVs at timepoints of Pre-HD and Post-HD in rat plasma, **(B)** Total endothelial (CD62e+) EV concentration, **(C)** Small endothelial (CD62e+) EV concentration, **(D)** Large endothelial (CD62e+) EV concentration, **(E)** Total platelet (CD41a+) EV concentration, **(F)** Small platelet (CD41a+) EV concentration, **(G)** Large platelet (CD41a+) EV concentration (n=7; Paired t-test, $*P < 0.05$, $**P < 0.01$)

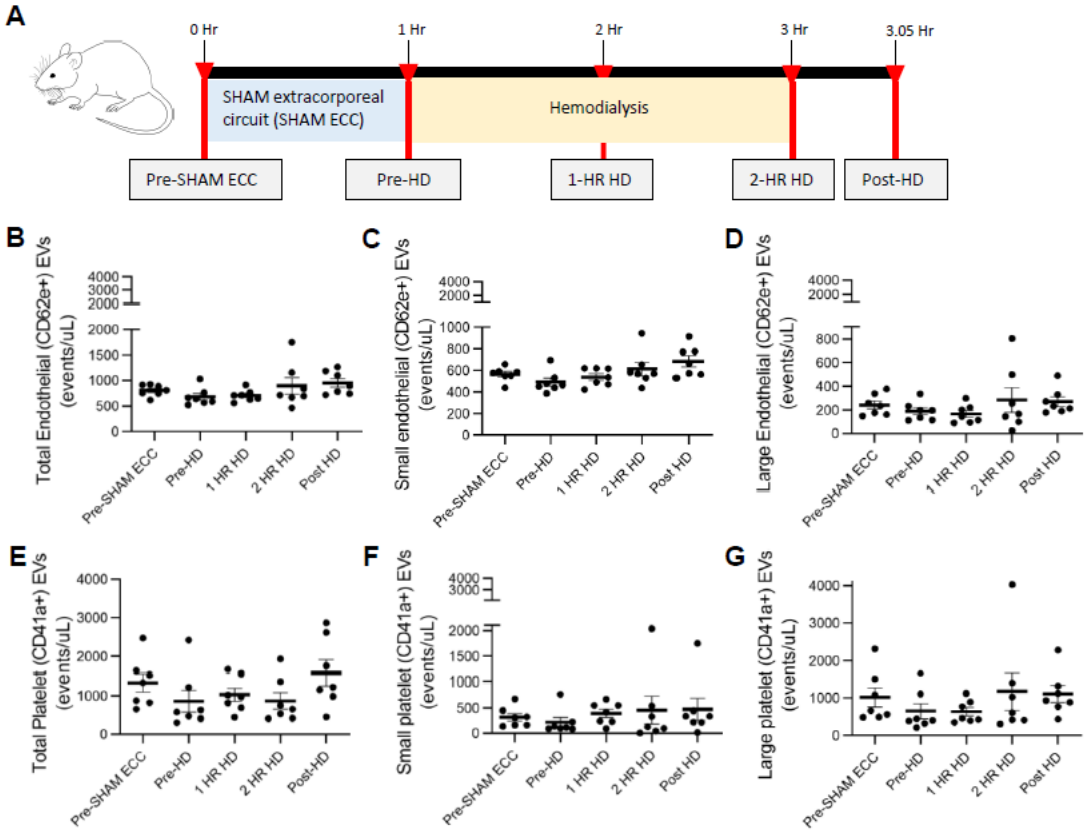

Figure 3.5 Rat plasma-derived small and large endothelial and platelet EV levels fluctuate over the course of Hemodialysis procedure

(A) Schematic of blood collection through the experiment, **(B)** Total endothelial (CD62e+) EV concentration in rat plasma, **(C)** Total platelet (CD41a+) EV concentration in rat plasma**, (D)** Small endothelial (CD62e+) EV concentration in rat plasma, **(E)** Small platelet (CD41a+) EV concentration in rat plasma, **(F)** Large endothelial (CD62e+) EV concentration in rat plasma, **(G)** Large platelet (CD41a+) EV concentration in rat plasma. (n=7; the error bars represent the means ± SEM, one-way ANOVA)

3.3.3 EVs may be adsorbing to the dialyzer

To determine whether the dialyzer sequesters EVs, we eluted the dialyzer post-HD following the rodent HD procedure. We found that more total platelet EVs (median: 591 events/uL, IQR: 378.4-1824, events/uL) than total endothelial EVs (median: 193.0 events/uL, IQR: 144.6-243.6, events/uL) adhered to the dialyzer membrane and extracorporeal circuit (Figure 3.6).

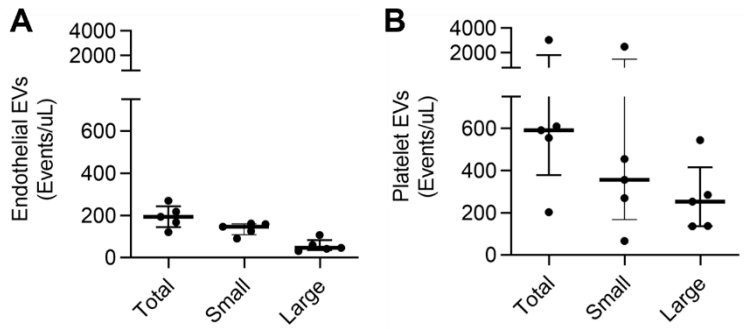

Figure 3.6 Endothelial and platelet EVs eluted from dialyzer and extracorporeal circuit

(A) Eluted endothelial (CD62e+) EVs, **(B)** Eluted platelet (CD41a+) EVs.

3.3.4 Association between intradialytic EDL-muscle hypo-perfusion and change in EV levels during HD

We examined muscle perfusion of the EDL within rats by using IVM during the HD procedure. Muscle perfusion was recorded at four time-points, pre-SHAM ECC, Pre-HD, 1 HR HD, and 2 HR HD. IVM data was analyzed through using an algorithm to quantify the absolute microvascular perfusion index represented as a number of identified points. A significant decline in muscle perfusion was observed from Pre-SHAM ECC (mean perfusion index: 148.4 ± 30.26) to the 2-HR HD time point (mean perfusion index: 85.67 ± 17.99 grid intersections) ($P<0.05$) (Figure 3.7 A). Furthermore, to understand the influence of EV size, we focused on small EVs, because our findings from the *in vitro* and *in vivo* experiments highlighted small EVs as being potentially important populations of EVs, which are likely missed by conventional FC. We noticed that as the percent change in perfusion declined from Pre-SHAM ECC to 2-HR HD, the percent change of small endothelial EV levels and small platelet EV levels increased (Figure 3.7 B, D). However, correlations between the change in perfusion and the change in small endothelial EV ($r=-0.39$, $P=0.45$) and small platelet EV ($r= -0.27$, $P=0.60$) levels were not statistically significant (Figure 3.7 C, E).

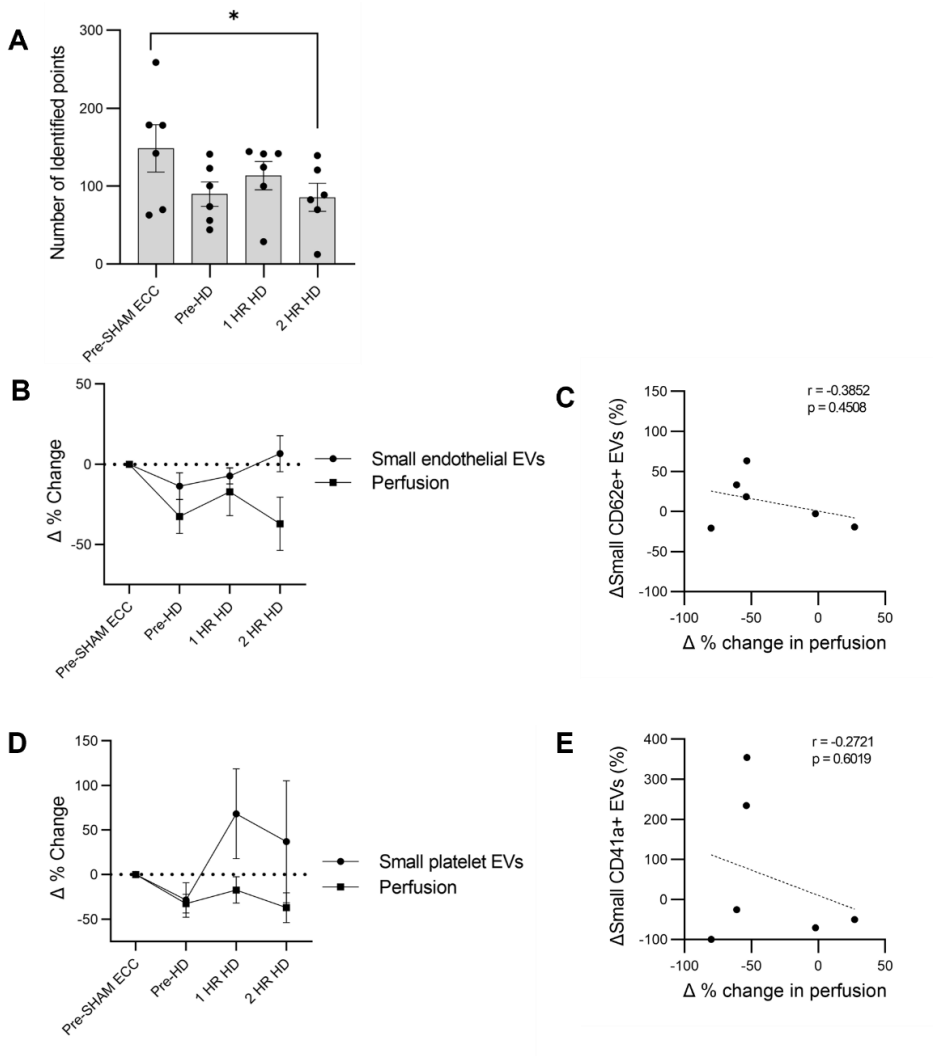

Figure 3.7 Tissue perfusion of the extensor digitorum longus (EDL) muscle decreases through HD

(A) Quantification of perfusion index (number of identified points) of the EDL muscle based on IVM data. (n=6; Friedman test with Dunnet correction for multiple comparisons, **P*<0.05), **(B)** The percent change of perfusion and small endothelial EVs from Pre-SHAM ECC to 2-HR HD time point, **(C)** Correlation between the change in perfusion (number of identified points) and total endothelial (CD62e+) EVs from Pre-SHAM ECC to 2-HR HD time point. **(D)** The percent change of perfusion and small platelet EVs from Pre-SHAM ECC to 2-HR HD time point. **(E)** Correlation between the change in perfusion (number of identified points) small platelet (CD41a+) EVs from Pre-SHAM ECC to 2-HR HD time point (n=6, Pearson correlation for normally distributed data).

3.3.5 Association between intra-HD hypotension and change in EV levels during HD

Throughout the in vivo HD experiments, the rats also had their MAP measured at Pre-SHAM ECC, Pre-HD, 1 HR HD, 2 HR HD, and Post-HD. We observed a decrease in MAP from Pre-SHAM ECC (80.32 ± 3.0 mmHg) to 1-HR HD (69.84±1.79 mmHg) ($P<0.05$), and to post-HD (54.26±5.03 mmHg) ($P<0.05$) (Figure 3.8 A). We focused on small endothelial and small platelet EV populations for these experiments. As blood pressure decreased through HD, small endothelial and small platelet EV levels increased (Figure 3.8 B, D). However, correlations between the change in MAP and change in small endothelial EVs ($r=-0.20$, $P=0.83$) and small platelet EV ($r= -0.54$, $P=0.34$) levels were not statistically significant (Figure 3.8 C, E).

