Cardiac Troponin-T Release within Small Extracellular Vesicles in Type 1 vs. Type 2 Myocardial Infarction

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Abstract

Distinguishing type 1 myocardial infarction (T1MI) vs type 2 myocardial infarction (T2MI) is challenging as both lead to cardiac troponin-T (cTnT) elevations. As the prognosis and treatments of T1/T2MI are distinct, accurate and timely diagnoses are crucial, but unavailable with standard cTnT testing. A cTnT release mechanism bound by small extracellular vesicles (sEV) was identified, possibly more associated with T2MI, which offers differentiating potential. Accordingly, 52 patient serum samples were collected, including healthy control, T1MI, and T2MI-Ischemic heart failure (HF) or non-ischemic hypertrophic cardiomyopathy (HCM). T1MI patients showed significantly higher absolute cTnT levels with sEVs containing 31.75±5.39% (mean±SEM). Comparatively, T2MI-HCM showed overall lower cTnT levels, and sEV proportion of 48.30±3.39%. The intermediate state, T2MI-Ischemic HF showed sEV cTnT to be present at 22.17±4.45%, more like T1MI. This early-stage analysis indicates that sEV cTnT displayed proportional variation across disease states consistent with alternate release profiles, which could assist in differentiating T1/T2MI.

Contributions

Preliminary research conducted within the laboratory of Dr. Peter Liu by Drs. Guo Hua Li, Guari Alkokar, and Mohammed Al-Khalaf generated the initial result of Cardiac Troponin-T localization within small extracellular vesicles. Further research conducted within the laboratory of Dr. Liu displayed an increase in the release of small extracellular vesicles in the context of cardiac remodelling. These preliminary findings have laid the groundwork for the examination of differential release patterns of cardiac troponin-T in various cardiac disease contexts. Human patient consent and sample collection was an integrated process with Sarah Plamondon, Dr. Derek So, and me. With training by the laboratory of Dr. Thomas Lagace, particularly Tanja Kosenko, I conducted small extracellular vesicle isolation via ultracentrifugation. Subsequent small extracellular vesicle characterization was conducted with guidance from Dr. Dylan Burger with the Zetaview 8.04.02 SP3 software located within the Burger laboratory. All western blot, immunoprecipitation, protein purification, and high sensitivity cardiac troponin-T analysis were solely conducted with guidance from Dr. Liyong Zhang and Qiujiang Du as needed. Mass spectrometry analysis of isolated samples was completed in collaboration with Drs. Anthony Gramolini and Uros Kuzmanov of the University of Toronto Cardiovascular Proteomics and Molecular Therapeutics laboratory with sample preparation and post-processing data analysis conducted at the University of Ottawa Heart Institute. Protocols followed were generated and adapted from previous Liu lab protocols as well as recent literature. Data was collected and subsequently analysed with the use of Microsoft Excel and Prism.

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Abbreviation List

Myocardial Infarction – MI
Cardiac Troponin-T – cTnT
Cardiovascular Disease – CVD
Cardiac Troponin-I – cTnI
Type 1 Myocardial Infarction – T1MI
Type 2 Myocardial Infarction – T2MI
Electrocardiogram – ECG
ST-Elevation Myocardial Infarction – STEMI
Non-ST-Elevation Myocardial Infarction – NSTEMI
Percutaneous Coronary Intervention – PCI
Coronary Artery Bypass Graft – CABG
Creatine Kinase – CK
High-Sensitivity Cardiac Troponin-T Assay – hs-cTnT
Left Ventricular Ejection Fraction – LVEF
Troponin C – TnC
Troponin I – TnI
Troponin T – TnT
Cardiac Troponin – cTn
Extracellular Vesicle – EV
Endosomal Sorting Complex Required for Transport – ESCRT
Vacuolar Protein Sorting-Associated Protein 4 – Vps4
Soluble N-ethylmaleimide-Sensitive Component Attachment Protein Receptor – SNARE
Small Extracellular Vesicle – sEV
Amyotrophic Lateral Sclerosis – ALS
Coronary Artery Disease – CAD
Sorbin and SH3 Domain Containing Protein 2 – SORBS2
Signal Transducer and Activator of Transcription 3 – STAT3
Vascular Endothelial Growth Factor – VEGF
Phosphate Buffered Saline – PBS
Immunoglobulin – IgG
Trichloroacetic-acid-Isopropyl Alcohol – TCA-IsoP
Dithiothreitol – DTT
Bovine Serum Albumin – BSA
PBS with Tween 20 – PBST
Liquid Chromatography Tandem Mass-Spectrometry – LC-MS/MS
Chronic Heart Failure with Ischemic Etiology – Ischemic HF
Hypertrophic Cardiomyopathy – HCM
Body Mass Index – BMI
Estimated Glomerular Filtration Rate – eGFR
New York Heart Association – NYHA
Left Anterior Descending Artery – LAD
Right Coronary Artery – RCA
Left Circumflex Artery – LCx
EV-Free Supernatant – SUP

1. Introduction

1.1. Burden of Cardiovascular Disease

Globally, cardiovascular disease (CVD) is a principal cause of mortality, reduced life expectancy, and quality of life while also imparting a significant cost to the healthcare system[1,2]. Over the course of forty years, from 1990 to 2019, cases of CVD have nearly doubled from 271 million to 523 million per year[1]. Additionally, deaths as a result of CVD have risen within this period from 12.1 million to 18.6 million annually[1]. Consistent with the global pattern of a high prevalence of CVD, North America is no exception[3-5]. Within Canada, CVD was the second leading cause of death in 2022 accounting for 30% of annual deaths, equivalent to a mortality of 197 deaths per 100, 000 in population, while 16% of total hospitalizations were a result of CVD[3,5]. Similarly, in the United States, the increasing trend of CVD is expected to prevail with an estimated 184 million people experiencing CVD by 2050, up from 45 million in 2020, continuing its role as a leading cause of death for both men and women[4,6]. Risk factors for CVD including hypertension, diabetes, and obesity are also expected to increase over this period, further contributing to its burden[4].

CVD serves as an umbrella term for many different diseases of the cardiovascular system. Hypertension afflicts a quarter of the population of North America, while 10% of adults have been diagnosed with coronary artery disease – including myocardial infarction (MI) and angina pectoris[6-10]. A history of stroke is present in 2% of the population and 6 million people in the United States and 750, 000 people in Canada have a diagnosis of heart failure (HF)[6-10]. While risk factors, including hypercholesterolemia, poor diet, inadequate physical activity, and smoking prevalence have declined and are expected to continue trending in this direction, CVD will prevail as a predominate health burden for the foreseeable future due to

the prevalence of additional comorbidities including diabetes, obesity, changes in dietary contents, mental stress, and an aging population[4,11].

1.2. Type 1 and Type 2 Myocardial Infarction

MI is one of the most notable CVDs as it afflicts a great number of individuals per year, while also being responsible for a significant number of CVD related deaths[4,6,11]. MI can be defined as the presence of acute myocardial injury indicated by the abnormal elevation of cardiac biomarkers, namely increases in cardiac troponin T (cTnT) or cardiac troponin I (cTnI) levels above a 99th percentile normal upper reference limit, along with a rise and fall of such troponin values in the setting of acute myocardial ischemia[12-14].

Clinical diagnoses of MIs can typically be separated into distinct subtypes; Type 1 MIs (T1MI) and Type 2 MIs (T2MI), along with Type 3 MIs where cardiac death occurs prior to biomarker detection, and Type 4 and 5 MIs which are resultant of cardiac procedures; percutaneous coronary intervention or coronary artery bypass graft surgery respectively [12,15,16]. Subsequent discussion in this thesis will focus on T1 and T2MIs.

A T1MI is resultant from atherothrombotic coronary artery disease which is triggered by a disruption, either rupture or erosion, of the atherosclerotic plaque (Figure 1A)[16,17]. Upon plaque rupture, the highly thrombogenic lipid core is exposed to the clotting factors in the blood, including platelets, red blood cells, and fibrin[16,17]. This leads to the subsequent formation of the thrombus within the coronary artery, obstructing blood flow and oxygen delivery to the distal cardiac tissue, resulting in cardiomyocyte death[16,17]. Further classification is integrated into a T1MI diagnosis by classifying electrocardiogram (ECG) findings into either ST-Elevation Myocardial Infarction (STEMI) or non-ST Elevation Myocardial Infarction

(NSTEMI)[18,19]. As the name suggests, with a STEMI, the ECG will display shifts and elevations of the ST-segment, with more profound shifts or multiple ECG leads being involved indicating the extent of the myocardial ischemia is more severe[16,18,19]. Conversely, in an NSTEMI, ST-segment depressions as well as T-wave inversions may be observed which indicates the reduction in blood flow and associated myocardial ischemia[16,18,19]. While treatment between STEMI and NSTEMI remain similar, typically percutaneous coronary intervention (PCI) or surgical revascularization (coronary artery bypass graft (CABG)), diagnosis is critical due to the absolutely urgent requirement of treatment for STEMI patients, within thirty minutes of initial medical contact for primary thrombolysis and ninety minutes for PCI[16,18-20]. While treatment must also be provided to NSTEMI patients in a timely manner, the time frame is longer in terms of days rather than minutes as the atherothrombotic obstruction of the coronary artery is generally incomplete; only roughly 25% of NSTEMI's present as a complete occlusion, and thus the time window for relief of ischemia is wider[16,18-21]. Epidemiologically, NSTEMIs have an increased prevalence which seems to be showing a moderate increase, while STEMI presentations are less common, and their prevalence has been recently observed to be decreasing[21-23].

Conversely, in a T2MI there is usually no association with the acute rupture of an atherosclerotic plaque leading to atherothrombosis[15,16]. Rather, T2MIs are defined more broadly as myocardial injury in the context of an imbalance between the oxygen supply provided to the myocardial tissue and the related demand[15,16]. This disparity in oxygen supply and demand may be a result of many variables which must be considered during clinical diagnostic process[15,16]. Notably, myocardial ischemia may arise due to a reduction in perfusion to the myocardial tissue caused by a coronary atherosclerotic plaque in the absence of its

rupture, full occlusion, or coronary artery spasm leading to a reduction in blood flow, coronary artery embolism or dissection, severe brady- and tachyarrhythmias, septic shock, amongst other causes[15,16]. Additionally, chronic release of cTnT indicating myocardial injury condition may also be observed in T2MI settings, primarily because of ongoing heart failure or cardiomyopathy (Figure 1B)[15,16]. The patient population which typically experience T2MI's differs significantly from those who suffer from T1MI[15,16,24-26]. Patients diagnosed with T2MI are generally older, largely female, with lower overall cTnT or cTnI levels, and present with more comorbidities including prior MI and PCI, heart failure, hypertension, chronic renal failure, and diabetes[15,16,24-26]. Due to the varied nature and etiology behind the development of a T2MI, there is not an established diagnostic criterion, or a set treatment algorithm that can be followed for each patient[15,16,24-26]. However, to control the increased myocardial demand, a beta-blocker may be administered to reduce the strain on the heart and thus limit the oxygen supply/demand imbalance[15,16,24-26]. Subsequent treatment recommendations follow the appropriate guidelines based on the underlying diagnosis which led to the T2MI[15,16,24-26].

The incidence of both T1MI and T2MI have seen significant reductions in recent years, with T1MIs decreasing from 202 to 84 per 100,000 from 2003 to 2012 and T2MIs decreasing from 130 to 78 per 100,000 in the same period[27]. These reductions display a shift in the prevalence of these forms of MI, now with a relatively equal incidence[27]. However, mortality rates vary between T1MI and T2MI, with patients diagnosed with T2MI displaying higher rates of inpatient, 30-day, and 1-year mortality as well as 30-day major cardiovascular events[27,28]. This increased mortality and adverse event rate is a result of elevated rates of comorbidities within the patient populations diagnosed with T2MI, a lack of definitive

diagnostic tool, or a direct etiological insight into cTnT elevation at the time of presentation[27,28].

With the etiology and outcomes of T1MI and T2MI being shown to vary, the clinical presentation remains relatively consistent between disease states which includes discomfort/pain in the chest, upper extremity, mandibular region, or epigastric region, as well as dyspnea, and fatigue with or without exertion[15]. Additionally, ST elevations are noted to be observed in certain T2MI cases, however, more often T2MIs are diagnosed as NSTEMI's, possibly leading to unnecessary or inappropriate treatment[15,28]. This overlap in presentation, diagnostic criteria, and risk factors, along with a lack of a gold standard method to differentiate these disease states makes identifying T1MI vs T2MI in an efficient and accurate manner a complex clinical challenge[15,16,28]. Studies have suggested that symptoms can vary between T1MI and T2MI, including chest pain being more prevalent in T1MI, and dyspnea, pulmonary crackles, leg edema, and cardiomegaly being more prevalent in T2MI[15,29]. However, these symptoms are not distinct to either disease state and are not able to be used solely as differentiating characteristics[15]. Biomarker as well as ECG assessments are crucial diagnostic parameters in each disease state, however, they do not vary significantly between T1MI and T2MI and thus also are not able to be utilized as a differentiator[15]. The most effective method to characterize these conditions is with the use of coronary angiography[15]. This also comes with drawbacks, in that it is an invasive imaging technique, accompanied by risk and co-morbidity, along with wasted resources and delayed proper diagnosis[15,16].

The initial working diagnosis in patients presenting with indicators of acute myocardial injury, outside of any clear contradictory clinical information, will be a T1MI[15,16]. Subsequent treatment planning and procedural care will follow the route of a T1MI, that being primary

PCI[15,16]. Moreover, physician accuracy in differentiating T1MI vs T2MI based on clinical information is as low as 60%[30]. Thus, improved diagnostic tools must be developed to assist in increasing the accuracy of T1MI and T2MI diagnoses for patients at high risk for death and complication, thereby improving subsequent patient management and treatment planning, anticipating better outcomes than observed today.

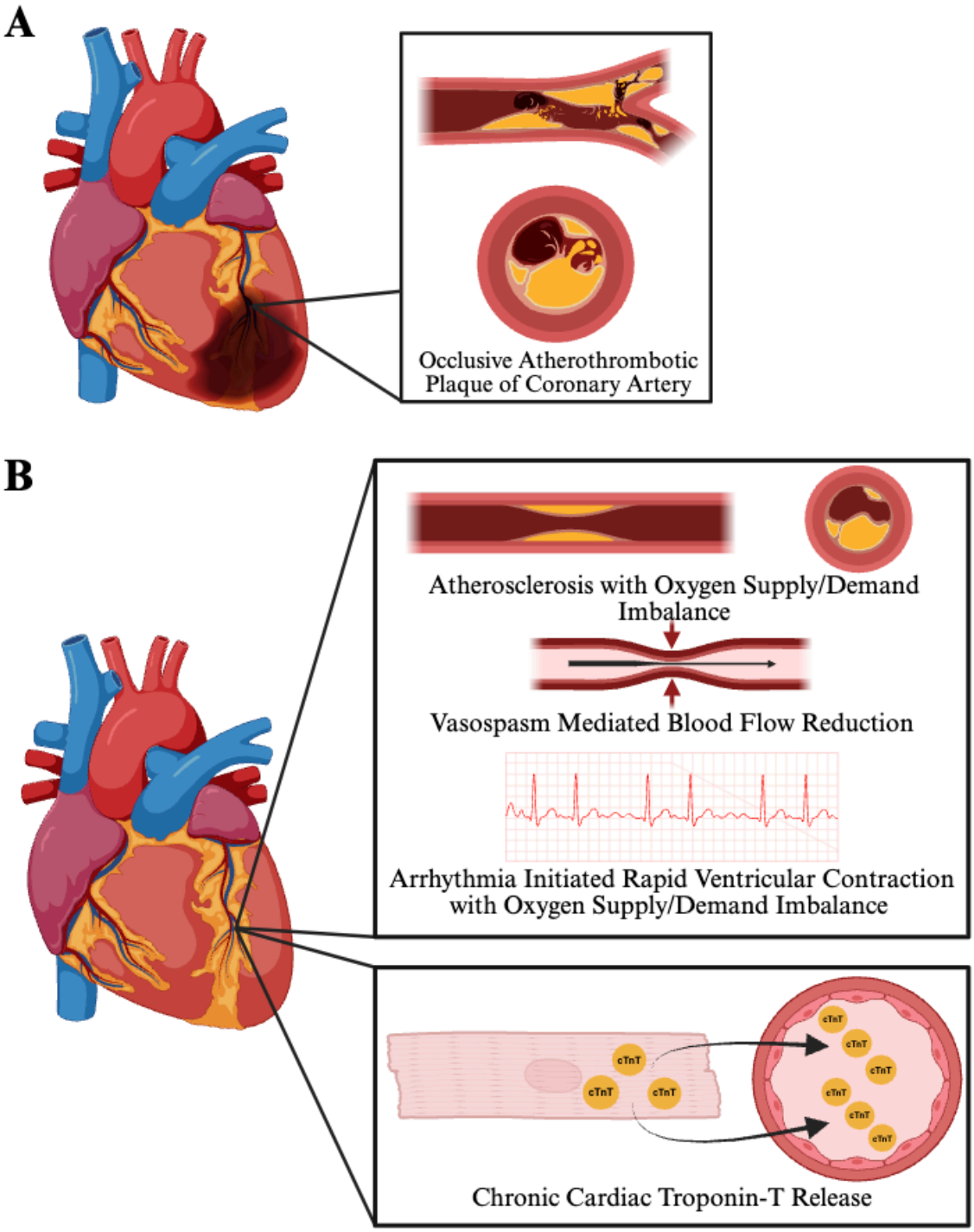

Figure 1 Type 1 and Type 2 Myocardial Infarction (A) Type 1 Myocardial Infarction. Resultant from the rupture of the atherosclerotic plaque and subsequent thrombosis, leading to distal myocardial ischemia. **(B)** Type 2 Myocardial Infarction. Resultant from various etiologies, namely fixed atherosclerotic plaque, vasospasm, arrhythmia, and chronic cTnT release. Figure developed using Biorender platform.

1.3. Biomarkers and Prognostic Markers of Myocardial Injury

During the diagnostic process when a patient presents with symptoms of MI, various examinations and laboratory tests are administered, notably ECG as well as biomarker analyses[16]. Biomarker assays are used to detect the evidence of myocardial injury as the lack of perfusion will result in cardiomyocyte membrane rupture and subsequent release of intracellular molecules freely into the bloodstream[16,31,32]. There are various molecules that can be used as biomarkers of MI, with creatine kinase (CK) and CK-MB, as well as the cardiac troponins, cTnT and cTnI, being the most popular and accurate[29,30]. Creatine kinase is an enzyme which is implicated in the energy production processes through transforming ATP and creatine into ADP and creatine-phosphate[32,33]. This enzyme consists of two subunits, M and B, which can dimerize in three different forms: CK-BB, CK-MM, and CK-MB[32,33]. The CK-MB form is the most common within muscle tissues, and thus in the myocardium[32-34]. Due to this increased specificity to the myocardial tissue, the CK-MB form is more commonly used as the cardiac biomarker[32-34]. However, CK-MB is still not solely cardiac specific as it is also present within skeletal muscle[32-34]. This can lead to increased levels during trauma or inflammation which will result in a reduction in the accuracy of myocardial injury assessments[32-34]. While CK-MB is capable of detecting large amounts of myocardial damage, it is limited in detecting less severe damage due to its high molecular weight, which reduces its sensitivity to detect all forms of myocardial injury, including early or stuttering presentation, leading to delays in definitive diagnosis[32-34]. Therefore, CK-MB assays have largely been replaced by the more sensitive troponin tests[32,35-37].

Currently, high sensitivity cTn's (hs-cTn) are the gold standard biomarker used in the diagnosis of acute MI[32,35-37]. Troponin molecules are present within myocytes, with TnT and

TnI having cardiac-specific isoforms which make them effective cardiac biomarkers, while Troponin C (TnC) is not solely specific to the cardiac tissue and thus is not readily utilized[32,35-37]. As both cTnT and cTnI are predominately found within the myocardial tissue, this provides an increased specificity; when a hs-cTnT/I result is above the 99th percentile upper reference limit, it will most likely be a result of myocardial necrosis[32,35-37]. Cardiac troponins are located within two different pools in cardiomyocytes, the cytosolic pool and in the contractile apparatus[32,38,39]. In the early phases of myocardial injury, the cytosolic pool of cTn's are released into the peripheral blood, while the cTn's within the contractile system follow later as the actin-myosin complexes must diffuse before being released[32,38,39]. In tandem with increased specificity of cTn's to the myocardium, sensitivity is also increased with novel hs-cTn assays as very small quantities of cTn can be detected; 2ng/L and 3ng/L in hs-cTnI and hs-cTnT respectively[32]. As such, with cTn's as a biomarker, less severe or early myocardial injury is detectable, generating a more accurate clinical diagnosis in a much more timely fashion[32]. Hs-cTn's are very effective diagnostic tools for acute MI, there are a number of non-cardiac disease states that may generate positive tests, including acute pulmonary edema and embolism, cardiotoxic drugs, chronic renal failure, pulmonary hypertension, and sepsis, which must be taken into consideration during clinical assessment[32,35-37]. To a certain extent, these are conditions that resemble T2MI[15,16].

Hs-cTn levels have also shown value as prognostic markers[40-42]. In patients diagnosed with acute coronary syndromes, a positive correlation was observed with increased cTn levels and adverse outcomes[40-42]. At 30-day, 6-month, and 1-year follow-ups, higher quantities of cTn were linked to elevated mortality rates[40-42]. Peak hs-cTn concentrations also correlate with reduced left ventricular ejection fractions (LVEF) and increased infarct size after acute MI[43,44].

In patients diagnosed with STEMI, elevated hs-cTnT values displayed a predictive reduction in LVEF and concomitant increase in infarct size, as would be expected with greater peak cTn release indicating the extent of myocardial injury is more severe[43,44].

1.4. Canonical Role of Cardiac Troponins

Troponin molecules are found within the striated muscle, this includes both the cardiac and skeletal muscles[45-47]. In each different muscle type, troponin plays an integral role in the regulation of Ca^{2+} during the contraction/relaxation cycle[45-47]. Troponin consists of three unique subunits, Troponin C (TnC), Troponin I (TnI) and Troponin T (TnT), each of which carry out distinct roles in muscular contraction[46,47]. Each subunit presents unique isoforms dependent on their tissue of origin, with TnC being an exception and lacking this specificity due to cardiac-TnC (cTnC) also being located within slow-twitch skeletal muscles[48]. Hereafter, the cardiac-specific troponins (cTns) will be discussed in detail.

cTnC is the subunit which binds directly to the Ca^{2+} molecule[47,49,50]. The cTnC molecule has one low-affinity binding site on its N-terminus which has high specificity for Ca^{2+} [47,49-51]. The cardiac troponin I (cTnI) subunit is the inhibitory subunit of the cTn complex[47,49,50]. There are two principal regions of the cTnI molecule, the switch peptide which is crucial for stabilizing the open conformation of the cTnC molecule when bound to Ca^{2+}, and the inhibitory region[49,52,53]. The latter interacts tightly with actin in the absence of Ca^{2+} to secure the position of tropomyosin thereby preventing myosin binding sites from being exposed and thus the binding of myosin to actin and the formation of cross-bridges[49,54]. The final subunit is cardiac troponin T (cTnT), which serves as the tropomyosin binding region, the fundamental

coordinating link between the cTn-tropomyosin-actin complex ensuring it remains contiguous[49,55].

During systole, cardiomyocyte depolarization results in an intracellular increase in the Ca^{2+} levels. This released Ca^{2+} then binds to the N-terminal binding site of cTnC which initiates the muscular contraction through actin (thin) filament activation[47,49-51]. Calcium binding initiates actin activation by reducing the stability of the closed conformation of the molecule and induces an open structure[47,49-51]. This exposes hydrophobic residues and results in an increased interaction with the cTnI subunit switch-peptide[47,49-51]. The increased interaction with the switch-peptide reduces the interaction of cTnI's inhibitory peptide with the actin molecule[47,49-51]. Subsequently, tropomyosin activity is increased, exposing myosin binding sites on the actin filaments, allowing for the formation of actin-myosin crossbridges[47,49-51]. Cross-bridge formation induces an allosteric impact by increasing the affinity of Ca^{2+} binding to cTnC, which induces further cross-bridge formation and myocyte contraction force[49,56-58]. Once the contraction is complete, Ca^{2+} is released from the cTn complex and re-sequestered into the sarcoplasmic reticulum, followed by a return of cTnC to its closed conformation and increased interaction of the inhibitory cTnI subunit with the actin molecule[49].

While cTn plays a crucial role in the contraction processes of the cardiomyocytes due to this interaction with the myofibril, alternate pools of cTn have been identified[38,39]. The great majority of cTn within the cardiomyocyte, as expected, is found bound in the contractile complex associated with the actin-myosin filaments[38,39]. However, pools of both cTnT as well as cTnI have been detected within the cytosol[38,39]. The cytosolic fractions account for roughly 2-4% of the total cTnI and approximately 6-8% of the total cTnT found within the

cardiomyocyte[38,39,48]. However, the definition of these as purely cytosolic pools has been debated as both cTnT and cTnI have relatively low solubilities in the hydrophilic environment of the cytosol[38,59]. It has since been hypothesized that a more accurate description would be cTn fractions loosely bound to the contractile myofibril complex[38,59].

