Examining SERCA2a Acetylation in the Diseased Human Heart

by:

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Abstract

Sarco(endo)plasmic reticulum calcium (Ca^{2+}) ATPase 2a (SERCA2a) regulates cardiac function by removing cytosolic Ca^{2+}. SERCA2a is altered by the post-translational modification, acetylation. However, the effect of acetylation on SERCA2a in the diseased human heart is not understood. Therefore, this case-control study examined SERCA2a acetylation in the right atrium (RA) of the diseased human heart. To accomplish this, human RA tissue samples were obtained from 61 patients undergoing cardiac surgery. Tissue collected was categorized based on heart disease type (i.e. heart valve disease (HVD) or coronary artery disease (CAD)), the absence or presence of systolic dysfunction (SD), and type 2 diabetes (T2D) status. The objectives of this study were to determine (1) if RA SERCA2a acetylation and SERCA function are altered by heart disease type; (2) if RA SERCA2a acetylation and function are altered by the presence of SD; (3) if RA SERCA2a acetylation and function are altered by T2D status. RA SERCA2a acetylation was altered by heart disease type, as there was a 53% difference in RA SERCA2a acetylation between patients with HVD or CAD ($p = 0.012$). A 31% difference in RA SERCA V_{max} was identified between patients with HVD or CAD ($p = 0.004$). RA SERCA2a acetylation was not altered by SD or T2D independently, but RA SERCA2a acetylation was increased by 3-fold in patients with combined SD and T2D, compared to patients with T2D alone ($p = 0.013$). SERCA V_{max} was 22% higher in the RA of patients with SD, compared to patients without SD ($p = 0.020$). The SERCA Hill coefficient was 29% greater in the RA of patients with T2D, compared to patients without T2D ($P = 0.021$). A positive relationship was found between RA SERCA2a acetylation and RA SERCA V_{max} using unadjusted linear regression analysis ($r = 0.36$; $r^2 = 0.13$; $p = 0.034$). Our study is the first to reveal differences in RA SERCA2a acetylation and SERCA function based on heart disease type, the presence of SD, and T2D status.

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Dedication

This thesis is dedicated to my family and it is a testament to the values you all instilled in me. Thank you for your sacrifices and constant support. To my father and mother, you are my role-models and your extraordinary work-ethic, determination, and discipline constantly inspire me to pursue meaningful goals.

List of Abbreviations

[Ca^{2+}] – Ca^{2+} concentration

ATP – Adenosine triphosphate

BMI – Body mass index

Ca^{2+} – Calcium

CABG – Coronary artery bypass grafting

CAD – Coronary artery disease

CCS – Canadian Cardiovascular Society

CHF – Congestive heart failure

COPD – Chronic obstructive pulmonary disease

CUPID – Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease

CVA – Cerebrovascular accident

CVD – Cardiovascular disease

DCM – Diabetic cardiomyopathy

ECC – Excitation contraction coupling

euroSCORE – European system for cardiac operative risk evaluation

FFR – Force frequency relationship

HAT – Histone acetyltransferase

$HbA1_c$ – Glycated hemoglobin

HDAC – Histone deacetylase

HF – Heart failure

HVD – Heart valve disease

ICU – Intensive care unit

K – Lysine

LAD – Left anterior descending

LoS – Length of stay

LV – Left ventricle

LVAD – Left ventricular assist device

LVD – Left ventricular dysfunction

LVEF – Left ventricular ejection fraction

MI – Myocardial infarction

NYHA – New York Heart Association

O_2 – Oxygen

OLETF – Otsuka-Long-Evans Tokushima Fatty

OR – Operating room

PCI – Percutaneous coronary intervention

PLN – Phospholamban

PMSF – Phenylmethylsulphonyl fluoride

PTM – Post-translational modification

PVD – Peripheral vascular disease

PVDF – Polyvinylidene difluoride

Q – Glutamic acid

R – Arginine

RA – Right atria

SD – Systolic dysfunction

SERCA – Sarco(endo)plasmic reticulum Ca^{2+} ATPase

SIRT – Sirtuin

SIRT1 – Sirtuin 1

SLN – Sarcolipin

SUMO1 – small ubiquitin-like modifier type

SR – Sarcoplasmic reticulum

STZ – Streptozotocin

T1D – Type 1 diabetes

T2D – Type 2 diabetes

TAC – Transverse aortic constriction

TIA – Transient ischemic attack

V_{max} – Maximum enzyme velocity

VP – Valve procedure

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Chapter 1: Literature Review

Heart Disease

Heart disease is a major public health concern, as it is the leading cause of death worldwide[1]. Nearly 300 Canadian adults over 20 years of age die from heart disease each day[2]. Looking ahead, the global incidence of heart disease is likely to increase, since the rates of important risk factors for the condition, like hypertension and obesity, continue to rise[3].

Heart Disease and Type 2 Diabetes

Type 2 Diabetes (T2D) affects more than 500 million people globally and the number of cases is expected to increase in the future[4]. T2D is a significant risk factor for heart disease, as people with diabetes have over twice the risk of developing heart failure (HF), compared to people without diabetes[5]. The Framingham Heart Study reported that diabetes independently increases risk of HF up to 2-fold in men and 5-fold in women, compared to age-matched controls[6]. The increased risk of HF in people with diabetes persists even after controlling for relevant confounders, such as age, hypertension, hypercholesterolemia, and coronary artery disease (CAD)[7].

The Role of Calcium in the Cardiac Cycle

The heart contracts in a rhythmic pattern and this process is regulated by the movement of calcium (Ca^{2+}) within cardiomyocytes. In cardiac muscle, a depolarizing stimulus arrives at the sarcolemma, spreads into the T-tubule region and opens L-type Ca^{2+} channels, leading to a flux of Ca^{2+} into the cytosol, stimulating excitation contraction-coupling (ECC). Ca^{2+} entry into the cytosol through L-type Ca^{2+} channels initiates further release of Ca^{2+} from the ryanodine

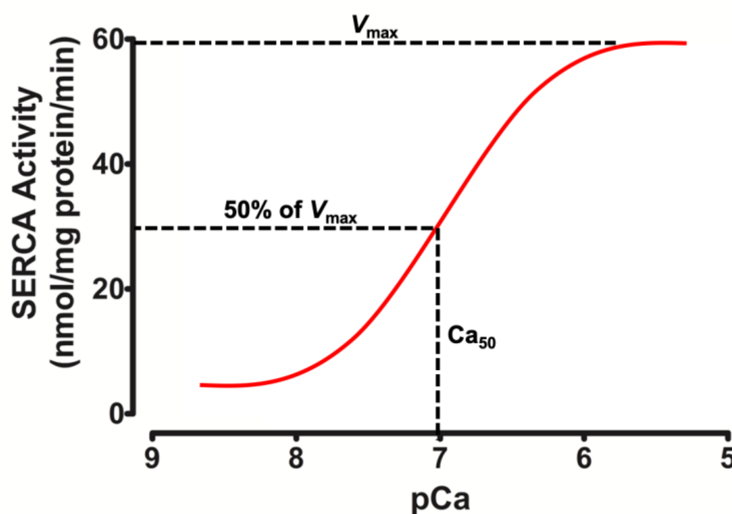
receptor 2, in an event known as Ca^{2+}-induced Ca^{2+} release[8]. This results in an approximately 10-fold increase in the concentration of free intracellular Ca^{2+} in the cytosol, facilitating the binding of Ca^{2+} to the troponin complex and exposing the myosin-actin binding site[9]. Exposing of the myosin-actin binding site allows for interaction between actin and myosin and subsequent cross-bridge cycling, where adenosine triphosphate (ATP) hydrolysis is coupled to the generation of the power stroke, leading to cardiac contraction and the ejection of blood from the ventricles; initiating systole. Following contraction, intracellular Ca^{2+} must be removed to initiate cardiac relaxation and ventricular filling during diastole. Four main transporters are responsible for removing Ca^{2+} from the cytosol of cardiomyocytes, the Na^{+}/Ca^{2+} exchanger, sarcolemmal Ca^{2+} ATPase, mitochondrial Ca^{2+} uniporter, and sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA)[9]. SERCA is the primary Ca^{2+} transporter that moves Ca^{2+} from the cytosol, back to the sarcoplasmic reticulum (SR) lumen in the mammalian heart[10]. As such, 92% of Ca^{2+} removal in the rodent heart and 70% of Ca^{2+} removal in the human heart is attributed to the sequestering activity of SERCA[10,11]

SERCA

SERCA is a 110 kDa transmembrane protein with three distinct regions, the cytoplasmic head, transmembrane helices containing the two Ca^{2+} binding sites, and luminal loops[12]. The cytoplasmic head of SERCA is composed of its actuator, phosphorylation, and nucleotide binding domains, each of which modulates SERCA function. The actuator domain provides the pivot for the translocation of Ca^{2+} from the cytosol back into the SR, and ATP hydrolysis occurs at the interface between the nucleotide and phosphorylation domains[13]. Each ATP hydrolyzed powers the translocation of two Ca^{2+} ions from the cytosol back into the SR lumen. The

hydrolysis of ATP is required for this process because an approximately 10,000-fold concentration gradient exists across the SR membrane at rest[13]. In fact, SERCA is the second greatest consumer of ATP in cardiomyocytes after the myosin ATPase[14]. The maximum enzyme velocity (V_{max}) of SERCA is the maximal rate at which SERCA transports Ca^{2+} (**Figure 1**)[15]. Higher V_{max} allows for faster Ca^{2+} re-uptake back into the SR lumen, increasing rates of muscular relaxation and contraction[16]. SERCA activity at submaximal Ca^{2+} concentrations ($[Ca^{2+}]$) is altered by changes in SERCA Ca^{2+} affinity[17]. Submaximal SERCA activity is described by the Ca_{50}, which is the $[Ca^{2+}]$ eliciting 50% of V_{max} (**Figure 1**)[18]. Submaximal SERCA activity is also described by the Hill coefficient, which is the slope of the relationship between $[Ca^{2+}]$ and enzyme activity for 10 – 90% of V_{max}[18]. Mammalian SERCA proteins are encoded by three different genes, ATPA1, ATPA2, and ATPA3, producing more than 10 SERCA isoforms[12,19]. The ATPA2 gene encodes SERCA2a, the pre-dominant cardiac SERCA isoform[12,20].

Figure 1. Example SERCA activity-pCa curve displaying SERCA V_{max} and Ca_{50}.



SERCA ATP hydrolysis is indicated by the Y-axis and the inverse logarithmic calcium concentration is indicated by the X-axis.

SERCA2a and Heart Disease

Cardiac contraction and relaxation become impaired in the pathological state that contributes to systolic and diastolic dysfunction, ultimately resulting in heart disease[21]. The impaired contraction and relaxation of the diseased heart is directly influenced by abnormal Ca^{2+} cycling in the myocardium, due to alterations to myocardial Ca^{2+} transport proteins[22–30]. Specifically, it is well-documented that heart disease is associated with reduced SERCA2a mRNA expression, protein level, and function, and this impairs myocardial contractile function[31–36]. For example, diminished transport of Ca^{2+} from the cytosol back into the SR lumen lowers the SR [Ca^{2+}], reducing the availability of Ca^{2+} ions for release to initiate cardiomyocyte contraction[37]. Furthermore, the removal of cytosolic Ca^{2+} is slowed, impairing cardiomyocyte relaxation[37].

Heart Valve Disease

The heart contains four valves, the aortic, pulmonary, mitral, and tricuspid valves. These valves promote coordinated blood flow through the heart during the cardiac cycle. Heart valve disease (HVD) describes a collection of conditions affecting the structure or function of one or more heart valves[38]. In the presence of abnormal valvular structure or function, the dynamics of flow are altered, imposing a hemodynamic stress on the myocardium in the form of pressure or volume overload[39]. This induces pathological changes in cardiac morphology, ventricular dysfunction, and eventually, the development of HF[40,41]. The heart valves are primarily affected by three distinct pathologies, valvular stenosis, valvular regurgitation, and valvular prolapse. Valvular stenosis refers to the narrowing of the valvular opening, reducing the outflow of blood through the valve. Valvular regurgitation occurs when the valve is unable to close effectively,

leading to blood flowing back through the valve when the valve is closed. Finally, valvular prolapse is the protrusion of the mitral or tricuspid leaflets into the atria during systole, leading to the back flow of blood into the atria.

Though the global epidemiology of HVD remains to be fully established, in the USA, the overall age-adjusted prevalence of HVD is estimated to be 2.50% of the population (95% CI 2.20 – 2.70%)[42]. In the same study, the prevalence of various types of HVD were found to be the following, aortic stenosis 0.40%, mitral stenosis 0.10%, aortic regurgitation 0.50%, and mitral regurgitation 1.70%[42]. Sex did not alter the prevalence of HVD, but it was greatly impacted by age, from <2.00% before 65 years of age to 8.50% in those aged 65 to 75 years, and 13.20% after the age of 75[42]. A similar age dependency has also been reported in European hospital-based surveys[43]. Thus, given the association between HVD and age, combined with the current rise in life expectancy, HVD has been referred to as the "next cardiac epidemic"[44].

The symptoms of aortic stenosis are angina pectoris, exertional syncope, and dyspnea and they tend to occur following a significant asymptomatic period[45]. The survival for patients with aortic stenosis is normal until the onset of symptoms, when survival rates rapidly decline[45]. Without surgical intervention, 75% of patients with aortic stenosis die within 3 years after the onset of symptoms[45]. Similarly, mitral stenosis has a progressive course that is initially slow, but rapidly accelerates[45]. Symptoms of mitral stenosis are exertional and paroxysmal dyspnea, orthopnea, and occasionally hemoptysis[45]. In aortic stenosis, patients may be asymptomatic until the development of severe ventricular dysfunction associated with dyspnea, orthopnea, and fatigue[45]. Mitral regurgitation is a state of prolonged volume overload, eventually leading to cardiac decompensation and pulmonary congestion[45]. Finally, symptoms of mitral prolapse include angina, dyspnea, and anxiety[45].

HVD is often first identified by the presence of distinct heart murmurs caused by altered valvular function[45]. For example, aortic stenosis produces a systolic heart murmur which radiates into the neck[45]. However, a definitive diagnosis of HVD is typically confirmed by echocardiography, which allows for visualization of anatomical valvular defects[46].

SERCA2a and Heart Valve Disease

Attempts have been made to determine the impact of HVD on cardiac SERCA2a. For example, cardiac SERCA2a mRNA expression was reduced in a porcine model of MR induced by volume overload (**Table 1**)[47]. Additionally, in a mouse model of AS induced by mild or severe transverse aortic constriction (TAC), left ventricle (LV) SERCA2a protein level was unaltered by aortic stenosis induced by mild TAC, but was diminished in aortic stenosis induced by severe TAC (**Table 1**)[48]. As well, cardiac SERCA2a protein level was reduced in sheep subjected to both myocardial infarction (MI) and mitral regurgitation, compared to sheep subjected to MI alone (**Table 1**)[49]. However, the effect of HVD on SERCA2a protein level in the human heart is inconclusive. For example, SERCA2a mRNA expression in LV tissue was lower in patients with HF and mitral regurgitation, compared to patients with mitral regurgitation alone (**Table 2**)[50]. While LV SERCA2a mRNA expression was unchanged in patients with severe isolated mitral regurgitation secondary to degenerative mitral regurgitation, compared to controls with no history of cardiovascular disease (CVD) (**Table 2**)[51]. The two studies previously discussed also assessed SERCA2a protein level in the hearts of patients with HVD. Leszek *et al.* found no differences in LV SERCA2a protein level between patients with HF and mitral regurgitation or patients with mitral regurgitation alone (**Table 2**)[50]. Similarly, Zheng *et al.* found no change in LV SERCA2a protein level in patients with severe isolated mitral regurgitation

secondary to degenerative mitral valve disease, compared to controls with no history of CVD (**Table 2**)[51]. Given the research presented, SERCA2a protein level may be reduced in HVD, as a result of pressure or volume overload; however, few studies have examined these outcomes in animal or human models of the disease. Moreover, no studies have measured cardiac SERCA function in animal or human models of HVD. Thus, the impact of HVD on SERCA2a and the role of SERCA2a in HVD are not clearly established.