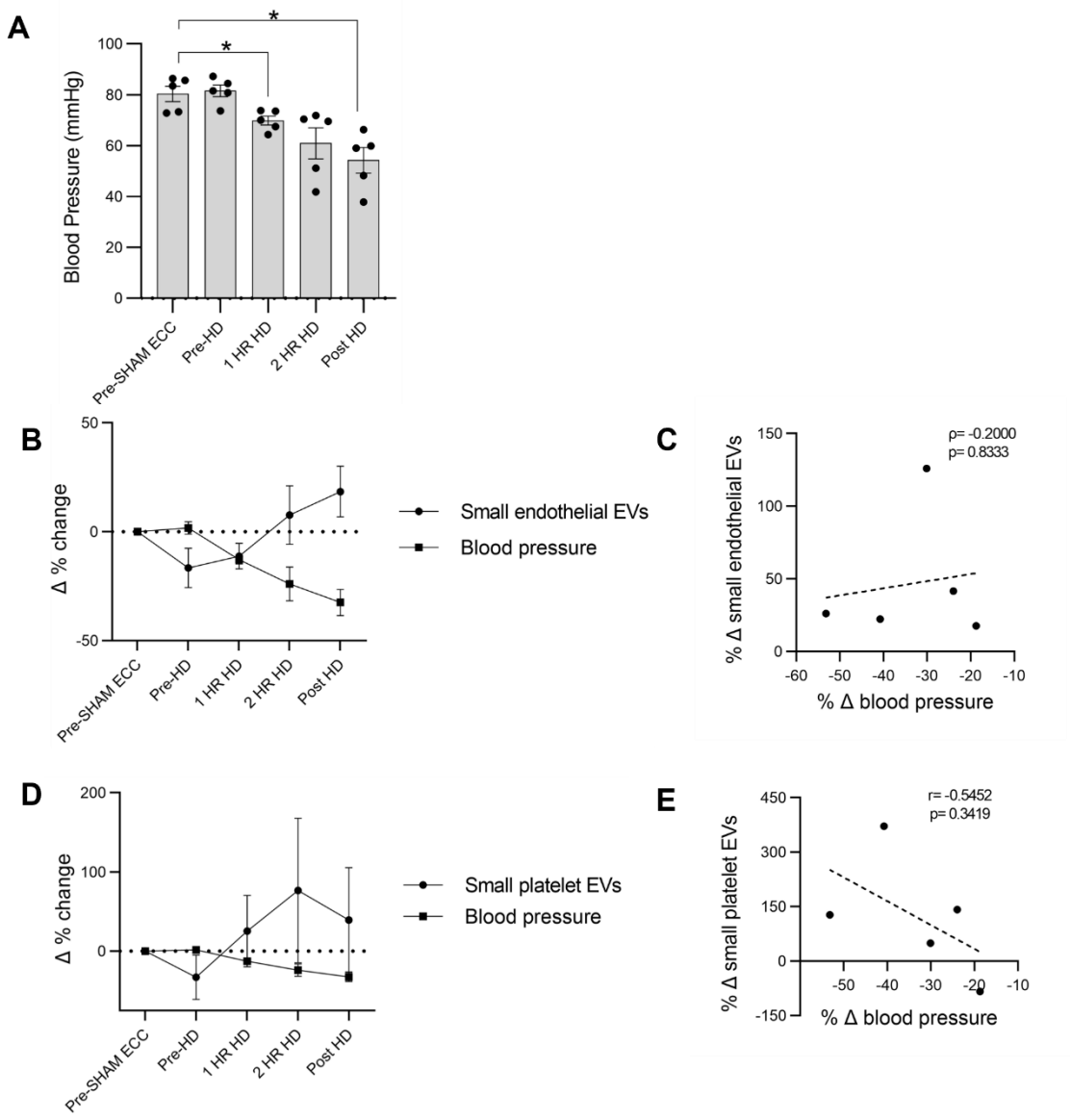

Figure 3.8 Animals experience a decrease in mean arterial blood pressure throughout HD

(A) Blood pressure at various timepoints of the animal experiment. (n=5, One-way ANOVA), **(B)** Percent change of blood pressure, and small endothelial (CD62e+) EVs levels from Pre-SHAM ECC to Post-HD time point, **(C)** Correlation between the change in blood pressure and small endothelial (CD62e+) EV levels from Pre-SHAM ECC to Post-HD time point, **(D)** Percent change of blood pressure, and small platelet (CD41a+) EV levels from Pre-SHAM ECC to Post-HD time point, **(E)** Correlation between the change in blood pressure and small platelet (CD41a+) EV levels from Pre-SHAM ECC to

Post-HD time point (n=5 Pearson correlation for normally distributed data, and Spearman correlation for non-normally distributed data).

3.4 Discussion

This study is the first to use a novel rat model of HD and a nFC for EV enumeration (concentration and size), to determine the potential utility of EVs as biomarkers of HD induced vascular injury. Through utilizing a nFC, designed specifically to resolve particles down to 80 nm, we found that the uremic milieu did not have a significant impact on EV concentration or size distribution. Though, our positive control, LPS, which is also a relevant stressor among HD patients, resulted in an increase in small-sized endothelial EVs. These findings indicate that small size EVs may be an important population, and further highlight the value of using nFC because it has the potential to enumerate these small sized EVs. Our *in vivo* results showed that there is an increase in endothelial (total, small) and platelet(total) EVs over the HD procedure, suggesting that EVs (total and small) may conceivably be an indicator of HD associated stress furthering the understanding of the impact of HD.

Our results show that small endothelial (CD62e+) EVs are indicative of endotoxic stress caused by LPS. This finding is relevant as previous studies have shown that small (<500nm) endothelial EV levels are associated with ischemic heart failure, coronary artery disease, and reductions in left ventricular ejection fraction.[45–47] Our findings highlight the importance of enumerating small EVs, which may have otherwise been overlooked by conventional flow cytometers that lack sensitivity below 300-500nm. These results also suggest that further subclassifications of EVs by size may be important rather than the broad categories such as microparticles (100-1000nm sized EVs). Our results also show that the overall uremic milieu (HD serum) did not have an impact on endothelial-derived EV levels compared to control. While past studies have found that individual uremic toxins (oxalate, indoxyl sulfate, p-cresylsulfate, and homocysteine) found in serum from HD patients, cause an increase in endothelial EV levels[25], to our knowledge our study is the first to utilize an nFC to assess the overall effect of the collective serum within cell culture to determine endothelial EV concentration and size.

Therefore, our results suggest that within a stationary cell culture system, pooled uremic serum may not be a strong enough factor.

Our use of a healthy rodent model of HD allowed us to independently assess the impact of HD treatment on EV concentration and size, without the confounding effects of renal kidney failure or uremia. Our results show that the rats had an increase in endothelial (total, and small) and platelet(total) EV levels over treatment, along with a decrease in EDL tissue perfusion and blood pressure. The results from the *in vivo* experiments also further highlighted that the increase in small endothelial EVs over HD, may be an important sub-population of EVs to investigate, as was observed in our *in vitro* work.

The increase in endothelial and platelet EVs over HD demonstrates the potential of HD imposing stress through several possible factors, such as bio-incompatibility of the dialyzer membrane (known to result in protein adsorption) promoting an inflammatory response of complement activation [48–50], leading to a rise in EV levels.[51] While we did utilize more biocompatible synthetic dialyzers (polysulfone) for these experiments, the dialyzer's hydrophobic properties are known to lead to greater protein adsorption which can further amplify an inflammatory response.[52] Other factors that may promote an increase in EV levels include mechanical/sheer stress.[53] These might include microbubbles causing platelet activation and aggregation, as well as exposure of blood to the roller pump segment in extracorporeal tubing.[54,55] Altogether, the findings from this rodent model are novel as we are the first to demonstrate the impact of HD on EV levels in a healthy animal along with direct visualization of the vasculature during the HD procedure.

Our results also showed that animals had a decrease in microcirculatory perfusion of their EDL muscle and a decrease in blood pressure through HD. These findings are significant as it further demonstrates that HD can induce ischemic injury, even without confounding factors such as CKD or kidney failure. These results confirm the findings of a past study involving Acute Kidney Disease patients, who in the absence of CKD-induced uremia, still experienced similar ischemic injury during HD.[39] Additionally, our results showed that there are weak correlations between the overall change in EV levels and overall

change in tissue perfusion. However, our findings regarding adsorption of EVs by the dialyzer may suggest that the change in EV levels over HD is being blunted and therefore it is difficult to make meaningful correlations with changes in EV levels. It is also important to note that the EVs are not being filtered out of the blood in the process of HD. The physical size of EVs (100-1000nm) are too large to pass through the dialyzer pores (~3.9 nm radius), and thus it is plausible that EVs are being adsorbed to the blood facing part of the membrane and not within the much larger surface area (trabecular material) of the fiber wall itself.

Our findings in regards to EV adsorption to the dialyzer are also consistent with a past study that detected EV-associated protein flotillin on dialyzer membranes post-HD.[32] Therefore, to further understand the changes in EV levels over HD, the effect of EV adherence to dialyzers must be considered since there are a variety of dialyzers used clinically, and they differ by factors such as composition, membrane thickness, surface area, pore size distribution, and pore density[56], all of which may further impact EV adsorption.

3.5 Limitations

There are some limitations with this study. While our study focused on determining the impact of the uremic milieu on EV levels, we acknowledge that our utilization of serum at a 2% concentration as a collective stimulus, may not be potent enough to elicit an effect in a static cell culture system. We chose 2% of pooled HD serum because the HUVEC cells used in this study were maintained with 2% FBS serum. We chose to expose cells to a percentage of serum that was similar to control conditions, so that we could make reasonable comparisons. Future studies may need to use endothelial cells maintained at higher FBS serum concentrations, to assess the effect of higher HD serum concentrations. Higher concentrations may also reduce potential dilution and buffering effect from the cell culture media. Moreover, we are aware that other factors of the uremic milieu such as uremic toxins, lowered pH, oncotic stress, or a change in potassium or sodium concentration may also need to be assessed to determine the extent of the uremic milieu and its impact on EV concentration and size distribution. Our

current study also utilized a healthy rodent model to understand the impact of HD, however, we understand that a CKD animal model would have allowed for further understanding and comparison of how HD with underlying CKD may impact EV levels, tissue perfusion, and hemodynamic stability of the animal. Although existing animal models of CKD involve either major surgical procedure (bilateral or 5/6 nephrectomy) and thus result in extensive stress to animals[40], or an adenine diet which creates heterogeneity of CKD outcome.[41] Therefore, the utilization of these models are currently not appropriate to use with our existing animal model.

3.6 Conclusion

In conclusion, EVs represent a practical biomarker for HD-induced injury as they can be easily quantified and characterized. More specifically, small endothelial EVs may have biological plausibility as markers of HD induced vascular injury and warrants further consideration. Future studies should continue to use appropriate and sensitive methodology capable of evaluating EVs within the entire size range of 100-1000nm, and especially below 500nm.

3.7 References

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Chapter 4

4 Endothelial and Platelet EVs as biomarkers of HD-induced vascular and cardiac injury

A modified version of this chapter is in preparation for publication.

Gomes et al., Endothelial and Platelet Extracellular Vesicles as a Measure of HD-Induced Microcirculatory Stress. *In preparation*. December 2022.

4.1 Introduction

Hemodialysis (HD) is a life-saving treatment for patients with kidney failure. However, patients undergoing HD are 10-20 times more likely to experience cardiovascular mortality than the general population.[1,2] Patients receiving HD may encounter complications such as episodic intradialytic hypotension (IDH), abnormal perfusion to various organs (heart, brain, liver, and kidneys), and damage to vulnerable vascular beds.[3–8] The repetitive nature of HD exposes patients to multiple episodes of circulatory stress, which leads to irreversible multi-organ injury, and well documented increased incidence of cardiovascular (CV) disease, stroke, and death.[5,9–12]

Various imaging-based studies have revealed HD-induced cardiac injury. Echocardiography studies have confirmed intradialytic Regional Wall Motion Abnormalities (RWMAs).[12] Other imaging modalities such as positron emission tomography (PET) with radiolabeled water, ($H_2{}^{15}0$-PET), have shown that patients experience a greater than 30% reduction in myocardial perfusion during HD.[13] Moreover these regions with reduced myocardial perfusion, were regions with matched myocardial wall motion abnormalities.[13] Overall, imaging modalities have provided valuable evidence, however, are costly and time-consuming to permit detection of HD-induced CV injury or monitor response to therapy, without waiting for tissue injury to have occurred. There is an urgent need for blood-based biomarkers of HD-induced vascular injury suitable for inclusion into conventional models of care. However, there are

currently no reliable biomarkers for HD patients that have successfully translated to clinical use.[14],[15]

Endothelial and platelet derived EVs (100-1000nm) are biomarkers of interest because they have been shown to directly and indirectly reflect vascular injury (endothelial dysfunction, increased inflammation, and increased coagulation).[16–22] More specifically, a pilot study completed among HD patients found that increased levels of endothelial EVs are a robust predictor of cardiac mortality and global mortality.[23] While this finding highlights the importance of using EVs as biomarkers amongst HD patients, to our knowledge, no study has investigated the association between EV levels (concentration and size) and intradialytic cardiac injury during corresponding HD sessions. By understanding whether EVs are direct indicators of HD tolerance, an EV based assay would have the potential to act as a tool for clinicians to actively monitor patients and even measure response to further treatments or interventions to improve HD tolerability.