1.5. Isoformic Variation Observed in Cardiac Troponin-T

Each troponin molecule within the cardiac tissue carries out a distinct and necessary role, however, there is variability that can be observed within these molecules, namely cTnT[60-62]. The gene which expresses cTnT is composed of 17 exons[60-62]. Two 5' exons which constitute the hypervariable region, exons 4 and 5 containing 15 and 30 nucleotides respectively, can be alternatively spliced during transcriptional processing[60-62]. Through this alternative splicing process, exons 4 and 5 can either be included or excluded from the final mRNA transcript[60-62]. The final mRNA transcript is translated to produce the cTnT protein including or excluding the respective amino acids of exon 4 and 5[60-62]. This alternative splicing process leads to the generation of four different cTnT isoforms; $cTnT_1$, $cTnT_2$, $cTnT_3$, and $cTnT_4$[60-62]. The $cTnT_1$ isoform contains both exon 4 and 5 post-transcriptionally (Figure 2A), $cTnT_2$ is missing exon 4 only (Figure 2B), $cTnT_3$ is missing exon 5 only (Figure 2C), while $cTnT_4$ is missing both exons 4 and 5 (Figure 2D)[60-62].

The inclusion/exclusion of these exons has also been shown to impact the functional capacities of cTnT isoforms, particularly with regard to Ca^{2+} sensitivity and activity of the tropomyosin-activated myosin ATPase[60-62]. Within the cTnT molecule, the variable N-terminal region was found to have a significant contribution to the Ca^{2+} sensitivity in force development[60-62]. This primarily acted through the alternatively spliced exon 5 and occurred in

a charge-dependent manner with a greater charge leading to an increased Ca^{2+} sensitivity[60-62]. Consequently, $cTnT_1$ and $cTnT_2$ express increased sensitivities to Ca^{2+} compared to $cTnT_3$ and $cTnT_4$ resulting in a reduced quantity of Ca^{2+} needing to be released to generate an effective contraction[60-62]. Additionally, in the absence of either exon 4 or 5, there is increased cooperativity with the *p*Ca force relationship[60-62]. Interestingly, each of the four cTnT isoforms generated similar maximal actin-tropomyosin-activated myosin ATPase activity[60-62]. While isoforms which contained exon 5, $cTnT_1$ and $cTnT_2$, showed an impaired ability to inhibit such ATPase activity[60-62]. Moreover, isoforms containing exon 5 also display reduced relaxation which may implicate exon 5 in preventing the inhibition of force generation and ATPase activity[60-62].

Interestingly, these isoforms are divergently expressed during development and in certain disease states[61-63]. During fetal development, the $cTnT_1$ and $cTnT_4$ isoforms are expressed highly, with $cTnT_2$ being expressed at a low level[61-63]. Throughout the perinatal cardiac development period, $cTnT_3$ expression increases while the isoforms expressed in the fetal state decrease, resulting in $cTnT_3$ being the only cTnT isoform present within the healthy adult human heart[61-63]. The alteration of this normal cTnT profile with the adult heart has been linked to heart failure and cardiac hypertrophy[61-64]. Primarily, there is a reappearance of the $cTnT_4$ isoform which was previously involved in fetal cardiac troponin complexes[61-64]. This cTnT isoformic shift to a fetal state is consistent with other observations during cardiac remodelling and hypertrophy processes[65-67]. The presence of reversions of gene expression patterns and molecular alterations that resemble a fetal phenotype impacting surface and internal structure as well as metabolic processes have been identified in these disease contexts[65-67]. The underlying cause of fetal gene reactivation and concomitant suppression of

adult genes is not entirely clear, with suggestions stating that it is a response to cope with the negative impacts of the cardiac remodelling process, however, this process results in further cardiac dysfunction[65-67].

Figure 2 Isoformic variation of Cardiac Troponin T. (A) $cTnT_1$ contains both exons 4 and 5 **(B)** $cTnT_2$ contains exon 5 while exon 4 is absent **(C)** $cTnT_3$ contains exon 4 but exon 5 is absent **(D)** $cTnT_4$ is absent of both exons 4 and 5. Tropomyosin (Tm), cTnI, and cTnC binding regions displayed on each cTnT isoform. Figure developed using Biorender platform.

1.6. Extracellular Vesicles and their Biogenesis

Extracellular vesicles (EVs) are small lipid bound vesicles which all cells release into the extracellular environment[68,69]. EVs can range in size from ~20nm to well over 5000nm[68,69]. Release can occur under the processes of normal cellular functioning; however, it is commonly observed to be upregulated in many pathologic contexts[68-70]. There are multiple subtypes of EVs that are identified and described based on their morphology, biogenesis, and mechanism of release from the cell: exosomes, microvesicles, apoptotic bodies, along with the more recently identified exophers and blebbisomes (Figure 3)[68-71].

Exosomes are the smallest EVs, typically ~40-160nm in diameter[68-71]. Exosomes are derived from the endocytic pathway and their formation begins with the invagination of the cytoplasmic membrane which leads to the creation of the early endosome[68-71]. During the maturation of the early endosome, multivesicular bodies will be generated within this vesicle[68-71]. Throughout the maturation process to a multivesicular body, the endosomal membrane will further invaginate on itself, leading to the creation of intraluminal vesicles within the maturing endosome[68-71]. Crucial in this process is the endosomal sorting complex required for transport (ESCRT) machinery, namely ESCRT-0, -I, -II, -III along with an associated AAA ATPase vacuolar protein sorting-associated protein 4 (Vps4) complex which recognizes and sorts ubiquitinated proteins into the intraluminal vesicles[68-73]. ESCRT-0 retains the ubiquitinated proteins in the late endosomal membrane, and ESCRT-I and -II initiate the invagination of the endosomal membrane[73,74]. Subsequently, ESCRT-III constricts the budding membrane and AAA ATPase Vps4 drives cleavage of the vesicle from the membrane[73,74]. Multivesicular bodies have two potential routes they can follow within the cell, targeting to the lysosome for degradation or trafficking and subsequent fusion with the plasma membrane to release its

contents[68-72,74]. However, it remains poorly understood what targets these vesicles to be degraded or released[68-72,74]. Studies have indicated this process may be dependent on posttranslational modifications of the cargo proteins, cellular conditions, or pathologic conditions[74-76]. Multivesicular bodies which do make their way to the plasma membrane to release their contents are transported via kinesin molecular motor proteins along intracellular microtubules[74-76]. Upon multivesicular body arrival at the plasma membrane, soluble N-ethylmaleimide-sensitive component attachment protein receptor (SNARE) protein machinery facilitates their subsequent fusion[74-76]. This fusion promotes the release of the intraluminal vesicles into the extracellular space, now defined as exosomes[74-76].

Microvesicles display a much greater range in size, spanning from ~20 nm all the way to 1000 nm[68-71,74]. However, microvesicles form in a manner that is not as well understood compared to exosomes[68-71,74]. Microvesicles follow a process of directly budding and separating from the plasma membrane[68-71,74]. Direct membrane release is not a uniform process as various mechanistic profiles have been identified[68-71,74]. Namely, the ESCRT complex has been implicated in vesicle production where high quantities of cell surface proteins are located, while acid sphingomyelinase has been identified in ceramide-dependant microvesicle formation[74,77-80]. Non-apoptotic blebbing of the plasma membrane has been identified, as well as cytoskeletal rearrangement induced membrane budding cleavage[74,77-80].

Apoptotic bodies are commonly the largest EVs with a size range of ~50-5000 nm, with many being observed on the larger end of the spectrum[68-71]. Apoptotic bodies are formed and released from dying cells[68-71]. Plasma membrane rupture and contraction result in a dissolution of the cellular membrane causing the formation of this EV subtype[68-71].

Exophers are large vesicles, ranging in size from ~1000 to over 5000nm[81,82]. These vesicles have been observed to contain organelles, large protein complexes, soluble proteins, along with various cytosolic molecules[81]. Their release in response to cellular stress is crucial to maintain homeostasis within the secreting cells by removing damaging protein aggregates or damaged organelles[83]. Exopher secretion follows a similar mechanism to microvesicles whereby they directly bud off the cell membrane and are released into the extracellular space[81,82].

Blebbisomes are very large membrane-bound vesicles which can reach sizes of ~20μm while also displaying their own blebbing activity[82]. They often contain active organelles including mitochondria; however, they lack an active nucleus[82]. Moreover, they display the capability to release and take up other extracellular vesicles including exosomes and microvesicles[82]. Functionally, blebbisomes contain a variety of proteins, mRNA, and miRNA signalling molecules, facilitating their role as communication units to modulate the extracellular space and corresponding cells[82].

A distinction must be made when discussing EVs collected from clinical samples. Since there is an overlap is size ranges between the different EV groups, and a lack of clear determination of the release mechanism of the vesicle, it is not possible to define vesicles collected within a size range solely as one of these categories. Moving forward, EVs typically found within the size range of exosomes (40-160nm), will be referred to as small extracellular vesicles (sEVs).

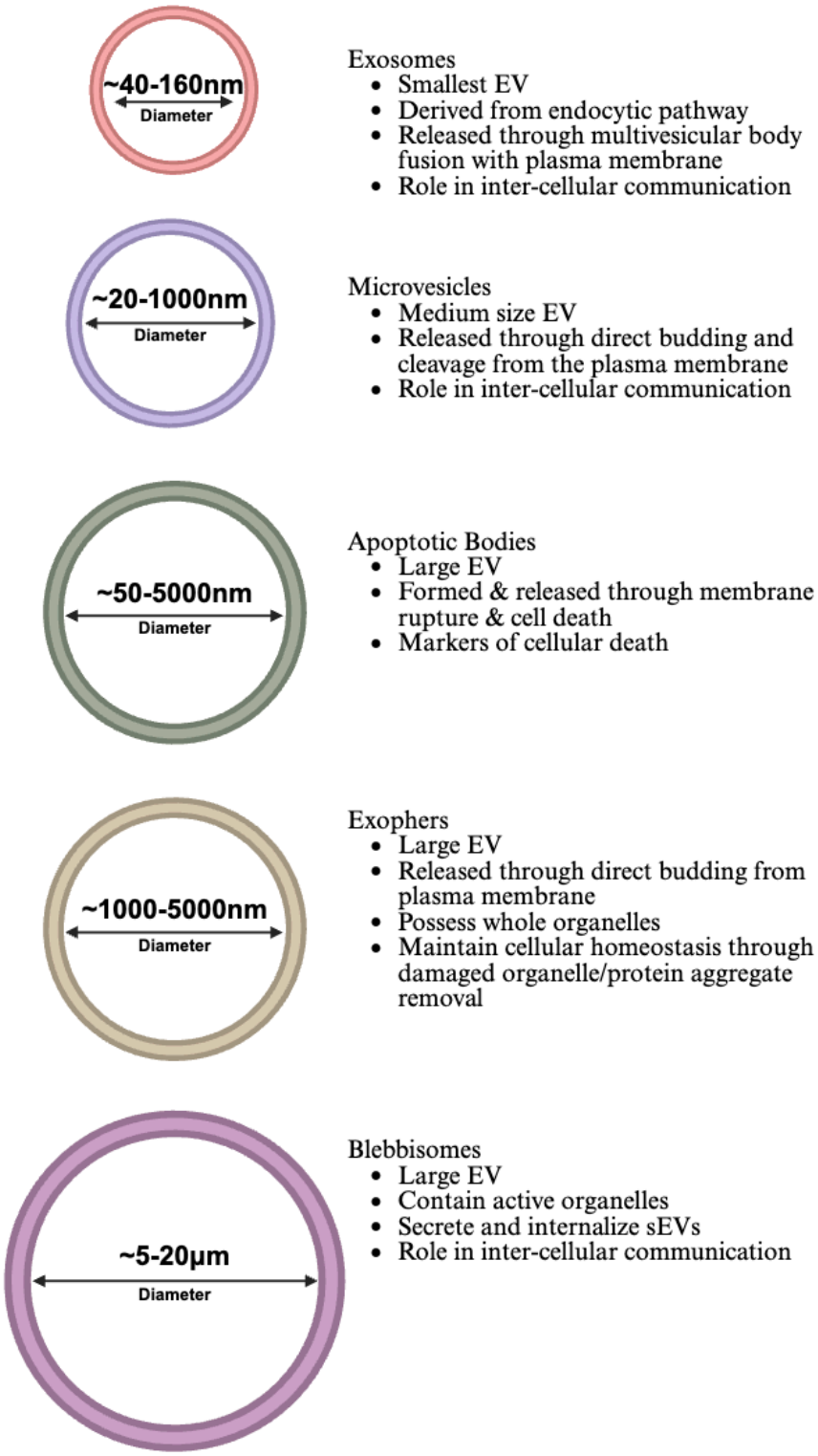

Figure 3 Characterization of the Various Types of Extracellular Vesicles. Exosomes, the smallest EV is derived from the endocytic pathway and plays a role in intercellular communication. Microvesicles, the middle-sized EV buds directly from the plasma membrane and also play a role in intercellular communication. Apoptotic bodies, the largest EV forms as a result of membrane rupture and their presence can be used as a marker of cellular death.

1.7. Contents and Roles of Extracellular Vesicles

EV contents vary based on subtype[68-71]. Primarily, the cargo that is found within EVs consists of lipids, nucleic acids including RNA, DNA, mRNAs, and miRNAs, along with proteins commonly associated with the plasma membrane, cytosol, or proteins involved in lipid metabolism[68-71]. Specific cargoes of both exosomes and microvesicles can vary based on the cell of origin and potential pathologic states and have not been wholly categorized outside of common marker proteins including ESCRT and its accessory proteins (ALIX, TSG101, HSC70, and HSP90β), trans-membrane proteins referred to as tetraspanins (CD9, CD63, and CD81), as well as other common proteins found within the cytosol and plasma membranes such as cytoskeletal components, heat shock proteins, and integrins[68-71]. However, the composition of apoptotic bodies has been more broadly defined due to its role in cellular death[68-71]. Common cargoes within these vesicles include intact organelles including the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and chromatin, as well as proteins associated with these organelles[68-71].

EV function was initially thought to be in cellular waste removal[68-71]. However, it has since been determined that both exosomes and microvesicles play a crucial role in the cell-cell communication processes through the extracellular space[68-71]. EV contents serve unique purposes in each different microenvironment in which they are released and potentially impart impacts throughout the body[68-71]. However, the specific molecules found within the EVs that are responsible and their mechanisms of action represent an exciting area of continued research[68-71]. Additionally, due to the nature of the production of apoptotic bodies, they have been identified and are commonly used as markers of cellular death[68-71].

1.8. Extracellular Vesicles in Disease and Therapeutics

The increased release of EVs during pathologic states has generated great interest in these vesicles as an important area of research[68,84]. EVs have been implicated in many different disease states ranging from neurodegenerative conditions such as Alzheimer's, Parkinson's, and Huntington's disease to various forms of cancers[85-87].

Within the brain, EVs can be produced by several cell types, namely neurons, oligodendrocytes, astrocytes, and microglia[85,88-90]. EV release into the neuronal extracellular space appears to mediate new synaptic formation and moderate the growth and development of neuronal cells and processes[85,91,92]. Consequently, EVs play crucial roles in the effective maintenance of the neuronal environment[85,91,92]. Thus, a pathologic release may have significant impacts on proper neuronal functioning[85,91,92]. In the context of neurodegenerative diseases, isolated EVs contain proteins, mRNA, and miRNA that are implicated in disease progression including β-amyloid and tau in Alzheimer's, α-synuclein in Parkinson's, TDP-43 in amyotrophic lateral sclerosis (ALS), as well as huntingtin and polyglutamine in Huntington's disease[85,93]. Additionally, due to the protective nature the EV provides its cargoes, these contents can spread and reach other cells exerting a peripheral impact of the disease[85,91-93].

EVs released from cancer cells also contain proteins, mRNA, and miRNA, however, in the pathologic state, these molecules may be oncogenic in nature[85]. Oncoproteins including epidermal growth factor receptor variant III, a constitutively active form of the epidermal growth factor receptor, have been identified within EVs[85,94]. Additionally, EV bound RNAs released from glioma cells have been shown to induce gene expression variations in cultured brain microvascular endothelial cells[85,95]. EVs released from tumors have also been implicated

in the diminished activation of immune cells[85,96]. EVs derived from mammary tumors were shown to inhibit the cytotoxic activity of the natural killer cells, preventing the immune defense targeting these cancer cells and allowing for their continued proliferation[96]. Tumor derived EVs have also been implicated in the advancement of metastasis through facilitating the process of angiogenesis through altering the gene expression profiles of endothelial cells to a pro-angiogenic state[85,97,98].

Disease associated EVs also present diagnostic and therapeutic potential. Namely, EVs have been extensively researched for the identification of biomarkers[84,85]. EVs are highly stable within bodily fluids including blood, urine, cerebrospinal fluid, and saliva which provides non-invasive access and diagnostic testing capability[84,85]. Biomarker analyses primarily target EV contents which may contain cargoes specifically upregulated in disease states[84,85]. Therapeutically, EVs derived from activated stem cells were observed to prevent apoptosis, which displayed great potential in limiting this process in addition to pro-regenerative impacts[84,85,99]. Moreover, EVs can be used as carriers of therapeutic agents, including chemotherapeutic drugs[84,85]. Due to their stable nature within the body and their ability to be modified by the addition of specific ligands, they can be administered and forced to target specific cell types[84,85,100]. This provides a more accurate and targeted treatment approach while aiming to minimize any unintended impact on healthy cells[84,85,100]. Presently, clinical translation of EVs as therapeutic carriers requires further research and refinement, but it provides exciting potential in targeted therapies[84,85,100].

1.9. Extracellular Vesicles and their Contents in Cardiac Disease

EVs play crucial roles in the progression, diagnosis, and therapeutic applications in cardiovascular disease[101-103]. In myocardial injury, EVs and their contents play a role in the inflammatory and coagulative processes, primarily through actions on endothelial cells, platelets, smooth muscle cells, as well as inflammatory cells[101,104]. In patients with stable CAD, EVs are released from endothelial cells and platelets[101,104]. miR-92a-3 was identified as a primary EV molecule and has been implicated in mediating angiogenesis in endothelial cells, indicating an attempt to overcome the ischemic nature of the CAD phenotype via EV release[101,104,105]. Additionally, EV release is increased with myocardial injury, suggesting utility as a biomarker[101-103,106]. Hypoxic cardiomyocytes appear to release EVs with high quantities of miRNA's, specifically miR-143 and miR-222, which are implicated in cardiomyocyte growth and angiogenisis[101,107]. Consequently, detection of these miRNA's could be used to indicate the presence of myocardial injury[101,107].

In the context of heart failure and cardiac remodeling, EVs and their contents have been shown to impart many different effects, primarily harmful[101]. Dysregulation of miR-21-5p in patients with heart failure prevents the canonical cardioprotective function of enhanced angiogenesis and cardiomyocyte survival leading to impaired cardiac functioning[101,108]. miR-21-3p has been implicated in promoting cardiac hypertrophy through upregulation of sorbin and SH3 domain containing protein 2 (SORBS2), a classically upregulated protein in cardiac hypertrophy, which is readily located within EVs in heart failure[101,109]. Further, inhibition of EV-bound miRNA activity diminishes the extent of hypertrophy[101,109]. Hypertrophic cardiomyocytes release EVs containing high quantities of heat shock protein 90 and interleukin-6[101,110]. This mediates the functioning of the transcription factor signal transducer

and activator of transcription 3 (STAT3) which is a primary regulator of the progression of cardiac hypertrophy and thus these EVs promote a hypertrophic phenotype[101,110].

1.10. Therapeutic Role of sEVs in Cardiac Disease

Therapeutically, EVs derived from mesenchymal stem cells have been shown to impart cardioprotective effects primarily through reducing oxidative stress, modulating the inflammatory response post-myocardial injury, and promoting cell viability[101-103,111-113]. However, there are challenges with this therapeutic approach which limits their translational capability, primarily rapid EV clearance from the heart and a limited distribution within the heart[114]. Research is progressing to overcome these obstacles through a biomaterials approach to increase targeting and longevity of EVs within the region of damaged cardiac tissue[114]. Additionally, EVs have been explored as therapeutic delivery systems in the context of cardiovascular disease[101-103]. Surface markers can be modified to specifically target cardiac cells of interest and deliver cargo, the cardiac specific therapeutic agent, directly to these cells [101-103]. While no therapeutic approaches using these methods have been approved for clinical use, there have been displayed improvements observed in *in-vivo* models[101-103,115-117]. In murine and rat models of MI, berberine and vascular endothelial growth factor (VEGF) delivery via EVs resulted in improved cardiac function via increased LVEF, while in an atherosclerotic murine model, EV fumagillin delivery displayed a reduction in atherosclerotic lesion size[101-103,115-117]. EVs as a therapeutic delivery mechanism continues to be investigated to improve specificity to cardiac tissue and decrease its clearance within the bloodstream to propel its use as a viable clinical tool[101-103].

1.11. Release of Cardiac Troponin-T within Small Extracellular Vesicles

The classically held belief regarding cTnT release in the context of myocardial injury was solely through a process of cellular death and associated membrane rupture[32,35-37]. While this remains consistent in the context of acute myocardial necrosis, common in patients presenting with T1MI, namely STEMI, this does not seem to be consistent in all cardiac disease states where there is a detectable increase in cTnT release (Figure 4). A novel release mechanism of cTnT was found to be dependent on sEVs. Patients who have established heart failure with a detectable increase in circulating cTnT, which represents a T2MI population, displayed a predominant cTnT release packaged within sEVs and not freely in the bloodstream. This was confirmed through an examination of the sEV fraction isolated from T2MI patient plasma samples via transmission electron microscopic imaging by Dr. GuoHua Li in the Liu Lab. Samples were fixed, sectioned, and subsequently conjugated with a cTnT specific immunogold labelled antibody. Electron microscopic images were collected, identifying the presence and localization of cTnT within these samples. In cTnT negative sEVs, there is no observed localization (Figure 4A). In the samples collected from T1MI patients, there was not a major detectable quantity of cTnT within the sEVs (Figure 4B). However, when the T2MI samples were analyzed there was a notable increase in the quantity of cTnT identified within the sEVs (Figure 4C, D). This was suggestive that there is a difference in the mechanism of release of cTnT between a T1MI and a T2MI.

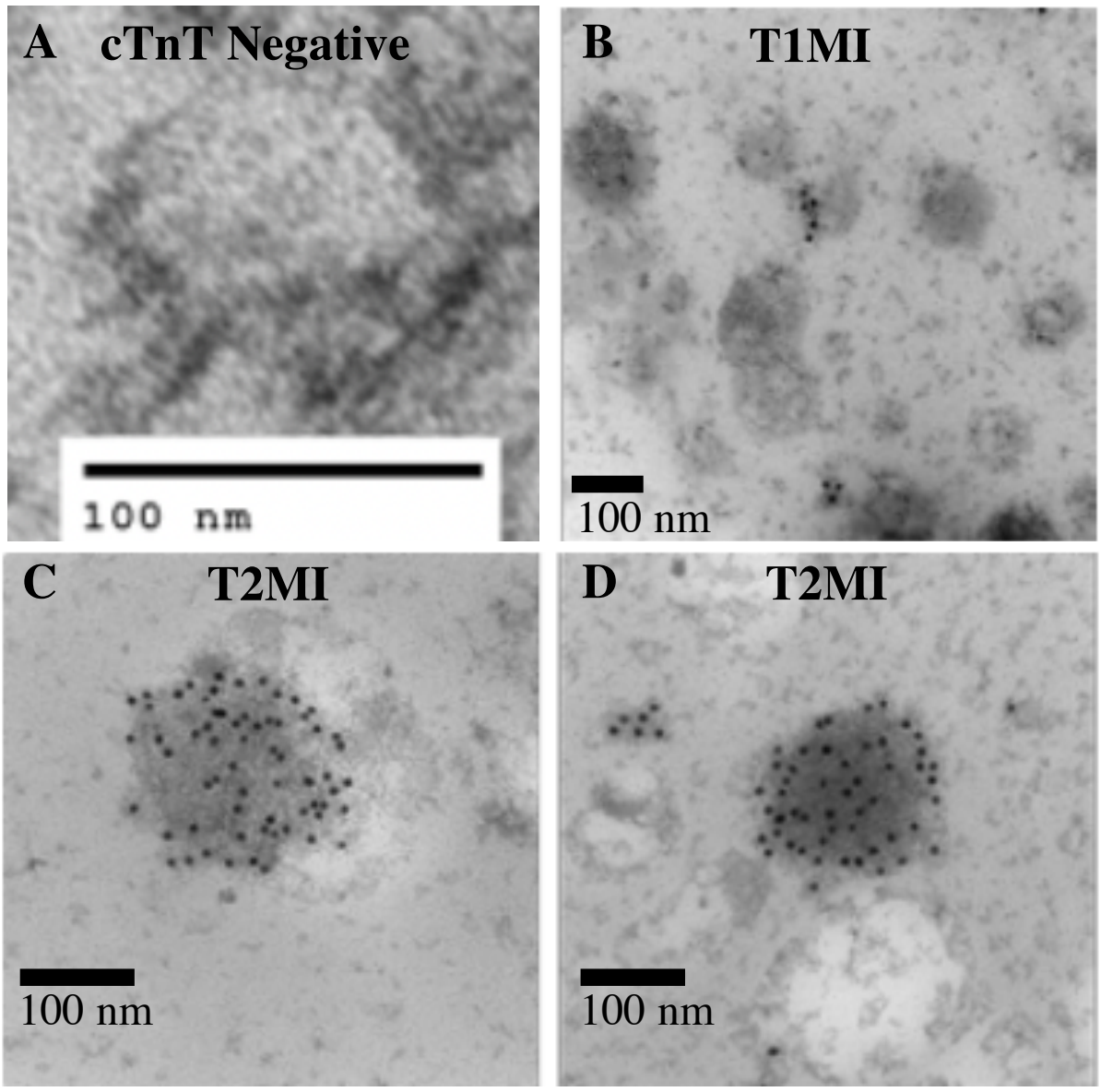

Figure 4 cTnT Release within sEVs. (A) Negative control displaying a sEV in the absence of cTnT. **(B)** T1MI sample displaying the lack of released cTnT found present within the released sEVs. **(C & D)** T2MI samples displaying the preferential release of cTnT within sEVs. Black dots correlate to immunogold labelled cTnT specific antibody and indicate the presence of cTnT.