Table 1. Changes to SERCA2a in animal models of HVD.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
Kawase *et al.* 2008.	Pig LV	Mitral regurgitation induced by volume overload	↓

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
van Deel *et al.* 2008.	Mouse LV	Aortic stenosis induced by mild TAC Aortic stenosis induced by severe TAC	Mild TAC unchanged Severe TAC ↓
Silveira *et al.* 2017.	Rat heart	Aortic stenosis induced by aortic banding	Unchanged
Beeri *et al.* 2010.	Sheep heart	MI alone MI + mitral regurgitation	MI + mitral regurgitation ↓

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
No published research			

Table 2. Changes to SERCA2a in human models of HVD.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
Leszek *et al.* 2006.	Human LV	Mitral regurgitation alone Mitral regurgitation + HF	Mitral regurgitation + HF ↓
Zheng *et al.* 2014.	Human LV	Severe isolated mitral regurgitation secondary to degenerative mitral valve disease	Unchanged

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
Leszek *et al.* 2006.	Human LV	Mitral regurgitation alone Mitral regurgitation + HF	Unchanged
Zheng *et al.* 2014.	Human LV	Severe isolated mitral regurgitation secondary to degenerative mitral valve disease	Unchanged

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
No published research			

Coronary Artery Disease

CAD generally refers to conditions involving blockage of the coronary arteries, which supply blood to the myocardium, resulting in myocardial ischemia[52]. Ischemia of the myocardium represents a state of inadequate oxygen (O_2) supply to meet the metabolic demand of cardiomyocytes in order to maintain normal cardiac function[53]. In the absence of O_2, cardiomyocytes must initiate anaerobic metabolism, leading to tissue acidosis from the production of lactic acid and reduced ATP availability[54]. When myocardial ischemia is prolonged, as in during a MI, cardiomyocyte death irreversibly alters cardiac function[53]. CAD is the principal cause of HF and almost two thirds of HF cases are attributed to myocardial ischemia resulting from CAD[55].

CAD is the most prevalent form of heart disease[53]. According to the most recent data from 2015, there were an estimated 110.50 million cases of CAD worldwide (95% CI: 100.68 – 121.80 million cases)[56]. The prevalence of CAD rapidly increases with age. For example, among persons 40 to 44 years of age, the prevalence of CAD was estimated at 290 cases per 100,000 (95% CI: 255 – 323 cases per 100,000). While this number increases 3-fold among persons 50 to 54 years of age to an estimated at 870 cases of CAD per 100,000 (95% CI: 783 –914 cases per 100,00). This number increases further in persons 75 to 79 years of age to an estimated 11,203 cases of CAD per 100,000 (95% CI: 9,610 – 13,178 cases per 100,00). Though in persons 80 years of age and over, the prevalence of CAD begins to decline, as it is estimated at 9,700 cases per 100,000 (95% CI: 8,733 – 10,738 cases per 100,000).

Blockage of the myocardial blood supply during CAD results in myocardial ischemia and produces the primary symptom of CAD, angina pectoris[52]. Though CAD is also associated with symptoms like anxiety, fatigue, dyspnea, heart palpitations, and dizziness[57].

CAD is diagnosed using a variety of modalities which identify myocardial ischemia resulting from blockage of the coronary arteries, either physiologically or anatomically. For example, electrocardiography and exercise-electrocardiography are used to identify changes in cardiac electroconductivity indicative of ischemic myocardial damage[58]. While cardiac magnetic resonance imaging, coronary catheterization and angiography, and coronary computer tomographic angiography are used to visualize blockages within the coronary arteries[59].

SERCA2a and Coronary Artery Disease

The changes to SERCA2a resulting from CAD in animal models are well-described. For example, ligation of the left anterior descending (LAD) coronary artery, resulting in myocardial ischemia comparable to CAD, reduces LV SERCA2a mRNA expression in rats (**Table 3**)[60,61]. Moreover, cardiac SERCA2a protein level is reduced in mice subjected to LAD coronary artery ligation (**Table 3**)[62]. As such, LAD coronary artery ligation is often reported to diminish cardiac SERCA2a protein level in rats (**Table 3**)[61,63–65]. However, no changes in cardiac SERCA2a protein level following coronary artery ligation in rats was reported by Plummer *et al.* (**Table 3**)[63]. CAD negatively impacts SERCA2a protein level in large animal models. For example, myocardial ischemia, resulting from blockage of the coronary arteries, diminished cardiac SERCA2a protein level in dogs (**Table 3**)[66]. As well, LV SERCA2a protein level was reduced in a porcine model of myocardial ischemia, induced by progressive stenosis on the LAD coronary artery (**Table 3**)[67]. Considering SERCA2a function, myocardial ischemia lead to a 74% reduction in SERCA function within the rabbit heart (**Table 3**)[68]. Furthermore, both Lee *et al.* and Toba *et al.* have found cardiac SERCA function is impaired in canine models of myocardial ischemia, resulting from blockage of the coronary arteries (**Table 3**)[66,69]. However, these results

are not well-replicated in humans with CAD. In patients with multi-vessel CAD undergoing coronary artery bypass grafting (CABG) surgery, Nef *et al.* found cardiac SERCA2a mRNA expression was increased, compared to patients undergoing surgery for atrial septal defects and healthy donor hearts unfit for transplantation (**Table 4**)[70]. Additionally, LV SERCA2a protein level was unchanged in patients with severe CAD, compared to patients with idiopathic cardiomyopathy and healthy donor hearts unfit for transplantation (**Table 4**)[71]. Though, Nef *et al.* found impairments in maximal cardiac SERCA activity in patients with multi-vessel CAD undergoing CABG surgery, compared to patients undergoing surgery for atrial septal defects and health donor hearts unfit for transplantation (**Table 4**)[70]. Considering the discussed literature, it is likely SERCA2a protein level and function are reduced in animal models of CAD. Though these findings are not well-replicated in the few studies that have examined human heart tissue.

Table 3. Changes to SERCA2a in animal models of CAD.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
Sallinen *et al.* 2007.	Rat LV	LAD ligation	↓ 54.80%
Zhang *et al.* 2016.	Rat LV	LAD ligation	↓

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
Zhang *et al.* 2018.	Mouse heart	LAD ligation	↓
Zhang *et al.* 2016.	Rat LV	LAD ligation	↓ 52%
Zhao *et al.* 2018.	Rat heart	LAD ligation	↓
Gui *et al.* 2018.	Rat heart	LAD ligation	↓
Plummer *et al.* 2018	Rat heart	LAD ligation	Unchanged
Toba *et al.* 1978.	Dog heart	Coronary artery blockage	↓
Xin *et al.* 2011.	Pig LV	LAD stenosis	↓

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
Kaplan *et al.* 1992.	Rabbit heart	Myocardial ischemia	↓ 74%
Toba *et al.* 1978.	Dog heart	Coronary artery blockage	↓
Lee *et al*. 1967.	Dog heart	Coronary artery blockage	↓

Table 4. Changes to SERCA2a in human models of CAD.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
Nef *et al.* 2006.	Human heart	Multi-vessel CAD	↑

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
Nef *et al.* 2006.	Human heart	Multi-vessel CAD	Unchanged
Hamdani *et al.* 2010.	Human LV	Severe CAD + no history of MI	Unchanged

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
Nef *et al.* 2006.	Human heart	Multi-vessel CAD	V_{max} ↓ 44%

Diabetic Cardiomyopathy

Diabetic cardiomyopathy (DCM) is a distinct form of heart disease that occurs in people with diabetes and is defined as ventricular dysfunction in the absence of hypertension and other forms of heart disease[72–75]. The pathogenesis of DCM is complex, beginning with myocardial insulin resistance, hyperinsulinemia, and hyperglycemia, leading to the development of adverse cardiac remodeling and fibrosis[76]. Eventually, these structural and functional changes to the myocardium result in systolic and/or diastolic ventricular dysfunction, ultimately progressing to HF[76].

The prevalence of DCM in the diabetic population remains to be determined. Though, in a study of 136 people 45 years or older with either type 1 diabetes (T1D) or T2D, it was reported that 16.90% of participants had systolic and/or diastolic dysfunction with no evidence of HVD, CAD, or congenital heart disease[77]. Similarly, diastolic dysfunction was reported to be present in 15.50% of participants in a study of 1093 people with T1D without known heart disease[78]. While in a study of 61 people with T2D without hypertension and CAD, diastolic dysfunction was reported to be present in 63 to 75% of participants, depending upon the method used to assess cardiac function[79].

In the early stages of DCM, the patient is usually asymptomatic[80]. However, as it progresses, people with DCM will begin to present with the typical signs and symptoms associated with HF, including fatigue, dyspnea, pulmonary and peripheral edema, and jugular vein distension[80]. The diagnosis of DCM requires the exclusion of hypertension, CAD, HVD, and congenital heart disease, and evidence of cardiac structural and functional abnormalities using echocardiography[80]. In its early stages, DCM is characterized by concentric ventricular hypertrophy and preserved ejection fraction (left ventricular ejection fraction (LVEF); ≥50%)[80].

As DCM advances, the ventricles dilate and systolic dysfunction (SD; LVEF <50%) develops[80]. However, recently it has been suggested that rather than occurring in succession, these two stages are actually distinct phenotypes of DCM[81].

SERCA2a and Diabetic Cardiomyopathy

Animal models have been extensively used to examine the impact of DCM on cardiac SERCA2a. For example, cardiac SERCA2a mRNA expression is diminished in mouse models of DCM induced by streptozotocin (STZ), which causes pancreatic β-cell dysfunction, hyperglycemia, and ventricular dysfunction (**Table 5**)[82–85]. While no change in cardiac SERCA2a mRNA expression was reported in a model of T2D using leptin deficient, db/db mice (**Table 5**)[86]. In STZ-injected rats, cardiac SERCA2a mRNA expression is consistently reduced (**Table 5**)[87–91]. As well, cardiac SERCA2a mRNA expression is reduced in Otsuka-Long-Evans Tokushima Fatty (OLETF) rats, a genetic model of advanced T2D, characterized by hyperglycemia and diastolic dysfunction (**Table 5**)[92]. However, LV SERCA2a mRNA expression was elevated in a model of DCM using Zucker Diabetic Fatty rats (**Table 5**)[93]. In the hearts of STZ-injected mice, SERCA2a protein level is consistently reduced (**Table 5**)[82–84,94–96]. Moreover, LV SERCA2a protein level is reduced in a model of T1D using Akita^{ins2} mice, which possess a mutation in the insulin 2 gene (**Table 5**)[97]. Similar to STZ-injected mice, cardiac SERCA2a protein level is consistently diminished in STZ-injected rats (**Table 5**)[88,89,91,98–104]. Cardiac SERCA2a protein level was also reduced in OLETF rats (**Table 5**)[92]. Only a single study has reported no change in SERCA2a protein level in STZ-injected rats, compared to control animals (**Table 5**)[105]. Additionally, in a model of DCM utilizing Goto-Kakizaki rats, characterized by hyperglycemia and ventricular dysfunction in the absence of obesity and

hypertension, cardiac SERCA2a protein level was unchanged (**Table 5**)[106]. STZ-injected rats also demonstrate impairments in maximal cardiac SERCA activity and additional kinetic properties (**Table 5**)[91,105]. Conversely, the effect of DCM on SERCA2a within the human heart is not well-described. Lamberts *et al.* and Bussey *et al.* found increased SERCA2a protein level in RA tissue obtained from patients with T2D undergoing CABG surgery without SD, compared to patients without T2D (**Table 6**)[107,108]. Additionally, Lamberts *et al.* found no change in LV SERCA2a protein level in patients with T2D undergoing CABG surgery without SD, compared to patients without T2D (**Table 6**)[108]. Therefore, significant evidence exists indicating SERCA2a protein level and function are reduced in animal models of DCM, particularly, in studies employing STZ-injected animals. However, no studies have examined the effect of diabetes on SERCA2a mRNA expression or function in the human heart.

Table 5. Changes to SERCA2a in animal models of DCM.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
Nagatomo *et al.* 2014.	Mouse LV	STZ-induced diabetes	↓
Trost *et al.* 2002.	Mouse heart	STZ-induced diabetes	↓
Sulaiman *et al.* 2010.	Mouse heart	STZ-induced diabetes	↓ 80%
Daniels *et al.* 2010.	Mouse LV	db/db mice	Unchanged
Qi *et al.* 2006.	Rat LV	STZ-induced diabetes	↓ 63.90%
Epp *et al.* 2013.	Rat LV	STZ-induced diabetes	↓ 36%
Lu *et al.* 2017.	Rat LV	STZ-induced diabetes	↓ 41%
Cheng *et al.* 2011.	Rat heart	STZ-induced diabetes	↓
Cheng *et al.* 2016.	Rat heart	STZ-induced diabetes	↓
Sakata *et al.* 2006.	Rat heart	OLETF rats	↓
Fredersdorf *et al.* 2012.	Rat LV	ZDF rats	↑

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
Nagatomo et al. 2014.	Mouse LV	STZ-induced diabetes	↓
Trost *et al.* 2002.	Mouse heart	STZ-induced diabetes	↓ 30%
Wold *et al.* 2006.	Mouse heart	STZ-induced diabetes	↓
Suarez *et al.* 2008.	Mouse heart	STZ-induced diabetes	↓ 60%
Sulaiman *et al.* 2010.	Mouse heart	STZ-induced diabetes	↓ 70%
Lei *et al.* 2018.	Mouse heart	STZ-induced diabetes	↓
LaRocca *et al.* 2012.	Mouse LV	Akita^{ins2} mice	↓
Qi *et al.* 2006.	Rat LV	STZ-induced diabetes	↓ 45.20%
Zhang *et al.* 2008.	Rat LV	STZ-induced diabetes	↓
Kranstuber *et al.* 2012.	Rat LV	STZ-induced diabetes	↓
Epp *et al.* 2013.	Rat LV	STZ-induced diabetes	↓ 21%
Lu *et al.* 2017.	Rat LV	STZ-induced diabetes	↓ 27%
Chang *et al.* 2019.	Rat LV	STZ-induced diabetes	↓
Vasanji *et al.* 2004.	Rat heart	STZ-induced diabetes	↓
Li *et al.* 2009.	Rat heart	STZ-induced diabetes	↓
Kain *et al.* 2011.	Rat heart	STZ-induced diabetes	↓
Cheng *et al.* 2016.	Rat heart	STZ-induced diabetes	↓
Sakata *et al.* 2006.	Rat heart	OLETF rats	↓
Ligeti *et al.* 2006.	Rat heart	STZ-induced diabetes	Unchanged
Bombicz *et al.* 2019.	Rat heart	Goto-Kakizaki rats	Unchanged

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
Ligeti *et al.* 2006.	Rat heart	STZ-induced diabetes	V_{max} ↓
Epp *et al.* 2013.	Rat LV	STZ-induced diabetes	V_{max} ↓ 32% Hill coefficient ↓ Ca_{50} ↑

Table 6. Changes to SERCA2a in human models of DCM.

SERCA2a mRNA Expression

Reference	Species/Tissue	Disease Model	Key Finding
No published research			

SERCA2a Protein Level

Reference	Species/Tissue	Disease Model	Key Finding
Lamberts *et al.* 2014.	Human RA	CAD without SD CAD without SD+T2D	T2D ↑ 64%
Bussey *et al.* 2015.	Human LV+RA	CAD without SD CAD without SD+T2D	LV unchanged RA T2D ↑

SERCA2a Function

Reference	Species/Tissue	Disease Model	Key Finding
No published research			

SERCA2a as a Therapeutic Target in Heart Disease

The diseased heart is characterized by contractile dysfunction resulting from reduced SERCA2a mRNA expression, protein level, and function[22,23,34,35,109]. Therefore, the restoration of cardiac function by rescuing SERCA2a level and/or function is a potential therapeutic strategy for the treatment of heart disease[110].