Conventional flow cytometry is the most common methodology used to enumerate EVs; however, this technique cannot reliably examine EVs below ~300-500nm in size. This represents a challenge, as the majority of EVs in human blood including those that may further reflect vascular injury, are below this limit.[24–26]

Therefore, to identify whether EVs have clinical utility among HD patients, and to assess the feasibility of an EV based assay, we used nanoscale flow cytometry (nFC), optimized to examine EV's down to 180nm in size, among human-based studies focused on HD-induced CV injury. Our use of nFC methodology was guided by our optimized pre-analytical guidelines for data linearity and sensitivity, which demonstrated that nFC is a refined methodology for EV research.[24] Additionally, through observing the importance of small size EVs and the overall effect of HD within an *in vivo* model, we demonstrated that EVs have biological utility, and this further supports their investigation among observational patient studies. Therefore, we hypothesized that circulating endothelial and platelet EVs (concentration and size) have utility as markers of subclinical CV injury, in HD patients.

To understand the clinical utility and significance of EV levels among patients, we conducted observational studies in patients receiving HD to determine the association between EV levels and HD-induced hypotension, ultrafiltration rate (UFR), and measures of indirect (RWMAs) and direct (myocardial perfusion) subclinical intradialytic cardiac injury. Additionally, to assess the feasibility of using an EV based assay in clinical HD settings, we compared EV levels from patients over a 2-week period to determine the change in EV levels in short-term.

4.2 Methods

4.2.1 Determination of EVs among patients undergoing HD

To investigate the impact that the HD procedure has on EV levels, we collected blood from patients undergoing HD and enumerated EVs pre- and post- HD. This experiment also allowed us to compare the change in EVs to our results from the rodent model in chapter 3. The details regarding methodology are listed below.

4.2.1.1 Patients and study approval

Patients' samples were obtained from three observational-based studies, which were all focused on observing the cardiovascular response to HD. These studies included patients from three single-center observational pilot studies: Assessing the impact of extended dialysis using the Theranova Dialyzer (REB #1589), Reducing hemodialysis induced recurrent brain injury to improve patients' lives (REB #109413), and Investigation of electrophysiological substrate of arrythmia in hemodialysis patients (REB #113905). For all three studies, study approval was provided by the University of Western Ontario Health Sciences Research Ethics Board. The study was conducted in compliance with the approved protocol, good clinical practice guidelines, the Declaration of Helsinki, and all applicable regulatory requirements. Informed consent was received from participants prior to inclusion in the study.

Patients were all from the London Health Sciences Centre Regional Renal Program (London, Ontario, Canada) and were enrolled into each of the studies after giving informed consent. The three studies were individually powered for specific sample sizes

to answer their respective research questions, and each included a baseline study visit. During the baseline study visit, no interventions or modifications to the conventional HD procedure were completed, which is why samples were only used from baseline study visits among all three of the studies.

The enrollment criteria for the three studies included patients over 18 years of age, with HD vintage (time on dialysis) > 3 months, and those who were receiving thrice weekly dialysis. Exclusion criteria for REB #1589 included patients who did not meet the inclusion criteria. Exclusion criteria for REB #109413 included patients who did not meet inclusion criteria and had established severe cognitive impairment with a Montreal Cognitive Assessment test (MoCA)<18 or with formal diagnosis of dementia, had previous clinical stroke, were taking drugs likely to blunt response to remote ischemic preconditioning (e.g., ciclosporin, ATP-sensitive potassium channel directed drugs) or dialyzing using lower limb vascular access. The exclusion criteria for REB#113905 included not meeting inclusion criteria, presence of a pacemaker and implantable cardioverter defibrillator, and prior diagnosis of chronic arrhythmia and/or on anti-arrhythmic drugs.

To determine the change in EV levels over HD, and the association of EV levels with ultrafiltration rate or blood pressure, 35 patients from the three studies were used. If patients completed more than one study, only their older baseline study visit was used so that each patient's data was exclusive. All the patients in the study with REB #113905 were used to determine the association between EVs and cardiac injury. Patients in this study also had a second study visit, directly one week after their baseline visit. Therefore, pre-HD samples between week one and week two were used to determine the change in EV levels over short-term within this patient subset. A summary and breakdown of the number of patients per study is provided in Table 4.1.

Table 4.1 Description of the number of patients per study

HD patient studies	Available number of patients per study	Number of patients used to assess change in EV levels over HD, and associations with ultrafiltration rate and blood pressure	Number of patients used for the association with cardiac injury	Number of patients used to determine the change in EV levels over short-term
Assessing the impact of extended dialysis using the Theranova Dialyzer (REB #1589)	**23**	22	0	0
Reducing hemodialysis induced recurrent brain injury to improve patients' lives (REB #109413)	**9**	9	0	0
Investigation of electrophysiological substrate of arrythmia in hemodialysis patients (REB #113905)	**10**	4	10	10
Total	**42**	**35**	**10**	**10**

4.2.1.2 Monitoring EV levels over short-term in HD patients

To observe whether circulating Pre-HD (directly before the start of the HD session) EV levels varied over short term, we assessed EV levels from 10 patients who were within the investigation of cardiac injury study (REB # 113905). We collected and compared endothelial and platelet EVs in the Pre-HD blood samples collected at the mid-week HD session during week 1 and the mid-week HD session during week 2 for the 10 patients.

4.2.1.3 Blood Collection

All blood samples were collected from patients on conventional HD (3-4 hour treatments/3 times a week) at the mid-week HD session. Blood was collected pre-HD (directly before the start of the HD session) and post- HD (directly after HD session was completed) using a 15-gauge needle. The first few mls of blood were discarded. The

remaining blood was collected within EDTA Vacutainers (BD Biosciences Inc.). These samples were processed within 30 mins to 1-hour post-collection by double centrifugation at 2,500 x g for 15 minutes to achieve platelet-free plasma (PFP). The plasma was aliquoted and stored at -80°C for later processing of EVs by nFC.

4.2.1.4 Plasma derived EV enumeration by nFC

EV analysis was performed using an Apogee A50-Micro Plus nanoscale flow cytometer (nFC) (Apogee Flow Systems, England) with autosampler, capable of EV resolution between ~80 to 1000nm. The set-up and specific machine settings have been described in Chapter 2 (section 2.2.1). Before sample analysis, silica calibration beads (Apogee Flow Systems Inc.) with diameter of 180nm, 240nm, 300nm, 580nm, 880nm, and 1300nm were used, and the machine was set-up as previously described.[24] Titrations of all detecting antibodies were performed, and dilutions were determined from the original concentration (µg/ml) as provided by the manufacturers. To determine endothelial EVs, we used an antibody against CD62e, e-selectin. Previous studies highlight the importance of CD62e as a marker of endothelial activation, and because it is solely located on endothelial cells, unlike most endothelial markers.[18,27] To assess platelet EVs, we used an antibody against CD41a as we have used previously.[24] To enumerate endothelial derived EVs from human plasma, 10 µL of plasma was incubated with 0.1 µg anti-CD62e-PE (P2H3 clone, monoclonal, catalog #12-0627-42, eBioscience). To enumerate platelet derived EVs from human plasma, 10 µL of plasma was incubated with anti-CD41a-AF488 (HIP8 clone, monoclonal, catalog #303724, Biolegend). All samples were also incubated with respective isotype matched antibodies IgG1k-PE (P3.6.2.8.1 Clone, catalog #12-4714-82, eBioscience), and IgG1k-AF488(MOPC-21 clone, catalog #400129, Biolegend) to determine any non-specific binding and autofluorescence within each sample. All samples were incubated at RT in the dark for 30 minutes. The samples were further diluted with PBS and run using the nFC with settings as previously described.[24] All experiments were completed in triplicate.

4.2.2 Comparing Pre-HD EV levels and cardiovascular measures

Pre-HD (directly before the start of the HD session) EV levels were correlated with important clinical measures including intra-HD hypotension, ultrafiltration rate, and subclinical measures of cardiac injury, which utilized echocardiography and Computed Tomography (CT)-perfusion imaging.

4.2.2.1 Blood pressure measurements

Blood pressure measurements were used to assess hemodynamic stability over HD treatment. More specifically, changes in systolic blood pressure were used to determine hemodynamic stability. Blood pressure was measured prior to HD treatment and then frequently throughout the treatment until the end of HD using a blood pressure cuff. All blood pressure measurements were taken from patients seated upright. The percent change in systolic blood pressure we used is defined by the change in systolic blood pressure from Pre-HD blood pressure to nadir systolic blood pressure.

4.2.2.2 Ultrafiltration rate

Ultrafiltration Rate (UFR) is defined as a composite metric of interdialytic weight gain (IDWG), treatment time, and post dialysis weight, which is calculated with each dialysis treatment.[28] Past research studies have shown that higher IDWG and short HD treatment times lead to higher UFR, which result in repetitive intra-HD hypotension episodes, and results in overall poor clinical outcomes. High UFR is associated with increased all-cause mortality and cardiovascular mortality among conventional HD patients.[29–31] Therefore UFR was used within this study as a measurement of risk assessment for comparison with EV levels.

4.2.2.3 Echocardiography

To understand whether EVs are an indirect measure of myocardial ischemia, echocardiography was performed to evaluate RWMAs at peak-stress of HD (15 to 30 minutes before the end of HD) for ten patients from the study assessing cardiac injury among HD patients (REB # 113905). A commercially available ultrasound (Vivid-q, GE Medical Systems, Soningen, Germany), was used to image a dynamic 2- and 4- chamber

parasternal view of the heart. Acquired images were retrospectively processed using a semiautomated 2D speckle tracking software (EchoPac, GE Healthcare). Three cardiac cycles were analyzed to quantify segmental longitudinal strain (out of 12 left ventricular segments). Through calculating segmental longitudinal strain, RWMAs were identified. RWMA represent a decrease in longitudinal strain by 20% compared to baseline strain values. They represent a loss of contractile function and further indicate indirect hypoperfusion.

4.2.2.4 Cardiac Computed Tomography (CT)-Perfusion

To determine whether EVs are a direct measure of myocardial ischemia, intradialytic CT-perfusion imaging was preformed to quantify blood flow at pre-HD and peak-stress of HD for ten patients from the study assessing cardiac injury among HD patients (REB # 113905). For CT acquisition, the patients laid on the CT bed in the feet-first supine position. To quantify perfusion, 32 scans were consecutively acquired to obtain the delivery of iodine contrast (Isovue 370, 0.7 mls per kg body weight) at a rate of 3-4 mls per second followed by a 30 ml bolus of saline. The dynamic images were processed using a mathematical model (three-dimensional non-rigid registration algorithm) to extract functional maps of the heart with absolute myocardial perfusion. For the quantification of global myocardial perfusion, the horizontal long axis view of the heart was used. Additionally, by using the short axis view, the perfusion maps were further segmented into 16 segments. These segments are established by the American Heart Association and correspond to myocardial perfusion of specific coronary arteries, which are responsible for perfusion of specific heart segments. Absolute blood flow values were quantified for each of the segments. A decrease of 40% or greater blood flow from pre-HD to peak HD was the threshold used to identify the number of hypo-perfused myocardial segments.

4.2.3 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). All continuous variables were presented as mean ± standard error of mean (SEM) or median

(interquartile range (IQR)), and categorical variables were expressed as percentages, unless otherwise specified. Continuous variables were assessed with parametric or non-parametric tests. To assess normal distribution, the Shapiro-Wilk test was used. Any continuous variables were analyzed using t-tests for normally distributed variables, and Mann-Whitney U test or Wilcoxon matched-pairs signed rank test was used for non-normally distributed variables. Ordinary One-Way ANOVA was used for analyses with multiple comparison, with ad-hoc Bonferroni correction. Friedman Test was used for multiple comparisons when data was not normally distributed, with Dunn's multiple comparisons test. Correlations were assessed utilizing the Pearson Correlation coefficient (r) for normally distributed variables and the Spearman correlation coefficient (ρ) for nonnormally distributed variables, with 95% confidence intervals, and P-value <0.05 considered as statistically significant.