Additional experimentation conducted in the Liu Lab by Dr. Li investigated this process within a cell culture model with cardiac HL-1 cell lines. Cells were exposed to the hypertrophy inducing stimuli phenylephrine (200nM) and contrasted with the cardiotoxic agent doxorubicin (10nM) after an incubation of 24-hours. Hypertrophic stimuli generated an increased release of sEVs compared to control (PBS) and doxorubicin treatment (Figure 5A). Notably, cTnT was found to be primarily encapsulated within sEVs in the hypertrophic setting and freely released in the cytotoxic setting (Figure 5B). Cell viability was not impacted after phenylephrine treatment, indicating the cTnT release was not a result of membrane rupture and cell death, while doxorubicin treatment showed a reduction in cell viability (Figure 5C). Moreover, in heart failure patients, sEV levels were observed to be elevated compared to healthy control (Figure 5D).

Further exploration of this novel release mechanism must be conducted to determine its potential use as a diagnostic tool in the differentiation of T1MI vs. T2MI as well as the translational capacity of such a tool to the clinical setting.

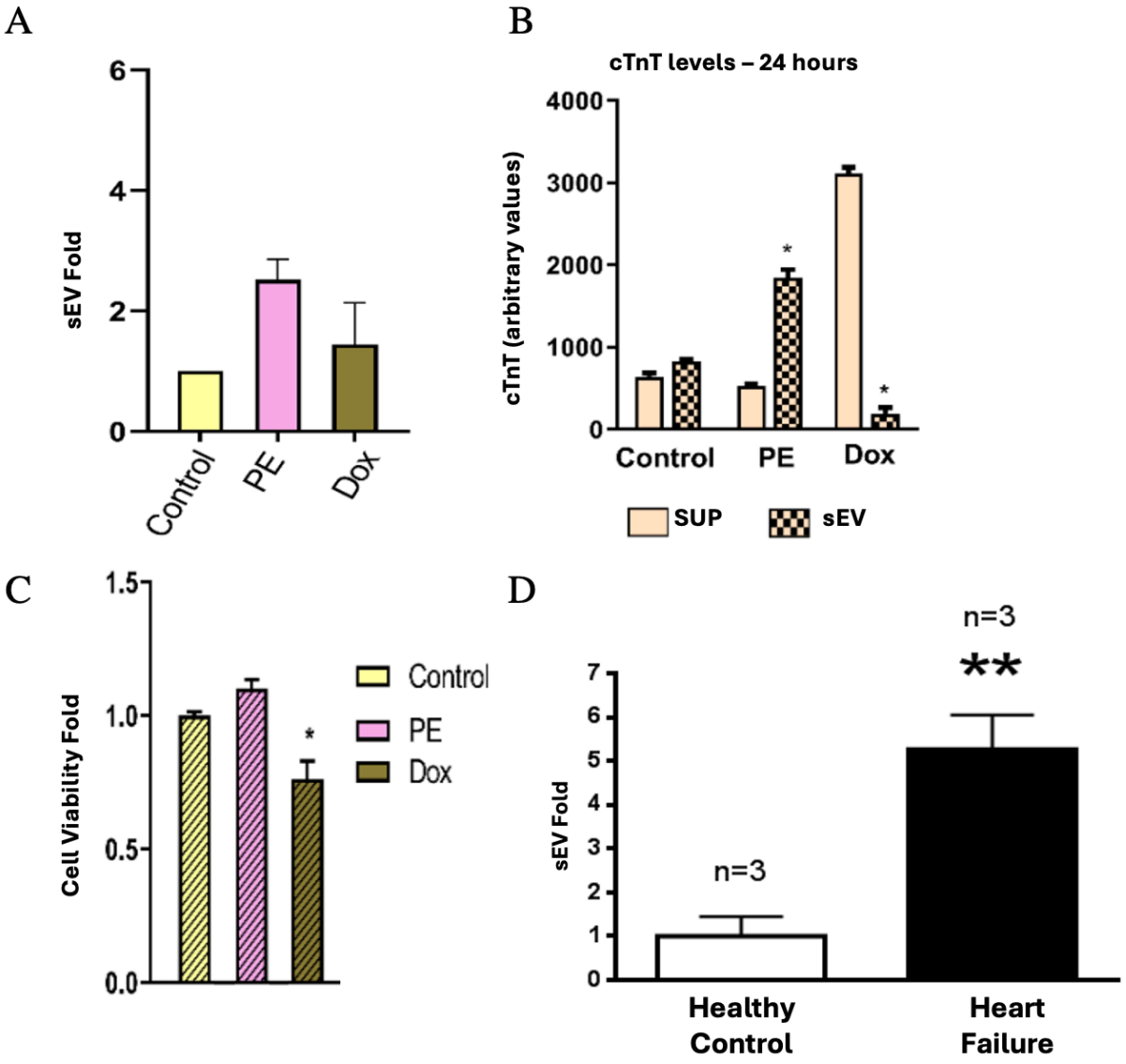

Figure 5 sEV and cTnT Release in Hypertrophic and Cardiotoxic Conditions. (A) sEV release shows an increase after cardiac hypertrophy treatment phenylephrine (PE) compared to control (PBS) and cardiotoxic doxorubicin (Dox) treatment. **(B)** cTnT values increase 24 hours after treatment with PE, primarily localized in sEVs whereas after Dox treatment primarily released freely (SUP). **(C)** Cell viability post treatment displayed reductions in Dox treated cells. **(D)** sEV release increased in Heart Failure serum compared to healthy control patients (n=3). Treatment included PBS alone, 200nM PE, and 10nM Dox. Analysis was carried out using a one-way ANOVA with significance of $p<0.05$.

2. Aim of the Project

As T1MI and T2MI present similarly in the clinical setting, it is crucial to generate an effective diagnosis to ensure the patient receives the appropriate treatment progression, which varies greatly between these disease states[15,16]. However, currently, there are no effective non-invasive diagnostic methods that are capable of effectively differentiating T1MI vs. T2MI[15,16]. As such, an assumption will commonly be made that the patient is presenting with a T1MI due to the rapid requirement for treatment[15,16]. Consequently, many patients presenting with T2MI will undergo unnecessary invasive procedures[15,16]. It is therefore crucial that rapid and effective non-invasive diagnostic techniques are developed to ensure patients receive the appropriate and clinically indicated treatment in a timely manner. It is hypothesized that the differential release profile of cTnT within sEVs along with a potential alternate isoform of cTnT within these sEVs in the context of T2MI will offer that diagnostic tool.

To answer this question, patient populations were identified to serve as investigational cohorts, including healthy control participants, T1MI consisting of patients presenting to the hospital with STEMI, and T2MI consisting of patients presenting with chronic cTnT leak in chronic heart failure with ischemic etiology and hypertrophic cardiomyopathy. To identify cTnT profiles within sEVs released in these different disease states, an effective sEV isolation and characterization protocol was optimized. As the gold standard technique for the isolation of sEVs, a serial centrifugation-ultracentrifugation technique was used[118]. Subsequent localization of cTnT within either sEVs or released freely into the bloodstream was determined in each disease state to identify whether there is a shift in the release pattern of cTnT. Lastly, isoformic determination was made to identify whether different forms of cTnT are released

more abundantly in either disease state as well as which isoforms are released within sEVs and which are released freely.

The outcome of this experimentation will allow for a broader understanding of the capacity that sEV bound cTnT can be used as a differentiating tool in T1MI vs. T2MI and whether there is the potential to translate this model to a clinical setting to help overcome the currently insufficient diagnostic capabilities in T1MI vs. T2MI.

3. Hypothesis

Within T1MI and T2MI, cTnT release will be unique to each disease state. A greater proportion of the released cTnT in the freeform in the highly ischemic T1MI context. While in the T2MI-HCM setting a greater proportion of released cTnT will be located within the sEVs. The intermediate condition of T2MI-Ischemic HF will have a proportion of cTnT in the sEVs lower than the T2MI-HCM group but greater than the T1MI group as the ischemia will be elevated in this population compared to the T2MI-HCM population but reduced compared to the T1MI setting. Differential proportions of the various cTnT isoforms will be present between the disease contexts.

4. Materials and Methods

4.1 Human Patient Sample Collection and Inclusion/Exclusion Criteria

This trial and human patient sample collection was conducted in compliance with the protocol (Section 8.1), principles laid down in the TCPS-2 guidelines, the declaration of Helsinki, and Good Clinical Practice guidelines where applicable. Further, this study received written and dated approval from the Ottawa Health Sciences Network Research Ethics Board

for protocol and consent form approval. This study is registered with Ottawa Health Sciences Network Research Ethics Board under the ID: #20140869 and ClinTrials.Gov under the ID number: NCT02347722.

Prior to patient participation and sample collection, written informed consent was obtained from each patient and complied with the above-mentioned clinical practice guidelines for patient care. No patient follow up was required, outside of 30-day, 60-day, 1-year, and 2-year chart follow up. Study involvement had no impact on the medical care provided to the patients and all normal diagnostic, procedural, and follow-up processes were undertaken in accordance with standard of care practices. Moreover, all patient identifier data was removed, and all sample analyses and investigation occurred in a de-identified and blinded manner.

4.1.1 Healthy Control

Patient inclusion criteria for healthy controls consisted of individuals between the ages of 50-85 years old, generally of a healthy status, and able to provide informed consent to supply a blood sample. Additionally, an equal number of male and female healthy volunteers were sought to provide an epidemiologically relevant population. Exclusion criteria included individuals who were not able to provide a blood sample, those with a history of cardiovascular disease including previous MI, heart failure, and valve disease, or those with major risk factors for developing cardiovascular disease including diabetes, hypertension, and dyslipidemia. Additionally, individuals were excluded if they had a diagnosis of major organ dysfunction including renal, hepatic, or pulmonary disease. Current smokers, individuals with a history of cancer (<10 years since treatment), individuals with a diagnosis of depression which requires medication, or those with any other conditions or treatments that may interfere with test results,

were excluded. Lastly, if a participant was involved in another research trial involving an investigational product within a 30-day period, they were excluded from the study (Table 1).

Once informed consent was obtained for healthy volunteer participants, a one-time blood draw was collected consisting of 33 ml of blood and subsequently processed as will be described in section 4.2. Additional measurements were collected during this period including blood pressure and heart rate. Demographic information was also collected for each participant including gender, age, ethnicity, past medical history, smoking history, alcohol consumption, current medications, and familial history of cardiovascular disease. No further follow-up was required for this participant population.

4.1.2 Type 1 Myocardial Infarction

Patient inclusion criteria consisted of all patients within the age range of 50-85 who were presenting with STEMI as confirmed via ECG analysis and elevated cTnT values. No major exclusion criteria were used outside of the patient not being able to provide a blood sample, patient not being able to provide consent (ex. patient being intubated, language barrier), or patients in which their inclusion in the study may put them at a greater risk as determined by the clinician providing medical care for the patient and obtaining the blood sample. Additionally, patients who were involved in a study involving an investigational product in the past 30-days were excluded to prevent potential interference (Table 1).

Following the collection of informed consent, blood was collected from the patient via an arterial line within the University of Ottawa Heart Institute catheterization lab. Collection was taken prior to the commencement of the percutaneous coronary intervention procedure to allow for the identification of biomarkers solely resultant from the coronary artery obstruction

and subsequent ischemic cardiac injury and not a result of the PCI procedure itself. In total, six collection tubes were filled, three BD Vacutainer K2E 10.8mg (Becton, Dickinson, and Company ref # 367863) for plasma collection, two BD Vacutainer Serum (Becton, Dickinson, and Company ref #367815), and one Tempus Blood RNA tube (Applied Biosystems ref # 4342792) and were subsequently processed (Section 4.2). Further patient health information including age, weight, past medical history, current medications, laboratory test results, or diagnostic testing was collected via Epic patient chart review.

4.1.3 Type 2 Myocardial Infarction

Patient inclusion criteria were patients between the ages of 50-85 who were admitted with heart failure symptoms, fitting a diagnosis of heart failure by the modified Framingham criteria: simultaneous presence of at least 2 major criteria or 1 major criterion along with 2 minor criteria not attributable to another disease. Major criteria are as follows; paroxysmal nocturnal dyspnea or orthopnea, neck vein distension, lung crackles, acute pulmonary edema, S3 gallop, increased venous pressure (>16 cm H_2O at right atrium), weight loss (>4.5 kg in 5 days in response to treatment), and echocardiographic left ventricular dysfunction. Minor criteria are as follows; bilateral ankle edema, nocturnal cough, dyspnea on exertion, hepatomegaly, pleural effusion, tachycardia (>120 beats/min), weight loss (>4.5 kg by factors other than treatment). Additional inclusion criteria include patients with a clear diagnosis of heart failure within the past 2 years and patients diagnosed with heart failure with preserved ejection fraction within the past 5 years. Exclusion criteria are patients who were unable to provide a blood sample, patients unable to provide consent, patients whose primary cause of heart failure is valve disease, patients with a life expectancy of less than 6 months or have

major co-morbidities, patients with significant disease or disorder which may put the patient at risk with participation in the trial, may influence the results of the trial, or impact the patients ability to participate as determined by the principal investigator. Additionally, patients who have participated in another research study involving an investigational product within a 30-day period, or if the diagnosis of heart failure with preserved ejection fraction has changed within the past 5 years are deemed ineligible for study inclusion (Table 1).

Patients admitted to the hospital or outpatient clinics were identified based on the criteria listed above and informed consent was collected to be involved in this study. Subsequently, patient blood samples were collected via an intravenous line. In total, the same six collection tubes were filled as in the Type 1 MI setting and were subsequently processed. Further patient health information including age, weight, past medical history, current medications, laboratory test results, or diagnostic testing was collected via Epic patient chart review.

Table 1 Patient Inclusion and Exclusion Criteria

Patient Group	Inclusion	Exclusion
Healthy Control	• Age 50-85 • Generally healthy • Able to provide informed consent	• Unable to provide blood sample • Cardiovascular disease history • Major cardiovascular disease risk factors (diabetes, hypertension, dyslipidemia) • Major organ dysfunction (renal, hepatic, pulmonary disease) • Current smoker • Cancer history (<10 years) • Depression (requiring medication) • Any other condition/treatment which may impact results • Participation in research with investigational product (<30 days)
Type 1 MI	• Age 50-85 • STEMI- confirmed by ECG & elevated cardiac biomarkers (cTnT, cTnI, CK-MB)	• Unable to provide blood sample • Unable to provide consent • Significant disease/disorder/co-morbidities where study participation may put patient at risk • Participation in research with investigational product (<30 days)
Type 2 MI	• Age 50-85 • Admitted to hospital with heart failure symptoms fitting diagnosis of heart failure (**Modified Framingham criteria**) • Diagnosed with heart failure within the past 2 years • Diagnosis of heart failure with preserved ejection fraction within past 5 years	• Unable to provide blood sample • Unable to provide consent • Primary reason for heart failure is valve disease • Significant disease/disorder/co-morbidities which study participation may put patient at risk • Life expectancy <6 months • Participation in research with investigational product (<30 days) • If heart failure diagnosis of heart failure with preserved ejection fraction changed in past 5 years

Modified Framingham Criteria- Simultaneous presentation of at least 2 major criteria or 1 major and 2 minor criteria

- **Major Criteria**- paroxysmal nocturnal dyspnea or orthopnea, neck vein distension, crackles/rales, acute pulmonary edema, S3 gallop, increased central venous pressure, weight loss >4.5kg in response to treatment, ECG left ventricular dysfunction

- **Minor Criteria**- bilateral ankle edema, nocturnal cough, dyspnea on exertion, hepatomegaly, pleural effusion, tachycardia, weight loss >4.5kg not in response to treatment

4.2 Blood Processing of Plasma, Buffy Coat, and Serum

Whole blood samples collected within the BD Vacutainer Serum were left to sit at room temperature for a thirty-minute period to facilitate coagulation of the blood sample. Simultaneously, whole blood samples collected with BD Vacutainer K2E 10.8mg for plasma collection and the Tempus Blood RNA tube were maintained on ice to prevent coagulation. After this thirty-minute period, serum and plasma tubes are centrifuged for ten minutes at 4°C at a rate of 1500 x g in an Eppendorf 5702 R benchtop centrifuge. Subsequently, plasma and buffy coat were collected from the plasma sample tubes, while serum was collected from designated serum sample tubes and transferred to clean 1.4mL storage tubes (Micronic MP32062). Plasma, serum, and buffy coat samples were then transferred to -80°C for storage for downstream analysis.

4.3 Serial Centrifugation-Ultracentrifugation Isolation of Small Extracellular Vesicles

This centrifugation-ultracentrifugation protocol abides by the standards set by the Minimal information for studies of extracellular vesicles 2023 (MISEV2023)[119]. Patient serum, 100 µl, was transferred to a clean 1.7 ml microcentrifuge tube (FroggaBio Graduated Microcentrifuge tubes #LMCT1.7BG). Subsequently, 100 µl of filtered 1x Phosphate Buffered Saline (PBS) was added to the 1.7 ml microcentrifuge tube containing the patient serum and resuspended via pipetting. Samples were centrifuged at 4°C for 20 minutes at 2000 x g in a Thermo Scientific Sorvall Legend Micro 21R microcentrifuge to remove remnant cellular fragments or cells which were still present within the sample. The supernatant was collected and transferred to a new clean 1.7 ml microcentrifuge tube, and the remnant pellet was discarded. Samples were centrifuged again (10,000 x g, 30 minutes at 4°C) in a Thermo

Scientific Sorvall Legend Micro 21R microcentrifuge to remove any leftover cellular debris. Again, pelleted debris was discarded, and the supernatant was transferred to a clean 1.5ml ultracentrifuge tube (Beckman Coulter Microfuge Tube Polypropylene #357448). Tubes were weighed within a 0.02g range to ensure ultracentrifuge balance and equitable force on each sample. Balanced ultracentrifuge tubes were then inserted into the Beckman Coulter Optima MAX-TL benchtop Ultracentrifuge using the TLA110 rotor (Beckman Coulter #366735) with Beckman Coulter microfuge adapters (#360951). Samples were ultracentrifuged for 120 minutes at 100,000 x g at 4°C. The supernatant was collected and transferred to a clean 1.7ml microcentrifuge tube and labelled as sEV free supernatant. The sEV pellet was resuspended in 100 µl filtered 1x PBS. Both the resuspended sEVs and the sEV free supernatant were then transferred to -80°C for storage until subsequent analysis (Figure 6).

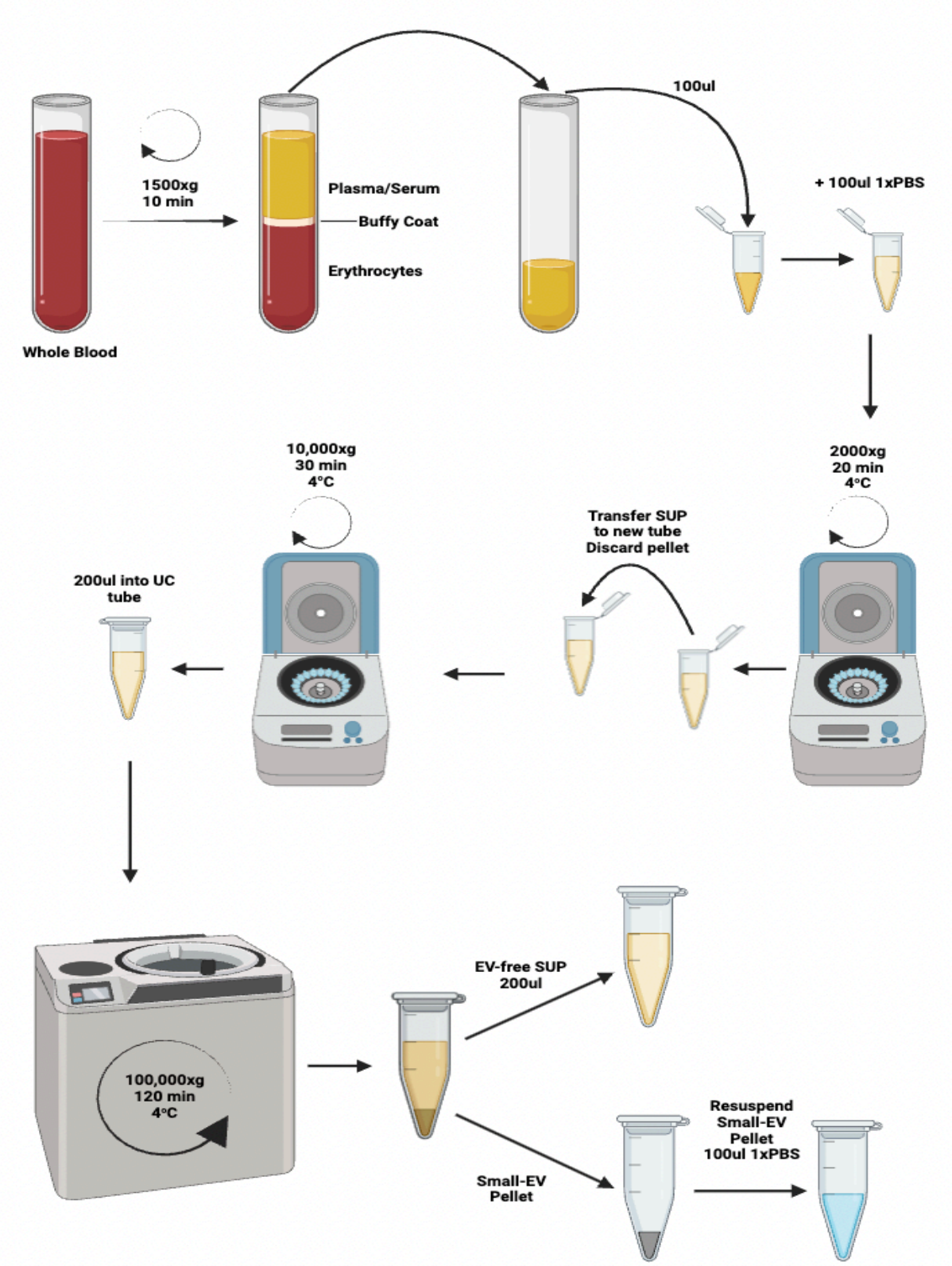

Figure 6 Serial Centrifugation-Ultracentrifugation Procedure. Protocol implemented in the isolation of sEVs from human patient serum samples. Figure developed using Biorender platform.

4.4 Protein Purification

An albumin and immunoglobulin (IgG) removal protocol was carried out to remove any potential contamination of these abundantly expressed proteins in the isolated serum fractions. The presence of these proteins may lead to the obstruction of analyses of other key proteins and thus impact the accuracy of the results[120]. To remove contaminants, 10 µl of the EV-free supernatant fraction and 5 µl of the resuspended sEV fraction was added to a clean 1.7 ml microcentrifuge tube containing 100 µl 1% trichloroacetic-acid-Isopropyl alcohol solution (1% TCA-IsoP). Following resuspension, samples were vortexed on high for 2 minutes and subsequently centrifuged for 5-minutes at 4°C and 1500 x g (Thermo Scientific Sorvall Legend Micro 21R microcentrifuge). Following centrifugation, the supernatant containing the albumin and IgG was discarded and the pellet was resuspended in 200 µl of 100% methanol. The sample was centrifuged again at 4°C, for 10 minutes at 3000 x g. The supernatant was again discarded, and the remaining pellet from the sEV-free supernatant fraction was resuspended with 50 µl filtered 1x PBS while the remaining pellet from the sEV fraction was resuspended with 25 µl of filtered 1x PBS (Figure 7).

For protein isolation, 20 µl of the sEV-free supernatant fraction and 10 µl of the sEV fraction were mixed with an equal volume of 1x RIPA buffer, separately. Following resuspension, samples were incubated on ice for 15 minutes. Samples were then heated to 95°C for 5 minutes and returned to incubate on ice for 5 minutes. Samples were centrifuged at 4°C for 15 minutes at 12,000 x g and the protein lysate containing supernatant was transferred to clean tubes. Notably, samples undergoing subsequent nanoparticle tracking analysis were not resuspended in RIPA lysis buffer to preserve the EV structure.