SERCA2a Gene Therapy

Gene therapy to enhance cardiac SERCA2a protein level has been reported to be an effective treatment to improve cardiac function. For example, the lentiviral-mediated gene transfer of SERCA2a improved LVEF and cardiac remodeling in a mouse model of HF[111]. In porcine models of HF, the adenoviral-mediated gene transfer of SERCA2a increased cardiac SERCA2a mRNA expression and protein level, preserved systolic function, and improved diastolic function and cardiac remodeling[47,67]. Enhancing SERCA2a mRNA expression has also been reported to be useful in treating the cardiac dysfunction associated with DCM. SERCA2a overexpression upregulated cardiac SERCA2a mRNA expression and protein level, improved cardiomyocyte contractility, and restored cardiac function in STZ-injected mice[82,95]. Finally, adenoviral-mediated SERCA2a gene transfer into the whole hearts of OLETF rats rescued cardiac SERCA2a mRNA expression, protein level, and cardiac function[92].

The efficacy of SERCA2a gene therapy for the treatment of heart disease has been studied in human clinical trials. The Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trials have aimed to restore SERCA2a protein level in patients with HF using adenoviral-mediated gene transfer[112–114]. In the phase 1 CUPID trial, 9 patients with severe HF received a single intracoronary infusion of an adeno-associated virus

(AAV) to overexpress SERCA2a to determine effective dose and patient outcomes[112]. The treatment demonstrated an acceptable safety profile 6- and 12-months post-treatment and patients receiving SERCA2a gene therapy displayed improvements in 4 clinically relevant domains, including symptoms, functional status, biomarkers, and LV function/remodeling[112]. Following the phase 1 trial, the phase 2a CUPID trial examined adenoviral-mediated SERCA2a gene therapy in 39 patients with severe HF[113]. It was found that patients treated with the SERCA2a AAV stabilized or improved, but patients receiving the placebo deteriorated over a 6- to 12-month period[113]. Furthermore, patients receiving SERCA2a gene therapy had a risk reduction for LV assist device (LVAD), heart transplantation, and death, of 60%, 56%, and 88%, respectively, 1-year after treatment[113].

Based on the success of the phase 2a CUPID trial, a multinational phase 2b trial, including 243 patients with moderate-to-severe HF was conducted[114]. However, no significant differences in efficacy endpoints between patients receiving SERCA2a gene therapy or placebo were identified in the phase 2b CUPID trial[114]. Though the clinical condition of study patients did not worsen[114]. The differences in outcomes between the phase 2a and phase 2b CUPID trials are reported to be due to reduced AAV transduction efficiency, as the proportion of empty viral capsids was significantly lower in the phase 2b CUPID trial (25%), compared to the phase 2a CUPID trial (85%)[115]. This dosing issue was attributed to changes in the manufacturing process[115]. The addition of empty viral capsids to AAV vector formulations reduces the activity of neutralizing antibodies, enhancing the transduction efficiency of active capsids following delivery[115]. Subsequently, the presence of AAV-delivered SERCA2a was assessed in cardiac tissue obtained from patients of the phase 2a and 2b CUPID trials requiring LVAD implantation, heart transplantation, or who had since passed away[114]. The amount of vector DNA in phase 2b

trial cardiac tissue was determined to be at the lower-end of the pharmacological dose-response curve[114]. Thus, it is likely patients of the phase 2b trial were expressing a smaller proportion AAV-delivered SERCA2a, compared to patients of the phase 2a tiral[114]. Despite the negative results of the phase 2b CUPID trial due to the identified dosing issue, the success of the phase 1 and 2a CUPID trials provide compelling evidence that SERCA2a is a promising target for the treatment of heart disease.

Targeting SERCA2a Regulatory Proteins in Heart Disease

Phospholamban (PLN) is a small, reversibly phosphorylated, 52 amino acid transmembrane protein, located in the cardiac SR, which binds to SERCA2a and regulates its function[11]. In its unphosphorylated state, PLN interacts with SERCA2a and diminishes its affinity for Ca^{2+}, reducing the amount of Ca^{2+} transported by SERCA2a back to the SR[11]. When phosphorylated, PLN dissociates from SERCA2a, relieving its inhibitory effect and increasing SERCA2a Ca^{2+} transport[11].

Through its ability to regulate SERCA2a function, PLN is capable of influencing cardiac function; thus, approaches that target PLN as a strategy to enhance SERCA2a function at submaximal Ca^{2+}-concentrations are in development. For example, the gene transfer of antisense PLN, resulting in the ablation of PLN protein content, improved cardiac contractility and Ca^{2+}-handling properties in cardiomyocytes isolated from failing human hearts[116]. As well, Sakai *et al*. developed a 30 nucleotide cell-penetrating aptamer that binds to PLN and impairs its ability to interact with SERCA2a, enhancing cardiac SERCA2a function and cardiomyocyte Ca^{2+} transients[117].

Pharmacological approaches which target PLN have also been developed to enhance cardiac SERCA2a function. Istaroxime is a luso-inotropic agent capable of displacing the interaction between SERCA2a and PLN, relieving the inhibitory action of PLN and stimulating SERCA2a function[118,119]. The clinical utility of istaroxime was assessed in the HORIZON – HF trial, which included 120 patients hospitalized with HF and severe SD (LVEF ≤35%)[120]. After controlling for age, sex, and baseline LVEF, istaroxime treatment improved cardiac hemodynamics, diastolic function, and cardiac remodeling[120]. This research provides a foundation for the development therapies to treat heart disease by attempting to increase endogenous SERCA2a function, without altering myocardial SERCA2a mRNA expression or protein level.

Post-Translational Modification of SERCA2a

Post-translational modifications (PTM) are signaling events that change the properties of a protein by adding or removing functional groups from one or more of the protein's amino acids[121]. PTMs alter a protein's function, localization, turnover, and interactions with other proteins[121]. SERCA2a is regulated by several PTMs, including glutathionylation, nitration, glycosylation, *O*-glycNAcylation, SUMOylation, and acetylation[122–127]. These PTMs of SERCA2a have previously been reviewed by Stammers *et al.*[128]. My thesis will focus on the acetylation of SERCA2a.

SERCA2a Acetylation

Acetylation is a PTM referring to the addition of an acetyl functional group to a lysine residue (K) within a target protein[129,130]. Acetylation is a reversible process regulated by histone

acetyl transferases (HAT) and histone deacetylases (HDAC), which acetylate and deacetylate target proteins, respectively[129,130]. SERCA2a undergoes acetylation at three lysine residues, K^{464}, K^{510}, and K^{533}, all of which are located in its cytoplasmic nucleotide binding domain[131]. Thus, it is possible acetylation may regulate the function of SERCA2a, but its effect remains to be determined.

The acetylation of SERCA2a was first suggested by Kho *et al.* in 2011 to impair cardiac SERCA2a function; however, the authors did not provide data to support that statement[124]. Since then, this group has published work reporting acetylation of SERCA2a is increased in the failing hearts of mice and pigs, and in failing human LV tissue obtained from patients with HF undergoing LVAD implantation or heart transplantation[127]. Further, this group reported acetylation of SERCA2a was associated with impaired SERCA2a function[127]. However, their data do not necessarily support this conclusion[127]. To examine the effect of acetylation on SERCA2a function, this group knocked down the HDAC, sirtuin 1 (SIRT1), in both *in vitro* and *in vivo* models, and found increased cardiac SERCA2a acetylation and diminished cardiomyocyte Ca^{2+}-handling properties[127]. Although intriguing, these findings are not sufficient to state that acetylation impairs cardiac SERCA2a function because SIRT1 is involved in numerous cellular processes within the heart, and its knockdown may lead to changes in SERCA2a function outside of the effects mediated by its acetylation status[132]. As well, despite assessment of cardiomyocyte Ca^{2+}-handling properties, SERCA2a activity was not directly measured; thus, it is not possible to conclude SERCA2a function was impaired. This group also used site-directed mutagenesis experiments to acetylate a single lysine residue of SERCA2a in HEK-293 cells and no changes to Ca^{2+} uptake, ATPase activities, or SERCA2a ATP binding affinity were revealed[127]. Contrary to impairing SERCA2a function, increased acetylation of

SERCA2a has also been found to be associated with improved SERCA2a function *in vitro*[133]. Therefore, while SERCA2a acetylation is increased in the failing animal and human heart, the effect of this PTM on SERCA2a is poorly understood because it has been linked to both impaired and improved SERCA2a function[127,133]. It is also unknown if cardiac SERCA2a acetylation is altered between patients with HVD or CAD, and if SD or T2D influence cardiac SERCA2a acetylation.

Preliminary research from our group has attempted to characterize the influence of site specific SERCA2a acetylation *in vitro*. More specifically, site-directed mutagenesis was utilized to modify all three acetylated-lysine sites within the SERCA2a protein[134]. This enabled the development of a triple acetylated-lysine SERCA2a mutant, where all acetylated-lysine sites were substituted for glutamic acid (Q) to create a SERCA2a$^{K464Q/K510Q/K533Q}$ mutant, and the development of an acetylated-lysine deficient SERCA2a mutant, where all acetylated-lysine sites were substituted for arginine (R) to create a SERCA2a$^{K464R/K510R/K533R}$ mutant [134]. Acetylated-lysine and glutamic acid are structurally alike and similarly charged[135]. Further, lysine and arginine are similarly charged, but arginine cannot be acetylated[135]. Thus, these substitutions are commonly used to study acetylated and acetylation-deficient proteins, respectively[135]. The SERCA2a mutants were transfected into Cos-1 cells and maximal SERCA activity (V_{max}) was found to be 35% lower in the triple acetylated-lysine mutant, compared to the acetylated-lysine deficient mutant[134]. In a different set of experiments, rat primary cardiomyocytes were incubated in high-glucose media for 18 hours to determine if SERCA2a is acetylated in hyperglycemic conditions, like that of diabetes[136]. Exposure to high-glucose media resulted in a 37% increase in SERCA2a acetylation and a 38% decrease in maximal SERCA activity, compared to control[136].

However, it must be stated that these findings were observed in a closed-system, *in vitro* model, which lacked the biological complexity of the diseased human heart.

Dr. Duhamel's research group has also sought to identify the enzymes responsible for SERCA2a acetylation and deacetylation *in vitro*[136]. To identify the HATs controlling SERCA2a acetylation in hyperglycemic conditions, cardiomyocytes were exposed to small molecule HAT inhibitors for 1 hour prior to 18 hours of incubation in high-glucose media[136]. Treatment of cardiomyocytes with the broad HAT inhibitor, anacardic acid, attenuated SERCA2a acetylation[136]. However, treatment with anacardic acid did not preserve SERCA function following exposure to high-glucose media[136]. CPTH2, a GCN5 inhibitor, was then used to determine if the HAT, GCN5 contributes to high-glucose-induced SERCA2a acetylation[136]. Treatment with GCN5 resulted in the attenuation of SERCA2a acetylation, but did not preserve SERCA function following exposure to high-glucose media[136]. No change in high-glucose-induced SERCA2a acetylation was observed following treatment of cardiomyocytes with C646, an inhibitor of the HAT, p300/CBP[136]. MG149 was used to determine if inhibition of the HATs, TIP60 and MOZ, prevented the increase in SERCA2a acetylation following high-glucose exposure, but as treatment with MG149 significantly reduced cell viability, SERCA2a acetylation status was not assessed in this condition[136]. Finally, to identify the class of HDACs controlling deacetylation of SERCA2a, cardiomyocytes were exposed to small molecule HDAC inhibitors for 18 hours[136]. Treatment of cardiomyocytes with nicotinamide, a class III HDAC inhibitor, resulted in a 36% increase in SERCA2a acetylation and a 33% decrease in SERCA V_{max}[136]. While treatment of cardiomyocytes with Trichostatin A, a class I and II HDAC inhibitor, did not alter SERCA2a acetylation or function[136]. Thus, it may be possible to control the

enzymes responsible for acetylating SERCA2a, but these experiments do not clearly reveal the effect of acetylation on SERCA2a function.

Chapter 2: Study Design and Methods

Statement of Problem

Acetylation of SERCA2a is upregulated in animal models of HF and in the failing human heart, but its effect on SERCA2a function is not understood. Preliminary research from our group indicates that diabetes may stimulate acetylation of SERCA2a. Furthermore, our preliminary research suggests the enzymes controlling SERCA2a acetylation can be targeted to regulate its acetylation status. Thus, altering SERCA2a acetylation may be a potential strategy to modulate endogenous SERCA2a function in the heart, without altering SERCA2a mRNA expression or protein level. However, the effect of acetylation on cardiac SERCA2a function must first be determined. As well, it is currently unknown if SERCA2a acetylation is elevated in the diseased hearts of patients with HVD or CAD, or in patients with HVD or CAD complicated with SD. It is also unknown if T2D exacerbates the acetylation of cardiac SERCA2a in the diseased human heart. My thesis research will examine these knowledge gaps.

Research Design

This study utilized a case-control design. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement guidelines for case-control studies were used to describe the research[137]. Appendix A details where each point on the checklist was addressed in this thesis.

Participants

According to institutional policies, all patients who undergo surgery at the St. Boniface Hospital sign a consent form allowing tissue materials and fluids, removed and discarded as a

normal part of surgery, to be used for research purposes. Therefore, my research utilized human RA tissue samples obtained from patients undergoing a valve procedure (VP) or CABG surgery at the St. Boniface Hospital. Informed consent was obtained from patients prior to cardiac surgery, either in-person at the St. Boniface Hospital or through the phone. The inclusion criteria for this study created a total of 8 patient groups (**Table 7**):

1. Patients undergoing a VP in whom LVEF was preserved within the normal range (50 – 65%) (HVD).
2. Patients undergoing CABG surgery, in the absence of a VP, in whom LVEF was preserved within the normal range (CAD).
3. Two additional patient groups similar to the two groups already described, but in whom LVEF was abnormal (SD; LVEF <50%) (HVD+SD; CAD+SD).
4. Four additional patient groups similar to those already described, but who also had T2D, as diagnosed by the patients' medical record and confirmed by a glycated hemoglobin (HbA_{1c}) of 6.5% or higher at the time of cardiac surgery (HVD+T2D; CAD+T2D; HVD+SD+T2D; CAD+SD+T2D).

Table 7. Patient groups by inclusion criteria.

Patient Group	Procedure	LVEF	T2D (Y/N)
HVD	VP	50 – 65%	N
CAD	CABG	50 – 65%	N
HVD+SD	VP	<50%	N
CAD+SD	CABG	<50%	N
HVD+T2D	VP	50 – 65%	Y
CAD+T2D	CABG	50 – 65%	Y
HVD+SD+T2D	VP	<50%	Y
CAD+SD+T2D	CABG	<50%	Y

***CABG**, coronary artery bypass grafting; **CAD**, coronary artery disease; **HVD**, heart valve disease; **VP**, valve procedure; **LVEF**, left ventricular ejection fraction; **SD**, systolic dysfunction; **T2D**, type 2 diabetes.*

Objectives

The objectives of this study were to:

1. Determine if RA SERCA2a acetylation and SERCA function are altered by heart disease type (i.e. HVD or CAD).
2. Determine if RA SERCA2a acetylation and SERCA function are altered by the presence of SD.
3. Determine if RA SERCA2a acetylation and SERCA function are altered by T2D status.

Tissue Collection

Human RA tissue samples of approximately 500 mg were obtained from patients undergoing cardiac surgery at the St. Boniface Hospital, placed in phenylmethylsulphonyl fluoride (PMSF) buffer (pH 7.5) containing 250 mM sucrose (Sigma-Aldrich, MO, USA), 5 mM HEPES (Sigma-Aldrich, MO, USA), 0.2 mM PMSF (Sigma-Aldrich, MO, USA), and 0.2% (*w/v*) NaN_3 (Sigma-Aldrich, MO, USA) on ice, and transferred to our laboratory in the St. Boniface Hospital Albrechtsen Research Centre. The surgical suite and research laboratory are located approximately 200 meters apart. At the laboratory, a portion of the RA tissue was immediately placed in a microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80°C for future use in co-immunoprecipitation experiments. The remaining RA tissue was diluted 1:10 (*w/v*) in ice-cold PMSF buffer and homogenized using an all-glass tissue grinder (Kimble Chase Life Sciences, TN, USA). The RA tissue homogenate was then aliquoted into microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80° C for future use in SERCA activity and Western blotting experiments. The total protein content of each RA tissue homogenate sample

was determined in duplicate with the Detergent Compatible™ Protein Assay (Bio-Rad Laboratories, CA, USA).