4.3 Results

4.3.1 HD is associated with an increase in patient plasma-derived endothelial EV (total and small) and platelet EV (large) levels

Our rodent study in chapter 3 showed that there is an increase in total and small endothelial and total platelet EV levels over HD. Therefore, to determine if this finding extends to human patients, we collected plasma samples from patients undergoing HD. Patient samples were obtained from patients from three different studies that were all focused on investigating HD-induced cardiovascular injury. All samples used were obtained from patients undergoing conventional HD. Further information regarding the number of patients used per study is included in the method section 4.2.1.1. Patient demographics are listed in Table 4.2. EV analysis was performed using an Apogee A50-Micro Plus nanoscale flow cytometer (nFC) (Apogee Flow Systems, England) capable of EV resolution between ~80 to 1000nm. We found an increase in total endothelial EVs from pre-HD (median (Interquartile Range (IQR)): 5128.0 (10277.0) events/uL) to post-HD (median (IQR): 15544.0(56088.0) events/uL) (P=0.013), and an increase in small endothelial EVs from pre-HD (median (IQR): 3213.0 (8056.0) events/uL) to post-HD (median (IQR): 3853.0 (7098.0) events/uL) (P=0.0063) endothelial EV levels, and no change in large endothelial EVs that was statistically significant (P=0.21) (Figure 4.1, A-

C). Thus, this increase in total and small endothelial EV levels we observed among patients, reflects what we observed in the rat study in chapter 3. There was no change in total platelet EVs and small platelet EVs from pre-HD to post HD (P=0.26, P=0.36, respectively) among patients (Figure 4.1, D-E). However, there was an increase in large platelet EVs from pre-HD (median (IQR): 3880.0 (5415.0) events/uL) to post-HD (median (IQR): 4717.0 (9984.0) events/uL) (P=0.048) (Figure 4.1F).

Table 4.2 Baseline Patient Characteristics

Variables	Patients (n=35)
Demographics	
Age (years)	63.1 ± 14.2
Sex (male/female), n/n	21/14
Weight (kg)	90.2 ± 18.4
Dialysis vintage (months), median (IQR)	41 (30-86)
Ultrafiltration rate (mL/kg/h)	7.7 ± 3.3
Access (Fistula/Central line), n/n	18/17
Etiology of kidney disease, % (n)	
Diabetic nephropathy	37 (13)
Glomerulonephritis	11 (4)
Hypertension/Hypertensive Nephrosclerosis	11 (4)
Reflux Nephropathy	9 (3)
IgA Nephropathy	11 (4)
Other	20 (7)
Biomarkers	
Creatinine (umol/L)	732.5 ± 225.0
Urea (mmol/L)	17.8 ± 4.8
Calcium (mmol/L)	2.2 ± 0.3
Albumin (g/L)	40.4 ± 3.2
WBC (10^3 cells/uL)	7.4 ± 2.6
Thrombocytes (10^3/uL)	227.2 ± 65.5
C-reactive protein (mg/L), median (IQR)	5.9 (3.4-17.9)

Values are mean ± SD, or % unless otherwise indicated

IQR: Interquartile range

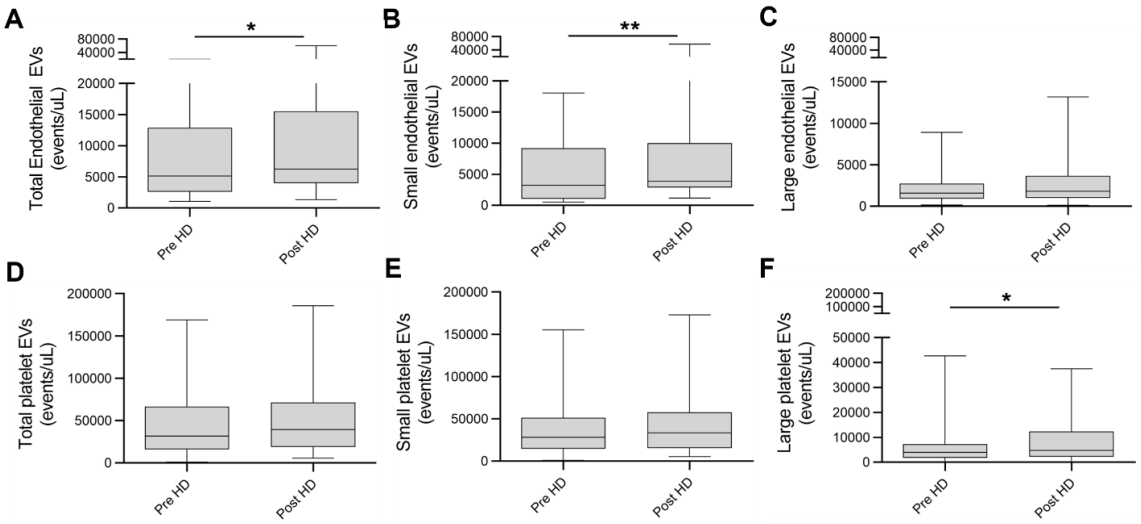

Figure 4.1 Human endothelial and platelet EV levels increase over hemodialysis

The EV concentration of EV subsets in human plasma Pre- and Post- HD **(A)** Total endothelial (CD62e+) EVs, **(B)** Small endothelial (CD62e+) EVs**, (C)** Large endothelial (CD62e+) EV, **(D)** Total platelet (CD41a+) EVs, **(E)** Small platelet (CD41a+) EVs, **(F)** Large platelet (CD41a+) EVs (n=35; Paired t-test, *$P < 0.05$, **$P < 0.01$)

4.3.2 HD patients' pre-HD endothelial EV (large) levels may be associated with UFR

To further understand the clinical significance of endothelial and platelet EV levels measured from patients at the beginning of HD (Pre-HD), we correlated them with UFR. We chose UFR as a measure to correlate with because high UFR is associated with increased all-cause mortality and cardiovascular mortality among conventional HD patients.[29–31] We found that large Pre-HD endothelial EV levels positively correlated with UFR (ρ=0.43, P=0.0064), and no other associations between specific EV subsets and UFR were found (Figure 4.2, A-F).

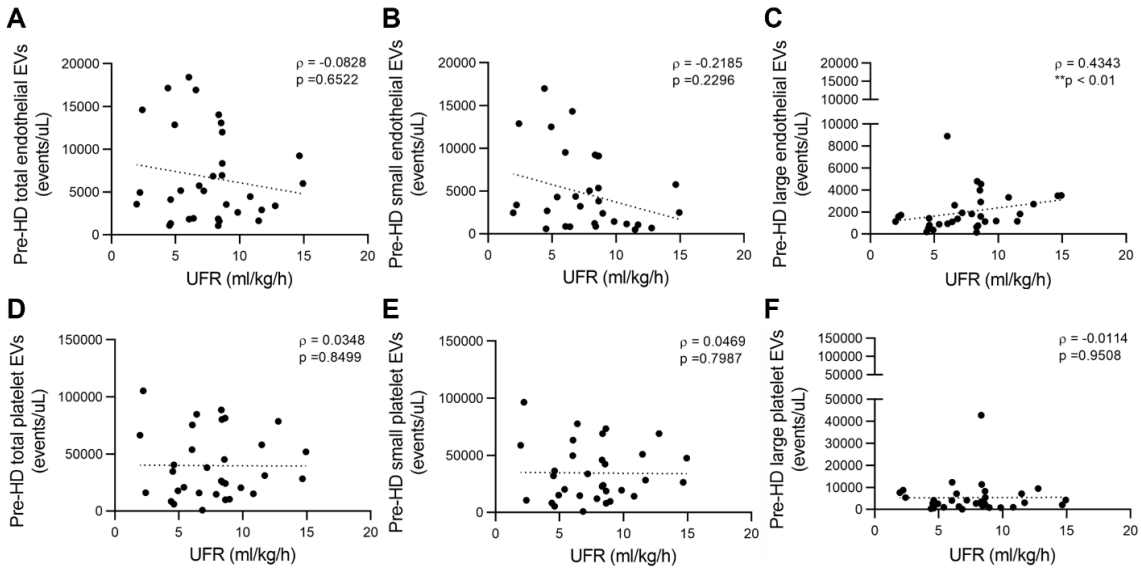

Figure 4.2 HD patients' pre-HD endothelial EV levels may be associated with UFR
Correlation between ultrafiltration rate and **(A)** total Pre-HD endothelial (CD62e+) EVs, **(B)** small Pre-HD endothelial (CD62e+) EVs, **(C)** large Pre-HD endothelial (CD62e+) EVs**, (D)** total Pre-HD platelet (CD41a+) EVs, **(E)** small Pre-HD platelet (CD41a+) EVs, **(F)** large Pre-HD platelet (CD41a+) (n=35, spearman correlation, $^{**}P < 0.01$)

4.3.3 HD patients' pre-HD platelet EV levels may be associated with change in intra-HD blood pressure

To further understand the clinical significance of endothelial and platelet EV (total, small, and large) levels measured from patients at the beginning of HD (Pre-HD), we correlated them with the change in intra-HD systolic blood pressure. We found significant association between percent change in intra-HD systolic blood pressure and pre-HD total (ρ=-0.41, P=0.019) and small (ρ=-0.39, P=0.026) platelet EV levels (Figure 4.3, D-F). There were no significant associations between changes in blood pressure and pre-HD endothelial EV levels (Figure 4.3, A-C).

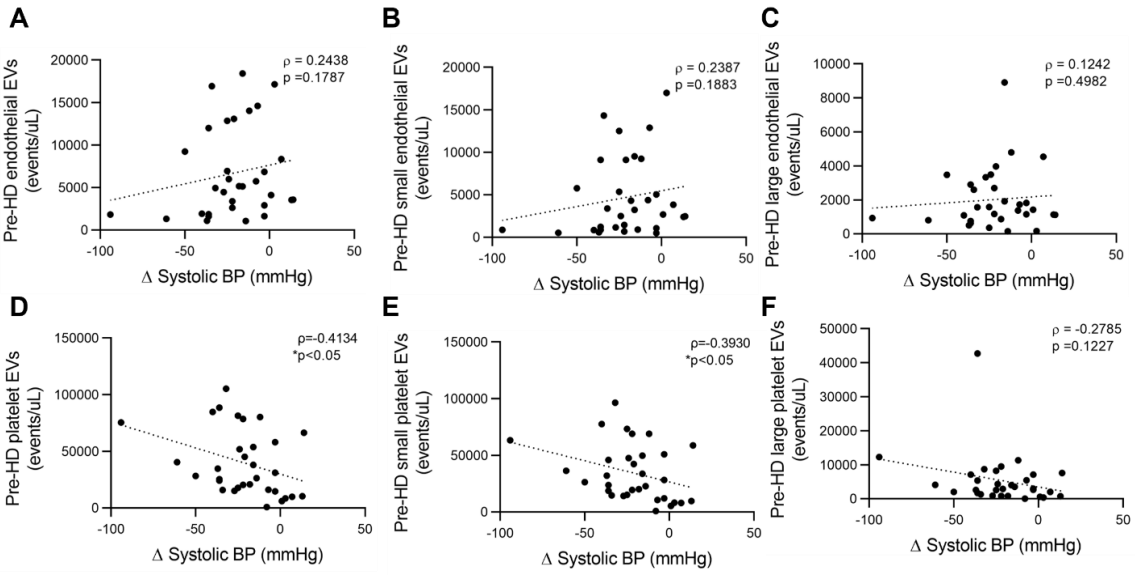

Figure 4.3 HD patients' pre-HD platelet EV levels may be associated with intra-HD hypotension

Association between change in systolic blood pressure(mmHg) and **(A)** total Pre-HD endothelial (CD62e+) EVs, **(B)** small Pre-HD endothelial (CD62e+) EVs, **(C)** large Pre-HD endothelial (CD62e+) EVs, **(D)** total Pre-HD platelet (CD41a+) EVs, **(E)** small Pre-HD platelet (CD41a+) EVs, **(F)** large Pre-HD platelet (CD41a+) EVs (n=35, spearman correlation, $*P < 0.05$)

4.3.4 HD patients' pre-HD endothelial EV levels are associated with increased RWMA at peak-stress of HD

To understand whether EV levels taken from patients at the beginning of HD (Pre-HD) are an indirect measure of myocardial ischemia, echocardiography was performed to evaluate RWMAs at peak-stress of HD (15-minutes before the end of HD) among patients. We compared endothelial and platelet EV (total, small, and large) levels with number of RWMAs at peak-stress of HD. We found significant correlations between the number of RWMAs and Pre-HD total endothelial EVs (r=0.82, P=0.0040), small endothelial EVs (r=0.79, P=0.0067), and large endothelial EVs (r=0.82, P=0.0034) (Figure 4.4, A-C). There were no significant associations between pre-HD platelet EV levels and the number of RWMAs (Figure 4.4, D-F).