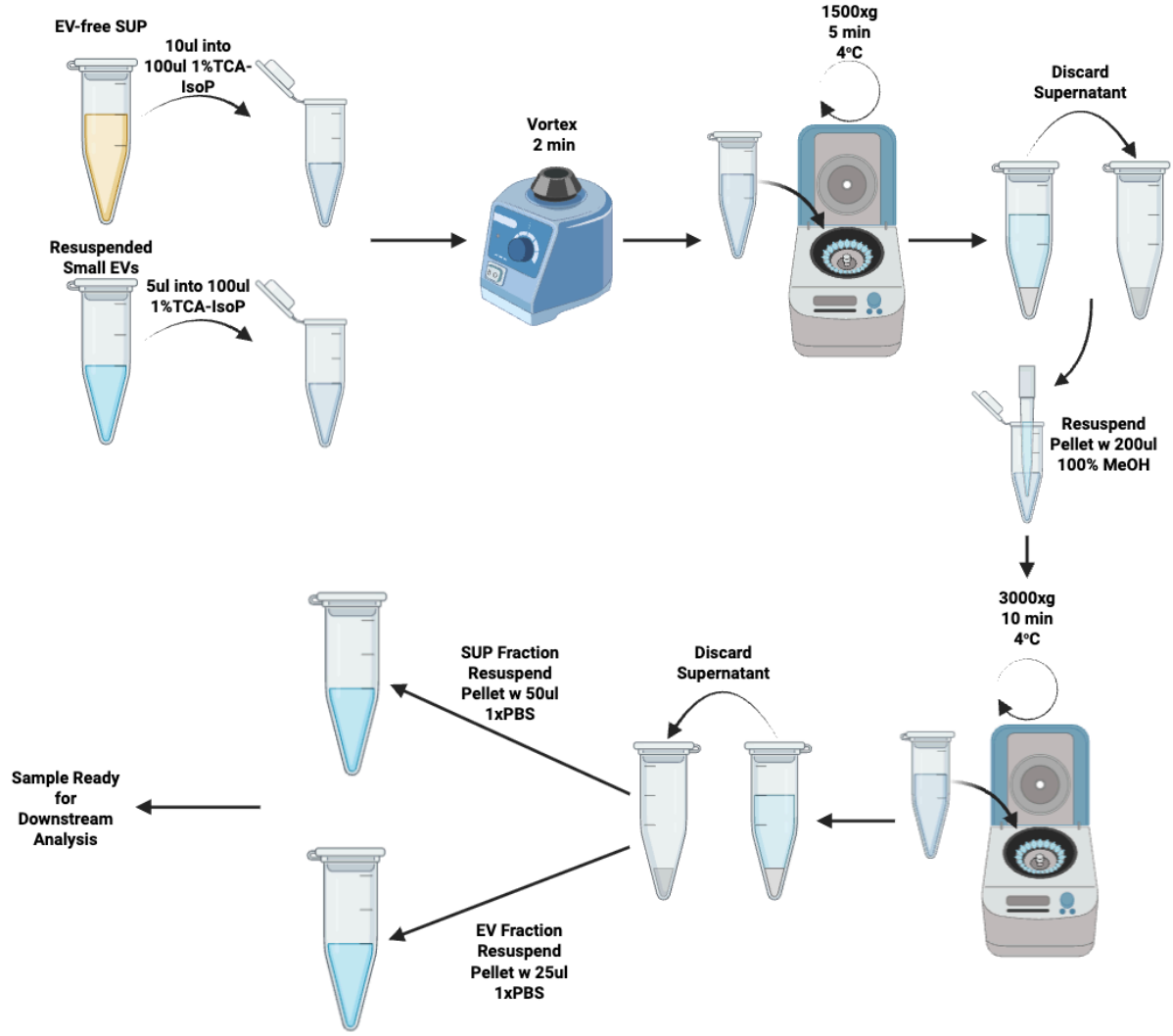

Figure 7 Protein Purification Procedure. Albumin and IgG removal protocol utilized to reduce sample contamination from abundantly present serum proteins. Downstream analyses are able to proceed in the absence of obstruction caused by the presence of these proteins. Figure developed using Biorender platform.

4.5 Nanoparticle Tracking Analysis

To characterize isolated sEVs, a nanoparticle tracking analysis procedure was completed at 21°C with Zetaview 8.04.02 SP3 software with the pre-acquisition parameters of shutter speed: 70, and 30 frames per second and post-acquisition settings of minimum brightness: 30, minimum tracelength: 15, minimum size: 20, and maximum size: 1000. Samples consisting of whole serum and isolated sEV fractions were diluted 1:200 in 1x PBS. A total volume of ~800 µl of sample was then loaded to the Zetaview system which uses various camera positions to acquire video in 11 sampling regions within the flow cell and subsequently generates an estimate of particle concentration within the sample. Following video acquisition, any images the Zetaview system suggested to be removed were subject to further analyses; this message commonly appears due to an insufficient number of particles present in the field and thus would not provide a representative readout. If more than 3 of the fields were suggested to be removed, the sample was rerun. Further analyses and graphing of Zetaview data was carried out using Prism 9.3 software.

4.6 Western Blot

Western blot analyses were completed to identify proteins of interest within sEV-free supernatant and sEV fractions isolated from whole serum. First, total protein quantifications were determined using the Qubit 2.0 Fluorometer (Invitrogen) and Qubit Protein Assay Kit as per manufacturer instructions (Thermo Fisher Scientific #Q33212). Briefly, assay tubes (DiaTEC #DIATEC610-3000) for each standard control were set up along with one tube for each sample being analysed. The Qubit working solution was prepared by diluting the Qubit Protein Reagent in the Qubit Protein Buffer in a 1:200 ratio. 200 µl of the working solution

was created for each standard and triplicate sample to be analysed for reproducibility. For each standard control, 190 µl of the working solution was added along with 10 µl of the standard control assay. For each sample, 198 µl of the working solution was added with 2 µl of the sample of interest. All standards and samples were then vortexed on high for roughly 5 seconds to sufficiently mix the sample and left to incubate at room temperature for 15 minutes. Sample protein concentrations were then calculated by analysis with the Qubit 2.0 fluorometer.

In a clean 1.7 ml microcentrifuge tube, 20 µg of sample protein was combined with 5 µl of 4x Bolt LDS Sample Buffer (Invitrogen #B0007), 2 µl dithiothreitol (DTT), and supplemented with filtered 1x PBS to a final volume of 20 µl. Samples were mixed via pipetting and boiled at 80°C for 10-minutes to denature the protein content. 2-(N-morpholino)ethanesulfonic acid (MES) running buffer was added to the Invitrogen Mini Gel tank, followed by the Bolt 4-12%, Bis-Tris Plus WedgeWell Gel 15 Well (Invitrogen #NW04125). A protein ladder was added to the first lane (6 µl, FroggaBio BLUelf prestained protein ladder #PM008-0500F), followed by 20 µl of each sample in the remaining wells. The gel was run at 100V for 60 minutes, followed by 30 minutes at 120V. The resolved proteins were electrophoretically transferred to a Pierce PVDF Transfer Membrane (Thermo Scientific #88520) in transfer buffer (Invitrogen Bolt Transfer Buffer #BT00061 and 10% methanol) for 60 minutes at 20V. Total protein was determined using No Stain Labeling Reagent (Thermo Fisher Scientific #100084996) following manufacturer protocol; a 20x dilution of No Stain Labeling Buffer in ddH_2O, followed by the addition of 20 µl of the No Stain activator and the derivatizer. The membrane was incubated in the No Stain reagent for 10 minutes with agitation and subsequently washed with ddH_2O 3 times for 2 minutes each, again with agitation. The membrane was imaged on a ChemiDoc XRS+ system (Bio-Rad, 1708265) with the ImageLab

5.1 Beta program using the stain-free blot setting to determine total protein content. Afterwards, bovine serum albumin (BSA) was dissolved in 0.1% PBS with Tween-20 (PBST) to create a 1% BSA blocking buffer. The membrane was incubated in 1% BSA blocking buffer for 60 minutes with agitation to prevent non-specific antibody binding to the membrane. Primary antibodies (albumin (abcam # ab207327, 1:1000), cTnT (Thermo Scientific # MA116687, 1:1000), ALIX (Invitrogen # PA5-52873, 1:1000), CD63 (Thermo Scientific # 10628D, 1:1000)) were diluted in 1% BSA blocking buffer solution and then added to the membrane to incubate on a shaker overnight at 4°C. The primary antibody was decanted, and the membrane was washed 3 times with 0.1% PBST for 10 minutes. Next, a horseradish peroxidase conjugated goat-anti-mouse IgG (Bio-Rad, 170-5047, 1:100,000) or goat-anti-rabbit IgG (Bio-Rad, 170-5046, 1:100,000) secondary antibodies were diluted in 1% BSA blocking buffer and added to the membrane based on the corresponding primary antibody used and incubated with agitation for 60 minutes. The membrane was washed again 3 times with 0.1% PBST for 10 minutes per wash and visualized via chemiluminescence (SuperSignal West Atto Ultimate Sensitivity Substrate (Thermo Fisher Scientific #A38556)). Briefly, 500µl of the substrate solution was added to the membrane, followed by 500µl of the stable peroxide solution, and ensured that the entire membrane had been exposed to the chemiluminescent atto solution and incubated for 5 minutes. The membrane was imaged using the ChemiDoc XRS+ system (Bio-Rad, 1708265) with the ImageLab 5.1 Beta program (BioRad) with the Chemiluminescent setting. Another image was taken to visualize the protein ladder using the colorimetric setting. The ladder image and the protein image were then merged to develop the final western blot image. Protein expression was contrasted with total protein and expressed as a fold change.

4.7 High-Sensitivity Cardiac Troponin-T Analysis

Isolated serum samples and aforementioned fractions were diluted 1:10 in filtered 1x PBS to a final volume of 500 µl. Diluted samples were transferred to Roche Elecsys assay cups. Samples were quantified using a Cobas e411 platform (Roche Diagnostics) and the clinical grade hs-cTnT elecsys assay (Roche Diagnostics #09315357190). The hs-cTnT assay has a lower limit of detection of 3 ng/L, well below the 99th percentile reference limit indicating myocardial necrosis of 14 ng/L, and all values below this limit were represented as <3 ng/L.

4.8 Cardiac Troponin-T Specific Immunoprecipitation

To generate a highly pure fraction of cTnT isolated from the sEV bound fractions and the freely released fractions, a cTnT specific immunoprecipitation protocol was established. Immunoprecipitation was carried out using Invitrogen Dynabeads Protein G for Immunoprecipitation kit protocol (Thermo Fisher Scientific #10007D). Per sample, 50 µl of magnetic beads were transferred to a clean 1.7 ml microcentrifuge tube and placed on a magnetic stand to remove the supernatant. Magnetic beads were then removed from the magnet and resuspended in 200 µl Ab Binding and Washing Buffer containing 5 µg of a cTnT specific antibody (Invitrogen #MA1-16687). The solution was then incubated at room temperature for 10 minutes to allow antibody binding. The supernatant was then discarded and the magnetic bead-cTnT antibody complex was resuspended in 200 µl Ab Binding and Washing Buffer. Samples were then added to the complex in corresponding tubes and incubated overnight at 4^{o}C to allow for thorough binding of endogenous cTnT to the complex. After overnight incubation, samples were placed on a magnet and the supernatant was removed. The magnetic bead-cTnT antibody-cTnT complex was resuspended with 200µl washing buffer three times.

After the final washing step, the cTnT containing complex was resuspended in 100µl washing buffer. The sample was placed on the magnet and the supernatant was again removed. The cTnT containing complex was then resuspended in 20 µl of Elution buffer. Two alternative approaches could be taken at this point, a denaturing or a non-denaturing approach. The denaturing approach was used for western blot identification of cTnT and proceeded as follows. To the samples resuspended in the Elution buffer, 10 µl of Bolt LDS Sample Buffer (Invitrogen #B0007) as well as 4 µl of DTT were added and sufficiently mixed via pipetting. The samples were then incubated at 80°C for 10-minutes, placed on the magnet, and the supernatant was loaded into the gel and subsequently analysed as described in Section 4.6. The non-denaturing approach was used for subsequent mass spectrometry analyses of cTnT isoform determination. Following resuspension in 20 µl Elution buffer, the sample was incubated at room temperature for 2 minutes. The sample was then placed on the magnet and the supernatant was collected and transferred to a clean tube and ready for downstream analysis.

4.9 Mass Spectrometry Analysis

To characterize the isolated cTnT either contained within the isolated sEVs or freely released, mass spectrometry analysis was conducted. In 40 µl of 8M urea, 10 µg of protein was resuspended and vortexed for 30 seconds and subsequently reduced with 2.5 mM DTT and incubated at 37°C for 60 minutes. 5 mM iodoacetamide dissolved in 50 mM ammonium bicarbonate was added to alkylate the sample which was incubated at room temperature in the dark for 30 minutes. 50 mM ammonium bicarbonate was added to dilute the sample 10-fold followed by sequencing grade trypsin in a 1:20 ratio to the total protein in the sample and

incubated at 37°C overnight. Formic acid (1%) was added to acidify the sample and inactivate the trypsin, as well as facilitate sample binding to the C^{18} matrix sample loading tube (Evotip C^{18}, Evosep #EV2001). 100 µl 1-propanol was transferred to each well of a 96 well microtiter plate, and the Evotip adapter rack and tips were then loaded on top of this plate to allow for the tips to soak in 1-propanol for 10 seconds, or until the tips were pale white in colour. Tips were then washed with 20 µl of solvent B; acetonitrile with 0.1% formic acid and centrifuged for 60 seconds at 700 x g. Tips were incubated again in 1-propanol until tips were pale white, after which 20 µl of solvent A (water with 0.1% formic acid) was added to each tip. Tips were again centrifuged for 60 seconds at 700 x g. After solvent A removal, 20 µl of the sample was added to each tip. Samples were ionized with a nanospray ion source (Proxeon) into the liquid chromatography tandem mass spectrometer (LTQ-Orbitrap Velos hybrid mass spectrometer, Thermo Fisher Scientific) for proteomic analysis.

4.10 Statistical Analysis

All statistical analysis was completed with Prism 9 software version 9.3.1. Analysis carried out to determine protein expression patterns was a one-way ANOVA test. Determination of cTnT content between disease states was analysed with the use of a two-way ANOVA with multiple comparisons. cTnT percentage located within sEVs between disease states was analysed with use of a one-way ANOVA with multiple comparisons. While fold change of proportion of cTnT located within sEVs was relative to healthy control cTnT levels and utilized a one-way ANOVA with multiple comparisons for statistical determination. Male vs. Female proportion of cTnT located within sEV determination in each disease state utilized a one-way ANOVA with subgroup testing. Lastly, concentration of cTnT located within sEVs

in STEMI samples with a comparative analysis on the culprit artery as well as patients presenting with one artery occluded or more than one artery occluded utilized a one-way ANOVA with multiple comparisons for statistical analysis. All values are reported as mean ± SEM, with n referring to sample size analysed, within each statistical analysis, significance being reached at a value of $p<0.05$. Subsequent graphical representation was also performed using Prism 9 software.

5. Results

5.1 Human Patient Clinical Characteristics

A total of 52 participants were identified to have met the inclusion and exclusion criteria described in Table 1 and were recruited to be study participants (Table 2). Of this population, 13 were healthy control participants, 11 were patients presenting to the hospital with a STEMI and thus included in the T1MI cohort. The T2MI cohort consisted of 13 patients who had a diagnosis of chronic heart failure with an ischemic etiology (Ischemic HF), while 15 had a diagnosis of hypertrophic cardiomyopathy (HCM). The T2MI- Ischemic HF group consisted of the oldest population (73 ± 3.01) (mean ± SEM) while the T1MI group consisted of the youngest population (62.27 ± 2.75), with T2MI- HCM (67.93 ± 3.46) in the middle. T1MI and T2MI- Ischemic HF groups were primarily composed of male patients at 81.81% and 76.92% respectively, while in the T2MI- HCM groups there was a relatively equal distribution of males and females recruited with 46.7% being males. hsTnT levels were measured in each disease state to identify the extent of ischemic cardiac injury where T1MI patients had the highest level (3210.82 ± 1418.94pg/mL) and T2MI- HCM patients had the lowest of the disease groups (63.05 ± 38.09pg/mL). T2MI- Ischemic HF patients had an

intermediate quantity (335.3 ± 82.68pg/mL). The healthy control reference group who were not experiencing any cardiac disease had low hsTnT levels (6.60 ± 1.33pg/mL). proBNP II levels were below the threshold of disease (150pg/mL) in the reference healthy controls (103.14 ± 30.80pg/mL). Elevations above the threshold of disease were observed in the T2MI-Ischemic HF (6952.39 ± 2328.96pg/mL) and T2MI- HCM (1866.25 ± 528.63pg/mL) groups indicating the presence of chronic HF in these populations, with the greater the quantity of proBNP II denoting more severe HF. Of the disease states T1MI displayed the lowest levels (398.45 ± 182.84pg/mL), although it was still above the threshold of disease.

Patient medical history was also recorded for the T1 and T2MI patient populations. Healthy control histories were not recorded as the presence of these diagnoses would have been exclusionary for their involvement. Dyslipidemia showed similar trends in the T1MI and T2MI- Ischemic HF patients with a diagnosis in 8 (72.73%) and 8 (61.54%) in these groups respectively. T2MI- HCM showed a diverging trend with 6 (40%) patients diagnosed. Atrial fibrillation was present in 0 (0%) of T1MI patients, while in the T2MI- HCM group 8 (53.33%) has a history. T2MI- Ischemic HF followed the HCM group in this setting with 6 (46.15%) patients. A history of CAD also showed elevated rates in both T1MI and T2MI- Ischemic HF conditions (11 (100%) and 13 (100%) respectively) whereas in the T2MI- HCM population, the rate was lower (5 (33.33%)). T2MI patients were characterized by the New York Heart Association (NYHA) HF classification do identify the severity of HF individuals were experiencing. Class 1, the lowest severity level, contained 0 (0%) T2MI- Ischemic HF patients and 1 (6.7%) T2MI- HCM patients. In class 2, 5 (38.5%) T2MI- Ischemic HF and 9 (60%) T2MI- HCM patients were designated. A diagnosis of Class 3 was observed in 5 (38.5%) T2MI- Ischemic HF and 5 (33.33%) T2MI- HCM patients. 3 (23.1%) T2MI- Ischemic HF

patients and 0 (0%) T2MI- HCM patients had a diagnosis of Class 4, the most severe classification. The study population primarily consisted of individuals from European/Caucasian backgrounds with 9 (81.81%), 13 (100%), and 14 (93.33%) in the T1MI, Ischemic HF, and HCM groups respectively. There was 1 (10%) Middle Eastern patient included in the T1MI cohort, while patients with Asian descent made up 1 (10%), and 1 (6.67%) in the T1MI, and T2MI- HCM populations.

Table 2 Patient Clinical Characteristics and History. Healthy Control (n=13), Type 1 MI (STEMI) (n=11), and Type 2 MI patients which consists of both chronic heart failure with an ischemic etiology (n=13) and hypertrophic cardiomyopathy (n=15). Clinical characteristics were collected during sample collection as well from patient chart review.

		Type 1 MI	**Type 2 MI**	
Variable (Mean ± SEM)	Healthy Control (n=13)	STEMI (n=11)	Ischemic HF (n=13)	HCM (n=15)
Age (years)	64. 85 ± 2.69	62. 27 ± 2.75	73 ± 3.01	67.93 ± 3.46
Male Sex (no., %)	7 (53.85)	9 (81.81)	10 (76.92)	7 (46.7)
Weight (kg)	72.44 ± 3.40	80.66 ± 5.32	83.22 ± 6.30	84.88 ± 3.16
Height (cm)	169.68 ± 3.31	174. 06 ± 3.22	168.54 ± 3.32	168.87 ± 2.13
BMI (kg/m^2)	25.05 ± 0.56	26.56 ± 1.51	28.97 ± 1.57	29.79 ± 1.04
hs-TnT (pg/mL)	6.60 ± 1.33	3210.82 ± 1418.94	335.3 ± 82.68	63.05 ± 38.09
proBNP II (pg/mL)	103.14 ± 30.80	398.45 ± 182.84	6952.39 ± 2328.96	1866.25 ± 528.63
LVEF (%)	NA	46.46 ± 3.71	35.8 ± 2.82	47.46 ± 5.21
Systolic Blood Pressure (mmHg)	134.92 ± 4.27	138.55 ± 9.63	118.69 ± 3.24	120.27 ± 3.70
Diastolic Blood Pressure (mmHg)	80.62 ± 1.57	79.18 ± 5.48	68.23 ± 2.99	75.2 ± 2.42
Creatinine (μmol/L)	NA	78.18 ± 6.43	89.54 ± 5.20	86.93 ± 6.45
eGFR (ml/min)	NA	111.27 ± 6.55	77.23 ± 5.73	82.53 ± 6.94
Hypertension (no., %)	NA	5 (45.45)	10 (76.92)	12 (80)
Diabetes (no., %)	NA	5 (45.45)	4 (30.77)	5 (33.33)
Dyslipidemia (no., %)	NA	8 (72.73)	8 (61.54)	6 (40)

Smoking History (no., %)	NA	6 (54.54)	12 (92.31)	11 (73.33)
AF (no., %)	NA	0 (0)	6 (46.15)	8 (53.33)
Coronary Artery Disease (no., %)	NA	11 (100)	13 (100)	5 (33.33)
Right Ventricular dysfunction (no., %)	NA	1 (9.09)	5 (38.46)	4 (26.7)
Diastolic Dysfunction (no., %)	NA	7 (63.63)	4 (30.8)	1 (6.7)
Valve Disease (no., %)	NA	0 (0)	9 (69.23)	3 (20)
Pulmonary Hypertension (no., %)	NA	0 (0)	4 (30.8)	1 (6.7)
NYHA Class 1 (no., %)	NA	0 (0)	0 (0)	1 (6.7)
NYHA Class 2 (no., %)	NA	0 (0)	5 (38.5)	9 (60)
NYHA Class 3 (no., %)	NA	0 (0)	5 (38.5)	5 (33.3)
NYHA Class 4 (no., %)	NA	0 (0)	3 (23.1)	0 (0)
Ethnic Background-European (no., %)	11 (84.62)	9 (81.81)	13 (100)	14 (93.33)
Ethnic Background-Middle East (no., %)	0 (0)	1 (10)	0 (0)	0 (0)
Ethnic Background-East Asian (no., %)	2 (18.18)	1 (10)	0 (0)	1 (6.67)

5.1.1 Culprit Arteries Implicated in STEMI Patients

To examine whether the location of coronary artery obstruction in STEMI patients impacted cTnT release, the culprit artery was recorded (Table 3). The most common arteries implicated in a STEMI are the left anterior descending coronary artery (LAD) corresponding to an anterior STEMI, while inferior STEMIs often have right coronary artery (RCA) and left circumflex artery (LCx) culprit arteries[121]. Seven (63.64%) STEMI patients presented with an LAD occlusion and a diagnosis of an anterior STEMI. In contrast, 4 (36.36%) of the STEMI patients were diagnosed with an occlusion in the RCA and a diagnosis of an inferior STEMI. Interestingly, no patients presented with an occlusion of the LCx. Additionally, 3 (27.27%) of the patients presented with more than 1 artery which displayed an occlusion greater than 70%, which would require further intervention later. Each patient was treated with emergent primary PCI with a drug eluting stent.

Table 3 Culprit Artery in STEMI. Primary causative arteries are identified as the Left Anterior Descending Artery, which was the culprit in 9 patients, Right Coronary Artery which was the culprit in 2 patients and Left Circumflex Artery which was not determined to be a culprit artery in any patient. 3 patients presented with >1 coronary artery stenosed at 70%. Each patient was treated with primary PCI + Stent.

Coronary Artery Implicated	# of STEMI Patients
Left Anterior Descending- 100% Occluded (no., %)	7 (63.64)
Right Coronary Artery- 100% Occluded (no., %)	4 (36.36)
Left Circumflex Artery- 100% Occluded (no., %)	0 (0)
> 1 Artery >70% Occluded (no., %)	3 (27.27)
Treatment with Primary PCI + Stent (no., %)	11 (100)

5.2 Characterization of Small Extracellular Vesicles

Nanoparticle tracking analysis was conducted on 3 T2MI- hypertrophic cardiomyopathy samples as this population displays the greatest quantity of EVs released, as described in section 1.10, and thus would provide a more thorough EV characterization. Whole serum samples displayed mean particle sizes of 134.45, 180.46, and 139.31 nm (Figure 8A). This is contrasted to the isolated sEV fractions which displayed mean particle sizes of 132.02, 136.94, and 152.03 nm (Figure 8B). The observed particle sizes are larger in the whole serum fractions as compared to the isolated sEV fractions, as well as the variation in detected particle size being greater in the whole serum fraction. The third sample does not show this similar trend as the isolated-EV fraction was shown to have larger particles with a greater variability. However, this fraction still met the 40-160 nm window, indicating this population of EVs still falls within the range of sEVs. Particle size measurements exhibit a separation of sEVs into the corresponding fraction from the whole serum.