Co-Immunoprecipitation

The Signal-Seeker™ Acetyl-Lysine detection kit (Cytoskeleton, CO, USA) was used to measure SERCA2a acetylation in RA tissue, according to the manufacturer's protocol. Raw RA tissue was lysed with ice-cold BlastR™ lysis buffer (Cytoskeleton, CO, USA) and DNA was removed by passing the lysate through the BlastR™ filter system (Cytoskeleton, CO, USA). Following dilution with BlastR™ dilution buffer (Cytoskeleton, CO, USA), the protein concentration of the lysate was determined with the Precision Red™ Protein Assay Reagent (Cytoskeleton, CO, USA) and assessed at 600 nm. The sample was then immunoprecipitated by incubating the lysate with acetylated-lysine affinity beads (Cytoskeleton, CO, USA) overnight at 4°C with rotation. The beads were then pelleted and washed 3 times with BlastR™ wash buffer. Bound acetylated-lysines were eluted using bead elution buffer (Cytoskeleton, CO, USA) and acetylated-SERCA2a was detected by Western blotting with an anti-SERCA2a primary antibody (catalogue no. 4388, Cell Signaling Technology, MA, USA). The antibody we used to detect SERCA2a accurately identifies the 110 kDa SERCA2a protein and it has been used extensively for the detection of SERCA2a by Western blotting[138]. The total amount of acetylated-lysine immunoprecipitated was detected by Western blotting with an anti-acetylated-lysine antibody conjugated to horseradish peroxidase (catalogue no. 19C4B2.1, Cytoskeleton, CO, USA). The total level of acetylated-SERCA2a was normalized to the total level of acetylated-lysine immunoprecipitated and expressed as arbitrary units. Acetylated-SERCA2a was measured in RA tissue from 4 – 5 patients per group by co-immunoprecipitation.

Ca^{2+}-dependent Kinetic Properties of SERCA

The Ca^{2+}-dependent kinetic properties of SERCA were measured in RA tissue homogenate using an NADH-linked, enzyme-coupled spectrophotometric assay, as previously described by Simondes and Van Hardeveld, and modified by Duhamel *et al.*[139,140]. This assay indirectly measured SERCA ATP hydrolysis over Ca^{2+} concentrations ($[Ca^{2+}]$) ranging from a pCa ($-\log_{10}([Ca^{2+}])$) of 7.94 to 5.86 by assessing the rate of NADH disappearance at 340 nm and 37° C for 1 hour using a FLUOstar® Omega microplate reader (BMG Labtech, GER). The reaction buffer (pH 7.0) contained 200 mM KCl (Fisher Scientific, MA, USA), 20 mM HEPES (Sigma-Aldrich, MO, USA), 15 mM $MgCl_2$ (Fisher Scientific, MA, USA), 10 mM NaN_3 (Sigma-Aldrich, MO, USA), 10 mM phosphoenolpyruvate (Roche Applied Science, GER), 5 mM ATP (Sigma-Aldrich, MO, USA), and 1 mM EGTA (Sigma-Aldrich, MO, USA). The SERCA-specific inhibitor, cyclopiazonic acid (CPA; 40 μM) (Sigma-Aldrich, MO, USA) was added to one reaction for each sample to determine basal ATPase activity[141]. SERCA activity was calculated based on the difference in the rate of ATP hydrolysis as stimulated by Ca^{2+} in the absence and presence of CPA. The data for each sample was then plotted, while blinded to patient group, to create a graph of SERCA activity versus pCa values using GraphPad Prism 5 (GraphPad Software, CA, USA). The following kinetic properties of SERCA were calculated: V_{max}, Ca_{50}, and the Hill coefficient. These kinetic properties of SERCA assess maximal and submaximal SERCA activity and SERCA Ca^{2+} affinity. The Ca^{2+}-dependent kinetic properties of SERCA were measured in RA tissue homogenate for all samples, in duplicate.

Western Blotting

Thirty (30) µg of RA tissue homogenate was diluted and denatured in 2x Laemmli sample buffer (Bio-Rad Laboratories, CA, USA). Samples were heated at 95° C for 5 minutes and then loaded onto Bio-Rad 4 – 15% Mini-PROTEAN® TGX Stain-Free™ precast gels (Bio-Rad Laboratories, CA, USA) to undergo SDS-PAGE. After electrophoresis, gels were activated using the Bio-Rad ChemiDoc™ MP imager (Bio-Rad Laboratories, CA, USA). This was followed by semi-dry transfer onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, CA, USA) using the Bio-Rad Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories, CA, USA). Membranes were then blocked with 5% (*w/v*) bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA) in Tris-buffered saline (pH 7.5) with 0.1% Tween® 20 (Sigma-Aldrich, MO, USA) (TBST) buffer for 1 hour at room temperature. Blots were incubated overnight at 4° C with the following primary antibodies diluted in 5% BSA in TBST buffer: anti-acetylated-lysine (catalogue no. 19C4B2.1, Cytoskeleton, CO, USA; 1:3000), anti-SERCA2a (catalogue no. 4388, Cell Signaling Technology, MA, USA; 1:1000), anti-PLN (catalogue no. 14562, Cell Signaling Technology, MA, USA; 1:500), and anti-phosphorylated-$PLN^{Ser16/Thr17}$ (catalogue no. 8496, Cell Signaling Technology, MA, USA; 1:500). Blots were washed for 10 minutes, 3 times, with TBST. Membranes were incubated in anti-rabbit secondary antibody conjugated to horseradish peroxidase (no. 7074, Cell Signaling Technology, MA, USA) diluted 1:5000 in 5% BSA in TBST buffer for 2 hours at room temperature and then washed for 10 minutes, 3 times, with TBST. Proteins were visualized using Clarity™ or Clarity Max™ Western ECL Substrate (Bio-Rad Laboratories, CA, USA) and the Bio-Rad ChemiDoc™ MP imager (Bio-Rad Laboratories, CA, USA). All images were analyzed using Image Lab software (Bio-Rad Laboratories, CA, USA). Relative protein levels were normalized to total protein with

stain-free imaging and expressed as arbitrary units. Phosphorylated-PLN was normalized to total-PLN to determine the phosphorylated-protein to total-protein ratio. RA tissue from 5 – 6 patients per group was analyzed by Western blotting.

Statistical Analyses

Three-way ANOVA was used to detect differences between patient groups based on heart disease type, absence or presence of SD, T2D status, and their interaction. The three-way ANOVA approach allows for the comparison of each patient group to each other and the identification of any main and interaction effects due to heart disease type, absence or presence of SD, and T2D status. A Tukey post hoc test was used to identify differences between specific means when significant differences ($p = <0.05$) were detected. An unadjusted linear regression analysis was performed to examine the relationship between RA SERCA2a acetylation and maximal RA SERCA activity. Statistical calculations were made using SPSS version 26 (IBM Corporation, NY, USA). This study was powered using maximal SERCA activity as its primary outcome variable, indicating a total sample size of 54 (7 per group) is needed to detect differences between patient groups (two tailed $\alpha = 0.05$; $\beta = 0.20$; Mean ± SD, Group 1, 107 ± 21; Group 2, 71 ± 17).

Chapter 3: Results

Patient Characteristics

Sixty-one (61) human RA tissue samples were obtained from patients who underwent cardiac surgery at the St. Boniface Hospital through January 2016 to August 2019. A total of 3527 cardiac surgeries were performed at the St. Boniface Hospital over this period, and 810 of these cardiac surgeries were VPs and 1612 were CABG surgeries. There were no significant differences in age, sex, or BMI between patient groups. Six (75%) patients from the HVD group, 8 (100%) patients from the HVD+SD group, 6 (85.71%) patients from HVD+T2D group, and 5 (83.33%) patients from the HVD+SD+T2D group underwent an isolated VP. While 2 (25%) patients from the HVD group, 1 (25%) patient from the HVD+SD group, and 1 (16.67%) patient from the HVD+SD+T2D group underwent combined valvular and aortic surgery. All patients with CAD underwent isolated CABG surgery. As expected, LVEF was significantly lower in the patient groups with SD, compared to the patient groups without SD (33.54% ± 8.51% vs. 60.55% ± 6.54%, p = <0.001). T2D diagnosis was confirmed for all patients assigned to the groups with T2D by reviewing each patients' medical record. Pre-surgical HbA_{1c} values for the HVD+T2D, CAD+T2D, HVD+SD+T2D, and CAD+SD+T2D patient groups were 8.17% ± 1.67%, 8.48% ± 1.59%, 8.08% ± 1.58%, and 7.39% ± 0.55%, respectively, further confirming the presence of T2D in these patients. All patients with T2D were prescribed medications for the treatment of diabetes. The characteristics of each patient group are outlined in Table 8.

Table 8. Patient characteristics.

	HVD (*n* = 8)	CAD (*n* = 6)	HVD+SD (*n* = 8)	CAD+SD (*n* = 9)	HVD+T2D (*n* = 7)	CAD+T2D (*n* = 10)	HVD+SD+T2D (*n* = 6)	CAD+SD+T2D (*n* = 7)
Preoperative								
Age (years)	70.50 ± 8.70	69.00 ± 10.97	68.13 ± 6.18	70.56 ± 7.50	67.14 ± 8.19	66.20 ± 5.87	67.83 ± 6.27	67.29 ± 7.02
Sex (female)	4 (50.00%)	0 (0.00%)	1 (12.50%)	0 (0.00%)	2 (28.57%)	2 (20.00%)	0 (0.00%)	1 (12.50%)
BMI (kg/m^2)	31.85 ± 7.28	31.36 ± 4.38	29.07 ± 3.98	30.34 ± 6.89	34.52 ± 4.46	32.16 ± 6.66	30.74 ± 7.13	26.24 ± 4.91
Prescribed Medications (#)	3.63 ± 2.67‡¥↟	4.33 ± 0.82	2.88 ± 2.03‡¥↟	4.63 ± 2.39	8.71 ± 4.23*†	8.30 ± 3.56*†	7.00 ± 2.10	9.14 ± 4.30*†
Hypertension Medications (% yes)	5 (62.50%)	6 (100.00%)	7 (87.50%)	6 (66.67%)	7 (100.00%)	10 (100.00%)	5 (83.33%)	6 (85.71%)
Lipid Medications (% yes)	2 (25.00%)	4 (66.67)	3 (37.50%)	5 (55.56%	6 (85.71%)	9 (90.00%)	2 (33.33%)	6 (85.71%)
Diabetes Medications (% yes)	0 (0.00%)‡¥✢↟	0 (0.00%)‡¥✢↟	0 (0.00%)‡¥✢↟	0 (0.00%)‡¥✢↟	7 (100.00%)*#†§	10 (100.00%)*#†§	6 (100.00%)*#†§	7 (100.00%)*#†§
LVEF (%)	61.38 ± 7.69†§✢↟	58.33 ± 4.55†§✢↟	35.00 ± 11.77*#†§	35.33 ± 7.71*#†§	58.29 ± 4.27†§✢↟	62.80 ± 7.71†§✢↟	34.33 ± 5.72*#†§	30.57 ± 8.34*#†§
Hypertension (% yes)	3 (37.50%)	6 (100.00%)	6 (75.00%)	9 (100.00%)	7 (100.00%)	9 (90.00%)	5 (83.33%)	6 (85.71%)
Dyslipidemia (% yes)	2 (25.00%)#¥	6 (100.00%)*	3 (37.50%)	6 (66.67%)	5 (71.43%)	9 (90.00%)*	4 (66.67%)	6 (85.71%)
HbA_{1c} (%)	-	-	-	-	8.17 ± 1.67	8.48 ± 1.59	8.08 ± 1.58	7.39 ± 0.55
Atrial Fibrillation (% yes)	1 (12.50%)	1 (16.67%)	4 (50.00%)	1 (11.11%)	2 (28.57%)	0 (0.00%)	3 (50.00%)	2 (28.57%)
PVD (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (10.00%)	1 (16.67%)	0 (0.00%)
CHF (% yes)	1 (12.50%)	0 (0.00%)	1 (12.50%)	0 (0.00%)	1 (14.29%)	1 (10.00%)	2 (33.33%)	3 (42.86%)
COPD (% yes)	0 (0.00%)	0 (0.00%)	1 (12.50%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Cardiogenic Shock (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Previous MI (% yes)	0 (0.00%)	1 (16.67%)	3 (37.50%)	3 (33.33%)	1 (14.29%)	2 (20.00%)	0 (0.00%)	3 (42.86%)
Previous CVA (% yes)	1 (12.50%)	0 (0.00%)	1 (12.50%)	1 (11.11%)	3 (42.86%)	1 (10.00%)	1 (16.67%)	4 (57.14%)
Previous TIA (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (12.50%)
Previous PCI (% yes)	3 (37.50%)	1 (16.67%)↟	1 (12.50%)↟	2 (22.22%)↟	2 (28.57%)	6 (60.00%)	2 (33.33%)	7 (100.00%)#†§
Previous Heart Surgery (% yes)	1 (12.50%)	0 (0.00%)	2 (25.00%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Renal Insufficiency (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	1 (16.67%)	0 (0.00%)
Renal Failure (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	1 (10.00%)	0 (0.00%)	0 (0.00%)
Urgent Operative Status (% yes)	0 (0.00%)	1 (16.67%)	2 (25.00%)	3 (33.33%)	2 (28.57%)	1 (10.00%)	0 (0.00%)	1 (12.50%)
Emergent Operative Status (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
EuroSCORE II (%)	2.19 ± 1.31	1.55 ± 1.76	2.40 ± 1.45	2.83 ± 1.99	2.28 ± 2.00	1.73 ± 1.73	8.42 ± 14.44	2.33 ± 0.70