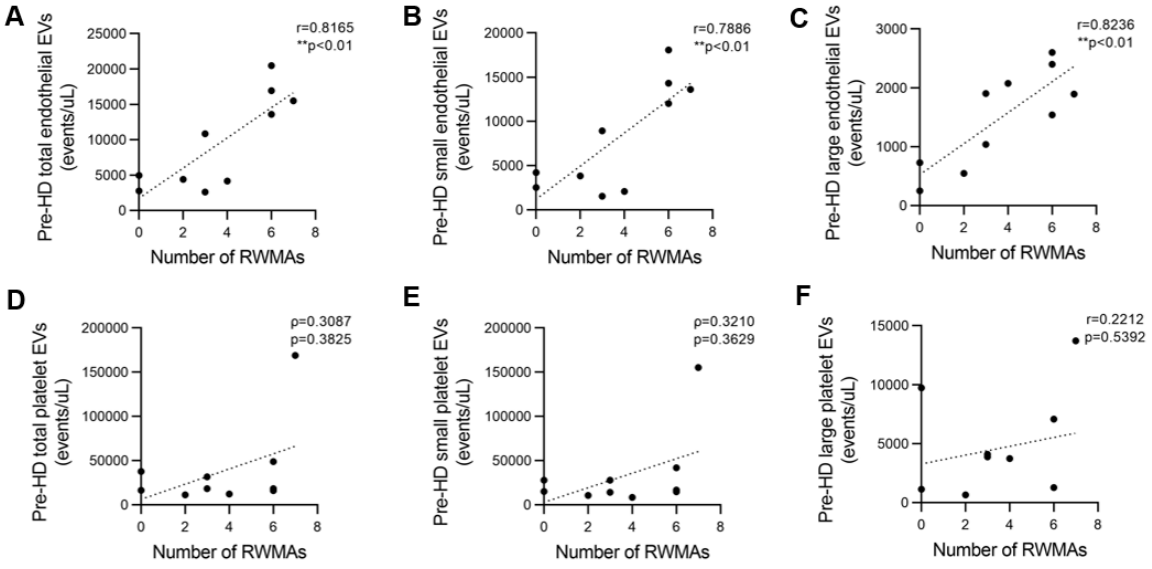

Figure 4.4 Pre-HD endothelial EVs are associated with intradialytic RWMAs at peak-stress of HD

Association between RWMAs at peak-HD and **(A)** total Pre-HD endothelial (CD62e+) EVs, **(B)** small Pre-HD endothelial (CD62e+) EVs, **(C)** large Pre-HD endothelial (CD62e+) EVs, **(D)** total Pre-HD platelet (CD41a+) EVs, **(E)** small Pre-HD platelet (CD41a+) EVs, **(F)** large Pre-HD platelet (CD41a+) EVs. (n=10, Pearson correlation for normally distributed data, and Spearman correlation for non-normally distributed data, $^{**}P < 0.01$)

4.3.5 HD patients' pre-HD endothelial levels may be associated with cardiac hypo-perfusion

To further understand whether endothelial and platelet EV (total, small, large) levels taken from patients at the beginning of HD (Pre-HD) are associated with measures of myocardial ischemia, we used intradialytic global and segmental myocardial CT-perfusion data. Through comparing pre-HD endothelial EV levels of those who were above and below the median of percent change in global perfusion (percent change from pre-HD to peak-stress HD, median: -18.03%), we observed no differences that were statistically significant (Figure 4.5, A-C). Utilizing segmental perfusion data and comparing pre-HD endothelial EV levels from patients that had greater or less than the median number of hypo-perfused segments (median:2), we found no differences that were statistically significant (Figure 4.5, D-F). There were no differences in pre-HD platelet EVs when assessing percent change in global perfusion and number of hypo-perfused myocardial segments (Figure 4.6).

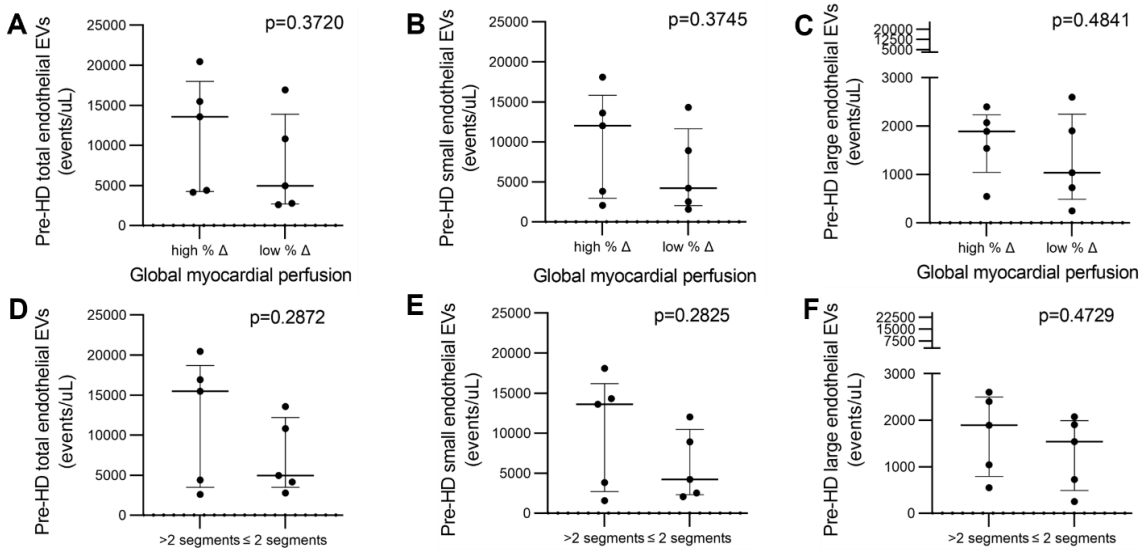

Figure 4.5 Comparison of Pre-HD endothelial EV levels between patients who experienced high or low global and segmental myocardial hypo-perfusion

Comparison of percent change in global myocardial hypo-perfusion. High % change represents a decrease in perfusion (-32.57% to -18.03%), and Low % change represents a decrease in perfusion (-18.03% to -7.15%) for **(A)** total Pre-HD endothelial (CD62e+) EVs, **(B)** small Pre-HD endothelial (CD62e+) EVs, **(C)** large Pre-HD endothelial (CD62e+) EVs. Comparison of number of hypo-perfused myocardial segments for **(D)** total Pre-HD endothelial (CD62e+) EVs, **(E)** small Pre-HD endothelial (CD62e+) EVs, **(F)** large Pre-HD endothelial (CD62e+) EVs (n=10, unpaired T-test)

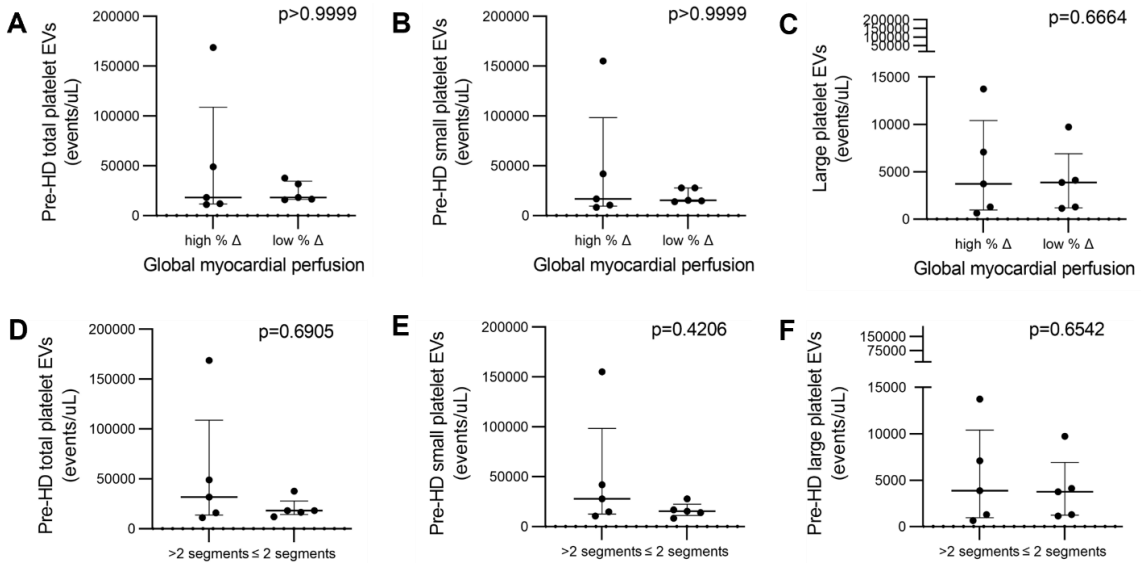

Figure 4.6 There is no difference in Pre-HD platelet EV levels between patients who experienced high or low global and segmental myocardial hypo-perfusion

Comparison of percent change in global myocardial hypo-perfusion. High % represents a decrease in perfusion (-32.57% to -18.03%), and Low % represents a decrease in perfusion (-18.03% to -7.15%) for **(A)** total Pre-HD platelet (CD41a+) EVs, **(B)** small Pre-HD platelet (CD41a+) EVs, **(C)** large Pre-HD platelet (CD41a+) EVs. Comparison of the number of hypo-perfused myocardial segments for **(D)** total Pre-HD platelet (CD41a+) EVs, **(E)** small Pre-HD platelet (CD41a+) EVs, **(F)** large Pre-HD platelet (CD41a+) EVs (n=10, unpaired T-test)

4.3.6 HD patients' pre-HD EV levels re-establish after a week of HD treatments

Furthermore, to observe whether circulating EVs taken at the beginning of HD sessions (Pre-HD) varied over short term, we assessed Pre-HD endothelial and platelet EV levels from 10 patients. We correlated endothelial and platelet EVs (total, small, large) in the Pre-HD blood samples collected at the mid-week HD session during week 1, and the mid-week HD session during week 2. There was a correlation between week one and week two of pre-HD total endothelial EVs ($r=0.82$, $P=0.0035$), pre-HD small endothelial EVs ($r=0.84$, $P=0.0026$), and pre-HD large endothelial EVs ($r=0.36$, $P=0.3002$) (Figure 4.7, A-C). We also found associations between week one and week two of pre-HD total platelet EVs ($\rho=0.84$, $P=0.0037$), pre-HD small platelet EVs ($\rho=0.90$, $P=0.0008$), and pre-HD large platelet EVs ($\rho=0.93$, $P=0.0003$) (Figure 4.7, D-F).