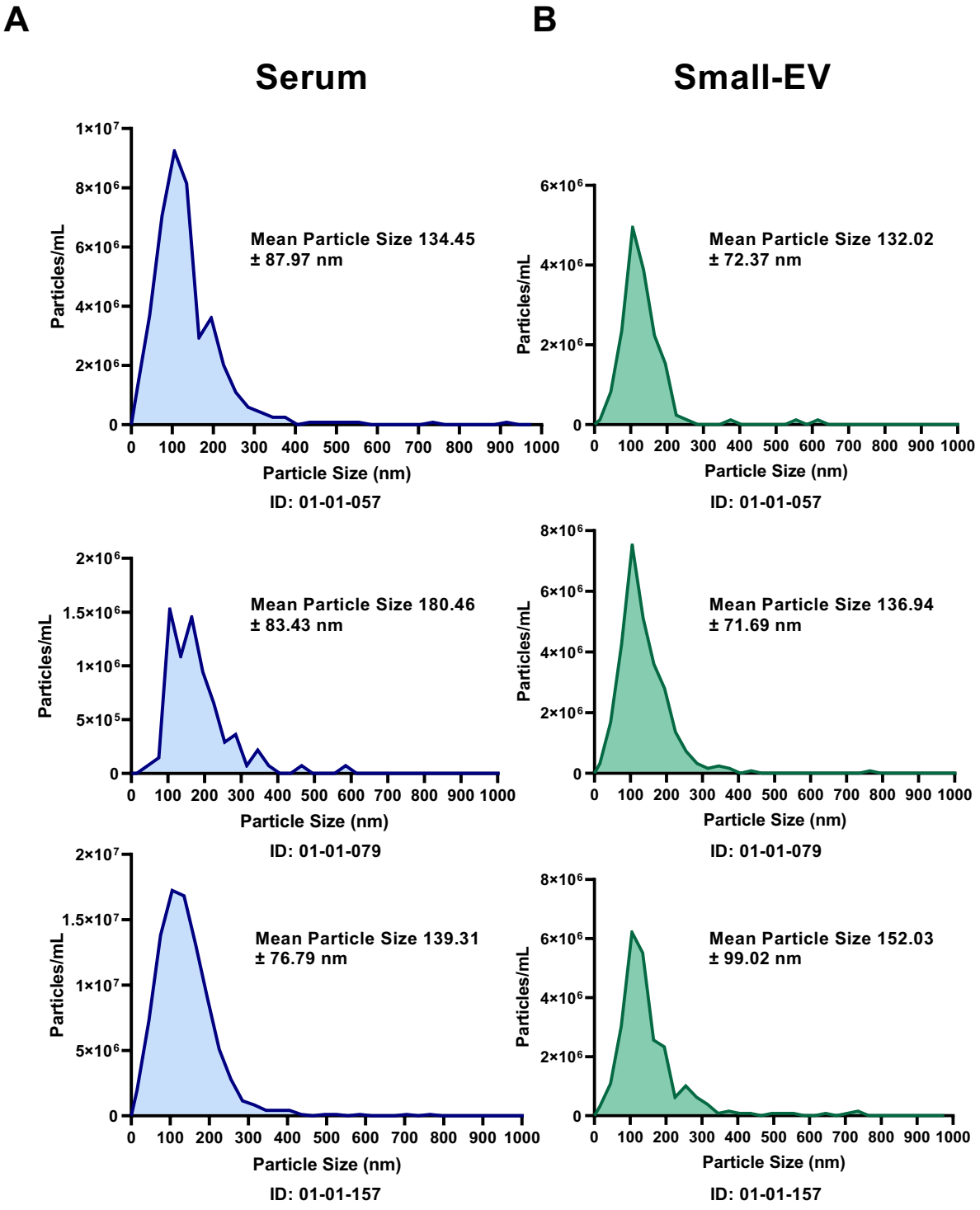

Figure 8 Particle Size Characterization of Isolated sEVs. (**A**) Particle characterization of whole patient serum of T2MI- hypertrophic cardiomyopathy patients. Mean and standard deviation values of 134.45 ± 87.97, 180.46 ± 83.43, 139.31 ± 76.79nm were observed respectively. **(B)** Particle characterization of isolated sEVs of the same patient samples. Mean and standard deviation values of 132.02 ± 72.37, 136.94 ± 71.69, and 152.03 ± 99.02nm were observed respectively.

To satisfy MISEV 2023 requirements, sEVs isolated from T2MI- hypertrophic cardiomyopathy human serum samples were characterized via protein analysis[119]. Protein expression of common sEV markers including the tetraspanin CD63 and ESCRT related protein, ALIX, were measured in whole serum, sEV-free supernatant, and the isolated sEV fraction. CD63 displayed corresponding bands in the 26 kDa range in each sample fraction. However, when quantified against the total protein loaded, there was an observed significant increase ($p<0.0001$) in the isolated sEV fraction compared to both the whole serum fraction as well as the sEV-free supernatant fraction (Figure 9A-C). ALIX expression profile displayed distinct corresponding bands within the 60-100 kDa region within the whole serum and sEV fractions while displaying minor bands in the sEV-free supernatant fractions. Quantification of ALIX expression showed a significant increase ($p=0.0445$) from the sEV-free supernatant fraction to the whole serum fraction. An increase was observed from the sEV-free supernatant fraction to the isolated sEV fraction as well, however, this increase did not reach statistical significance ($p=0.0554$) (Figure 9D-F). Taken together with the nanoparticle tracking analysis data described previously, serial centrifugation-ultracentrifugation is an effective method to isolate sEVs.

The expression pattern of cTnT was also investigated in the same context. Observable corresponding bands were detected around the 36 kDa region in the whole serum as well as sEV fractions, while faint expression was observed in the sEV-free supernatant fraction. A significant increase was displayed from the sEV-free supernatant fraction to the isolated sEV fraction ($p=0.0234$). An increase from the sEV-free fraction was observed in the whole serum, however, it did not reach statistical significance ($p=0.0672$) (Figure 9G-I).

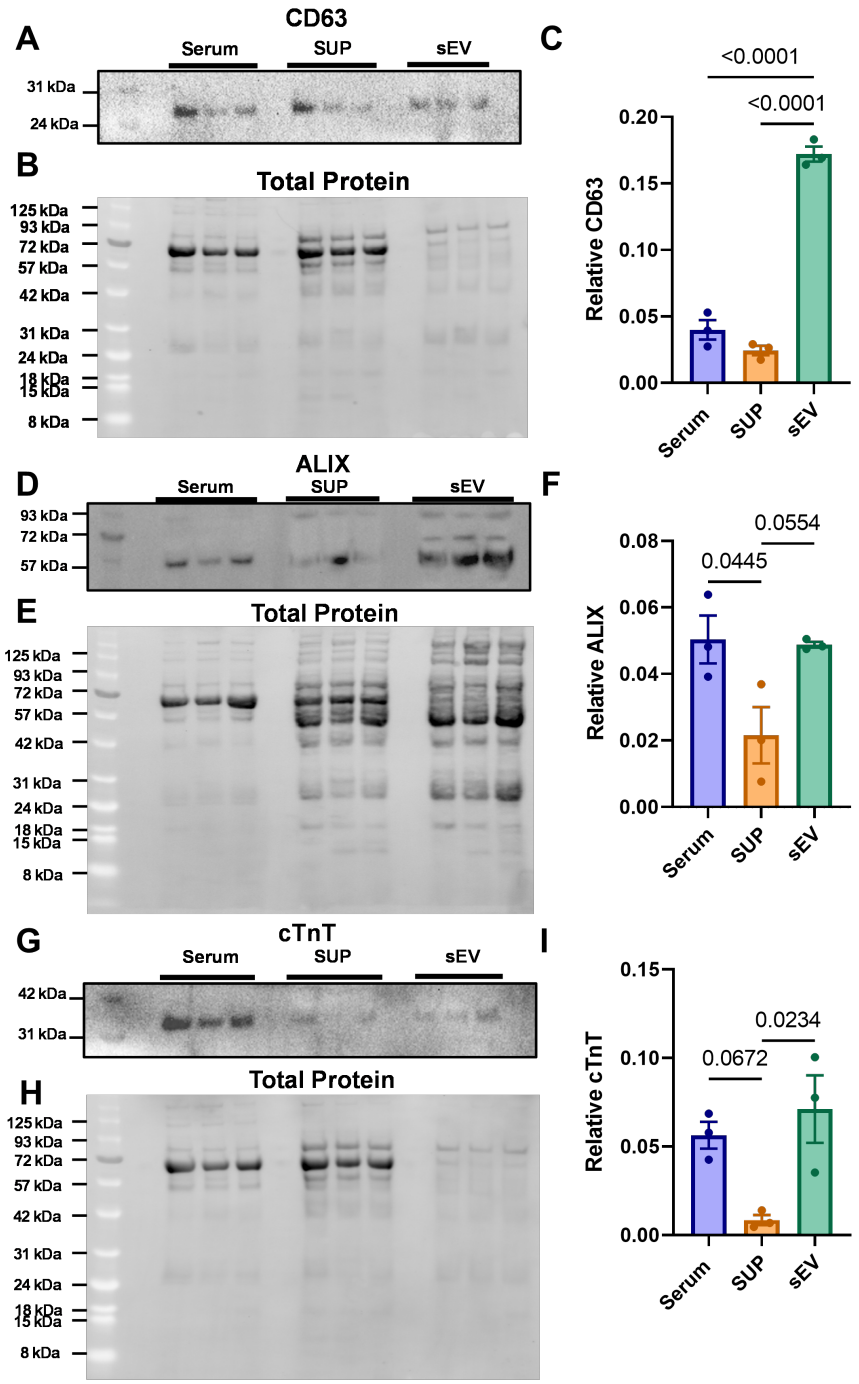

Figure 9 Protein Characterization of Isolated sEVs. (A-C) CD63 expression within T2MI-hypertrophic cardiomyopathy patient serum samples, total protein control, and quantification respectively. Expression is significantly increased in the isolated sEV (green) fraction compared to both whole serum (blue) and sEV-free supernatant (SUP) (orange) ($p<0.0001$). **(D-F)** ALIX expression, total protein control, and quantification respectively. Expression is significantly increased in whole serum compared to sEV-free fraction ($p=0.0445$) and increased from sEV-free fraction to sEV fraction, however, not significantly ($p=0.0554$). **(G-I)** cTnT expression, total protein control, and quantification respectively. cTnT is significantly increased from sEV-free fraction to sEV fraction ($p=0.0234$) and increased from sEV-free to whole serum not reaching significance ($p=0.0672$). $n=3$ for each expression analysis with significance being determined using a one-way ANOVA with significance of $p<0.05$.

5.3 Proportion of cTnT Quantified within sEVs Differs between T1 and T2MI

Determining the pattern of cTnT release, either freely or within sEVs between T1MI and T2MI- HCM, and comparing this release with the intermediate T2MI- Ischemic HF condition provides further understanding of the mechanism of disease and its progression. cTnT levels were measured with a clinical grade Roche hsTnT assay within isolated sEVs and sEV-free supernatant fractions from healthy control, T1MI, T2MI- Ischemic HF, and T2MI- HCM patients and expressed as percentages of total cTnT released.

The T1MI patient population was quantified to contain 31.75 ± 5.39% of total cTnT released within sEVs and 68.25 ± 5.39% in the freeform. The T2MI- HCM patient population displayed a trend in proportion with a greater amount of sEV bound cTnT with 48.30 ± 3.39% and correspondingly 51.70 ± 3.39% in the freeform. Whereas the T2MI- Ischemic HF group displayed 22.17 ± 4.45% in the sEV and 77.83 ± 4.45% in the freeform, similar to the rate that was observed in the T1MI setting. Correspondingly, significant increases in the proportion of sEV bound cTnT were observed in the T2MI- HCM population from both the T1MI and T2MI- Ischemic HF groups ($p=0.0169$ and $p<0.0001$ respectively). Moreover, between the T1MI and T2MI- Ischemic HF group no statistical differences were observed again indicating a similar sEV bound cTnT release profile (Figure 10A & B). Fold changes of the proportion of sEV bound cTnT were also measured compared to the healthy control population. A fold change of 0.59 ± 0.10 was observed in the T1MI population, with T2MI- HCM patients having a fold change of 0.89 ± 0.063, while T2MI- Ischemic HF patients displayed a fold change of 0.41 ± 0.082 again following a trend more similar to T1MI. This was observed to be significantly reduced in both the T1MI and T2MI- Ischemic HF groups compared to the T2MI- HCM group ($p=0.0169$ and $p<0.0001$ respectively). Again, no significant difference was observed between

the T1MI and T2MI- Ischemic HF patient populations (Figure 10C). Respective fold changes compared to healthy control of the released cTnT proportion located in the freeform were also analysed across disease states. T1MI populations displayed a fold change of 1.49 ± 0.12, while T2MI- HCM displayed a fold change of 1.13 ± 0.07. The T2MI- ischemic HF group again followed a similar trend to T1MI with a fold change of 1.70 ± 0.10. A significant increase in the proportion of cTnT quantified in the freeform was observed in the T1MI group compared to the T2MI- HCM group ($p=0.0169$). Additionally, the T2MI- Ischemic HF group displayed a significant increase compared to the T2MI- HCM group ($p<0.0001$) but was not significantly different compared to the T1MI setting (Figure 10D).

Individual patient cTnT release profiles were used to examine the presence of inter-patient variability in cTnT release within each disease group. In healthy control patients, there is very little variation in the release profile as seen by a very consistent split with a ratio of 0.5 for both sEV and freeform cTnT (Figure 10E). In T1MI patients, there is greater variation in the release profile which is indicative of high levels of inter-patient variability in the proportion of cTnT released within sEVs (Figure 10F). The T2MI- Ischemic HF group also showed some level of variation again indicating the presence of inter-patient variability (Figure 10G). In the T2MI- HCM context, there was a relatively consistent spread of sEV bound cTnT around the 0.5 mark. However, one patient (ID: 01-01-167) displayed a ratio substantially lower (Figure 10H).

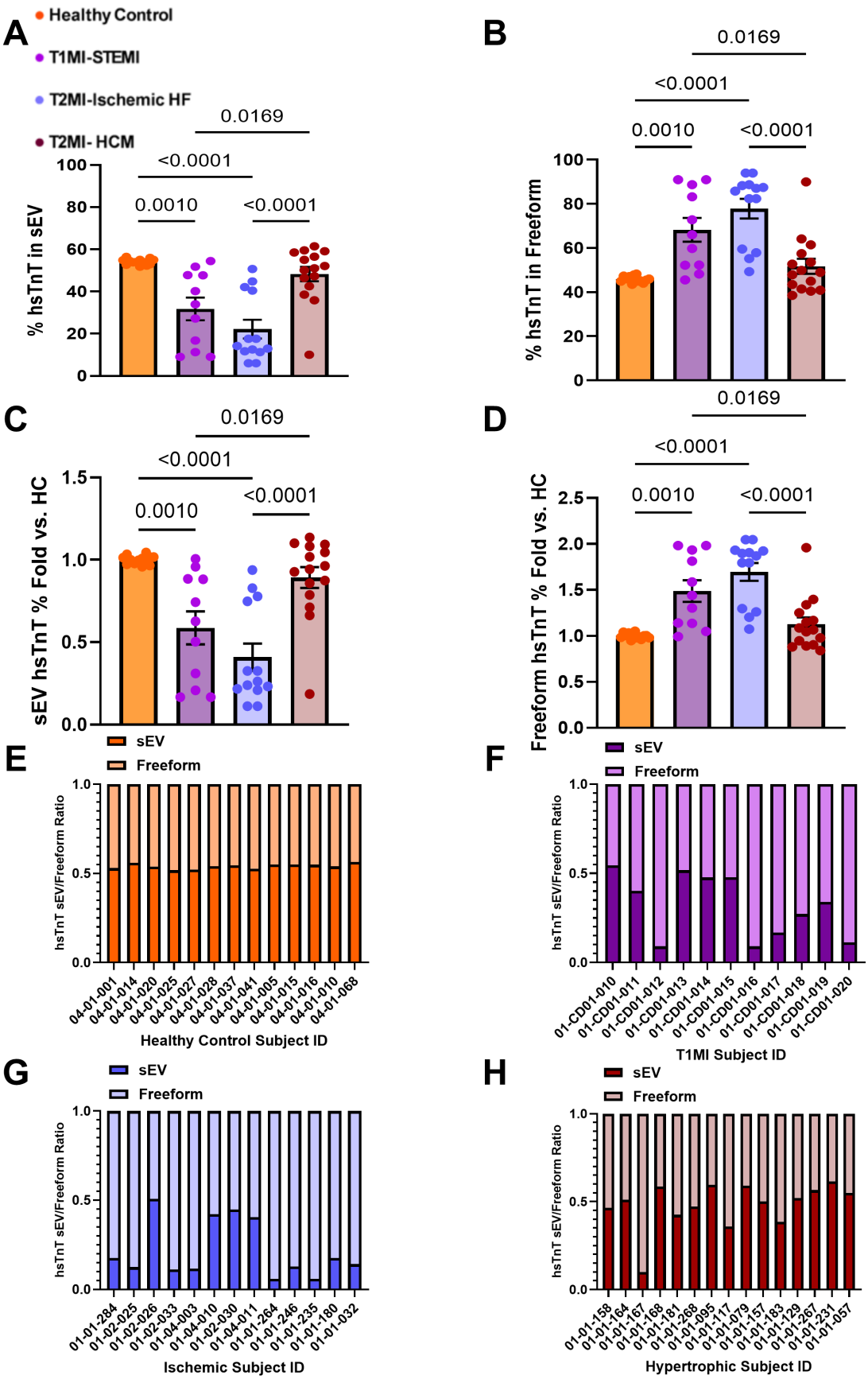
A
Healthy Control
T1MI-STEMI
T2MI-Ischemic HF
T2MI- HCM
% hsTnT in sEV
0.0169
<0.0001
0.0010
<0.0001
B
% hsTnT in Freeform
0.0169
<0.0001
0.0010
<0.0001
C
sEV hsTnT % Fold vs. HC
0.0169
<0.0001
0.0010
<0.0001
D
Freeform hsTnT % Fold vs. HC
0.0169
<0.0001
0.0010
<0.0001
E
sEV
Freeform
hsTnT sEV/Freeform Ratio
04-01-001
04-01-014
04-01-020
04-01-025
04-01-027
04-01-028
04-01-037
04-01-041
04-01-005
04-01-015
04-01-016
04-01-010
04-01-068
Healthy Control Subject ID
F
sEV
Freeform
hsTnT sEV/Freeform Ratio
01-CD01-010
01-CD01-011
01-CD01-012
01-CD01-013
01-CD01-014
01-CD01-015
01-CD01-016
01-CD01-017
01-CD01-018
01-CD01-019
01-CD01-020
T1MI Subject ID
G
sEV
Freeform
hsTnT sEV/Freeform Ratio
01-01-284
01-02-025
01-02-026
01-02-033
01-04-003
01-04-010
01-02-030
01-04-011
01-01-264
01-01-246
01-01-235
01-01-180
01-01-032
Ischemic Subject ID
H
sEV
Freeform
hsTnT sEV/Freeform Ratio
01-01-158
01-01-164
01-01-167
01-01-168
01-01-181
01-01-268
01-01-095
01-01-117
01-01-079
01-01-157
01-01-183
01-01-129
01-01-267
01-01-231
01-01-057
Hypertrophic Subject ID

Figure 10 cTnT Localization within sEVs vs Freeform. (A) Proportion of hsTnT within sEVs in Healthy Control (orange), T1MI (purple), T2MI- Ischemic HF (blue), and T2MI- HCM (red) patients. Significant increase was observed from T1MI to T2MI- HCM ($p=0.0169$). As well, between T2MI-HCM showed significant reductions to T2MI- Ischemic HF ($p<0.0001$). No significant differences were observed between T1MI and T2MI- ischemic HF. **(B)** Proportion of hsTnT within freeform in each disease state. Significant differences were observed between T1MI and T2MI- HCM ($p=0.0169$), and T2MI- Ischemic HF and T2MI- HCM ($p<0.0001$). No difference observed between T1MI and T2MI-ischemic HF. **(C)** Fold change of proportion of hsTnT within sEV compared to healthy control with T1MI displaying 0.59 ± 0.10, T2MI- HCM displaying 0.89 ± 0.062, and T2MI- Ischemic displaying 0.41 ± 0.082 where significant differences were observed between T2MI- HCM and both T1MI and T2MI- Ischemic HF ($p=0.0169$ and $p<0.0001$ respectively). T1MI and T2MI- ischemic did not display a significant difference. **(D)** Fold change of proportion of hsTnT in the freeform compared to healthy control, with T1MI displaying 1.49 ± 0.12, T2MI- HCM displaying 1.13 ± 0.07, and T2MI- ischemic HF with 1.70 ± 0.10 with significant differences observed between T2MI- HCM and T1MI ($p=0.0169$) and T2MI- Ischemic HF ($p<0.0001$). T1MI and T2MI- ischemic did not display a significant difference. **(E-H)** Individual patient hsTnT localization in **(E)** healthy control, **(F)** T1MI, **(G)** T2MI- Ischemic HF, and **(H)** T2MI- HCM. Statistical analysis performed to determine significance was a 2-way ANOVA with multiple comparisons where significance was reached at $p<0.05$.

5.3.1 Female Sample Size limited determination of Sex-based difference in sEV cTnT

It was next sought to evaluate potential sex specific differences in the release of cTnT within sEVs. In the healthy control patient population, there were 7 males and 6 females. The male patients displayed a proportion of sEV hsTnT of 54.35 ± 0.50% compared to the females where it was 53.83 ± 0.64%, which was not a significant difference (Figure 11A). In T1MI males (n=9) had a mean proportion of sEV bound cTnT of 34.78 ± 5.99% whereas in the female (n=2) population it was observed to be 18.15 ± 9.09% (Figure 11B). Due to a lack of females within the patient population, statistical significance could not be determined. The T2MI-Ischemic HF population had values of hsTnT within sEVs in males (n=10) of 18.83 ± 4.70% while in the female population (n=3) the hsTnT values were observed to be 33.29 ± 10.19%; this difference was not statistically significant (Figure 11C). Both males (n=7) and females (n=8) in the T2MI- HCM population showed similar distribution of hsTnT within the sEVs with 45.08 ± 6.30% and 51.12 ± 3.30% respectively, which was not a significant difference (Figure 11D). Collectively, no differences in sEV bound cTnT content were observed between sexes within each patient population.

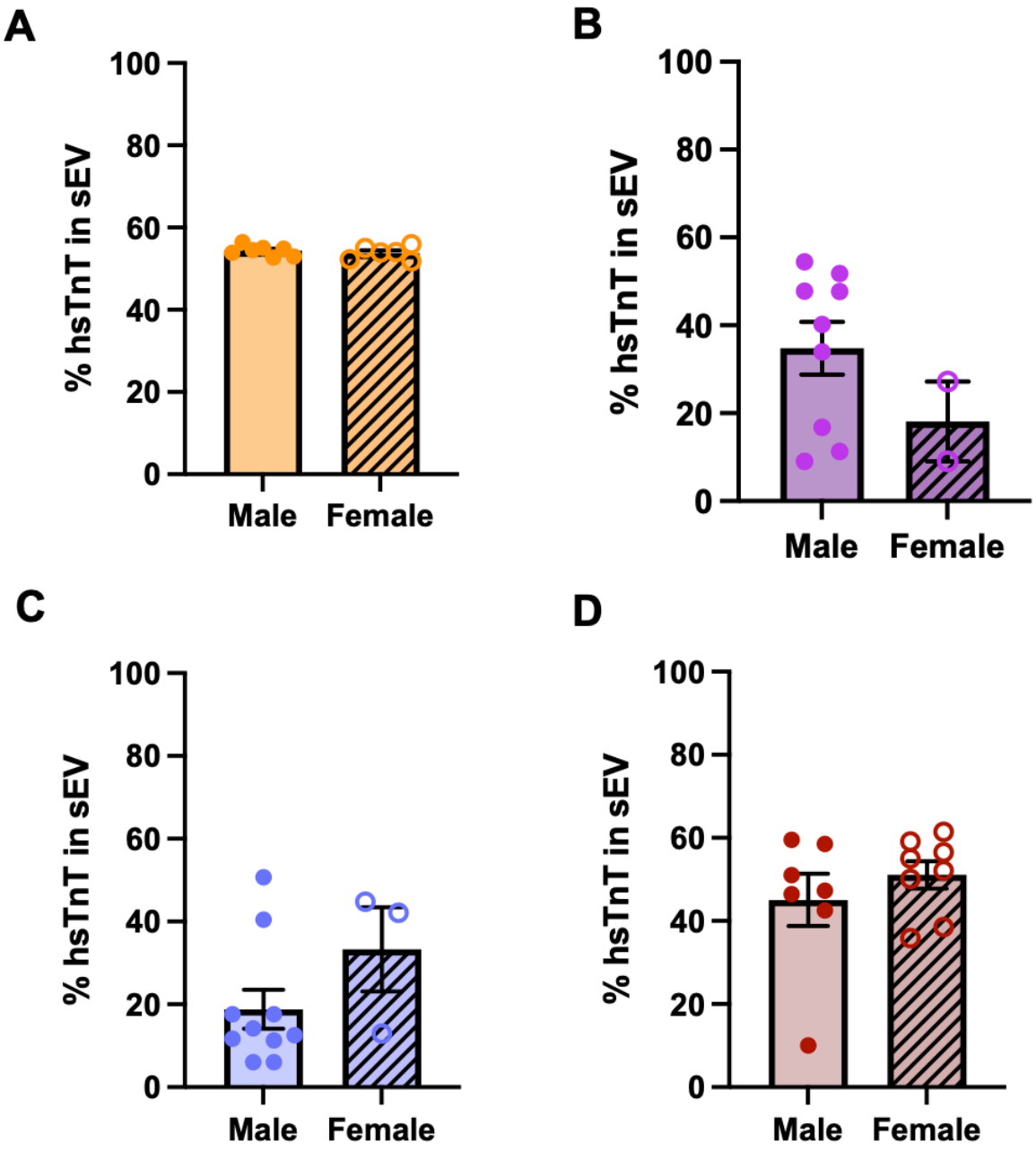

Figure 11 Male vs. Female cTnT Localization within sEVs. cTnT proportion within sEVs between males and females in **(A)** Healthy Control participants 54.35 ± 0.50% and 53.83 ± 0.64% respectively, in **(B)** T1MI participants with 34.78 ± 5.99% and 18.15 ± 9.09% respectively, in **(C)** T2MI- Ischemic HF patients with 18.83 ± 4.70 and 33.29 ± 10.19% respectively, and in **(D)** T2MI- HCM patients with 45.08 ± 6.30% and 51.12 ± 3.30% respectively. In all groups, differences were not observed to be statistically significant. All values represented as % of total hsTnT. Statistical analysis carried out to determine significance was a one-way ANOVA with subgroup comparisons where significance was reached at $p<0.05$.