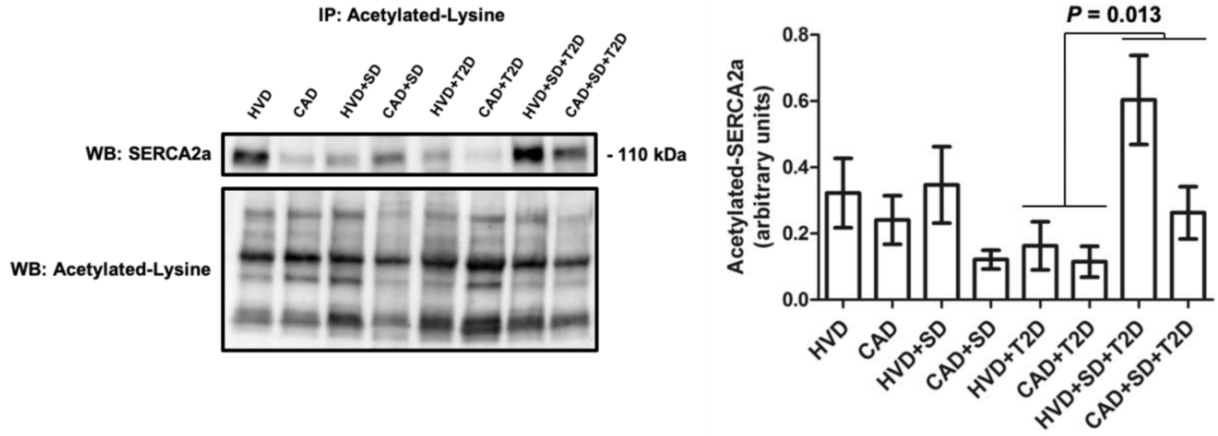
NYHA Class								
I (% yes)	1 (12.50%)	1 (16.67%)	1 (12.50%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (12.50%)
II (% yes)	2 (25.00%)	1 (16.67%)	0 (0.00%)	3 (33.33%)	2 (28.57%)	0 (0.00%)	0 (0.00%)	1 (12.50%)
III (% yes)	5 (62.50%)	1 (16.67%)	4 (50.00%)	3 (33.33%)	2 (28.57%)	1 (10.00%)	3 (50.00%)	1 (12.50%)
IV (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (28.57%)	0 (0.00%)	1 (16.67%)	0 (0.00%)
Unclassified (% yes)	0 (0.00%)	3 (50.00%)	3 (37.50%)	3 (33.33%)	1 (14.29%)	9 (90.00%)	2 (33.33%)	3 (42.86%)
CCSA Grade								
I (% yes)	0 (0.00%)	0 (0.00%)	1 (12.50%)	1 (11.11%)	0 (0.00%)	1 (10.00%)	1 (16.67%)	0 (0.00%)
II (% yes)	0 (0.00%)	2 (33.33%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	3 (30.00%)	1 (16.67%)	1 (12.50%)
III (% yes)	1 (12.50%)	1 (16.67%)	1 (12.50%)	1 (11.11%)	0 (0.00%)	4 (40.00%)	1 (16.67%)	1 (12.50%)
IV (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	1 (10.00%)	0 (0.00%)	1 (12.50%)
Unclassified (% yes)	7 (87.50%)	3 (50.00%)	6 (75.00%)	5 (55.56%	7 (100.00%)	1 (10.00%)	3 (50.00%)	4 (57.14%)
Operative								
Isolated CABG (% yes)	0 (0.00%)	6 (100.00%)	0 (0.00%)	9 (100.00%)	0 (0.00%)	10 (100.00%)	0 (0.00%)	7 (100.00%)
Isolated VP (% yes)	6 (75.00%)	0 (0.00%)	8 (100.00%)	0 (0.00%)	6 (85.71%)	0 (0.00%)	5 (83.33%)	0 (0.00%)
CABG + Aortic Surgery (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
VP + Aortic Surgery (% yes)	2 (25.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (14.29%)	0 (0.00%)	1 (16.67%)	0 (0.00%)
Postoperative								
Returned to OR (% yes)	1 (12.50%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (28.57%)
Intubation Time (hours)	3.30 ± 2.43	3.69 ± 1.72	6.93 ± 8.34	37.79 ± 69.06	8.73 ± 12.64	5.50 ± 1.36	69.13 ± 123.83	14.16 ± 17.92
Re-intubated (% yes)	1 (12.50%)	0 (0.00%)	1 (12.50%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	1 (16.67%)	0 (0.00%)
ICU LoS (hours)	1.51 ± 1.59	1.55 ± 0.86	6.02 ± 10.70	3.58 ± 3.95	6.84 ± 13.09	1.17 ± 3.65	4.49 ± 7.01	3.58 ± 3.11
Hospital LoS (days)	7.75 ± 2.25	8.50 ± 2.88	12.75 ± 7.85	13.44 ± 10.37	14.43 ± 21.08	9.10 ± 2.73	15.33 ± 15.71	13.71 ± 8.02
Delirium (% yes)	2 (25.00%)	0 (0.00%)	1 (12.50%)	1 (11.11%)	1 (14.29%)	0 (0.00%)	0 (0.00%)	1 (12.50%)
In-Hospital Mortality (% yes)	1 (12.50%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
30 Day Readmission (% yes)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (11.11%)	2 (28.57%)	2 (20.00%)	1 (16.67%)	0 (0.00%)

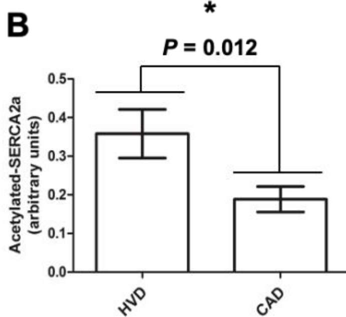
***BMI,** body mass index; **CABG,** coronary artery bypass grafting; **CAD,** coronary atery disease; **CCS,** Canadian Cardiovascular Society; **CHF,** congestive heart failure; **COPD,** chronic obstructive pulmonary disease; **CVA,** cerebrovascular accident; **EuroSCORE,** European system for cardiac operative risk evaluation, **HbA_{1c},** hemoglobin $A1_c$; **HVD,** heart valve disease; **ICU,** intensive care unit; **LoS,** length of stay; **LVEF,** left ventricular ejection fraction; **MI,** myocardial infarction; **NYHA,** New York Heart Association; **OR,** operating room; **PCI,** percutaneous coronary intervention; **PVD,** peripheral vascular disease; **SD,** systolic dysfunction; **TIA,** transient ischemic attack; **T2D,** type 2 diabetes; **VP,** valve procedure. * indicates significantly different from Group 1. # indicates significantly different from Group 2. † indicates significantly different from Group 3. § indicates significantly from Group 4. ‡ indicates significantly different from Group 5. ¥ indicates significantly different from Group 6. ✢ indicates significantly different from Group 7. ⇞ indicates significantly different from Group 8. Continuous variables expressed as mean ± standard deviation and compared using a one-way ANOVA.*

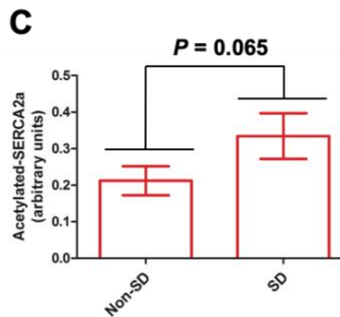
Right Atrial SERCA2a Acetylation in the Diseased Human Heart

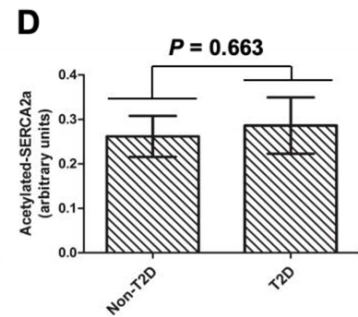
It is important to state that a control sample of RA tissue from a healthy human heart was not included in the co-immunoprecipitation experiments for the detection of acetylated-SERCA2a because all of the samples were obtained from patients undergoing cardiac surgery. Thus, comparison to a healthy control condition is not possible. RA SERCA2a acetylation was detected in samples from all patient groups. RA SERCA2a acetylation was altered by heart disease type, as there was a 53% difference in RA SERCA2a acetylation between patients with HVD or CAD ($p = 0.012$) (**Figure 2B**). The presence of SD and T2D did not alter RA SERCA2a acetylation alone (**Figure 2C**; **Figure 2D**). While an interaction between the presence of SD and T2D status was identified, as SERCA2a acetylation was increased by 3-fold in in the RA of patients with combined SD and T2D, compared to patients with T2D alone ($p = 0.013$) (**Figure 2A**).

Figure 2. Right atrial SERCA2a Acetylation.







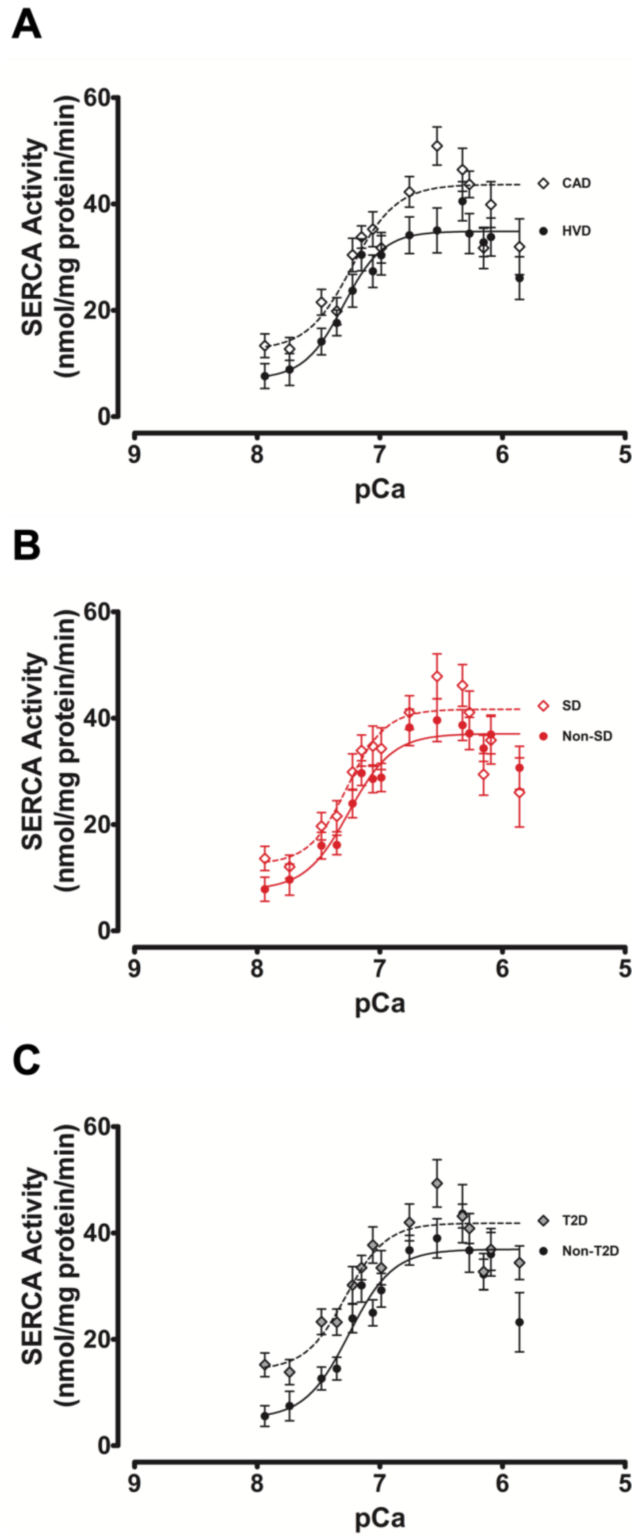


SERCA2a acetylation in the RA of patients with HVD; CAD; HVD and SD; CAD and SD; HVD and T2D; CAD and T2D; HVD, SD, and T2D; and CAD, SD, and T2D. ***A****) Representative Western blot and graphical representation of RA acetylated-SERCA2a following immunoprecipitation of acetylated-lysines and detection of SERCA2a by Western blotting, indicating any interaction effects (n = 4 – 5 per group).* ***B)*** *Graphical representation of the main effect of heart disease type on RA SERCA2a acetylation (HVD: n = 17; CAD; n = 17).* ***C)*** *Graphical representation of the main effect of SD on RA SERCA2a acetylation (Non-SD: n = 17; SD: n = 17).* ***D)*** *Graphical representation of the main effect of T2D on RA SERCA2a acetylation (Non-T2D: n = 18; T2D: n = 16). * indicates significant difference (p = <0.05). Graphs are presented as the mean ± standard error. Compared using a three-way ANOVA and a Tukey post hoc test.*

Right Atrial SERCA Function in the Diseased Human Heart

The Ca^{2+}-dependent kinetic properties of SERCA were not measured in RA tissue from a healthy human heart because all samples were obtained from patients undergoing cardiac surgery. Thus, comparison to a healthy control condition is not possible. RA SERCA activity was assessed over pCa concentrations ranging from 7.94 to 5.86 (**Figure 3**). A 31% difference in maximal RA SERCA activity was identified between patients with HVD or CAD ($p = 0.004$) (**Figure 4B**). SERCA V_{max} was 22% higher in the RA of patients with SD, compared to patients without SD ($p = 0.020$) (**Figure 4C**). While T2D status did not impact RA SERCA V_{max} (**Figure 3D**). Type of heart disease, the presence of SD, and T2D status did not alter RA SERCA Ca_{50}, which is the [Ca^{2+}] eliciting 50% of V_{max} (**Figure 4F**; **Figure 4G**; **Figure 4H**). However, an interaction between heart disease type, presence of SD, and T2D status was identified, as the Ca_{50} of patients with HVD, SD, and T2D was 51% lower, compared to patients with CAD and T2D ($p = 0.003$), indicating a difference in SERCA Ca^{2+} affinity between these groups (**Figure 4F**). The Hill coefficient, which quantifies the slope of relationship between SERCA activity and [Ca^{2+}] for 10 – 90% of V_{max}, was 29% higher in patients with T2D, compared to patients without T2D ($p = 0.021$) (**Figure 4L**).

Figure 3. Right atrial SERCA activity-pCa curves.

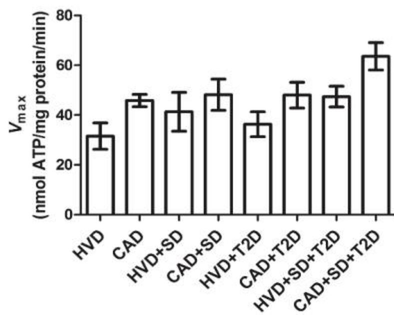


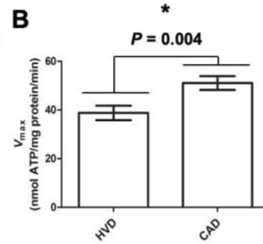
*SERCA activity-pCa curves displaying SERCA ATP hydrolysis over Ca^{2+} concentrations ranging from a pCa of 7.94 to 5.86 from the RA of patients with (**A**) HVD or CAD; (**B**) without SD or with SD; (**C**) without T2D or with T2D.*

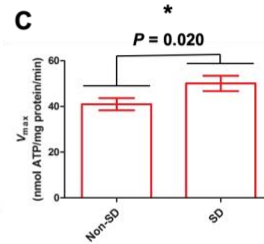
Figure 4. Right atrial SERCA function.