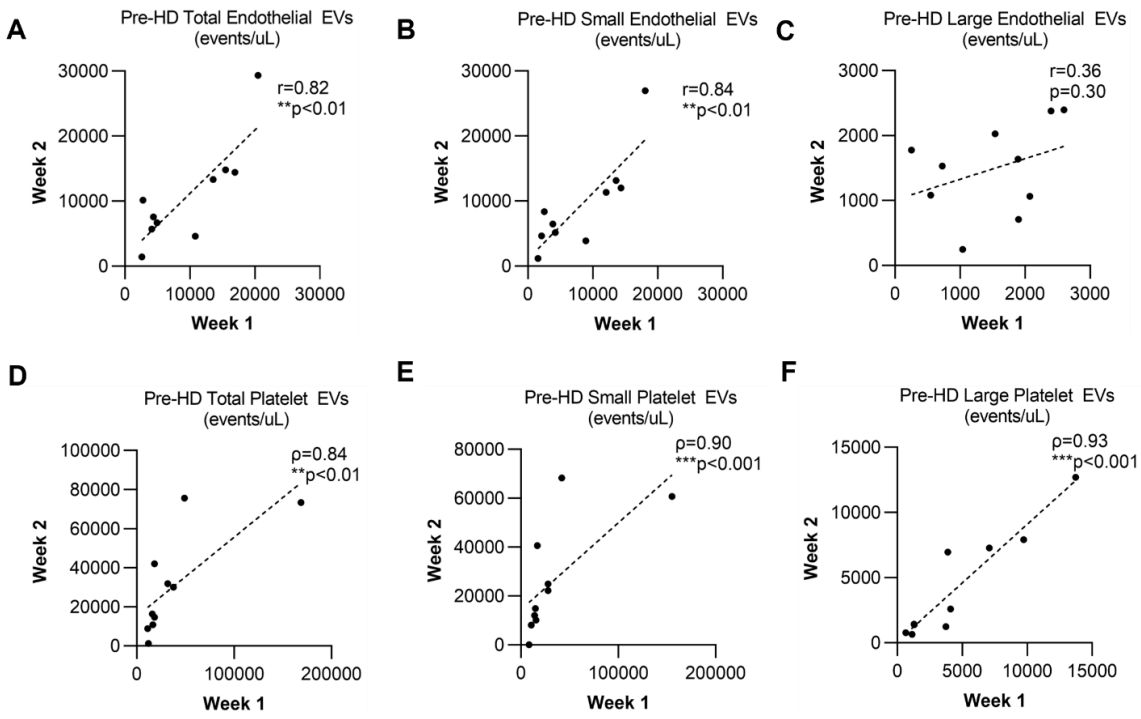

Figure 4.7 There is an association between Pre-HD EV levels measured over short-term

Correlation of week one and week two of **(A)** total Pre-HD endothelial (CD62e+) EVs, **(B)** small Pre-HD endothelial (CD62e+) EVs, **(C)** large Pre-HD endothelial (CD62e+) EVs, **(D)** total Pre-HD platelet (CD41a+) EVs, **(E)** small Pre-HD platelet (CD41a+) EVs, **(F)** large Pre-HD platelet (CD41a+) (n=10, Pearson correlation for normally distributed data, and Spearman correlation for non-normally distributed data, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

4.4 Discussion

This is the first study which utilizes nanoscale flow cytometry to report the clinical utility of endothelial and platelet EVs (concentration and size) as biomarkers for HD-induced intradialytic cardiac injury. Our results showed that HD produced an increase in overall and small endothelial EVs over HD, which was consistent with our findings from our previous *in vivo* experiments in rats. Moreover, Pre-HD endothelial EV levels correlated with critical measures such as intradialytic RWMAs, and Pre-HD platelet EV levels correlated with HD-induced intra-HD hypotension.

Our results show that there are increases in endothelial EVs (total and small) and platelet EVs (large) over HD. These findings are consistent with past studies which also found increases in EV levels over HD.[32–35] Though there exist other studies that have reported no change or a reduction in EV levels over HD.[36–39] These discrepancies may result from using different EV markers (inducible or constitutive) of interest as well as methodology or study design. We chose an inducible marker of cell activation, E-selectin (CD62e), because high levels of CD62e+ EVs, have been associated with cardiovascular events in patients with stroke history. [27] This is compared to using constitutive markers such as CD31+/CD42-, which were not predictive of cardiovascular outcomes.[27] Thus, it is plausible that an inducible marker may better reflect HD-associated endothelial activation and injury. Moreover, De Laval *et al*. is the only study besides ours to use CD62e and report an increase in endothelial EVs over HD as we have here.[34] Additionally, our combination of CD62e and sensitive nanoscale flow cytometry methodology for EV size is valuable because even past studies among coronary artery and pulmonary hypertension patients have emphasized that specifically small sized (<500nm) CD62e+ EVs are expressed at higher levels and may predict poor clinical outcomes.[40,41]

Additionally, our study only used samples from mid-week HD sessions, whereas some studies which found decreases or no changes in EV levels over HD treatment, used early-week HD (defined by the long interdialytic period) plasma samples.[36,37,42] The long interdialytic period is associated with higher patient risk[43], due to factors such as volume status and electrolyte and acid-base status.[44] These may be confounding factors that

influence EV levels, which is why the mid-week HD samples we used may be more appropriate.

In addition to understanding the change in EV levels, we also wanted to understand what clinical implications these levels had for patients during their HD sessions. Our results showed that pre-HD large endothelial EV levels positively correlated with UFR. To our knowledge, only one other study has investigated the relationship between change in EVs and UFR. They found no significant differences, suggesting that UFR does not impact EV clearance over HD.[39] Our contrary decision to examine an association with UFR is because of its consideration as a potentially important factor for patient risk-assessments as higher UFR is associated with greater mortality.[29,31]

Our results also showed that patients with higher pre-HD platelet EV (total and small) levels had a greater decrease in blood pressure through HD. Hypotension is an important HD-related outcome because of its association with all-cause and cardiovascular mortality.[45,46] Previous studies have suggested that endothelial and platelet EVs are involved in regulation of blood pressure through the modification of vascular tone[47], however, to our knowledge we are the first to investigate the relationship between Pre-HD EVs (concentration and size) and intra-HD hypotension. In a study on sepsis (an inflammatory state that results in intense vasodilation and subsequent hypotension) patients exhibited higher levels of platelet EVs compared to non-septic patients[48]. Therefore, it is plausible that pre-HD platelet EV levels could also be used to risk stratify patients who are more susceptible to HD-induced hypotension.

Pre-HD endothelial EVs (total and small) also correlated with RWMAs experienced at peak-stress of HD. RWMAs are a contributor to the development of heart failure and increased mortality among HD-patients.[12] While a previous study has shown that pre-HD endothelial EV levels may be a robust independent predictors of cardiovascular mortality and all-cause mortality [23], to our knowledge, no study has investigated the association between pre-HD EV levels and intradialytic cardiac injury during corresponding HD sessions. Our observed correlation between Pre-HD endothelial EVs (total, small, and large) and intradialytic RWMAs are novel findings and show that Pre-HD endothelial EV

levels may risk-stratify patients who are susceptible to direct cardiac injury during HD. Past studies among heart failure patients have also shown that endothelial EVs may even predict future cardiovascular events[50], which further supports that endothelial EVs may even be useful biomarkers for heart failure risk-stratification.

When assessing pre-HD EV levels of those who experienced myocardial hypo-perfusion (segmental and global), we found no differences that were statistically significant. However, the overall trends, while not significant, were consistent with RWMA data, showing that those who had higher Pre-HD endothelial EV levels experienced more global and segmental myocardial hypo-perfusion.

Our results also show that patients had similar pre-HD endothelial and platelet EV levels within short-term blood sampling a week apart. This novel finding, previously uninvestigated, suggests that over a short-term period, weekly blood-sampling of patients may not be necessary. However, evaluation over longer time periods is required.

4.5 Limitations

There are some limitations in this study. We acknowledge that using patient samples from three different observational studies, which were powered for sample sizes to answer their own respective research questions related to HD-induced cardiovascular injury, may have not resulted in an adequate sample size for our investigation. Due to the lack of pilot studies investigating EV levels among HD patients with sensitive methodology (nFC) and intradialytic HD injury, there was no existing suitable pilot study data that would provide us with the details to determine appropriate sample size. Therefore, the results from this study will allow us to calculate a powered sample size for future studies. Another limitation within this study is that we did not elute dialyzers post-HD to confirm the findings we saw amongst our previous *in vivo* experiments with rats. However, past studies suggest that HD membranes adsorbed EV-associated protein, flotillin[39], which suggests that EVs are adhering to the dialyzer membranes. Future studies should elute dialyzers to investigate the impact of EV adherence to dialyzers and to properly account for how it may impact the change in EV levels over HD.

Additionally, we acknowledge that our focus on cardiac injury might be limiting since other vascular beds within the brain, liver, and kidneys also experience differing degrees of HD-induced hypo-perfusion and injury. Therefore, in the future, EV levels may need to be compared with other intradialytic organ-based injury as well.

4.6 Conclusion

In conclusion, the results from our study suggest that circulating endothelial and platelet EVs (concentration and size) have clinical utility among HD patients and may be associated with HD-induced cardiac injury. Additionally, the evaluation of EV levels taken directly at the beginning of HD sessions, and their association with important intradialytic measures showed that an endothelial and platelet EV based assay may have utility as a relatively inexpensive and efficient tool. It has the potential to monitor HD-induced cardiac injury, and thus warrants further investigation.

4.7 References

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Chapter 5

5 Overall Discussion

5.1 Summary

HD is a life-saving treatment for those with ESRD, however it is associated with complications such as hemodynamic instability and microvascular endothelial dysfunction, which lead to irreversible multi organ injury and increased risk of cardiovascular disease and death.[1–3] This type of vascular injury has been observed through gold-standard imaging methods.[4–6] However, these imaging modalities are complex, expensive, can be invasive, and require specialized equipment and technicians. Therefore, the overall aim of this thesis has been to develop and evaluate the clinical utility of endothelial and platelet derived EV biomarkers.

The first project focused on methodology to optimize nanoscale flow cytometry for data linearity and high throughput EV analysis. The second project focused on assessing the biological utility of EVs as biomarkers of HD-associated injury. By utilizing *in vitro* and *in vivo* methodology, we understood how the uremic milieu and HD modality itself affect EV concentration and size. The third project focused on the clinical applicability of an EV based assay. Through obtaining plasma samples from patients in observational HD studies and correlating EV levels with important measures and intradialytic cardiac injury, we determined whether EV concentration and size had clinical significance among HD patients.

The novel findings of this overall research study have shown us that an EV based assay does have clinical utility among HD patients. It has the potential to be used as an indicator for HD-induced vascular and organ-based injury, and therefore may offer clinicians and scientists a tool to assess and monitor patients. This work will guide future research studies to calculate appropriate sample sizes for further biomarker validation and development. This discussion chapter will be used to revisit and summarize the project

objectives, major findings, clinical impact, overall limitations, and future research directions.

5.1.1 Project 1: Analytical Considerations in Nanoscale Flow Cytometry of Extracellular Vesicles

A variety of methods exist for the enumeration and characterization of EVs. However, enumeration of EVs by commonly used conventional FC and other methods are limited as they are unable to detect EVs smaller than ~300-500 nm. Therefore, it is valuable to use specialized methodology such as a nFC, as it can enumerate EVs as small as ~80-180 nm. While nFC is a suitable tool to use, there are a lack of protocols that have assessed its potential for data linearity, as well as established pre-analytical guidelines. The objectives of this project included:

- To assess for massive coincidence (swarm effect) and linearity by validating flow rate and dilution effect among standards and plasma samples.
- To determine the effect of plasma centrifugation, plasma storage, and freezing and thawing of samples for EV analysis.

The important findings of this project included:

- We refined technical details regarding detection limits of nFC and the optimal range of settings on this instrument for analysis of EVs.
- We determined that the nFC produces linearity in results. The expected concentration of EVs is lost when flow rate is too low, when the events of interest are close to the size of noise, when the sample is incorrectly processed by centrifugation, and when the sample is not diluted.
- We established a pre-analytical pipeline which included appropriate flow rate (1.5µL/min), dilution factor (between 1/160 to 1/40), appropriate centrifugation for whole blood (twice at 2500 x g for 15 minutes), and storage conditions of plasma samples (ideally -80°C).

Through this project, we developed important guidelines that will allow us to use the nFC within a clinical laboratory environment to investigate EVs. Optimizing machine parameters and utilizing standard operating procedures are essentially a prerequisite that will allow for inter-laboratory reproducibility and validation of EVs as biomarkers.[7,8] Moreover, the findings from this project suggest that research groups investigating EVs must be transparent regarding the capabilities of their FCs, the bead composition and sizes used for calibration, and data linearity validation. This transparency will allow for a clearer understanding of the sizes of EVs investigated and comparisons made. Overall, this specific project is clinically significant because these guidelines can be applied to the study of EVs among various pathophysiological states.

5.1.2 Project 2: Utilizing *In Vitro* and *In Vivo* Methodology to Investigate the Biological Plausibility of Endothelial and Platelet EVs as Biomarkers of HD-Induced Vascular Injury

Past studies have demonstrated that endothelial and platelet derived EVs (100-1000 nm) are biomarkers that directly and indirectly reflect vascular injury in the form of endothelial dysfunction, increased inflammation, and increased coagulation.[9–15] However, previous studies have not clearly demonstrated the biological plausibility of EVs as biomarkers of HD-induced vascular injury using composite study design (*in vitro* and *in vivo*) and appropriate methodology (nFC) to enumerate EVs by concentration and size distribution (especially <500nm). The objectives of this project included:

- To assess the impact of the uremic milieu as a factor leading to endothelial (CD62e+) EV release and EV size distribution.
- To assess the impact of HD on endothelial (CD62e+) and platelet (CD41a+) EV (concentration and size) within a rodent model of HD.

The important findings of this project included:

- Small sized endothelial EVs (<500 nm) may be indicative of endotoxic stress.
- The collective uremic milieu did not impose a change in EV levels or size distribution *in vitro*.

- Rat plasma derived endothelial (CD62e+, total ($P<0.05$), and small ($P<0.01$)) and platelet (CD41a+, total ($P<0.05$)) EV levels increase over HD treatment.