5.3.2 Culprit Artery Showed no difference in sEV Bound cTnT

To identify the impact of whether the culprit artery implicated in the T1MI altered the release profile of cTnT within the sEVs, patients were segregated based on their culprit artery diagnoses, and subsequent analysis via proportion of cTnT localization was carried out. A total of 7 patients were diagnosed with a LAD culprit artery. This patient population exhibited 28.90 ± 7.14% of recovered hsTnT within the sEVs. The remaining 4 patients presented with right coronary artery culprits. Within this population, 36.75 ± 8.68% of hsTnT was found to be located within the sEVs. This difference in sEV bound cTnT was not statistically significant (Figure 12A).

Additionally, whether patients presented with multiple arteries occluded more than 70% was examined to identify the impact of coronary artery disease on the release of cTnT within sEVs. In total, 8 patients presented with only one artery that was occluded at a rate greater than 70%, while 3 presented with multiple arteries greater than 70% occluded. Patients with only one artery occluded had a release of 31.54 ± 6.49% of cTnT within the sEV, while the population with more than one artery occluded had sEV bound cTnT content of 32.31 ± 11.84%, which was not statistically significant (Figure 12B).

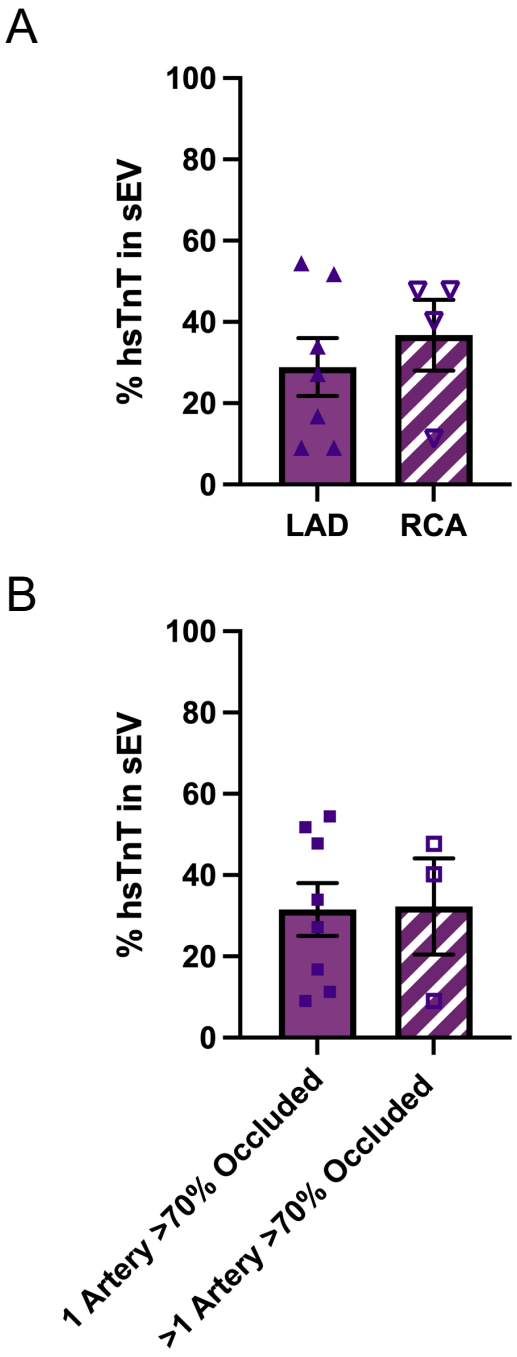

Figure 12 cTnT Proportion within sEVs based on Culprit Arteries in T1MI. cTnT quantity located within sEVs based on **(A)** culprit artery in T1MI and **(B)** severity of coronary artery disease in T1MI patients as determined by whether or not they presented with more than 1 artery with an occlusion greater than 70%. **(A)** No statistical difference in sEV bound cTnT was observed between patients presenting with an LAD or RCA culprit artery with 28.9 ± 7.14% and 36.75 ± 8.69% respectively. **(B)** patients with more than 1 artery occluded at 70% also did not show significant differences compared to only 1 artery occluded with sEV cTnT contents of 31.54 ± 6.49% and 32.31 ± 11.84% respectively. hsTnT values are represented as % of total. Statistical analysis carried out to determine significance was an unpaired T-Test where significance was reached at $p<0.05$.

5.4 cTnT Isoformic Determination Requires Further Refinement

When analyzing sEVs from human serum samples, highly abundant proteins within the serum may hinder this process and obstruct subsequent analysis[120]. An albumin removal protocol was implemented to minimize such impacts and increase the precision of downstream analyses. Albumin expression levels were measured via western blot analysis in whole serum, sEV-free supernatant, and sEV fractions post albumin removal. Distinct bands corresponding to albumin were observed at roughly 65 kDa in both the whole serum and sEV-free supernatant fraction, while bands were essentially non-existent in the sEV fraction (Figure 13A & B). Significant reductions were observed when comparing the expression of albumin in both the whole serum and sEV-free supernatant fractions to the sEV fraction with $p=0.0024$ and $p=0.0004$ respectively. No significant difference was observed between the whole serum and sEV-free supernatant fractions (Figure 13C).

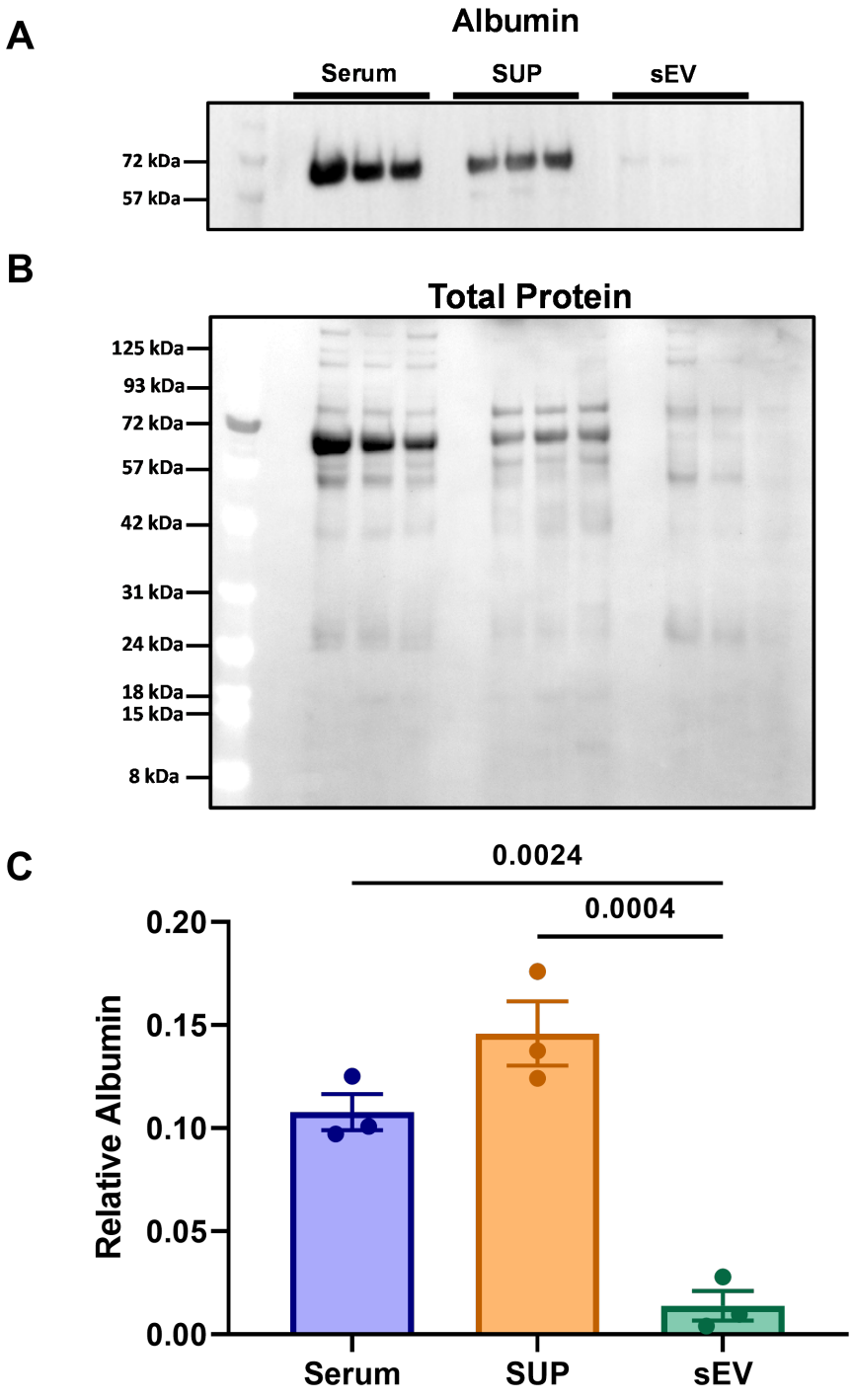

Figure 13 Patient Serum Albumin Removal. **(A)** Protein expression of albumin post albumin removal in whole serum, sEV-free supernatant (SUP), and sEV fractions. **(B)** Total protein control of loaded samples. **(C)** Quantification of albumin expression in whole serum (blue), sEV-free supernatant (orange), and sEVs (green). Significant decrease observed in sEV fraction compared to whole serum and sEV-free supernatant ($p=0.0024$ and $p=0.0004$). $n=3$ for each analysis with significance determined by one-way ANOVA with multiple comparisons with $p<0.05$.

To generate a highly pure fraction of isolated cTnT within each fraction, a cTnT specific immunoprecipitation was conducted. In each fraction, whole serum, sEV-free supernatant (SUP), and sEV distinct bands corresponding to cTnT were identified at the size range of 49 kDa as well as 27 kDa (Figure 14A). Within the whole serum and the sEV fractions, the signal was stronger indicating a greater concentration of cTnT within these fractions as compared to the sEV-free supernatant fraction where the signal intensity was reduced. When protein expression was quantified against the total protein loaded (Figure 14B), a relative increase in cTnT was observed in both the whole serum and sEV fractions. The sEV-free supernatant fraction showed the lowest content of cTnT (Figure 14C). This is indicative that post-immunoprecipitation, cTnT located within the isolated sample fractions has been extracted and concentrated.

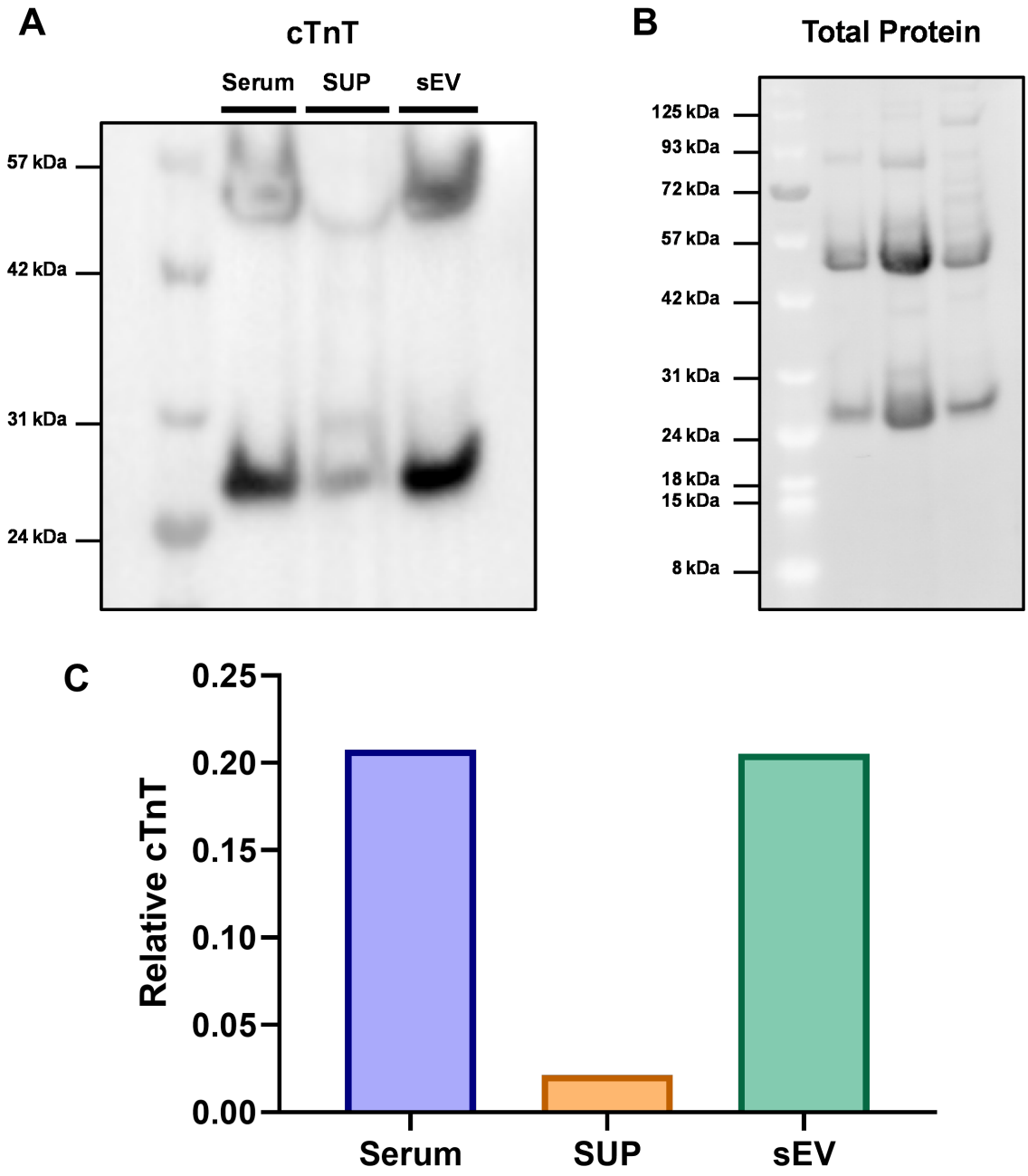

Figure 14 Purification of cTnT. (A) Protein expression of cTnT post immunoprecipitation from whole serum, sEV-free supernatant (SUP), and sEV fractions from T2MI-hypertrophic cardiomyopathy patient sample. Distinct band corresponding to cTnT were observed at 49kDa and 27kDa in whole serum and sEV fractions, while faint bands at the same weights were observed in the SUP fraction. **(B)** Total protein of sample loaded. **(C)** Quantification of cTnT expression in whole serum (blue), sEV-free supernatant (orange), and sEVs (green). Increased levels were observed in the whole serum and sEV fractions as compared to the sEV-free supernatant fraction. Statistical analysis not conducted as n=1 for each fraction.

6. Discussion

Circulating levels of cTnT increase in patients presenting with either T1MI or T2MI and currently, there are no non-invasive diagnostic measures to rapidly differentiate these disease states due to overlapping detectable cTnT in the serum of both types of MI. Presumptive diagnoses of T1MI are often conferred on all patient's with positive cTnT due to the need for rapid treatment[15]. This results in many more T2MI patients undergoing unnecessary invasive procedures[15]. A novel release mechanism of cTnT within sEVs has been identified which may offer potential as a diagnostic tool capable of differentiating T1 vs. T2MI. Additionally, it is not clear what isoform distribution of cTnT is located within the sEVs compared to the freely released form. Therefore, this thesis aims to profile the form and release (freeform of sEV bound) of cTnT between T1MI and T2MI.

A cohort of 52 patients consisting of healthy controls (n=13), T1MI- STEMI (n=11), T2MI- non-ischemic hypertrophic cardiomyopathy (n=15) and intermediate form of T2MI- Chronic HF with ischemic etiology (n=13) were included in the study. Across study groups, many variables remained consistent including age and weight. In contrast, BMI trended up from T1MI patients, to T2MI- Ischemic HF, to T2MI HCM. T1MI having the lowest BMI of the disease groups is expected, as the majority of patients who will present with a STEMI have a BMI range of 25-30kg/m^2 as is observed with this population having a mean of 26.56 ± 5.02kg/m^2 [122]. In T2MI patients in both the ischemic HF and HCM groups, increased BMI levels are associated with worse disease progression[123,124]. With study inclusion being dependent on patient presentation to the hospital, it is more likely to obtain a patient population with a more severe form of disease. Consequently, patients with a higher BMI were included at a greater rate due to their increased disease severity. The T1MI sample population had an

80/20 split for male/female, however, in patients presenting with STEMI epidemiological trends indicate males account for roughly 75% of this population, while females make up the minority of 25%[125]. Similarly in ischemic HF, the prevalence skews towards males having elevated rates compared to females, which the roughly 75% male population in the ischemic HF group displays[126]. While both the T1MI and T2MI- ischemic HF groups follow the epidemiological trend, females are significantly underrepresented in the study. In the HCM group, epidemiological trends still display an increased proportion of males being diagnosed, at roughly 60% of diagnoses[127]. However, it has been observed that females experience worse prognoses compared to males[127]. With study inclusion again being dependent on hospital presentation, female patients may be more likely to require care due to more severe disease progression, which could explain the 50/50 split observed in the HCM group. Levels of hs-TnT were very low in healthy control populations. This was an expected result as cTnT should not be readily released in patients who are not experiencing any cardiac injury[13]. T1MI patients had the greatest levels of hs-TnT of all groups, likely the result of significant acute death of cardiomyocytes associated with STEMI[128]. While ischemic HF displayed lower hs-TnT levels than T1MI, it displayed much elevated hs-TnT compared to HCM patients. This can be explained through ischemic HF having an ischemic etiology which will result in small amounts of ongoing myocardial necrosis, especially during heart failure decompensation which brought the patients to the hospital and being enrolled in the current study[129,130]. Conversely, the primary etiology of HCM is left ventricular myocardial thickening which should not be accompanied by acute myocardial necrosis and associated cTnT release[129,130]. Nevertheless, cTnT release is observed in HCM as we have here. This is consistent with the hypothesis that the cTnT release in cardiomyopathies is due to a different process than acute ischemia, supported further by our

observation of higher cTnT content in sEVs being actively released. As expected in the healthy control population, proBNP II levels were within the normal range of 125 pg/mL for individuals under the age of 75[131]. Both ischemic HF (6952.39 ± 8397.18pg/mL) and HCM (1866.25 ± 2047.37pg/mL) populations had proBNP II levels well in excess of this 125 pg/mL range, indicating a diagnosis of heart failure and thus their inclusion in the T2MI patient populations. The ethnic demographic in each study group heavily favoured individuals from European/Caucasian backgrounds; in all cases, over 80% of the study population identified as a member of this group. Overall epidemiological data suggests that other ethnic populations including Black, Asian, and South-Asian have elevated levels of cardiac disease[132,133]. As such, the study data lacks diversity in the patient population which will need to be enhanced to define the generalizability of the results to all demographic groups. Underrepresentation of these populations is likely a result of the predominant ethnic background in the general population of Ottawa and surrounding communities, where the study populations were recruited from, being primarily of European/Caucasian descent.

Interesting results were also observed based on the medical history of patients included in each study group. All healthy control participants showed no major medical histories associated with the development of cardiovascular disease, as these were all exclusionary criteria. Most patients in both T2MI populations had previous diagnoses of hypertension. In both ischemic HF and HCM, hypertension has been implicated in the development and worsening of the disease state[134,135]. Nearly half of the patients presenting with T1MI had hypertension as an accompanying comorbidity. This agrees with the epidemiological prevalence of hypertension at around 40% in patients presenting with STEMI[136]. Diabetes incidence was higher than what would be expected in the T1MI and HCM populations where

expected rates are roughly 20% and 10-15% respectively[137,138]. Elevated rates may be resultant of more severe disease in patients with diabetes as a comorbidity, and thus an increased need for hospital presentation, a requirement for study inclusion[137,138]. Diabetes rates in the ischemic HF group agrees with the epidemiological rate of 20-40%[139]. Dyslipidemia was prevalent in both T1MI and ischemic HF groups which is expected as it is a major risk factor in each disease state due to the significant role it plays in the development of atherosclerosis and coronary artery disease[140,141]. Smoking history was elevated in each population, unsurprisingly, due to the increased risk of cardiovascular disease which accompanies cigarette smoking[142].

Continued efforts to increase the patient sample size will be crucial to determine the generalizability and potential broad impact of the results. A particular focus must be placed on recruiting a greater number of female participants, namely in the T1MI as well as the ischemic HF groups, to allow for the identification of any sex differences in the release pattern of cTnT. Similarly, the ethnic background demographic would benefit from an increase in diversity. As such, a focus should be placed on recruiting individuals from Middle Eastern, Asian, and African backgrounds which were vastly underrepresented in the patient population collected. This would allow for a determination of whether these results hold true across a variety of clinical settings.

Characterization of the sEV isolated fractions from patient serum samples followed MISEV 2023 guidelines by examining both the particle size range isolated as well as the protein determination of the vesicles[119]. It is crucial to employ multiple methods of EV characterization as no one method can meet all requirements due to the heterogeneity of EV sizes, molecular contents, and the lack of EV-specificity in the measurement techniques that are implemented to characterize EVs[119]. Analyses via nanoparticle tracking and western blot

revealed a particle size within the desired range of sEVs, 40-160nm, and an increased expression of sEV specific markers, CD63 and ALIX. This suggests that the serial centrifugation-ultracentrifugation technique that was employed for the purposes of sEV isolation was successful at separating these vesicles from the rest of the serum sample. Consequently, this allowed for an effective comparison between the cTnT contents located within the sEVs and the cTnT content that was found freely released into the serum. Without effective sEV isolations, accurate quantifications of cTnT in each fraction would not be possible. The data may then not display the true release pattern observed in these disease contexts and the validity of the use of sEV bound cTnT as a potential diagnostic tool would be limited or potentially overlooked.

Isolating and characterizing sEVs does not come without challenges, particularly during the visualization of sEV markers and contents via western blot analysis. The protein content within sEVs is significantly lower than that of serum which contains significant amounts of albumin and lipoproteins, which makes subsequent detection of the sEV specific proteins difficult[143]. To overcome this obstacle, a greater quantity of protein within the sEV fractions must be loaded to facilitate a more distinctive visualization. While clear distinctions on the protein content found within the whole serum, sEV-free supernatant fractions, and sEV fractions can be made, visualization is not as clear as when analyzing serum proteins. Future equalization may be considered to generate more discernable bands in the sEV fraction by further reducing serum quantity loaded or increasing sEV lysate loaded. Additionally, sEV isolation techniques are not completely successful at separating every sEV within the sample[119]. As such, as is observed in Figures 9A and D, small quantities of signal from sEV specific markers were observed in the sEV-free supernatant fractions. Consequently, in

downstream analysis, minute quantities of contents located within the sEV-free supernatant may be attributable to sEVs. In future other isolation techniques could be implemented which may increase the purity of the sEV fractions collected, however, when the specificity of the technique increases the total yield of sEVs collected will decrease[119]. Correspondingly, when the yield is increased, the purity of the isolated sample will decrease[119]. The imperfection of these techniques increases the challenges associated with studying sEVs and their related contents and roles[119]. Further refinement is imperative to ensure highly pure fractions of sEVs can be obtained without the sacrifice of a reduction in yield.

To determine the diagnostic potential of cTnT release within sEVs between T1MI and T2MI, concentrations of cTnT found in both the sEV and freeform fractions were measured. To exhibit potential as a diagnostic tool, a significant difference in the mechanism of release must be identified, as observed by significant variations in the localization of the released cTnT. There are significant differences observed between the disease groups with the proportion of cTnT released within the sEVs, where T2MI- HCM displayed elevated levels compared to T1MI. The intermediate T2MI- Ischemic HF setting displaying a similar release profile to the T1MI setting. This indicates that the mechanism of release varies between these disease states. This alternate release profile offers potential to then differentiate these disease contexts based on the distribution of isolated cTnT. In each setting, cTnT was quantified within sEVs which could be explained as follows. With sEVs being understood to play a vital role in the cellular communication process, sEVs may be constitutively released from cardiomyocytes to exert a maintenance function[68-71]. This is supported by the presence of cTnT within sEVs in the healthy control patients who are not experiencing any cardiac related injury or disease. What role these vesicles, and the cTnT packaged within them, play remains to be understood.