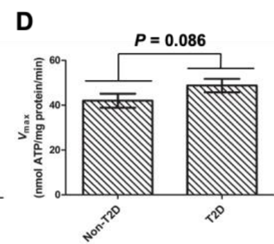
SERCA V_{max}

A



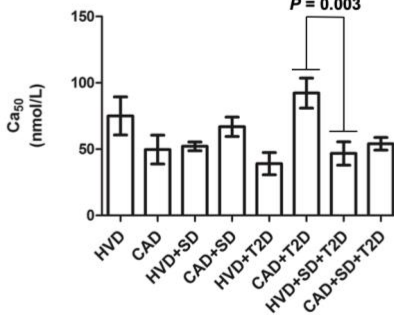


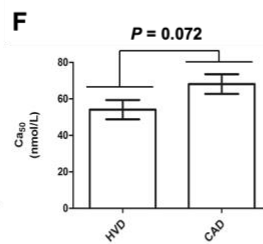


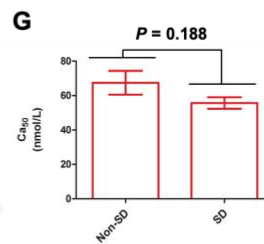


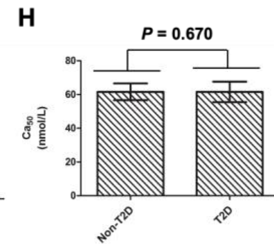
SERCA Ca_{50}

E



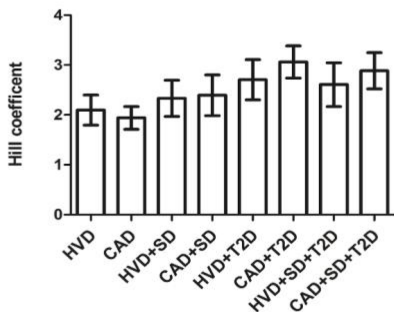


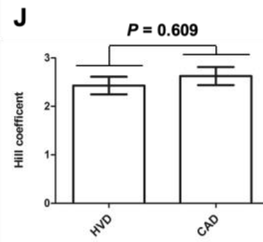


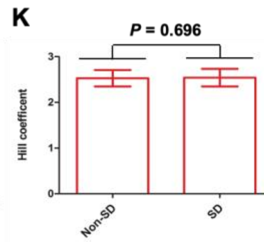


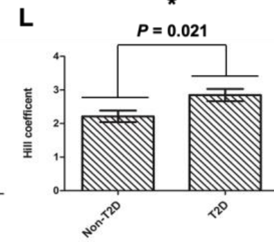
SERCA Hill coefficient

I









Ca^{2+}-dependent kinetic properties of SERCA from the RA from patients with HVD; CAD; HVD and SD; CAD and SD; HVD and T2D; CAD and T2D; HVD, SD, and T2D; and CAD, SD, and T2D. ***A)*** *Graphical representation of V_{max} indicating any interaction effects (n = 6 – 10 per group).* ***B)*** *Graphical representation of the main effect of heart disease type on V_{max} (HVD: n = 27; CAD: n = 32).* ***C)*** *Graphical representation of the main effect of SD on V_{max} (Non-SD: n = 30; SD: n = 29).* ***D)*** *Graphical representation of the main effect of T2D on V_{max} (Non-T2D: n = 29; T2D: n = 30).* ***E)*** *Graphical representation of Ca_{50} indicating any interaction effects (n = 6 – 9 per patient group).* ***F)*** *Graphical representation of the main effect of heart disease type on Ca_{50} (HVD: n = 28; CAD: n = 28).* ***G)*** *Graphical representation of the main effect of SD on Ca_{50} (Non-SD: n = 28; SD: n = 28).* ***H)*** *Graphical representation of the main effect of T2D on Ca_{50} (Non-T2D: n = 28; T2D: n = 28).* ***I)*** *Graphical representation of the Hill coefficient indicating any interaction*

effects (n = 6 – 10 per group). ***J)*** *Graphical representation of the main effect of heart disease type on the Hill coefficient (HVD: n = 27; CAD: n = 32).* ***K)*** *Graphical representation of the main effect of SD on the Hill coefficient (Non-SD: n = 30; SD: n = 29).* ***L)*** *Graphical representation of the main effect of T2D on the Hill coefficient (Non-T2D: n = 29; T2D: n = 30). * indicates significant difference (p = <0.05). Graphs are presented as the mean ± standard error. Compared using a three-way ANOVA and a Tukey post hoc test.*

Relationship between SERCA2a acetylation and SERCA V_{max} in the Right Atrium of the Diseased Human Heart

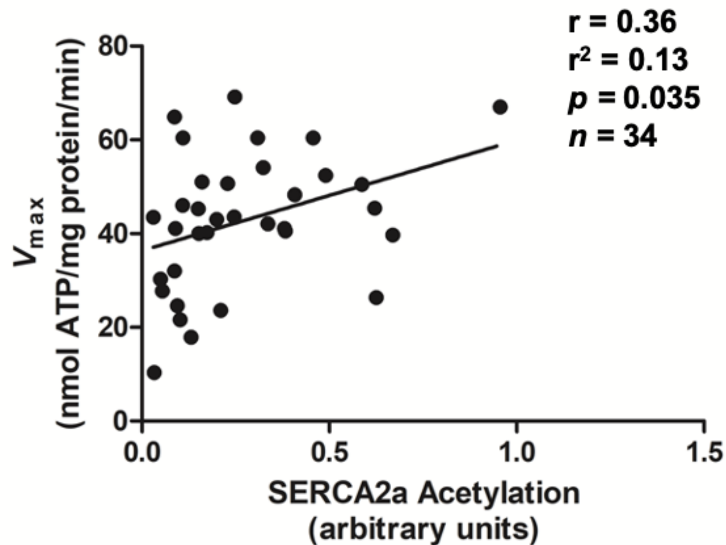
The relationship between SERCA2a acetylation and maximal SERCA activity in RA tissue was examined by unadjusted linear regression analysis, and a positive relationship was found ($r = 0.36$; $r^2 = 0.13$; $p = 0.034$) (**Table 9**; **Figure 5**). We then performed a linear regression analysis between RA SERCA2a acetylation and maximal RA SERCA activity, adjusting for clinical and experimental parameters; however, that approach did not identify any additional significant relationships.

Table 9. Model summary of the unadjusted linear regression analysis between right atrial SERCA2a acetylation and maximal right atrial SERCA activity.

Model Summary

				Change Statistics				
r	r^2	Adjusted r^2	SE of the Estimate	r^2 Change	F Change	df1	df2	Sig. F Change
0.36	0.13	0.11	13.57	0.13	4.87	1	32	0.035

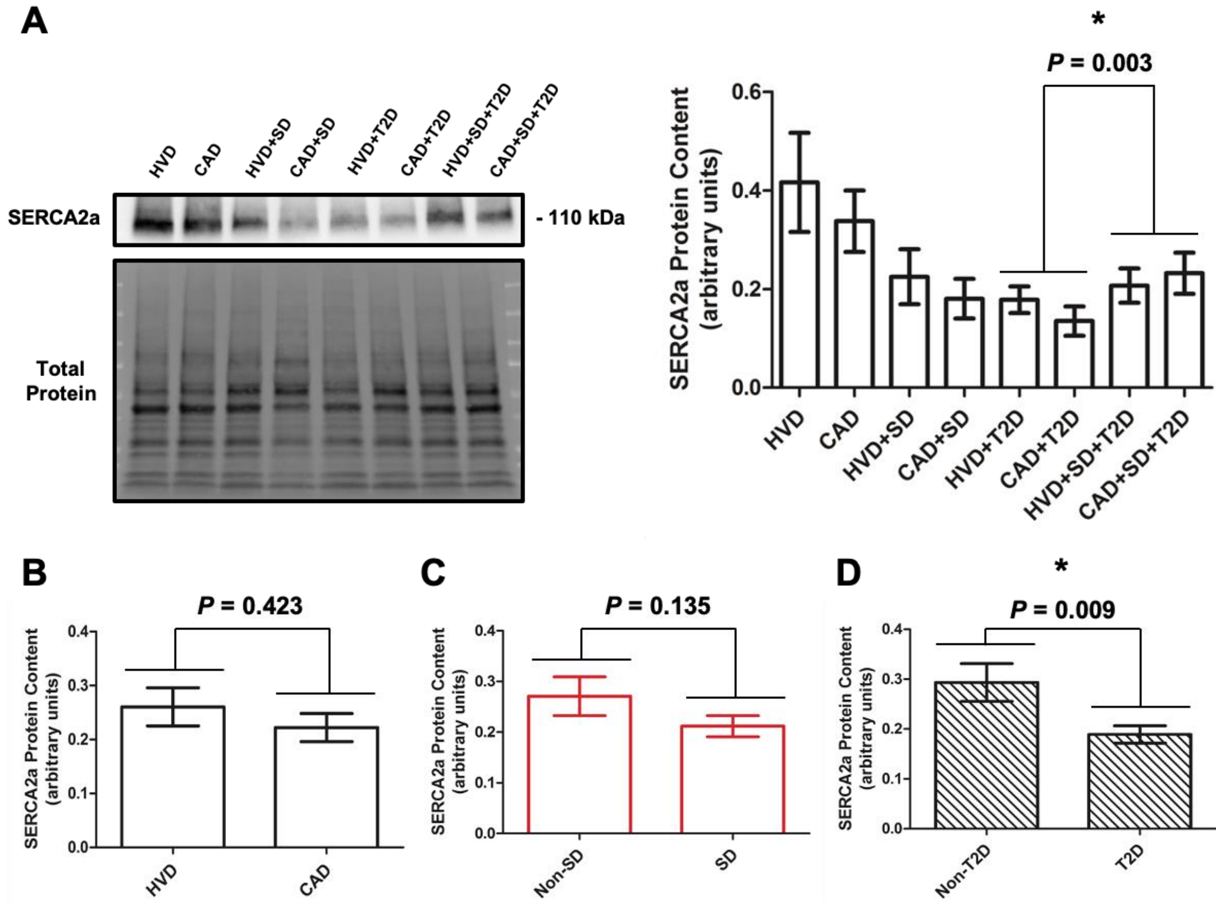
Figure 5. Unadjusted linear regression analysis between right atrial SERCA2a acetylation and maximal right atrial SERCA activity.



Right Atrial SERCA2a Protein Level in the Diseased Human Heart

A control sample of RA tissue from a healthy human heart was not included in the Western blotting experiments because all samples were collected from patients undergoing cardiac surgery. Thus, comparison to a healthy control condition is not possible. The protein level of SERCA2a in RA tissue was unaltered by heart disease type or by the presence of SD (**Figure 6B; Figure 6C**). However, RA SERCA2a protein level was reduced by 36% in patients with T2D, compared to patients without T2D ($p = 0.009$) (**Figure 6D**). An interaction between the presence of SD and T2D status was identified, as SERCA2a protein level was 40% higher in the RA of patients with combined SD and T2D, compared to patients with T2D alone ($p = 0.003$) (**Figure 6A**).

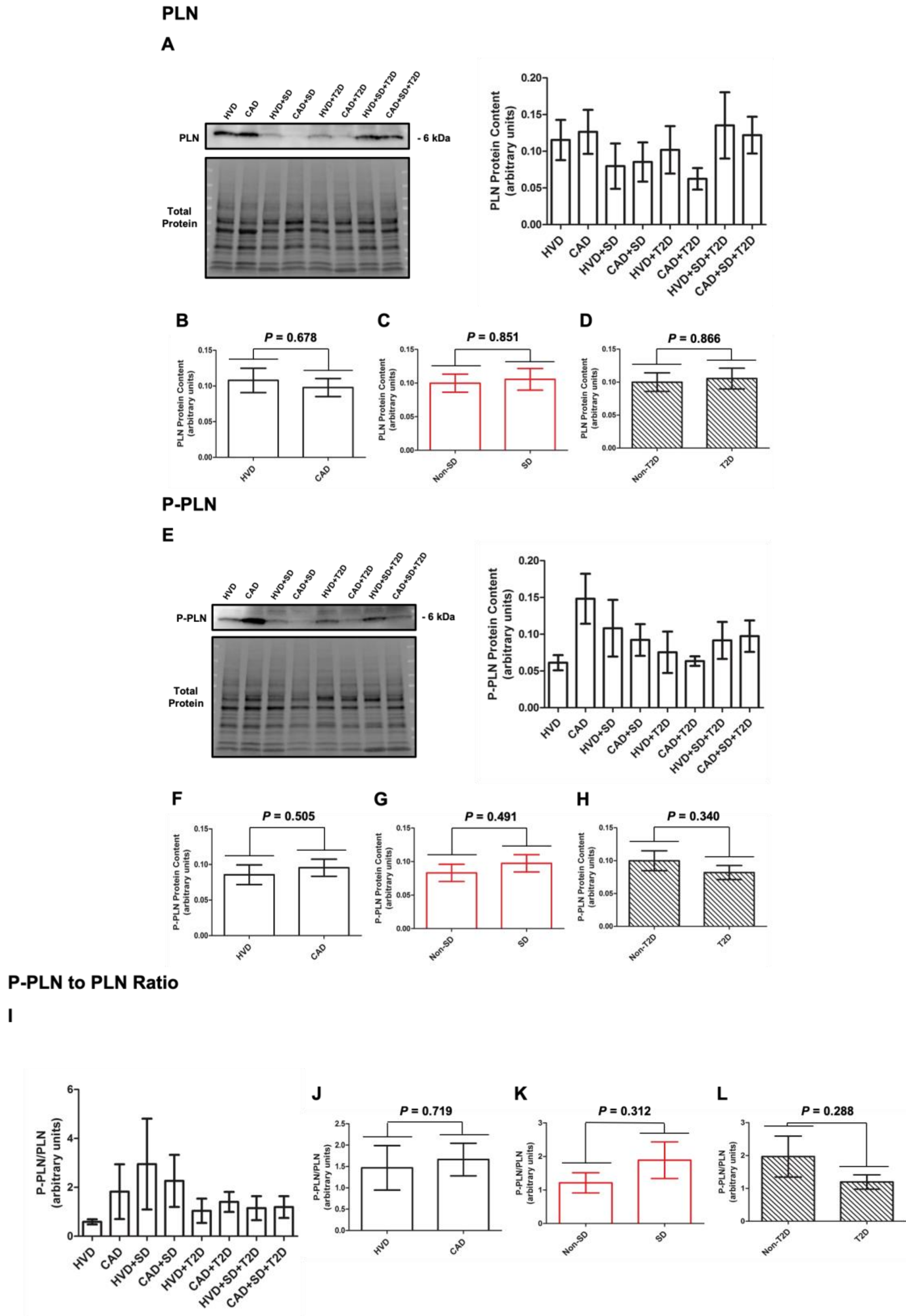
Figure 6. Right atrial SERCA2a protein level.



RA SERCA2a protein level from patients with HVD; CAD; HVD and SD; CAD and SD; HVD and T2D; CAD and T2D; HVD, SD, and T2D; and CAD, SD, and T2D. ***A)*** *Representative Western blot and graphical representation of RA SERCA2a protein level indicating any interaction effects (n = 6 per group).* ***B)*** *Graphical representation of the main effect of heart disease type on RA SERCA2a protein level (n = 24 per group).* ***C)*** *Graphical representation of the main effect of SD on RA SERCA2a protein level (n = 24 per group).* ***C)*** *Graphical Representation of the main effect of T2D status on RA SERCA2a protein level (n = 24 per group). * indicates significant difference (p = <0.05). Graphs are presented as the mean ± standard error. Compared using a three-way ANOVA and a Tukey post hoc test.*

RA PLN and phosphorylated-PLN protein levels and the phosphorylated-PLN to PLN ratio were not altered heart disease type, the presence of SD, or by T2D status, nor were there any interaction effects (**Figure 7**).

Figure 7. Right atrial PLN and phosphorylated-PLN protein level and the phosphorylated-PLN to PLN ratio.



RA PLN and P-PLN protein level and the P-PLN to PLN ratio from patients with HVD; CAD; HVD and SD; CAD and SD; HVD and T2D; CAD and T2D; HVD, SD, and T2D; and CAD, SD, and T2D. ***A)*** *Representative Western blot and graphical representation of RA PLN protein level indicating any interaction effects (n = 5 – 6 per group).* ***B)*** *Graphical representation of the main effect of heart disease type on RA PLN protein level (HVD: n = 23; CAD: n = 23).* ***C)*** *Graphical representation of the main effect of SD on RA PLN protein level (Non-SD: n = 22; SD: n = 24).* ***D)*** *Graphical representation of the main effect of T2D on RA PLN protein level (Non-T2D: n = 22; T2D: n = 24).* ***E)*** *Representative Western blot and graphical representation of RA P-PLN protein level indicating any interaction effects (n = 5 – 6 per group).* ***F)*** *Graphical representation of the main effect of heart disease type on RA P-PLN protein level (HVD: n = 23; CAD: n = 23).* ***G)*** *Graphical representation of the main effect of SD on RA P-PLN protein level (Non-SD: n = 22; SD: n = 24).* ***H)*** *Graphical representation of the main effect of T2D on RA P-PLN protein level (Non-T2D: n = 22; T2D: n = 24).* ***I)*** *Graphical representation of the RA P-PLN to PLN ratio indicating any interaction effects (n = 5 – 6 per group).* ***J)*** *Graphical representation of the main effect of heart disease type on the RA P-PLN to PLN ratio (HVD: n = 23; CAD: n = 23).* ***K)*** *Graphical representation of the main effect of SD on the RA P-PLN to PLN ratio (Non-SD: n = 22; SD: n = 24).* ***L)*** *Graphical representation of the main effect of T2D on the RA P-PLN to PLN ratio (Non-T2D: n = 22; T2D: n = 24). Graphs are presented as the mean ± standard error. Compared using a three-way ANOVA and a Tukey post hoc test.*

Chapter 4: Discussion

We sought to determine if SERCA2a acetylation and SERCA function are altered in the diseased human heart. SERCA2a acetylation was detected in RA tissue from all patient groups. Our study is the first to reveal differences in RA SERCA2a acetylation and SERCA function between patients with HVD or CAD, but we cannot state the direction of these changes because healthy human RA tissue was not included in this study. We have further observed that RA SERCA2a acetylation is altered by SD and T2D in the diseased human heart, as RA SERCA2a acetylation was the highest in patients with combined SD and T2D and increased by 3-fold, compared to patients with T2D alone. RA SERCA function was found to be altered by SD and T2D, since SERCA V_{max} was increased by 22% in patients with SD, compared to patients without SD, and the SERCA Hill coefficient was increased by 29% in patients with T2D, compared to patients without T2D. A positive relationship between SERCA2a acetylation and maximal SERCA activity was found using unadjusted linear regression analysis, and this novel observation suggests acetylation is associated with enhanced SERCA2a function in the RA of the diseased human heart. While SERCA2a acetylation has been previously reported to be increased in the failing human heart, no other group has determined if a relationship exists between SERCA2a acetylation and SERCA2a function in the diseased human heart[127]. RA SERCA2a, PLN and phosphorylated-PLN protein levels, and the phosphorylated-PLN to PLN ratio were examined, and no differences were found due to heart disease type, the presence of SD, or T2D status, indicating the changes to RA SERCA function were not due to SERCA2a protein level or the PLN-mediated regulation of SERCA2a. However, we were unable to determine if confounding factors, like PTM's other than acetylation, may have changed between patient groups and therefore, contributed to the observed changes to RA SERCA function.