- The dialyzers adsorbed both endothelial (CD62e+) and platelet (CD41a+) EVs, and therefore the change in EV levels through HD may be blunted.

- Intradialytic muscle perfusion ($P<0.05$) and mean arterial blood pressure ($P<0.05$) decreased over HD. There were no associations between change in intradialytic muscle perfusion and change in EV levels (small endothelial, CD62e+, EVs, r= -0.39, P=0.45; small platelet, CD41a+, EVs, r= -0.27, P=0.60). There were no associations between change in blood pressure and change in EV levels (small endothelial, CD62e+, EVs, r= -0.20, P=0.83; small platelet, CD41a+, EVs, r= -0.54, P=0.34).

Overall, this project demonstrated that small EVs are important indicators of inflammatory stress and HD-induced injury. These findings highlight that appropriate methodology is necessary to detect small sized EVs and suggests that conventional flow cytometers may be under-reporting this important population of EVs. The *in vivo* work demonstrated that HD, independent of animal renal pathology or CKD-related uremia, caused an increase to endothelial and platelet EV levels. This increase in endothelial and platelet EV levels may be the result of HD-related mechanical and shear stress, activation and aggregation of platelets to the blood roller pump or extracorporeal tubing, and bioincompatibility of the dialyzer membrane. [4–6,16–18] Our research also highlights that the dialyzer's adsorptive properties in relation to EVs is an important factor to investigate. To summarize, this project allowed us to elucidate the biological plausibility of using EVs as biomarkers for HD-induced vascular injury and we therefore suggest that future research should investigate EV sub-populations, as well as HD dialyzers to understand their impact on EV adsorption.

5.1.3 Endothelial and Platelet EVs as biomarkers of HD-induced vascular and cardiac injury

To understand the clinical utility of an endothelial and platelet EV-based assay for HD-induced cardiac injury, we utilized HD patient samples from observational studies. Using

these samples, we determined whether EV levels correlated with changes in blood pressure, ultrafiltration rate, intradialytic RWMAs, and intradialytic myocardial perfusion. The objectives of this project included:

- To determine the impact of HD on endothelial (CD62e+) and platelet (CD41a+) EV (concentration and size) levels among HD patients.
- To investigate the association between endothelial (CD62e+) and platelet (CD41a+) EV (concentration and size) levels and HD-induced intradialytic myocardial injury.

The significant findings of this project included:

- Endothelial (CD62e+, total ($P<0.05$) and small ($P<0.01$)) and platelet (CD41a+, large ($P<0.05$)) EV levels increased over HD.
- Pre-HD, large endothelial (CD62e+) EV levels correlated with UFR ($\rho=0.43$, $P=0.0064$).
- Pre-HD, total ($\rho= -0.41$, $P=0.019$), and small ($\rho= -0.39$, $P=0.026$) platelet (CD41a+) EV levels correlated with a change in systolic blood pressure over HD.
- HD patients pre-HD total ($r=0.82$, $P=0.025$), small ($r=0.77$, $P=0.043$), and large ($r=0.83$, $P=0.022$) endothelial (CD62e+) EV levels correlated with intradialytic RWMAs.

Altogether, this project demonstrated that an endothelial and platelet EV based assay may have clinical utility as a biomarker of HD-induced cardiac injury. We found an increase in endothelial (CD62e+) and platelet (CD41a+) EVs over HD, similar to our findings in project 2, which further suggests that the HD modality itself impacts EV levels. Moreover, we found that EV levels enumerated at the beginning of HD (Pre-HD) were associated with intradialytic hemodynamic instability and cardiac injury. Our results are novel because they show how endothelial (CD62e+) and platelet (CD41a+) EVs measured immediately before HD may indicate which patients are more susceptible to

HD-induced cardiovascular injury. These findings also suggest that using an EV based assay has potential to substitute for more expensive imaging modalities and may allow for more frequent monitoring of patients over a longer-term. However, we also note that while our study focused on analyzing EVs in categories of total (100-1000 nm), small (<500 nm) and large (>500 nm), further studies are needed to make additional conclusions regarding the clinical implications of each subset.

5.2 Overall Limitations

There are limitations with our overall study. The first project of this study assessed the nanoscale flow cytometer's potential for data linearity, as well as established pre-analytical guidelines to use this machine for high-throughput EV analysis. We focused primarily on standards and platelet (CD41a+) EVs for this project and did not carry out the investigation with focus on other cell-derived EVs. We focused on platelet (CD41a+) EVs because they are the most abundant EV in the plasma. By refining pre-analytical guidelines with focus on these EVs, we limit the potential of massive platelet EV-artifact creation in plasma, and thus have purer samples to investigate other cell-derived EVs. Additionally, EVs of all cellular origins have similar membrane composition (except for cell-specific antigens)[19], which means that they are technically enumerated in the nFC in similar manner. Additionally, inter-laboratory comparisons with other nFCs should be carried out for further machine validation.

The second project of this study involved the investigation of the biological utility of endothelial (CD62e+) and platelet (CD41a+) EVs (concentration and size distribution) as biomarkers of HD-induced vascular injury. We acknowledge that while our study showed that the uremic milieu (pooled 2% HD serum) may not impact EV levels, other higher concentrations of serum may need to be tested. Moreover, endothelial cells cultured in a single monolayer may not recapitulate some of the sheer stress of flowing blood through vessels. Therefore, future studies may also need to focus on using cell culture within microfluidic devices[20] and assess the impact of the uremic milieu and other uremic factors. Additionally, while our rat model provided insightful information regarding the effect of a single-HD session, it may be beneficial to compare with rats that were exposed

to multiple HD-sessions to further show the impact of chronic conventional HD. While currently there are no comparable rat models, it is likely that the design of such a study would not receive animal ethics approval due to the likelihood of severe injury to animals. Therefore, while animal models are beneficial to further understand HD and the effect on pathophysiology, we acknowledge the challenges which impact their ability to accurately reflect HD-patients.

The third project of this study focused on whether EVs are biomarkers of HD-induced cardiovascular injury among patients. Our sample size for this project included patients from three studies, which were powered for sample sizes to answer their own respective research questions relating to HD-induced cardiovascular injury. Thus, the overall sample size used for our investigation in this project may not have been adequate. However, our existing results highlight the usefulness of EVs with the utilization of appropriate EV methodology (nFC) and comprehensive study design, which has not yet been completed. Our overall study therefore provides the necessary information for future sample size calculations. Moreover, our investigation regarding cardiac injury is informative, but may be limiting as HD is also known to cause injury in other organs such as the brain, liver and kidneys. Therefore, investigation of EVs and other HD associated organ injury is needed.

5.3 Overall Conclusion

Through conducting a translational composite research study which focused on refining methodology, determining biological utility, and assessing clinical applicability, this project suggests that an EV based assay has clinical utility among HD patients. Ideal biomarkers are non-invasive, easily measured, inexpensive, and produce rapid results.[21] We believe an EV based biomarker fulfills these properties. There are many opportunities afforded by using the nFC for EV enumeration. This methodology requires only 10 µL of a patient's plasma sample, which is subsequently diluted 40 to 160-fold. Moreover, minimal (20 to 150 ng) pre-conjugated antibody is usually needed per sample, with sample results acquired within 3-4 minutes of nFC sample analysis. Therefore, this assay is beneficial to use because it is cost effective, efficient, and is relatively easy to

implement within a clinical setting, especially with the appropriate pre-analytical guidelines we established. Furthermore, the research findings from this thesis demonstrate that endothelial and platelet EVs have biological utility as markers of HD-induced microcirculatory stress and have the potential to stratify patients who may be more susceptible to HD-induced injury. By having a high throughput assay to assess and monitor patients, it also has the potential to guide clinicians and researchers to develop better treatments and interventions for HD to improve patient health.

5.4 Future Directions

5.4.1 EVs as bioactive molecules of intercellular communication

While this thesis demonstrates that EVs may be promising biomarkers, we acknowledge that EVs are also bioactive molecules with cargo (lipids, RNA, protein) and participate in cell-to-cell communication. [22–24] EVs are known to have "real-time" molecular signature of the physiological state of the parental cells and microenvironment they are released from. Various *in vitro* and *in vivo* studies have shown that the material delivered by plasma derived EVs to recipient cells can modulate inflammation, oxidative stress, thrombogenesis, vascular senescence, or endothelial dysfunction.[25–29]

Within the context of CKD, *in vitro* studies have found that EVs which are released from cells exposed to uremic substances, promote functional abnormalities in endothelial progenitor cells and TGF-beta mediated proliferation of vascular smooth muscle cells.[30,31] Furthermore, EVs taken from ESRD patients have been known to impair NO release and affect endothelium relaxation, which further influences endothelial dysfunction.[32] More specifically, micro-RNA (miRNA) within EVs may promote endothelial dysfunction and vascular injury. It was found the miR-92a- involved in atherosclerosis and cardiovascular disease is found within endothelial EVs from uremic patients.[33] However, it has also been found that EVs could potentially induce anti-inflammatory responses in recipient cells, as patients with unstable angina had EVs enriched with miR-19b, which exerts antithrombotic function through inhibiting tissue factor expression in endothelial cells.[34] Therefore, while this thesis has only viewed EVs within the context of their role as biomarkers, future projects are needed to understand

how EV levels, their cargo, and recipient cells may be affected. In the context of our research findings, it is plausible that the EVs (enumerated at the Pre-HD time-point among patients) and their cargo in combination with the stress of the HD-procedure further promotes endothelial injury, and thus lead to injury such as intradialytic RWMAs. Our next steps for this project have included submitting our observational patient study samples for analysis of miRNA data. We hope that this information will allow us to further understand EVs in the context as biomarkers and bioactive molecules.

To further understand the important role of EVs as biomarkers and bioactive molecules, our group is also planning to update our existing nFC with EV sorting machinery. Being able to sort EVs by size or antigen marker may allow us to further characterize and understand EV sub-populations, as well as determine the value of their cargo.

5.4.2 EVs may be biomarkers of responsiveness to HD intervention and other organ-based injury

Despite the negative side effects of HD, there are studies focused on HD related interventions to improve treatment. Some interventions include intradialytic exercise, and remote ischemic preconditioning. These interventions are known to improve vascular tolerability to subsequent HD-induced stress. It is imperative that future studies investigate whether EVs are biomarkers of responsiveness to interventions to further validate their clinical utility.

Intradialytic exercise (IDE) is an intervention that is accomplished through cycling on a low-impact stationary bike during HD. This form of exercise improves cardiac function by increasing left ventricular ejection fraction, reducing systolic pulmonary artery pressure, and right ventricular size.[35,36] Intradialytic exercise has also been shown to improve oxygenation, aerobic capacity, physical function, and arterial stiffness.[35,37,38] Some studies have assessed the EV phenotype of HD patients who underwent IDE. Smith et al. found that intradialytic exercise does not affect the phenotype of EVs but rather beneficially alters their proinflammatory function.[39] Although Highton et al. found that even after six months of regular IDE, there were no effects to EV levels and no changes to thrombotic or inflammatory status.[40] While studies regarding IDE and EVs have

produced contradictory findings, these studies primarily focused on EV levels in relation to inflammatory function, rather than HD-induced organ injury. The McIntyre lab has currently completed an exploratory, single group, observational study that investigates the impact of IDE on cardiac injury, investigating key features such as RWMAs and myocardial perfusion. This would be an ideal study to assess our biomarker assay and test the utility of EVs as biomarkers of responsiveness to intervention.

Remote ischemic preconditioning (RIPC) is an intervention that utilizes transient non-lethal episodes of ischemia to reduce the effect of subsequent larger insults.[41] RIPC can be achieved by applying brief episodes of ischemia, using a blood pressure cuff on a limb such as the arm or leg, remote from a target organ. Successful clinical studies have shown that RIPC provides protection against cardiac ischemia.[42] The McIntyre lab has recently finished a parallel-arm controlled trial to study whether RIPC intervention over the course of a year impacts brain perfusion, brain white matter ultrastructural changes, microglial inflammation, and neurocognitive assessment. Through utilizing the samples from this study, we would be able to understand whether EVs are biomarkers of responsiveness to RIPC, as well as determine the clinical utility of EVs as biomarkers of HD-induced brain injury.

Therefore, through utilizing our assay and assessing patients that have undergone HD interventions to improve vascular tolerability, it might allow us to further understand the clinical utility of EVs as biomarkers of injury and their potential as biomarkers of responsiveness to intervention.