However, furthering knowledge in this area could provide greater insight into inter-cellular communication and transport within the heart and potentially beyond, as well as a possible progression of disease. sEVs may also display a maximum quantity of cTnT that can be packaged and when that limit is reached, additional cTnT will be released freely from the cell. This could provide an explanation for the observations of the T2MI- Ischemic HF group where the majority of cTnT is seen released freely from the cell without the presence of significant myocardial necrosis, while a similar level to the other groups is released in the sEV. In patients presenting with stable heart failure, the pattern of cTnT release matching an acute MI setting would not be expected[130]. Therefore, a model of release where there is a limit in cTnT packaging into sEVs with any extra being released freely would potentially provide an explanation for this unanticipated release pattern.

Considerations must be made when identifying the cause of the proportional differences that were observed. During an MI, sEV release is also increased in cells other than cardiomyocytes, including endothelial and immune cells[144]. It is crucial to identify which cells are releasing the sEVs which contain cTnT. With the mechanism of sEV cargo loading being known to depend on incorporating intracellular molecules, it would suggest that as only the cardiomyocytes possess cTnT, they would be the cells responsible for the release[68-73]. To ensure this is the case, an immunofluorescence co-staining of released sEVs with other cardiomyocyte specific markers such as alpha and beta-sarcoglycan and desmin could be conducted[145]. It has also been described that sEVs possess a corona of proteins attached to its exterior[146]. This corona may trap proteins located freely within the serum, such as cTnT[146]. This would indicate that the cTnT is not released within the sEVs, rather free cTnT in the serum is incorporated into the sEV corona. A co-staining process of cTnT with specific sEV

membrane markers, tetraspanins (CD9, CD63, or CD81) could be used to identify whether the detected cTnT is bound within the sEV or attached to its exterior. After release, sEVs are primarily cleared from the body through phagocytosis by macrophages[147]. With the varying levels of sEVs being observed in the different disease states, it was hypothesized that the rate of release was increased in the T2MI setting. However, it is possible that the rate of release remains constant, but the rate of removal is reduced in T2MI. To determine whether there is any variation in macrophage activity, an examination of specific molecules produced by active macrophages, including cytokines TNF-α, IL-1β, and IL-6 would be undertaken[148]. Additionally, identification of macrophage markers CD14, CD68, and CD163 could be used to identify whether the concentration of macrophages differs across disease states, thus contributing the differences in sEV concentration[149].

Interestingly, it was observed that the T2MI- Ischemic HF population showed similar trends in the release of cTnT to patients experiencing a T1MI, where there was a predominant release within the freeform as compared to within the sEVs. As significant ischemic injury and myocardial necrosis are hallmarks of an acute MI, it is expected that the majority of cTnT would be released freely into the blood. However, as the ischemic HF group displayed the same trend, it is suggested that a similar level of myocardial necrosis occurred in these patients. As these are stable HF patients, a chronic level of cardiomyocyte death of this magnitude would be unlikely as it would impart severe negative impacts on the health of the patient and their cardiac functioning[150]. This would then suggest the presence of an additional mechanism of release of cTnT from the cardiomyocyte that does not require membrane rupture but is not packaged into a sEV. Additionally, this observation provides insight into ischemic cardiomyopathies possessing symptoms and presentations of both classical cardiomyopathies

as well as acute ischemic cardiac injury. Each patient within this group possessed a diagnosis and classic symptoms of heart failure as described in Table 1, but now also displays a characteristic release profile of cTnT that was believed to be heavily associated with the acute myocardial ischemic injury of a T1MI. Classically, factors associated with acute myocardial injury would not be considered in patients with chronic ischemic cardiomyopathy[151]. This suggests that ischemic cardiomyopathies may present as a hybrid condition where patients have aspects of both chronic and acute myocardial injury.

It must be noted that there is the presence of inter-patient variability within each disease group, outside of the healthy controls which showed remarkable consistency in the release profile of cTnT. The consistency in the healthy control group likely stems from very low levels of cTnT being released in this context which therefore does not allow for much variation. In cases of elevated cTnT, variation can be observed, which is likely a result of each patient possessing unique characteristics, patient histories, comorbidities, and genetic factors that may impact the progression and presentation of these cardiac diseases[152]. As such, to effectively account for and minimize the impact of this variability within patient groups, a larger sample size must be collected.

The presentation of cardiovascular disease varies between males and females, with differences in presentation, progression, and treatment being identified[153,154]. While the underlying etiology behind these sex-based differences are not wholly elucidated, it does present an important clinical challenge[153,154]. Consequently, diagnostic tools must be investigated and developed in a manner that identifies and accounts for any sex-based variations[155]. Coordinately, a disaggregated investigation of the release of cTnT within sEVs was conducted based on patient sex. In healthy controls and both T2MI populations, no

significant differences were observed, indicating that males and females have the same pattern of release of cTnT within the sEVs. But this is in the context of extremely low levels of cTnT in healthy controls, and the relevance to disease settings cannot be assumed. In the T1MI population, there appears to be a larger difference, however, due to the epidemiological rates of T1MI, only 2 females were included in this analysis. Accordingly, it was not possible to identify whether this was a significant shift, or if this is a product of inter-patient variability. To generate a greater understanding of the presence or absence of sex-related disparities, a much larger female patient population must be collected and analysed.

In T1MI patients, which artery is found to be the culprit artery has been shown to impact the extent of myocardial injury and clinical outcomes[156,157]. It was observed that patients presenting with an LAD lesion have a greater extent of myocardial ischemia and death, correlating to worse outcomes as compared to patients presenting with a lesion in the RCA or LCx[156,157]. As such, a determination of whether there is a differential release pattern of cTnT was sought. Regardless of whether there was an LAD or RCA lesion, cTnT release exhibited the same pattern indicating that the culprit artery, and thus the location of the infarction does not alter the release mechanism of cTnT. In both contexts, significant ischemic injury will take place, whereby cTnT release will heavily favour the freeform because of consequent membrane rupture and cell death, as was seen. While no patients in this population presented with an occlusion of the LCx, as it is the least common between the LAD, RCA, and LCx, it would be expected to display a similar pattern due to possessing the same mechanisms of disease[158].

Patients presenting with multiple coronary arteries occluded have a more severe form of coronary artery disease, which has been implicated in worse outcomes and more extensive

ischemic injury during T1MI[159,160]. Patients diagnosed with more than 1 coronary artery with an occlusion of 70% or greater and patients presenting with only 1 artery showing such occlusion were identified to determine the presence of a differential release pattern of cTnT. The 70% occlusion was selected as this is the point where there is a clinical indication to intervene on the artery and insert a stent to restore blood flow[161]. There was not a significant difference between the quantity of cTnT released within sEVs based on whether the patient had 1 or more coronary arteries occluded. This is indicative that the extent of CAD the patient presents with does not alter the release profile of cTnT in the ischemic context of a T1MI. This is likely a result that the primary factor for the acute release of cTnT in this setting is an ischemic cardiomyocyte injury[13]. Thus, with only a one-time sample being collected following T1MI, the release pattern should follow the trend where freeform cTnT predominates, as was observed[13]. In a chronic setting, there is potential for variations in cTnT release to arise. With patients experiencing more severe forms of CAD, an observed progression to ischemic cardiomyopathy and related cardiac remodeling may occur[162]. Thus, during this development, it is unclear whether cTnT may be released primarily in sEVs or whether this cTnT is involved in the disease progression. Future sampling of chronic-CAD patients would provide further insight into the release and potential role of cTnT in patients with multivessel disease compared to single vessel disease before an acute event occurs.

With cellular communication being a primary characteristic of sEVs, it generates the question as to what role the sEVs which contain cTnT are playing on the surrounding cardiomyocytes, and potentially beyond. As such, further investigation should take place in this realm to explore the potential impacts and associated mechanisms. Experimental examination could be undertaken as follows. HL-1 cells treated with phenylephrine generate

large quantities of sEVs containing cTnT as was described in section 1.10. Additional treatment of HL-1 cells with cTnT knocked out will generate large quantities of sEVs, without the cTnT cargo. These sEVs will be collected and administered to cultured cardiomyocytes. An all-encompassing catalog of molecules, including proteins, miRNAs, and mRNAs found within these sEVs will be crucial to ensure the only physiological difference is the presence of cTnT. This would allow for a determination of whether the packaged cTnT imparts an impact on the recipient cells, or whether there are other molecules present within the sEVs facilitating a biologic impact. Notable markers and pathways of cardiac hypertrophy including Nppb, Nppa, β-myosin heavy chain, calcineurin/NFAT, and GATA4/p300 will be measured to identify potential remodeling impacts of the cTnT containing sEVs[163,164]. Additionally, immune markers associated with myocardial injury and remodeling will be assessed, including TNF-α, IL-6, and IL-1β[165]. Interestingly, cognitive impairments are common in patients post-MI or patients experiencing cardiac remodeling[166,167]. Additionally, sEVs are capable of passing through the blood-brain barrier and potentially exerting impacts on the brain[168]. As such, investigating the impact of these sEVs on neuronal cells, namely hippocampal cells as this region is primarily implicated in cognition, may provide further insight into cardiac related cognitive impairments[169]. Cellular markers commonly associated with cognitive impairment including C-reactive protein, neurofilament light, and glial fibrillary acidic protein will be measured to identify potential impacts[170-172]. This investigation will provide a greater understanding of the potential far-reaching roles and impacts of these sEVs.

It is hypothesized that a different cTnT isoform proportion may be released within the sEVs compared to what is freely released. Further, the presence of a unique vesicle bound isoform could offer potential as a biomarker to aid in the diagnosis of T2MIs. Mass

spectrometry analysis was employed to determine the contents of sEVs isolated from T1MI and T2MI patient serum samples. However, sEV protein identification was not able to be determined due to the presence of serum proteins within the isolated sEV fraction, namely albumin and immunoglobulins. With sEV protein content being significantly lower compared to serum proteins, an extensively pure fraction of sEVs must be collected as any small amount of serum protein will overpower the signal and prevent the sEV contents from being identified[120]. As sEV yield is reduced as the purity of the isolation technique increases there is a challenge to isolate a sufficient fraction of sEVs to be analysed by mass spectrometry without serum protein contamination[119]. This suggested that a serial centrifugation-ultracentrifugation technique alone was not sufficient in isolating sEVs without the presence of serum contaminants. An optimization of the isolation technique and sEV refinement was necessary. An albumin/IgG removal protocol was implemented with the aim to remove any serum albumin and IgG that was present after ultracentrifugation, which was shown to effectively remove a significant quantity of albumin. Thus, it could have positive impacts on the purity of the sEV fraction and the capacity of subsequent proteomic analysis. A cTnT specific immunoprecipitation was carried out to collect only cTnT proteins from each sample fraction. This technique showed an effective isolation of cTnT molecules from both the sEV-free supernatant as well as the sEV specific fractions. This ensured that when mass spectrometry was conducted, a pure fraction of cTnT was being analysed without serum contaminants obstructing. Future outlooks will focus on incorporating each of these steps into the sEV isolation protocol to generate a highly pure fraction of cTnT. This will allow for an effective determination of the presence of any isoformic variation.

As was described in sections 1.8 and 1.9, sEVs offer great potential as biomarkers of disease as well as in therapeutic settings[68,84]. Thus, generating a thorough understanding of the contents found within the sEVs released in these disease states may provide prospective novel biomarkers that could advance diagnostic capabilities. As well, this may contribute to furthering the insight into the role these vesicles play in the progression of disease and potentially offer therapeutic targets to diminish this advancement. However, to accomplish this, there are various challenges that must be overcome. Namely, interpatient variability will exist, with each patient potentially presenting with a slightly different combination of contents found within the isolated sEVs[173]. As such, a large sample size must be collected and analysed to overcome any variability presented by these patients to provide a greater overall understanding of the contents. Additionally, as has been discussed, serum contaminants may pose issues when analysing sEV contents[173]. Correspondingly, it is crucial to ensure the sEV isolation process is extensively precise to prevent the presence of serum contaminants.

7. Conclusion

As T1MI and T2MI present similarly in the clinical setting but require different treatment routes, accurate diagnoses are crucial[15,16]. Currently, there are no established non-invasive methods capable of effectively differentiating these disease states in a timely manner[15,16]. The reality is that many T2MI patients will undergo unnecessary and potentially higher risk invasive procedures for T1MI, due to the rapid requirement for intervention in case it is a T1MI[15,16]. The novel release mechanism of cTnT within sEVs showed promise as a potential diagnostic technique to overcome these challenges. We showed in this study that the release of cTnT within sEVs displayed significant differences in the proportion isolated

between classic T1MI of STEMI patients, with classic T2MI- non-ischemic HCM patients. T2MI- ischemic HF patients with a hybrid intermediate condition resembles more closely T1MI by sEV cTnT release pattern, which is novel. Consequently, utilizing the release profile of sEV bound cTnT may offer potential to differentiate disease states and thus possesses diagnostic capability. Further refinement and analysis of the cTnT within each fraction may display isoformic proportion changes, which provides continued promise in this research as a diagnostic tool. This presents a new area of research which focuses on understanding the links between this highly ischemic cTnT release pattern and the disease progression of ischemic cardiomyopathy.

8. Appendix

8.1 Study Protocol

Evaluation of Existing Biomarker Candidates to Accurately Classify the Etiology of Heart Failure

This study has been registered at http://www.ClinicalTrials.gov under ID number NCT02347722

Protocol version Nov 14, 2024

Funding for this project is provided by Genome Canada

Principal Investigator: Dr. Peter Liu (University of Ottawa Heart Institute)
Co-Investigators: Dr Derek So (University of Ottawa Heart Institute)
Dr. Sharon Chih (University of Ottawa Heart Institute)

Dr. Lisa Mielniczuk (University of Ottawa Heart Institute)
Dr. Ellamae Stadnick (University of Ottawa Heart Institute)

This is a peer reviewed, investigator-initiated trial, funded by Genome Canada as a partnership grant with matching funds from Roche Diagnostics and University of Ottawa Heart Institute. The funder will have no role in study design, collection, management, analysis, and interpretation of data. The funding partner Roche Diagnostics will be given 60 days to review any manuscripts prior to publication.

Composition, roles, and responsibilities of:

The coordinating centre: The principal investigator (PI) site at the University of Ottawa Heart Institute (UOHI) will be the coordinating centre. The study will commence at the PI site (UOHI) and other sites will be added if recruitment goals cannot be met within the target time.

Advisory committee: This committee will be made up of representatives from Roche Diagnostics, Duke Clinical Research Institute and UOHI.

Data management team: The PI will create the data base in conjunction with the Cardiovascular Research Methods Centre at the UOHI, which will be responsible for data management and analysis.

Data monitoring committee: This committee will consist of 3 individuals, an expert in cardiology, an expert in statistics and an expert in diagnostics. This committee will report to the advisory committee.

Heart failure biomarker panel: This panel will consist of cardiologists specialized in heart failure diagnosis and treatment. The panel will be responsible for classifying heart failure from the results of the biomarker tests.

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1. INTRODUCTION

1.1 Background and rationale

Around half a million people in Canada live with heart failure and due to the aging population, this number is expected to increase annually. There are several different classifications of heart failure. Since clinical treatment varies for the different etiologies of heart failure, it is imperative to diagnose the etiology. For example,

patients with systolic heart failure benefit from therapy with ACE inhibitors, beta blockers and mineralocorticoid receptor antagonists. However, those with diastolic heart failure don't seem to benefit from these therapies. Patients with systolic heart failure and ischemia may benefit from revascularization. However, which patients should undergo expensive workup evaluation is not clear. Diagnosing the etiology of heart failure involves costly and often invasive diagnostic procedures. Using biomarkers to identify and classify heart failure could decrease our reliance on diagnostic procedures, benefit patients and reduce costs for the health care system. It could also pave the way to better treatment.

To date, Roche Diagnostics, UOHI and the University of Toronto have identified eight novel biomarker candidates for heart failure characterization. Three of these biomarkers, IGFBP7, Nogo-C and SIRPA, have been found to be elevated in certain classifications of heart failure. Several studies have shown elevated levels of IGFBP7 in patients with diastolic heart failure. Recent studies in patients with LV hypertrophy due to hypertension identified 3 times higher levels of Nogo-C as compared to controls. Other studies measured SIRPA at 3 times higher levels in patients with dilated cardiomyopathy versus those with ischemic disease. This is the first of several planned studies that will further clinical evaluation of the biomarkers, with the intent of developing a heart failure biomarker panel and an accompanying clinical development program to translate the findings from basic research to clinical benefit of patients.

2. OBJECTIVES

2.1 Goal: To effectively use biomarkers to diagnose the etiology of heart failure.

2.2 Objective: To validate the clinical accuracy of novel blood-based biomarkers in correctly classifying patients into (a) ischemic-systolic or (b) idiopathic dilated-systolic or (c) diastolic heart failure etiologies first from retrospective cohorts, followed by prospective evaluation.

2.3 Hypotheses: Novel blood-based biomarkers will correctly identify the etiology of heart failure in patients.

2.4 Trial Design: This is a cohort study consisting of 2 patient cohorts. The *first cohort* will be studied retrospectively, using existing blood samples from biobanks at Duke Clinical Research Institute and Roche Diagnostics. Biomarkers will be measured and matched with the etiology of previously diagnosed heart failure by a biomarker panel of heart failure experts. In the *second cohort*, biomarkers will be measured and used to diagnose the etiology of heart failure for each subject. This diagnosis will then be compared to the diagnosis from results of the usual diagnostic tests. We will also include an active control group that will be compared to the heart failure cohort. These participants will have a diagnosis of acute myocardial infarction (MI) or stable coronary artery disease (CAD). Subjects will be recruited primarily from Ottawa. However, if enrollment goals cannot be met, additional Canadian sites will be added, following approval of the protocol amendment by the REB.

3. METHODS

3.1 Participants, interventions, and outcomes

3.1.1 Study setting

This study will be conducted in academic hospitals. Blood samples from the first cohort were collected at Duke Clinical Research Institute and in Europe. A final decision has not been made in regard to which sites will be involved in the recruitment of the second cohort. We will start with recruitment at UOHI and the Ottawa Hospital (TOH). Other Canadian sites will be added if recruitment goals cannot be met.

3.1.2 Inclusion criteria

- Patient admitted with heart failure symptoms, fitting the diagnosis of heart failure by the modified Framingham criteria (fill in Framingham criteria below)
  OR
- Patient diagnosed with heart failure within the past 2 years with a clear diagnosis of heart failure documented in the medical records OR
- Patient diagnosed with heart failure with preserved ejection fraction (HFpEF) within the past 5 years Modified Framingham Criteria: Simultaneous presence of at least 2 major criteria or 1 major criterion in conjunction with 2 minor criteria.

Major criteria:

- Paroxysmal nocturnal dyspnea or orthopnea
- Neck vein distention
- Crackles/Rales (>10 cm from the base of the lung)
- Acute pulmonary edema
- S3 gallop
- Increased central venous pressure (>16 cm H_2O at right atrium)
- Weight loss >4.5 kg in 5 days in response to treatment
- Echocardiographic left ventricular dysfunction

Minor criteria:

- Bilateral ankle edema
- Nocturnal cough
- Dyspnea on exertion
- Hepatomegaly
- Pleural effusion
- Tachycardia (heart rate >120 beats/min)
- Weight loss >4.5 kg caused by heart failure where factors other than treatment of CHF could have contributed to the weight loss

Minor criteria are acceptable only if they cannot be attributed to another medical condition (such as pulmonary hypertension, chronic lung disease, cirrhosis, ascites, or the nephrotic syndrome).

3.1.3 Exclusion criteria

- Patients unable to provide blood sample
- Patients unable to provide consent
- Patients whose primary reason for heart failure is valve disease
- Patient with life expectancy of less than 6 months or has major co-morbidities (HF secondary to chemotherapy is acceptable if life expectancy is greater than 6 months).
- Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the trial, or may influence the result of the trial, or the participant's ability to participate in the trial.
- Participants who have participated in another research trial involving an investigational product in the past 30 days.
- If the heart failure diagnosis for HFpEF patients has changed within the past 5 years (i.e. the patient has been diagnosed with ischemia, valve disease or other condition which has worsened the heart failure symptoms)

3.1.3. 2 Inclusion criteria for the active control group/comparator cohort

- Patients with a diagnosis of acute MI (within 1-5 days), confirmed by ECG and/or elevated troponin values. OR
- Patients with a history of stable coronary artery disease (CAD) with no ER visit or hospitalization in the past 3 months prior to study enrollment

3.1.4 Interventions

No interventions are planned for this study.

The *first cohort* will consist of blood samples from 300 patients diagnosed with heart failure. Informed consent for use of blood for future research has been obtained from all subjects at the respective sites. These blood samples have already been collected and are stored in biobanks at Duke Clinical Research Institute and Roche Diagnostics. Samples will be shipped to UOHI from the biobanks for analysis once ethics approval has been obtained. The samples will be tested for specific biomarkers. Biomarker results will be returned to the originating sites for matching with research data and analysis. This entire *retrospective cohort* consists of 100 patients each with (1) ischemic cardiomyopathy, defined as left ventricular ejection fraction (LVEF)<40%, proven coronary artery disease with angiography and associated evidence of ischemia on treadmill testing or perfusion scan; (2) dilated cardiomyopathy defined as LVEF<40%, absence of coronary artery disease or other known secondary causes such as valvular disease or toxin exposure and (3) diastolic heart failure defined as LVEF≥50%, objective evidence of heart failure (CXR or physical evidence of edema or elevated JVP) and preferably evidence of diastolic dysfunction on echocardiogram. Blood samples will be analyzed for novel biomarkers including IGFBP7 and SIRPA. We will be seeking biomarker candidate(s) alone or in combination which can predict each category of heart failure etiology with over 85% accuracy and the lowest levels of reclassification. A panel of heart failure experts will be assembled, and the appropriate cut-off values

determined based on Receiver Operating Characteristic (ROC) and predictive modeling. Once this goal has been achieved, the results will be used to diagnose the recruited patients in the prospective study. This diagnosis will be compared to the clinical diagnosis.

The *second cohort (prospective)* will consist of 470 patients admitted to hospital or outpatient clinics with symptoms of heart failure and a diagnosis of heart failure within the past 2 years. Patients will have blood samples drawn and tested for different biomarkers including IGFBP7 and SIRPA. Using the biomarker values, their heart failure etiology will be predicted by the assembled clinical team based on the algorithm of the first cohort in an objective manner. The patients will undergo definitive etiological workup as usual, including echocardiography, coronary angiography, and a myocardial perfusion scan to establish the actual etiology of the heart failure. The predictive accuracy of the new heart failure panel will be compared to clinical assessment.

Cost-modeling in terms of the potential savings in avoiding unnecessary coronary angiographies or perfusion scans in a typical mixed cohort of heart failure patients based on the marker determined etiology will be performed.

This trial will only diagnose the etiology of heart failure. No interventions or concomitant care are restricted during the diagnostic (study) period.

The comparator cohort/ active control group will include a cohort of 20-40 patients admitted a diagnosis of acute MI and 20-40 patients with a history of stable coronary artery disease (CAD).

For the stable CAD group, we consider accessing the University of Ottawa Heart Institute Biobank's samples and associated clinical data.

3.1.5 Outcomes

Primary outcome: accuracy of heart failure panel to predict etiology of heart failure using biomarkers.

Secondary Outcome: cost savings.

3.1.6 Analysis metric

One time measurement of biomarkers compared to usual diagnostic procedures.

3.1.7 Participant timeline

For the second cohort (prospective), subjects will be diagnosed with usual diagnostic testing. Once the biomarker results are available, a biomarker-based diagnosis will be made by a panel of experts who are blinded to the clinical diagnosis. A complete clinical diagnosis for heart failure takes about 3 days for in-patients and up to 2 months for out-patients. Subject participation is complete once diagnosed. However, patient involvement of study procedures will only consist of the consent process, a short interview to collect demographics, medical history, risk factors for heart disease and current treatment information. Patients will have their heart rate and blood pressure recorded (may be done by clinical staff) and will have a onetime blood draw. The total time involvement for the subject should be no more than 30-45 minutes. Recruitment for the study is expected to take a total of 2 years. We will do a chart review for outcomes at 30 days, 6 months and yearly post hospital discharge for up to ten years, as long as we have the resources to complete the follow ups. Medical records reviews will record any hospitalizations, related diagnosis,

diagnostic tests results and the date and cause of death. Patient records will be reviewed in existing electronic medical records including Epic and ICES. This will also be applicable for the active control group.
For the participants in the acute MI comparator group, we will aim to collect blood additional blood samples. The first and second samples will be collected pre- and post-intervention/coronarography, ideally in the Cath lab. The third sample will be collected before the patient is discharged home from the hospital (within 48h -3 days), while a fourth sample will be collected approx. one-month post-discharge. The study team will coordinate the last sample with a clinic follow up visit, when possible.