Table 10. The main effects of our study by heart disease type, systolic dysfunction, and type 2 diabetes, and their interaction.

Outcome	Key Finding
SERCA2a Acetylation	53% difference (p = **0.012**)
V_{max}	31% difference (p = **0.004**)
Ca_{50}	↑ 22% (p = 0.072)
Hill coefficient	Unchanged
SERCA2a Protein Level	Unchanged
PLN Protein Level	Unchanged
P-PLN Protein Level	Unchanged
P-PLN to PLN Ratio	Unchanged
Outcome	**Key Finding**
SERCA2a Acetylation	↑ 60% (p = 0.065)
V_{max}	↑ 22% (p = **0.020**)
Ca_{50}	Unchanged
Hill coefficient	Unchanged
SERCA2a Protein Level	Unchanged
PLN Protein Level	Unchanged
P-PLN Protein Level	Unchanged
P-PLN to PLN Ratio	Unchanged
Outcome	**Key Finding**
SERCA2a Acetylation	Unchanged
V_{max}	↑ 17% (p = 0.086)
Ca_{50}	Unchanged
Hill coefficient	↑ 29% (p = **0.021**)
SERCA2a Protein Level	↓ 36% (p = **0.009**)
PLN Protein Level	Unchanged
P-PLN Protein Level	Unchanged
P-PLN to PLN Ratio	Unchanged
Outcome	**Key Finding**
SERCA2a Acetylation	↑ 3-fold (p = **0.013**) SD+T2D vs. T2D alone
Ca_{50}	↓ 51% (p = **0.003**) HVD+SD+T2D vs. CAD+T2D
SERCA2a Protein Level	↑ 40% (p = **0.003**) SD+T2D vs. T2D alone

Heart Disease Type and Right Atrial SERCA2a Acetylation and SERCA Function

The first objective of our study was to determine if RA SERCA2a acetylation and SERCA function are altered by heart disease type. Previously, Gorski *et al*. reported acetylation of SERCA2a was increased in failing human LV tissue obtained from patients with HF undergoing LVAD implantation or heart transplantation[127]. However, these LV tissue samples were not categorized by heart disease type; as such, it is unknown if cardiac SERCA2a acetylation is altered between patients with HVD or CAD. We found that RA SERCA2a acetylation is altered by heart disease type, as a difference in SERCA2a acetylation was identified in RA tissue between patients with HVD or CAD. In addition, we found RA SERCA function was altered by heart disease type, since a difference in maximal RA SERCA activity between patients with HVD or CAD was found. However, healthy human RA tissue was not included in this study; therefore, we cannot state the direction of these changes in RA SERCA2a acetylation and SERCA function.

No differences in RA SERCA2a protein level were found between patients with HVD or CAD in our study. This finding is in accordance with the literature, as previous studies have proven inconclusive regarding the effects of HVD or CAD on SERCA2a protein level in the human heart. For example, Leszek *et al*. assessed cardiac SERCA2a protein level in patients with HVD, and reported SERCA2a protein level was unchanged in patients with mitral regurgitation and HF, compared to patients with mitral regurgitation alone[50]. In another study examining SERCA2a protein level in HVD, Zheng *et al*. reported LV SERCA2a protein level was unchanged in patients with severe isolated mitral valve disease, compared to patients with no history of CVD[51]. In terms of the relationship between CAD and SERCA2a protein level in the human heart, Nef *et al*. reported no change in cardiac SERCA2a protein level in patients with

multi-vessel CAD, compared to patients undergoing surgery for atrial septal defects and healthy donor hearts unfit for transplantation[70]. Though a different study reported that LV SERCA2a protein level was higher in patients with severe CAD, compared to patients with idiopathic dilated cardiomyopathy[71]. Thus, our finding of no differences in RA SERCA2a protein level between patients with HVD or CAD is not surprising considering the disparate conclusions of previous studies assessing cardiac SERCA2a protein level in patients with HVD or CAD. As such, the observed changes in maximal RA SERCA activity are not due to differences in SERCA2a protein level between patients with HVD or CAD, suggesting direct modulation of cardiac SERCA2a function, possibly resulting from acetylation.

Systolic Dysfunction and Right Atrial SERCA2a Acetylation and SERCA Function

Next, we determined if RA SERCA2a acetylation and SERCA function are altered by the presence of SD. SERCA2a acetylation has been reported to be increased in failing human LV tissue by Gorski *et al.*, but the LVEF data of this patient population was not provided[127]. Though it is probable these patients had SD since Gorski *et al.* obtained severely diseased cardiac tissue from patients with HF undergoing LVAD implantation or heart transplantation[127]. In our study, no differences in RA SERCA2a acetylation were found between patients with or without SD alone. It is possible our results differ from those of Gorski *et al.* because the cardiac tissue we used to assess SERCA2a acetylation was significantly less diseased, as the patients we obtained tissue from did not require LVAD transplantation or heart transplantation. However, we did identify that SERCA V_{max} in RA tissue was greater in patients with SD, compared to patients without SD. We did not detect a difference in RA SERCA2a protein level in patients with SD, compared to patients without SD. This outcome is in accordance with a previous study which

reported no differences in SERCA2a protein level in RA tissue obtained from HF patients with a mean LVEF of 28 ± 5% undergoing heart transplantation, compared to RA tissue obtained from non-failing hearts[142]. Therefore, the increase in maximal RA SERCA activity found in patients with SD is not the result of increased RA SERCA2a protein level in this patient group; again, suggesting direct modulation of cardiac SERCA2a function, either by acetylation or other mechanisms.

Type 2 Diabetes and Right Atrial SERCA2a Acetylation and SERCA Function

The final objective of our study was to determine if RA SERCA2a acetylation and SERCA function are altered by T2D status. SERCA2a acetylation has not been previously assessed in the diseased hearts of patients with diabetes. Though preliminary research from our group has found that exposing rat primary cardiomyocytes to high-glucose media, like that of diabetes, increases SERCA2a acetylation[136]. No differences in RA SERCA2a acetylation were found due to T2D independently in our study. Although, we did observe that RA SERCA2a acetylation was upregulated by 3-fold in patients with combined SD and T2D, compared to patients with T2D alone. This patient group was likely the most diseased of our study, as they possessed both SD and T2D. The finding of increased RA SERCA2a acetylation in these patients is important because it suggests acetylation of cardiac SERCA2a accompanies increased levels of cardiac dysfunction. A possible explanation for the significant increase in RA SERCA2a acetylation in patients with combined SD and T2D is diminished myocardial sirtuin (SIRT) level and/or activity. SIRTs are HDACs that deacetylate numerous cellular proteins and are reported to be perturbed by diabetes, leading to reduced SIRT level and/or activity in the diabetic heart[143]. There is also evidence that acetylation of cardiac SERCA2a may be regulated by SIRT1[127]. Thus,

SIRT level and/or activity may have been diminished in the RA of patients with SD and T2D, resulting in upregulated SERCA2a acetylation. Additionally, RA SERCA function was found to be altered by T2D status, as the SERCA Hill coefficient was increased in patients with T2D, compared to patients without T2D. The Hill coefficient quantifies the slope of the relationship between SERCA activity and [Ca^{2+}] for 10 – 90 of V_{max}[18]. Thus, the observed change corresponds to a steeper SERCA activity slope and indicates enhanced SERCA enzymatic function in the RA of patients with T2D. This change to the SERCA activity slope is displayed on Figure 3C, which shows RA SERCA activity in patients with and without T2D over pCa concentrations ranging from 7.94 to 5.86.

We cannot state that cardiac SERCA2a is acetylated to a greater extent in patients with DCM since describing the patient group with combined SD and T2D in our study as suffering from DCM is not appropriate. This is because DCM is a distinct form of heart disease that occurs in patients with diabetes and is defined as ventricular dysfunction in the absence of hypertension and other forms of heart disease[72–75]. While these patients had T2D and ventricular dysfunction, they also possessed heart disease in the form of either HVD or CAD, which prohibits describing these patients as having DCM[72–75].

Our study found a reduction in RA SERCA2a protein level in patients with T2D, compared to patients without T2D. This finding conflicts with other studies that have assessed SERCA2a protein level in the RA of patients with T2D. Lamberts *et al*. and Bussey *et al*. reported an increase in RA SERCA2a protein level in cardiac surgery patients with T2D, compared to cardiac surgery patients without T2D [107,108]. However, our findings likely differ from the two previous studies because we obtained RA tissue from patients with or without T2D undergoing a VP or CABG surgery, with or without SD. But in the aforementioned studies,

human cardiac tissue was acquired only from patients with or without T2D undergoing CABG surgery, without SD[107,108]. It is possible that by obtaining RA tissue samples from a greater diversity of cardiac surgery patients, we were able to identify a reduction in RA SERCA2a protein level associated with T2D. Surprisingly, even though SERCA2a protein level is a significant determinant of maximal SERCA activity in the heart, we did not find a corresponding reduction in RA SERCA V_{max} in patients with T2D[144]. Instead, we observed enhanced RA SERCA enzymatic function in patients with T2D, leading us to speculate that cardiac SERCA2a function was directly influenced. We also found that RA SERCA2a protein level was increased in patients with combined SD and T2D, compared to patients with T2D alone. Such an outcome concerning RA SERCA2a protein level in patients with combined SD and T2D has not been previously reported in the literature and is in contrast to the main effect of T2D on RA SERCA2a protein level identified in this study.

Relationship Between Right Atrial SERCA2a Acetylation and SERCA2a Function in the Diseased Human Heart

Acetylation has been linked to cardiac SERCA2a functional changes[124,127,133]. For example, acetylation has been suggested by Gorski *et* al. to impair cardiac SERCA2a function, but the experimental results of their study do not directly support such a conclusion[127]. Contrary to the findings of Gorski *et al.*, Meraviglia *et al*. reported that treatment of cardiomyocytes with a HDAC inhibitor, increased SERCA2a acetylation and enhanced SERCA2a function *in vitro*[133]. Preliminary findings from Dr. Duhamel's research group has also used *in vitro* approaches to manipulate the acetylation status of SERCA2a, resulting in changes to SERCA2a function[134,136]. However, these experiments did not clearly reveal the effect of acetylation on SERCA2a[134,136].

Therefore, the effect of this PTM on cardiac SERCA2a function is not understood. Here, we show for the first-time that a difference in RA SERCA2a acetylation exists between patients with HVD or CAD, and this change in RA SERCA2a acetylation status occurs alongside alterations in RA SERCA function. However, because we were not able to include RA tissue from a healthy human heart as a control, we are unable to draw conclusions regarding the direction of this relationship in patients with HVD or CAD.

Table 11. Summary of published research assessing SERCA2a acetylation and the effect of acetylation on SERCA2a function.

Reference	Model	SERCA2a Acetylation Change	SERCA2a Functional Change
Meraviglia *et al.* 2018.	Treated rat cardiomyocytes with an HDAC inhibitor	↑	↑
Gorski *et al.* 2019.	Failing human and animal LV tissue	↑	Not measured
	Knock down of SIRT1	↑	↓
	Site-specific acetylation of K^{492}	↑	Unchanged

While we did not identify alterations to RA SERCA2a acetylation due to SD or T2D independently, RA SERCA2a acetylation was found to be upregulated in patients with combined SD and T2D, compared to patients with T2D alone. However, there were no corresponding changes to RA SERCA function in patients with combined SD and T2D, compared to patients with T2D alone. Although, alterations to RA SERCA function were found in patients with SD and in patients with T2D independently. Furthermore, we identified an increase in RA SERCA2a protein level in patients with SD and T2D, compared to patients with T2D alone. Thus, the higher level of RA SERCA2a acetylation in patients with SD and T2D may be due to the increased RA SERCA2a protein level in this patient group, allowing for more SERCA2a protein

to be acetylated. Though, this increase in RA SERCA2a acetylation could also be due to the fact that the hearts of these patients with combined SD and T2D exhibited significant pathology.

To further understand the functional influence of acetylation on cardiac SERCA2a, we performed an unadjusted linear regression analysis to examine the relationship between RA SERCA2a acetylation and maximal RA SERCA activity. We found a positive relationship between RA SERCA2a acetylation and RA SERCA V_{max}, identifying that SERCA2a acetylation is associated with enhanced SERCA2a function in the RA of the diseased human heart. Linear regression analysis has not been previously used to examine the relationship between SERCA2a acetylation and SERCA2a function in the diseased human heart. This result should be considered in the context of our study, which exclusively used diseased human RA tissue and lacked a comparison to a healthy control. An even stronger correlation between RA SERCA2a acetylation and maximal RA SERCA activity may have been identified had we included healthy human RA tissue in our analyses.

In the failing human heart, SERCA2a function is reported to be increased in the RA, while SERCA2a function is reportedly diminished in the LV[142]. Furthermore, the RA myocardium exhibits a positive force frequency relationship (FFR) in the failing human heart, whereas a negative FFR is observed in LV myocardium of the failing human heart[142]. Our study supports these findings, as we observed maximal SERCA activity was greater in the RA of patients with SD, compared to patients without SD. We also observed differences in RA SERCA function between patients with HVD or CAD and due to T2D status. Higher SERCA V_{max} allows for faster Ca^{2+} re-uptake from the cytosol back into the SR lumen, leading to increased rates of muscular relaxation and contraction[15]. As well, cardiac SERCA activity is correlated with the FFR in both the RA and LV of the human heart[142]. Together these findings indicate the

activation of a compensatory mechanism to stimulate the contractile function of the RA by enhancing SERCA2a function in the diseased human heart, and this compensatory mechanism may be the acetylation of cardiac SERCA2a.

PLN is located in the SR and it is a critical regulator of cardiac SERCA2a function[11]. When PLN is unphosphorylated, it binds to SERCA2a and reduces its Ca^{2+} affinity, lowering the amount of Ca^{2+} transported by SERCA2a[11]. Phosphorylation of PLN inhibits its interaction with SERCA2a, increasing SERCA2a Ca^{2+} transport[11]. We found RA PLN and phosphorylated-PLN protein levels and the phosphorylated-PLN to PLN ratio were unaltered by heart disease type, the presence of SD, or by T2D status, nor were there any interaction effects. This suggests the changes we found in RA SERCA function due to heart disease type, the presence of SD, and T2D status are the result of mechanisms outside of the PLN-mediated regulation of SERCA2a.