5.4.3 Translation of EVs as biomarkers in clinical practice

Over the past decade, interest in EVs as circulating biomarkers has grown.[43] However, few EV biomarkers have been translated to clinical use. There is a disparity in the number of novel EV biomarkers that have been reported in the literature and the amount that are approaching or have progressed to clinical use.[44] An informative review by Yekula et al. highlights the pathway and the four phases to EV biomarker discovery and eventual clinical translation (Figure 5.1).

During phase one of EV biomarker discovery, biomarkers can be discovered either serendipitously, through focused study, or through large scale multiparametric screening of several EV biomarkers. This phase is important because limitation in aspects such as study design, methodology used, biofluid used (plasma or serum, saliva, nasal secretions, cerebrospinal fluid, and urine), sample quality, EV analysis, and data interpretation can contribute to the failure of biomarkers ever reaching clinical utility.[44]

During phase two, biomarker verification is accomplished to identify promising candidates for clinical use. Biomarkers must be verified across several studies within well-defined patient cohorts that are different from the original discovery cohort. Additionally, selection of appropriate matched controls based on the target population allows for further verification. Biomarker verification confirms biomarker potential, as well as establishes the role of the biomarker and whether it is for diagnosis, monitoring, and/or prognosis.[44]

For phase three, biomarker validation, biomarkers will have to be analytically and clinically validated. Validation includes determining assay accuracy, precision, sensitivity, specificity, linearity of results, reportable range, positive predictive value, and negative predictive value. Moreover, the validation stage helps to determine variables such as age, sex, race, and other factors that may impact biomarker discovery.[44]

The final phase is clinical translation. For this phase, laboratories that have been developing the biomarker over the previous stages must ensure that Good Laboratory Practice and Good Manufacturing Practices, which are standards regulated by the FDA and the International Organization of Standardization practices, are followed. This allows for eventual certification of laboratory standards. For biomarkers to progress to commercialization, the verified and validated assay needs to comply with additional rules and regulations. Commercialization can occur through marketing the assay for distribution as *in vitro* diagnostics, which are assays that can be purchased and used within the clinical setting of hospitals and laboratories. Alternatively, assays can be promoted as laboratory developed tests for clinical use where the assay is eventually used by one lab.[44]

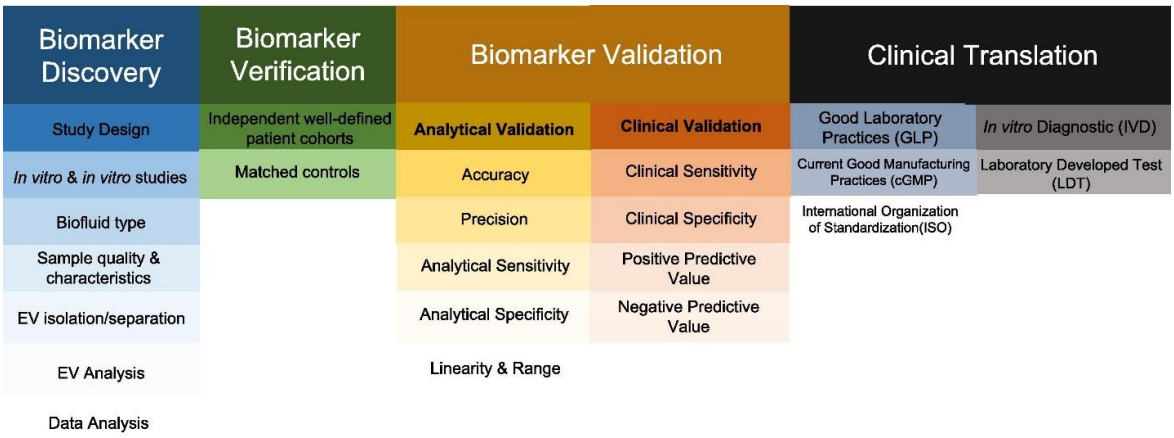

Figure 5.1 The pathway of EV biomarker discovery to clinical translation

There are four phases involved which include biomarker discovery, biomarker verification, biomarker validation, and clinical translation. Note: This figure was originally published online in *Methods. Yekula, A., Muralidharan, K., Kang, K.M., Wang, L., Balaj, L., and Carter, B.S. From laboratory to clinic: Translation of extracellular vesicle based cancer biomarkers. 2020; 177, 58-66. © 2020 Elsevier Inc.* This figure is being reproduced for education purposes only and not for any commercial use. This figure is included in the Ph.D. dissertation with attribution.[44]

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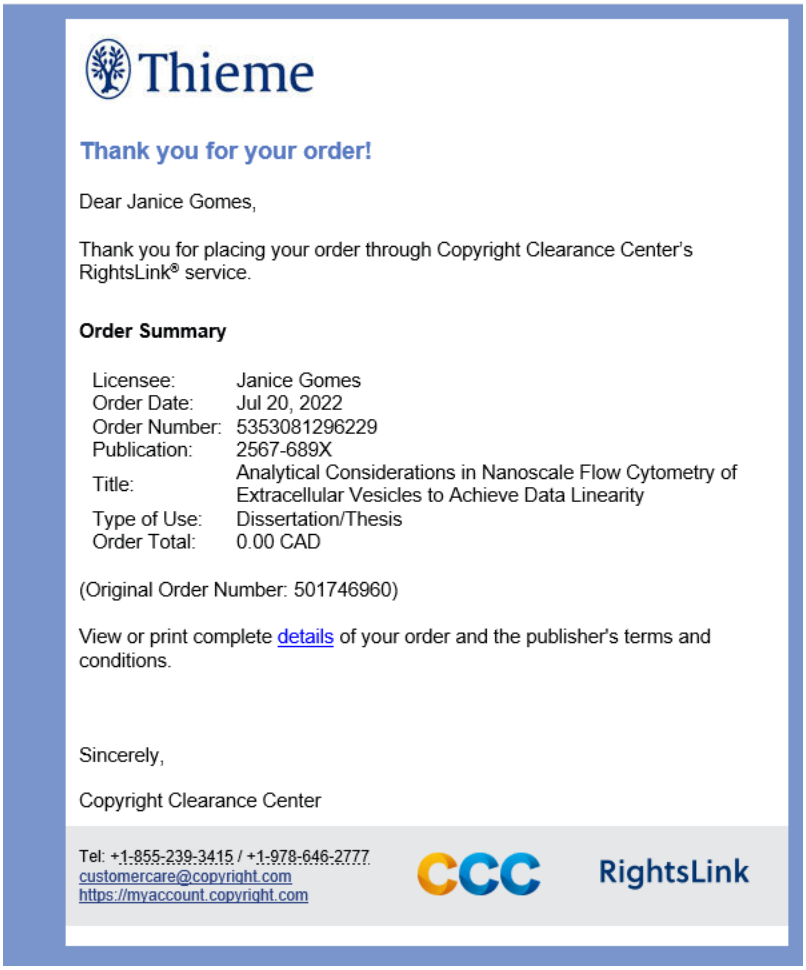
5.6 Appendices

Appendix A 1: Permission to Reproduce Gomes *et al.*, 2018 from *Thrombosis and Haemostasis*



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1. **Gomes, J**., Janssen, B.G.J., Zhang, Y.M., Hur, L., Penny, J.D., Dayarathna, T., Salerno, F.R., Pasternak, S., and McIntyre, C.W. (2022) Endothelial and Platelet Extracellular Vesicles as a measure of Hemodialysis-Induced microcirculatory stress. *(In preparation for submission)*
2. Anazodo, U., Wong, D.Y., Theberge, J., Dacey, M., **Gomes, J**., Penny, J.D., Ginkel, M.V., Poirier, S.E., and McIntyre, C.W. (2022) Application of Intradialytic Magnetic Resonance Imaging and Spectroscopy Demonstrates Hemodialysis-Related Acute Brain Injury. *Journal of the American Society of Nephrology (submitted)*
3. Schorr, M., Zalitach, M., House, C., **Gomes, J.,** Wild, C.J., Salerno, F.R., and McIntyre, C.W. (2022). Cognitive Impairment Early After Initiating Maintenance Hemodialysis: A Cross Sectional Study. *Front Neurol,* 15(13): 1-9.

4. **Gomes, J**.*, Lucien, F.*, Cooper, TT., Kim, Y., Williams, KC., Liao, T., Kaufman, L., Lagugne-Labarthet, F., Kenyon, O., Boysen, J., Key, NE., McIntyre, CW., and Leong, HS. (2018). Analytical Considerations in Nanoscale Flow Cytometry of Extracellular Vesicles to Achieve Data Linearity. *Thromb Haemost,* 118(9): 1612-1624.
5. Teatero, S., Neemuchwala, A., Yang, K., **Gomes, J.**, Athey, TBT., Martin, I., Demczuk, W., McGeer, A., and Fittipaldi, N. (2017) Genetic evidence for a novel variant of the pilus island 1 backbone protein in group B Streptococcus. *J Med Microbiol*. doi: 10.1099/jmm.0.000588.
6. Burns, KE., Jacob SK., Aguirre, V., **Gomes, J.,** Mehta, S., and Rizvi, L. (2016) Stakeholder Engagement in Trial Design: Survey of Visitors to Critically Ill Patients Regarding Preferences for Outcomes and Treatment Options during Weaning from Mechanical Ventilation. *Ann Am Thorac Soc*, 13(11), 1962-1968.
7. Teatero, S., McGeer, A., Li, A., **Gomes, J.,** Seah, C., Demczuk, W., Martin, I., Wasserscheid, J., Dewar, K., Melano, RG., and Fittipaldi, N. (2015). Population Structure and antimicrobial resistance of invasive serotype IV and group B Streptococcus, Toronto, Ontario, Canada. *Emerg Infect Dis*, 21(8), 585-591.

Abstracts and Presentations

1. **Gomes J**., Janssen, B., Penny, J., Hur, L., Pasternak, S., and McIntyre, C.W. Development of an Extracellular Vesicle-based biomarker of hemodialysis induced vascular injury. Pathology Research Day, London, Canada, 2022 (Poster Presentation)
2. **Gomes J**., Janssen, B., Dayarthna, T., Pasternak, S., and McIntyre, C.W. Development of an Extracellular Vesicle-based biomarker of hemodialysis induced vascular injury. Pathology Research Day, London, Canada, 2021 (Poster Presentation)
3. **Gomes, J.,** Grant, C., Qirjazi, E., Pasternak, S., and McIntyre, CM. Development of a microparticle-based bio-marker of hemodialysis induced vascular injury. *London Health Research Day, London, Canada, 2019. (Poster Presentation)*
4. **Gomes, J.,** Grant, C., Qirjazi, E., Pasternak, S., and McIntyre, CM. Development of a microparticle-based bio-marker of hemodialysis induced vascular injury. *Pathology Research Day, London, Canada, 2019. (Poster Presentation)*
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6. **Gomes, J**., Lucien, F., Kim, Y., McIntyre, CM., and Leong, HS. Development of a microparticle-based tool as an indicator of the effectiveness of dialysis treatment. *London Health Research Day, London, Canada, 2018. (Poster Presentation)*
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treatment. *Pathology Research Day, London, Canada, 2018. (Poster Presentation)*

8. **Gomes, J**., Lucien, F., Kim, Y., McIntyre, CM., and Leong, HS. Non-linearities in nanoscale flow cytometry of extracellular vesicles and standard. *International Society of Extracellular Vesicles Scientific Meeting, Toronto, Canada, 2017. (Poster Presentation)*
9. **Gomes, J**., Lucien, F., Kim, Y., McIntyre, CM., and Leong, HS. Development of a microparticle-based tool as an indicator of the effectiveness of dialysis treatment. *Pathology Research Day, London, Canada, 2017. (Poster Presentation)*
10. **Gomes, J**., Lucien, F., Kim, Y., McIntyre, CM., and Leong, HS. Non-linearities in nanoscale flow cytometry of extracellular vesicles and standard. *London Health Research Day, London, Canada, 2017. (Poster Presentation)*
11. **Gomes, J.**, and Brown, JCL. Do ageing theories based on animal studies apply to plants? The effects of exogenous H2O2 on annual and perennial flax. *Canadian Society of Zoologist Scientific Meeting, London, Canada, 2016. (Oral Presentation)*
12. **Gomes, J.,** Teatero, S., Low, D.E., McGeer, A., Fittipaldi, N. Distribution of pilus island among invasive Group B *Streptococcus* from the Greater Toronto Area. *Laboratory, Medicine, Pathobiology Summer Student Day, University of Toronto, 2013. (Poster Presentation)*