3.1.8 Sample size:

In previous studies, known biomarkers have been validated in cohorts of 150 to 300 patients. If identification of new biomarkers is part of the objective, more samples are required. We are suggesting 450 patients for the prospective study. Dr. George Wells will provide a final sample size calculation.

3.1.9 Recruitment

Prospective Cohort 2: Subjects will be recruited from UOHI and TOH. If during the course of study recruitment, trends show that recruitment cannot be completed in 2 years, other centers will be added. Centers will be added only after an amendment to the ethics committee has been approved. We will start with the University Health Network in Toronto and McMaster University in Hamilton. Other centers like University of Calgary, and health centers in Edmonton and/or Montreal may also be approached, depending on how many centers are required to reach our recruitment target in 2 years. 300 patients are admitted to UOHI with heart failure every year. Any patients that are admitted with symptoms of heart failure or were diagnosed with heart failure within the past 2 years will be targeted for recruitment. Patients diagnosed with heart failure with preserved ejection fraction will be recruited if they were diagnosed within the past 5 years and did not have a change in their heart failure diagnosis within that time. Due to the low risk and time commitment of this study, it is anticipated that a high percentage of eligible patients will join the study. For the comparator group - patients diagnosed with an MI, will be recruited at the time of the scheduled coronarography at the UOHI. Patients with a diagnosis of stable CAD will be enrolled from the outpatient clinics or as inpatient, if admitted at UOHI for a different diagnostic and meet the inclusion criteria for the study.

3.2 **Data collection, management, and analysis**

3.2.1 Data collection methods

Existing blood samples for the retrospective cohort will be analyzed for novel biomarkers including IGFBP7 and SIRPA and correlated with the classification of heart failure from the matched research data.
The second cohort will consist of patients diagnosed with heart failure within the past 2 years and patients diagnosed with MI or stable CAD. They will be recruited from outpatient clinics, nursing units and imaging departments at UOHI and TOH. After written informed consent has been obtained, 33 ml of blood will be drawn and

analyzed for biomarkers. Blood samples will be drawn as close to admission as possible before interventions are started. Efforts will be made to coordinate the blood draw with clinical blood draws to reduce the risk and discomfort to the patient. For the MI and CAD group the blood sample will be collected at the time of coronarography or hospital visit, if possible. The patient demographics, medical history, risk factors and current treatment record will be obtained by interviewing the patient and reviewing the patient's health records. Blood pressure and heart rate will be recorded. The patient will continue with usual care and diagnostic procedures for heart failure including blood work, echocardiography, coronary angiography and cardiac perfusion scan. All results from the diagnostic procedures will be recorded as part of the study, however, no diagnostic procedures will be requested specifically for the study. Once the diagnostic procedures have been completed, the patient's participation in the study is complete.
For the samples retrieved from the UOHI Biobank, all sample donors have been recruited and consented by the UOHI Biobank according to the REB approved Biobank protocol #20140276-01H. Once transferred, Dr Peter Liu will be the custodian of the de-identified samples and data for the entire duration of the study.

Identifiers that will be collected are the patient's gender, name, date of birth, medical records number, health card number and email address. These identifiers are required for access to existing internal and external databases for retrieval of relevant medical information such as past medical history, diagnostic tests and treatment. The email address is required for contact purposes. Any secondary use of information will first be submitted to and approved by the REB.
Blood samples will be processed and analyzed in our lab at the UOHI using automated platform-based ELISA techniques by trained personnel.
If specific tests are required or future analysis is planned, and the UOHI lab does not have the capacity to process the test or analysis, the de-identified samples will be securely transferred to external labs (in Canada or outside Canada) for processing. Material and data agreements will be executed for all the data and samples exchanges.
Biomarker results will be correlated with the etiology of the clinical diagnosis of heart failure.
A heart failure panel will be setup to identify the etiology of heart failure from the biomarker results prior to looking at the diagnostic test results. The diagnosis resulting from the biomarkers alone will be compared to the diagnostic test results. The biomarkers from the heart failure cohort will be compared with the biomarkers from the MI and stable CAD group. For the data associated with the stable CAD samples from the UOHI biobank, that includes standard data collected by the biobank at the time of sample collection: age, sex, height, weight, BMI, blood pressure, heart rate, history of cardiovascular disease and risk factors for cardiovascular disease, current medications, cigarette smoking history, alcohol consumption, exercise level, family history of cardiovascular disease, ethnicity and first language spoken. Additional data required for the protocol will be obtained by biobank staff from reviewing the patient's electronic health record (EPIC).

3.2.2 Data management

Subject identity will be protected by coding all data with a unique study ID code. Blood samples will be de-identified by labelling all samples with a barcode. The biomarker results will be paired with the de-identified research data during data analysis. The Cardiovascular Research Methods Centre at UOHI will be responsible for data verification and analysis. The database will be designed by the PI in conjunction with the Cardiovascular Research Methods Centre at UOHI. All data collected for the study will be entered into the database and stored on the secure institutional network server that is password protected. No identifying information will be entered on the database. Electronic case report forms (CRF) will be used for data recording.

In the future, de-identified samples and/or data may be shared with external parties provided a material and/or data sharing agreement is in place between participating parties.

3.2.3 Statistical methods

For the first patient cohort (retrospective): A panel for heart failure etiology will be assembled, and the appropriate cut-off values determined for specific biomarkers, based on Receiver Operating Characteristic (ROC) and predictive modeling.

The predictive accuracy of the new heart failure panel will be compared to clinical assessment, all against the gold standard using C-statistic and reclassification test.

Cost-modeling in terms of the potential savings in avoiding unnecessary coronary angiographies or perfusion scans in a typical mixed cohort of heart failure patients based on the marker determined etiology will be performed.

3.3 Monitoring

3.3.1 Data monitoring

An independent data monitoring committee will be set up and will consist of 3 experts, one from each field: cardiology, statistics and diagnostics. The data monitoring committee will review biomarker and diagnostic results.

An interim analysis is not planned.

Since this is a diagnostic study, no stopping guidelines are planned for.

3.3.2 Harms

This is a non-interventional trial. Potential harm to the patient from procedures is minimal. Blood drawing carries a small risk of discomfort, bleeding or bruising and rarely infection at the site. This risk will be minimized by ensuring that all blood is drawn by qualified staff using appropriate techniques.

Risk of breach of confidentiality/privacy is low. Specific identifiers are required to access existing data bases in order to validate patient medical history and treatment records. This risk will be minimized by storing all identifying information separately on a secure network with password protection. No identifying information will leave the UOHI. For analysis, only de-identified biomarker results and de-identified medical information will be linked to each other. Patients cannot be identified from the biomarker results alone.

3.3.3 Auditing

This is an investigator-initiated trial not regulated by Health Canada and will be monitored internally. Internal auditing may take place. Any auditing will be conducted under the supervision of Dr. Liu and/or his research staff.

4. ETHICS AND DISSEMINATION

4.1. Research ethics approval

This trial will be conducted in compliance with the protocol, principles laid down in TCPS-2 guidelines, the Declaration of Helsinki and Good Clinical Practice (GCP as defined by the International Conference on Harmonization (ICH), where applicable. Before study initiation, the Investigator must have written and dated approval from the OHSN-REB for the protocol and consent form.

4.2. Protocol amendments

Any protocol amendments will be communicated to the REB, co-investigators and other study sites (if applicable). Any amendments will require ethics approval.

4.3. Consent procedure

Prior to patient participation, written informed consent will be obtained from each patient and comply with the TCPS-2 guidelines, Declaration of Helsinki and applicable local regulations. The original signed consent will be retained on file and a copy given to the patient.

When patients are admitted to the hospital or outpatient clinic with symptoms of heart failure or a diagnosis of heart failure within 2 years, they will be approached for this study. All patients admitted to UOHI and TOH with symptoms of heart failure will be screened for eligibility. We will also screen patients booked for imaging in all of the imaging departments. We will request lists of all patients who have their ejection fraction (EF) assessed and have an EF <50%. The imaging departments that are unable to generate lists by EF, will be asked to provide a list of all scheduled patients. We will use these lists to screen patients for eligibility, ensuring first that they have consented to research contact. Eligible patients will be approached for the study. First contact will be made by hospital staff within the circle of care. Patients who have consented to research contact will be contacted by the study coordinator directly. If the patient is interested in participating in this study, the research coordinator will explain the study in detail. The patient will be given the opportunity to review the provided study information, ask questions and discuss the study with family members before making a decision to participate. If the patient decides to participate in the study, written informed consent will be obtained by the study coordinator. This process will be documented in the patient case report forms (CRF). Patients with a history of a recent MI (within 1-5 days) will be approached for study enrollment at the time of coronarography/angiogram or UOHI clinic visit or during hospital admission if inpatient.

4.4. Confidentiality

Once the consent form is signed, a unique study ID code that contains no identifiers will be assigned to the participant and used on all patient records and samples related to the study. The master file, a document containing the identifying information of the patients with a link to the study ID codes, will be kept in a separate folder from the study documents on a secure computer network. The patient's signed consent form and source documents will be stored in a separate folder in a locked filing cabinet in a locked office. Results collected from diagnostic tests will be de-identified by blacking out the patient identifying information and adding the study ID code. All data will only be accessible by Dr. Peter Liu, his research team and designated personnel at the Cardiovascular Research Methods Centre.

The Cardiovascular Research Methods Centre will be able to link the biomarker values with the patient's de-identified research data for data analysis. Raw data will not be shared with anyone outside the research team at UOHI. No identifying information will leave UOHI.

After the study has been completed the paper files may be transferred to a secure storage system used by UOHI for study records. Study records will be kept for a minimum of 10 years, after which time the paper records will be securely shredded and the electronic records deleted. Blood samples will be destroyed unless a separate consent has been signed by the participants to store samples in a biobank.

4.5. Declaration of conflict of interests

Dr. Peter Liu has a potential conflict of interest in that he is part of the University of Toronto/University of Ottawa team that shares the intellectual property on some of the novel biomarkers.

4.6. Access to data

Dr. Peter Liu, his research staff and the Cardiovascular Research Methods Centre at the UOHI will have access to the final trial dataset. If other study sites are added during the course of this study, other site investigators will have access to the datasets originating from their sites only. Otherwise, only study results will be shared. No contractual agreements limit access to the dataset for UOHI investigators. Periodic requests will be made to access all CHF samples stored at the UOHI biobank, along with the associated research and clinical data, to further validate biomarkers of heart failure patients.

4.7. Ancillary and post-trial care

No post-trial care is anticipated beyond usual clinical care. This is a low risk study for diagnostic and observation purposes. It is not anticipated that patients will suffer any harm from trial participation.

4.8. Dissemination policy

Results will be presented in-house and at conferences and will be published in peer reviewed journals. Results will be presented as means for each of the 2 cohorts. No individual participant will be identified in any presentations or publications. Public access to the full protocol, dataset or statistical code will not be granted.

Authorship eligibility will adhere to the guidelines established in peer reviewed journals.

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Addendum to Protocol #20140869: Evaluation of Existing Biomarker Candidates to Accurately Classify the Etiology of Heart Failure

The purpose of this study is to isolate biomarkers that will, on their own or in combination, diagnose the etiology of heart failure. In order to identify the relevant biomarkers, we need to compare biomarkers of healthy volunteers to those of heart failure patients. We propose an addendum to the study which will enroll a cohort of healthy volunteers to serve as controls. All aspects of the protocol will also pertain to this cohort except for the differences described below.

Design: We will recruit 80 healthy, age matched volunteers to serve as controls for this study. The heart failure population is generally represented by people between the ages of 50 to 85. However, there are eligible patients with heart failure under the age of 50 participating in the study. We will recruit 20 subjects for each of 4 age groups; under 50, 50-60, 61-70, 71-85. We will make an effort to enroll an equal number of males and females. We expect to recruit mostly from UOHI staff for the first 2 age groups and volunteers from the community for the other age groups. To target UOHI staff, we will send an email to all staff and ask for volunteers. Advertisements to target volunteers will be placed in prominent areas at UOHI where staff and visitors can see them. They will also be posted at TOH (Civic and General campus), fitness and sports centres and senior's community centres throughout the cities of Ottawa and Gatineau. Patients that attend the heart failure clinic are often accompanied by their spouse. If the spouse is healthy, he/she could be suitable as a control subject. When recruiting patients in the outpatient heart failure clinics, we will ask spouses that accompany the patients if they would like to participate as a control subject.

Inclusion Criteria:

- Age between 18 to 85 years old
- Generally healthy
- Able to give informed consent

Exclusion Criteria:

- Subject unable to provide blood sample
- History of cardiovascular disease
- Major cardiovascular risk factors (diabetes, hypertension, dyslipidemia)
- Major organ dysfunction (renal, hepatic, pulmonary)
- Current smoker
- History of cancer (unless 10 years post treatment and deemed cured)
- Depression, requiring medications
- Any other condition or treatment that can interfere with test results
- Participation in a research trial involving an investigational product in the past 30 days.

Methods:

After explaining the study to the volunteer and obtaining informed consent, we will draw one blood sample for biomarkers consisting of 33 ml of blood. We will measure the blood pressure and heart rate. We will record the gender, age, ethnicity, past medical history, smoking history, alcohol consumption, current medications and family history of cardiovascular disease. This will complete the involvement of the volunteer. No follow-up visits are required.

Private identifying information: We will collect the name and contact information of all participants to file in the master list. This information is required in case we need to clarify information during biomarker analysis. No other identifiers are required.

8.2 Patient Participant Informed Consent Form

PARTICIPANT INFORMED CONSENT FORM

Title of Study: Evaluation of Existing Biomarker Candidates to Accurately Classify the Etiology of Heart Failure. (HF-CAUSE) Protocol #20140869
Local Site Principal Investigator (PI): Dr. Peter Liu 613-696-7351
Sponsor (or Funding Agency): Genome Canada, Roche Diagnostics and University of Ottawa Heart Institute.
Participation in this study is voluntary. Please read this Participant Informed Consent Form carefully before you decide if you would like to participate. Ask the study doctor and study team as many questions as you like. We encourage you to discuss your options with family, friends or your healthcare team.

Why am I being given this form?
You are being asked to participate in this research study because you have been admitted to the hospital with symptoms of heart failure or have been diagnosed with heart failure within the past 2 years or you have been diagnosed with a heart attack or have a history of heart disease (coronary artery disease).

Why is this study being done?
Nearly half a million people in Canada suffer from heart failure. Heart failure is a condition in which the heart loses the ability to pump enough blood to the body's tissues. An aging population will result in more people developing heart failure every year. There are several types of heart failure and treatment is not the same for each type of the disease. This makes it very important that the correct diagnosis is made before treatment is started. Currently, several tests are useful in diagnosing the type of heart failure a person has. These include blood tests, ultrasound of the heart, imaging of the arteries around the heart and scans that determine the health of the heart muscles. These tests can be time consuming, invasive and expensive.
Over the past few years, we have found several biomarkers in the blood that may help us diagnose the type of heart failure people have. Biomarkers are proteins or chemicals that show the existence of a disease, how the disease evolves or how treatment affects the disease. In this study, we want to take a closer look at some of these biomarkers and see if they can be used to correctly diagnose different types of heart failure. If we are successful, diagnosing heart failure will become much easier, quicker and less expensive. We also want to see if biomarkers can predict complications associated with heart failure. We would also want to compare the biomarkers found in heart failure with the biomarkers found in cases where people have a heart attack or blockages in their blood vessels in the heart (coronary artery disease).
This study is taking place at the University of Ottawa Heart Institute and the Ottawa Hospital. We want to enroll 470 patients in this study.

How is the study designed?
This study is designed to find a better way to diagnose heart failure.

What is expected of me?

If you agree to participate in this study, the study coordinator will ask you a few questions in regards to your medical history and treatments. You will have about 2 tablespoons or 33 ml of blood drawn. Nothing else is required of you. Your doctor will order the usual tests that will diagnose the type of heart failure you have or type of blockages in your heart vessels. All of the tests ordered by your doctor to diagnose your condition are part of usual care. We will record the results of your tests and use them together with your blood results to see how accurate the biomarkers are at diagnosing the type of heart failure you have.

Will my samples or research data be used in future research?

Your blood samples will be measured for biomarkers at the University of Ottawa Heart Institute. Some of your blood may be left over after the measurements are done. The University of Ottawa Heart Institute would like to store this left over blood in their biobank for future research.

Blood can be frozen and safely stored in a biobank for many years. A biobank is a storage facility that stores blood samples along with medical information for future research. Both your blood samples and medical information will be de-identified before storage. This means that all information that links them to you will be removed. A unique ID code will be used for identification, which will allow researchers to link your blood and medical information to each other. This is necessary to get meaningful information from your blood samples. No personal identifying information will be stored in the biobank.

If you agree to have your de-identified blood samples and medical information stored in the biobank, they will be used for future research on biomarkers. Because we don't know what kind of research will be available in the future, it is possible that your samples could also be used for genetic research. Rules at the University of Ottawa Heart Institute require study doctors to get permission from the research ethics board before using your samples in future research. Your blood samples will not be released for any research without written permission from the research ethics board. You have a choice of whether or not you permit your blood samples and research data to be stored in the biobank. You can still participate in this study if you decide not to have your blood stored in the biobank.

How long will I be involved in the study?

Recruitment for the entire study will take about 2 years. The interview and blood sample will take about half an hour to complete. We will review your medical records at 30 days, 6 months, and then every year after you go home from the hospital until your time of passing. This will allow us to assess if biomarkers are able to predict the progression and complications of heart failure and to compare with the biomarkers of heart disease due to blockages in the blood vessels in the heart You will not be required to return for any additional visits and will not be routinely contacted. However, we may contact you from time to time to collect additional information from you if your medical record shows that you have had a problem with your heart.

Your participation in the study may be stopped for any of the following reasons:

- The study doctor feels it is in your best interest.
- You do not follow the study staff's instructions.

What are the potential risks I may experience?

Blood Sample Risks

You may experience some temporary discomfort when the blood sample is taken. There is a small risk of bruising, infection or swelling at the site where the needle is inserted, and some people may feel faint or dizzy. This is no different from having blood drawn for usual testing.
If you agree to have your blood stored for future research, there is a remote possibility that you could be identified from the blood samples or your medical information. Because both your de-identified medical information and blood samples will only be linked to each other and no longer linked to you specifically, this risk is extremely low. The University of Ottawa Heart Institute will take all reasonable steps to keep your research information confidential. Should someone not involved in the research find out that your blood was used in a genetic research study, or if you choose to share your results (if they are provided to you), there is a possibility that this could affect your insurance or employment.
We will collect personal identifiers; your name, gender, date of birth, health card number, hospital record number, and email address. These identifiers will be securely stored indefinitely and will only be accessible by your study team. Your study doctor will store these identifiers in case they are needed in the future. Examples of why these identifiers would be needed are, if you decide to remove your blood samples and medical records from the biobank, or if future research makes it necessary for the study doctor to contact you.
If you do not want your left over samples stored in the biobank, we will record your name, date of birth, gender, hospital record number, health card number and email address. We need this information to access your medical records that are stored on different confidential databases. We will keep this personal information confidential as described below. We will keep your email address on file in case we need to contact you in regards to your medical information.

Can I expect to benefit from participating in this research study?

You will not receive any direct benefit from participating in this study. Your participation may allow the researchers to find a simpler way of diagnosing types of heart failure. This may benefit future patients.

Do I have to participate? What alternatives do I have?

You can choose not to participate in this study. If you choose not to participate, you will continue with the usual test procedures. Your participation in this study is voluntary. You may decide not to be in this study, or to be in the study now, and then change your mind later without affecting the medical care, education, or other services to which you are entitled or are presently receiving at this institution.

If I agree now, can I change my mind and withdraw later?

You may withdraw from the study at any time without any impact on your current or future care at this institution.

- If you withdraw your consent, the study team will no longer collect your personal health information for research purposes, unless it is needed for review of safety.
- You may also request to have your data withdrawn from the study completely by making a request to Dr. Peter Liu in writing.

What compensation will I receive if I am injured or become ill in this study?

In the event of a study-related injury or illness, you will be provided with appropriate medical treatment and care. Financial compensation for lost wages, disability or discomfort due to an injury or illness is not generally available. You are not waiving any of your legal rights by agreeing to participate in this study. The study doctor and the University of Ottawa Heart Institute still have their legal and professional responsibilities.

Will I be paid for my participation or will there be any additional costs to me?

You will not be paid for participation and there will be no costs to you associated with this study.

If the biomarkers are proven effective, they may be approved for use and the tests made available for sale internationally for doctors to use. If this occurs, the University of Ottawa Heart Institute, The University Health Network (Toronto) and Roche Diagnostic may benefit from this development. You will not share in any profits arising from commercialization.

How is my personal information being protected?

- All personal health information (PHI) and your personal identifying information (PII), such as your name, address, date of birth, health card number, hospital record number, etc. will be kept confidential.
- Release of your PHI/PII information will only be allowed if it is legally required.
- As a participant, you will be assigned a coded study number that will be used throughout the study on all your study records and blood samples.
- It is not anticipated that any documents or samples will leave the University of Ottawa Heart Institute. All samples will be analyzed at the institute. All data will be handled by the Cardiovascular Research Methods Centre at the University of Ottawa Heart Institute.
- A Master List provides the link between your identifying information and the coded study number. This list will only be available to Dr. Peter Liu and his research team and will not leave this site.
- The Master List and coded study records will be stored securely.
- For audit purposes only, your original medical records may be reviewed under the supervision of Dr. Liu's staff by representatives from:
    - the Ottawa Health Science Network Research Ethics Board (OHSN-REB), and the University of Ottawa Heart Institute.
- You will not be identified in any publications or presentations resulting from this study.
- Research records will be kept for 10 years, as required by the ethics board.
- At the end of the storage time, all paper records will be shredded and all electronic records will be securely deleted. Blood samples will be destroyed. If you agree to store your samples in the biobank, the samples and medical records will be stored indefinitely.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

This research study can be found on the above listed website by using the clinical trial registration number NCT02347722.

Do the investigators have any conflicts of interest?

Dr. Peter Liu has a potential conflict of interest in that he is part of the University of Toronto/University of Ottawa team that shares the intellectual property on some of the biomarkers.

What are my responsibilities as a study participant?

It is important to ask your study coordinator or doctor if you have any questions or concerns during this study.

Will I be informed about any new information that might affect my decision to continue participating?

You will be told in a timely fashion of any new findings during the study that could affect your willingness to continue in the study. You may be asked to sign a new consent form.

Who do I contact if I have any further questions?

If you have any questions about this study, or if you feel that you have experienced a study-related injury or illness, please contact Dr. Peter Liu at 613-696-7351 extension or the study staff at 613-696-7000 extension 10945.

The Ottawa Health Science Network Research Ethics Board (OHSN-REB) has reviewed this protocol. The Board considers the ethical aspects of all research studies involving human participants at the University of Ottawa Heart Institute. If you have any questions about your rights as a study participant, you may contact the Chairperson at 613-798-5555, extension 16719.

Title of Study: Evaluation of Existing Biomarker Candidates to Accurately Classify the Etiology of Heart Failure. (HF-CAUSE) Protocol #20140869

Consent to Participate in Research

- I understand that I am being asked to participate in a research study about biomarkers to diagnose types of heart failure
- This study was explained to me by _____.
- I have read, or have had it read to me, each page of this Participant Informed Consent Form.
- All of my questions have been answered to my satisfaction.
- If I decide later that I would like to withdraw my participation and/or consent from the study, I can do so at any time.
- I voluntarily agree to participate in this study.
- I will be given a copy of this signed Participant Informed Consent Form.

_____ _____ _____

Participant's Printed Name Participant's Signature Date

Investigator or Delegate Statement

I have carefully explained the study to the study participant. To the best of my knowledge, the participant understands the nature, demands, risks and benefits involved in taking part in this study.

_____ _____ _____

Investigator/Delegate's Printed Name Investigator/Delegate's Signature Date

Assistance Declaration

Was the participant assisted during the consent process? ❑ Yes ❑ No

❑ The consent form was read to the participant/substitute decision-maker, and the person signing below attests that the study was accurately explained to, and apparently understood by, and consent was freely given by the participant/substitute decision-maker.

❑ The person signing below acted as a translator for the participant/substitute decision-maker during the consent process. He/she attests that they have accurately translated the information for the participant/substitute decision-maker, and believe that the participant/substitute decision-maker has understood the information translated.

_____ _____ _____

Name of Person Assisting (Print) Signature Date

Ottawa Hospital Research Institute
Institut de recherche de l'Hôpital d'Ottawa

UNIVERSITY OF OTTAWA HEART INSTITUTE
INSTITUT DE CARDIOLOGIE DE L'UNIVERSITÉ D'OTTAWA

uOttawa

<u>Title of Study</u>: Evaluation of Existing Biomarker Candidates to Accurately Classify the Etiology of Heart Failure. (HF-CAUSE) Protocol #20140869
<u>Permission for Storage of Blood Samples for Future Discoveries</u>

While my blood samples are provided for the analysis of biomarkers related to heart failure, I understand that new biomarkers may be tested on my stored blood samples in the future. Future research may also include genetic research. I agree to have my de-identified blood stored for future research. I also permit the linking of my biomarker information with my de-identified medical information to make sense of the information provided by the biomarker measurements.

Yes ____ No ____

_____ _____ _____

Participant's Printed Name Participant's Signature Date

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