Our findings regarding RA PLN and phosphorylated-PLN protein levels and the phosphorylated-PLN to PLN ratio are similar to those previously reported in the literature. For example, PLN protein level has been reported to be unchanged in patients with severe isolated MR secondary to degenerative to mitral valve disease, compared to patients with no history of CVD[51]. As well, there was no difference in LV PLN protein level between patients with severe CAD or idiopathic dilated cardiomyopathy[71]. The previously discussed studies of Lamberts *et al.* and Bussey *et al.* assessed RA PLN protein level in cardiac surgery patients, with and without T2D, and found RA PLN protein level was lower in patients with T2D, compared to patients without T2D [107,108]. Again, our study obtained RA tissue samples from a more diverse cardiac surgery patient population than the studies of Lamberts *et al.* and Bussey *et al.*; thus, we may have been unable to detect a reduction in RA PLN protein level in patients with T2D[107,108]. It is also possible the human RA tissue samples used in our study may have possessed more variation,

limiting our ability to detect changes to PLN and phosphorylated-PLN protein levels between patient groups.

In the only study which had previously assessed SERCA2a acetylation in the diseased human heart, LV SERCA2a acetylation was increased in failing human LV tissue obtained from patients with HF undergoing LVAD implantation or heart transplantation, compared to healthy human tissue[127]. Additionally, acetylation of cardiac SERCA2a was found to be increased in murine and porcine models of HF[127]. This group proposed acetylation impairs the function of cardiac SERCA2a, but their experimental results do not necessarily show direct modulation of cardiac SERCA2a function by acetylation[127]. Moreover, *in vitro* models have linked cardiac SERCA2a acetylation to enhanced cardiac SERCA2a function[133]. Thus, the effect of acetylation on cardiac SERCA2a function is not understood. Based on the findings of our study, it is difficult to state with certainty if the changes we found in RA SERCA2a acetylation in the diseased human heart are responsible for the observed differences in RA SERCA function. Though, as SERCA2a function is enhanced in the RA of the diseased human heart, and this increased function is not explained by endogenous SERCA2a protein levels or PLN-mediated regulation, it may be possible acetylation of SERCA2a directly modulates this functional change[142]. This is supported by our novel unadjusted linear regression analysis which found a positive relationship between RA SERCA2a acetylation and maximal RA SERCA activity in the diseased human heart.

Alternatively, it is possible that acetylation of cardiac SERCA2a increases with level of cardiac dysfunction, but rather than modulating the function of cardiac SERCA2a directly, it is instead associated with a different cellular process. For example, cross-talk between acetylation and another PTM, SUMOylation, is known to occur and this may be a part of a coordinated

mechanism for the differential regulation of SERCA2a in the diseased human heart[145]. SERCA2a SUMOylation occurs through the actions of the small ubiquitin-like modifier type (SUMO1) protein, which binds to K^{480} and K^{585} residues of SERCA2a and increases its ATPase activity[124]. In fact, SERCA2a SUMOylation has been found to be critical for preserving SERCA2a function and stability in the diseased heart[124]. Evidence suggests SERCA2a acetylation may occur reciprocally with SUMOylation, and since SUMOylation increases SERCA2a ATPase activity, this may explain the positive relationship between SERCA2a acetylation and maximal SERCA activity we observed in the RA of the diseased human heart[124].

Limitations

Comparison to a true control condition was not possible in this study because healthy human RA tissue was not used in any of our analyses. However, there is still merit in examining the clinical relevance of SERCA2a acetylation in the diseased human heart attributed to different pathologies, and to determine if T2D influences cardiac SERCA2a acetylation in these conditions. Next, RA tissue was used in our experiments rather than LV tissue, which may limit the apparent translational capacity of our findings. However, contractile function of the RA and LV are correlated with SERCA function in the healthy and diseased human heart[142]. Further, diabetes severity is correlated with contractile function in all four chambers of the human heart[146]. The decision to use RA tissue in this study was made based on the fact that it was feasible to obtain a larger number of tissue samples from patients undergoing cardiac surgery; whereas, it would have been challenging to obtain LV tissue because it is not routinely sampled at the St. Boniface Hospital, as sampling of LV tissue is deemed to place the patient at undue risk of bleeding. An exception to this is that LV tissue can be secured during LVAD implantation,

wherein a core of LV tissue is removed from the apex of the heart during this procedure. Though the number of patients undergoing LVAD implantation at the St. Boniface Hospital is relatively low (*n* = 6 per year), which limited the feasibility of this approach for our intended purpose. Another alternative approach would have been to obtain LV tissue from patients undergoing heart transplantation surgery and healthy donor hearts unfit for transplantation, as this strategy has been used to assess changes to SERCA2a in the human LV[70,71,127]. However, we did not have access to such tissue because heart transplantation surgery is not currently performed in Manitoba. In the future we could explore collaborations with Canadian heart transplantation centres, such as the Mazankowski Alberta Heart Institute or the University of Ottawa Heart Institute. An additional limitation of this study was that human RA tissue samples were obtained from an actual population of cardiac surgery patients who possessed numerous comorbidities associated with the need to undergo cardiac surgery. This likely resulted in substantial variation between RA tissue samples, potentially limiting our ability to accurately assess experimental outcomes. All patients with T2D in this study were prescribed medications for the treatment of diabetes. Insulin has been reported to stimulate cardiac SERCA2a activity, so it may be possible the changes to RA SERCA function we identified in patients with T2D were influenced by prescribed insulin therapy[93]. A final limitation of this study was that we did not assess sarcolipin (SLN) protein level within the RA tissue, as we could not procure an adequate commercially available SLN antibody. SLN is a 31 amino acid protein found in the SR which is structurally similar to PLN and is capable of regulating SERCA2a function in the human heart[147]. SLN might be particularly important for controlling cardiac SERCA2a function, especially in the human atria[148]. Thus, alterations to RA SLN level may have contributed to the changes we found in RA SERCA function due to heart disease type, presence of SD, and T2D status.

Future Directions

SERCA2a is an important regulator of cardiac performance and increasing its mRNA expression, protein level, and function in the diseased human heart have been studied as potential therapies for heart disease[110,112–114,120]. Acetylation of SERCA2a is upregulated in the diseased human heart, but its effect on cardiac SERCA2a is not completely understood because it has been linked to both impaired and enhanced cardiac SERCA2a function. Therefore, research assessing the direct impact of this PTM on SERCA2a function is necessary to determine its relevance. Future studies utilizing site-directed mutagenesis approaches to manipulate the acetylation status of SERCA2a, in combination with the assessment of SERCA2a enzyme kinetics, ATP binding affinity, and protein structure both *in vitro* and *in vivo* are warranted. Specific efforts should be focused on assessing the impact of acetylation on SERCA2a function in models which mimic the biological complexity of the diseased human heart.

Conclusion

We discovered a positive relationship between SERCA2a acetylation and maximal SERCA activity in the RA of the diseased human heart. Our study also revealed for the first-time differences in RA SERCA2a acetylation and SERCA function based on heart disease type, presence of SD, and T2D status. The changes in RA SERCA function we found occurred without corresponding changes to RA SERCA2a, PLN, and phosphorylated-PLN protein levels, and the phosphorylated-PLN to PLN ratio. These observations suggest acetylation may modulate SERCA2a to enhance its function in the RA of the diseased human heart.

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Appendix A: STROBE Statement Guidelines for Case-Control Studies Checklist

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	i
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	i
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	11-31
Objectives	3	State specific objectives, including any prespecified hypotheses	38
Methods			
Study design	4	Present key elements of study design early in the paper	36
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	36-38, 43
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	36-37
		(*b*) For matched studies, give matching criteria and the number of controls per case	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	38
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	39-42
Bias	9	Describe any efforts to address potential sources of bias	40
Study size	10	Explain how the study size was arrived at	42
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	42
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	42
		(*b*) Describe any methods used to examine subgroups and interactions	42
		(*c*) Explain how missing data were addressed	N/A
		(*d*) If applicable, explain how matching of cases and controls was addressed	N/A
		(*e*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	43
		(b) Give reasons for non-participation at each stage	N/A
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	43
		(b) Indicate number of participants with missing data for each variable of interest	44
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	44

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Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A
		(*b*) Report category boundaries when continuous variables were categorized	N/A
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	42, 52
Discussion			
Key results	18	Summarise key results with reference to study objectives	57-67
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	68-69
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	70
Generalisability	21	Discuss the generalisability (external validity) of the study results	68-70
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	ii

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Appendix B: Informed Consent Package

Research Participant Consent Form – In-Person

Dr. Todd Duhamel
Institute of Cardiovascular Sciences,
St. Boniface General Hospital Albrechtsen Research Centre
R4012 - 351 Tache Ave, Winnipeg, MB, Canada, R2H 2A6.
Phone (204) 235-3589. Email: tduhamel@sbrc.ca

RESEARCH PARTICIPANT CONSENT FORM

Title of Study: Examining SERCA2a acetylation in the diabetic heart

Principal Investigators: Dr. Todd Duhamel
University of Manitoba
Albrechtsen Research center, St. Boniface General Hospital,
R4012 - 351Taché Avenue, Winnipeg, Manitoba, R2H 2A6
Phone: 204-258-1031

Sponsor: Heart and Stroke Foundation

Study Institution: St. Boniface Hospital
Institute of Cardiovascular Sciences
Albrechtsen Research center
351 Taché Avenue, Winnipeg, Manitoba, R2H 2A6

You are being asked to participate in a Clinical Trial (a human research study). You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. Please ask me to explain any words or information that you do not clearly understand.

PURPOSE OF STUDY

This Clinical Trial is being conducted to examine specific proteins found in the human heart. We want to understand how they interact under certain disease conditions. Through this research we hope to learn more about the causes of heart disease and to develop new strategies to improve cardiac function in certain disease states.

STUDY PROCEDURES

In our study, we are looking at certain proteins in the human heart in order to understand how they work under certain conditions. During your cardiac surgery there will be extremely small pieces of heart tissue (<0.5 cm^2) removed and discarded as part of normal cardiac surgical procedures. This information was previously provided to you in your surgical consent document (article 7). This discarded tissue would provide us with valuable tissue that we need for our research. We will collect this cardiac tissue when it becomes available during your surgical procedure. The Cardiac tissue that is collected will be stored until testing can be performed. Your cardiac surgeon is aware of this research study.

Additionally, we will be completing a very brief review of medical information from your hospital records to verify the characteristics of your cardiac condition for the research study. Your involvement in this study will not impact the quality of care you receive during your admission to St. Boniface Hospital for your cardiac surgery. Participation in this research study will only be for this one point in time. There will not be further contact regarding your participation in this research study from our part unless you contact us for further questions you may have about your participation in this study.

RISKS AND DISCOMFORTS

There is no additional risk to participate in this study beyond that which was already explained to you by your hospital surgical staff prior to your consent for your surgical procedure.

BENEFITS

By participating in this study you will be providing clinical researchers the opportunity to gather valuable tissue that will help the study doctors and researchers understand the impact of disease on cardiac function. We hope the information learned from this study will benefit other patients with heart disease by enabling researchers to develop new therapies for heart disease in the future.

COSTS

There will be no cost for participation in this study.

PAYMENT FOR PARTICIPATION

You will receive no payment or reimbursement for any expenses related to taking part in this research study.

CONFIDENTIALITY

Information gathered in this research study may be published or presented in public forums; however, your name and other identifying information will not be used or revealed. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Medical records will only be examined by the study research staff. The University of Manitoba Research Ethics Board and St. Boniface General Hospital may review records related to the study for quality assurance purposes and to ensure safe practices are being followed.

VOLUNTARY PARTICIPATION/WITHDRAWAL FROM THE STUDY

Your decision to take part in this study is voluntary, as is your withdrawal. You may withdraw your participation and information from this study at any time by contacting research study coordinator David Kent @ 204-237-2985.

MEDICAL CARE FOR INJURY RELATED TO THE STUDY

If you should become physically injured as a result of any research activity, you will be provided with any immediate necessary treatment. You are not waiving any of your legal rights by signing this consent form or releasing the investigator(s) from their legal and professional responsibilities.

QUESTIONS

You are free to ask any questions that you may have about this research and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the principal investigator Dr. Todd Duhamel @ 204-235-3589 or study coordinator David Kent @ 204-237-2985. For questions about your rights as a research participant, you may contact The University of Manitoba, Bannatyne Campus Research Ethics Board Office at (204) 789-3389.

STATEMENT OF CONSENT

1. I have understood this Information and Consent provided, and freely and voluntarily agree to take part in this research study.
2. I have received an explanation of the purpose and duration of the study, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to research personnel. My questions were answered to my satisfaction.
3. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.
4. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.
5. By giving consent, I am aware that none of my legal rights are being waived.

Name of Participant: ___________________________ Date: ____________________
(day/month/year)

Participant Signature: _________________________

"Thank you very much for your participation in our research study"

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name: ___________________________ Date:____________________
(day/month/year)

Signature: _________________________ Role in the study: _______________________

Research Participant Consent Form – Telephone

Dr. Todd Duhamel
Institute of Cardiovascular Sciences,
St. Boniface General Hospital Albrechtsen Research Centre
R4012 - 351 Tache Ave, Winnipeg, MB, Canada, R2H 2A6.
Phone (204) 235-3589. Email: tduhamel@sbrc.ca

RESEARCH PARTICIPANT TELEPHONE CONSENT FORM

Title of Study: Examining SERCA2a acetylation in the diabetic heart

Principal Investigators: Dr. Todd Duhamel
University of Manitoba
Albrechtsen Research center, St. Boniface General Hospital,
R4012 - 351Taché Avenue, Winnipeg, Manitoba, R2H 2A6
Phone: 204-258-1031

Sponsor: Heart and Stroke Foundation

Study Institution: St. Boniface Hospital
Institute of Cardiovascular Sciences
Albrechtsen Research center
351 Taché Avenue, Winnipeg, Manitoba, R2H 2A6

"Hello Mr./Mrs./Ms.________________, my name is ________________. I am a research coordinator with the Cardiac Sciences Program at St. Boniface Hospital. You have previously consented to be contacted about upcoming research studies in the Cardiac Sciences Program. Based on our information you are scheduled for cardiac surgery in the coming days. As part of our continued research, I was wondering if you had a moment to hear about a new study that may be of interest" ☐ **Yes** ☐ **No**

You are being asked to participate in a Clinical Trial (a human research study). You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. Please ask me to explain any words or information that you do not clearly understand.

PURPOSE OF STUDY

This Clinical Trial is being conducted to examine specific proteins found in the human heart. We want to understand how they interact under certain disease conditions. Through this research we hope to learn more about the causes of heart disease and to develop new strategies to improve cardiac function in certain disease states.

STUDY PROCEDURES

In our study, we are looking at certain proteins in the human heart in order to understand how they work under certain conditions. During your cardiac surgery there will be extremely small pieces of heart tissue (<0.5 cm^2) removed and discarded as part of normal cardiac surgical procedures. This information was previously provided to you in your surgical consent document (article 7). This discarded tissue would provide us with valuable information that we need for our research. We will collect this cardiac tissue when it becomes available during your surgical procedure. The Cardiac tissue that is collected will be stored until testing can be performed. Your cardiac surgeon is aware of this research study.

Additionally, we will be completing a very brief review of medical information from your hospital records to verify the characteristics of your cardiac condition for the research study. Your involvement in this study will not impact the quality of care you receive during your admission to St. Boniface Hospital for your cardiac surgery. Participation in this research study will only be for this one point in time. There will not be further contact regarding your participation in this research study from our part unless you contact us for further questions you may have about your participation in this study.

RISKS AND DISCOMFORTS

There is no additional risk to participate in this study beyond that which was already explained to you by your hospital surgical staff prior to your consent for your surgical procedure.

BENEFITS

By participating in this study you will be providing clinical researchers the opportunity to gather valuable tissue that will help the study doctors and researchers understand the impact of disease on cardiac function. We hope the information learned from this study

will benefit other patients with heart disease by enabling researchers to develop new therapies for heart disease in the future.

COSTS

There will be no cost for participation in this study.

PAYMENT FOR PARTICIPATION

You will receive no payment or reimbursement for any expenses related to taking part in this research study.

CONFIDENTIALITY

Information gathered in this research study may be published or presented in public forums; however, your name and other identifying information will not be used or revealed. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Medical records will only be examined by the study research staff. The University of Manitoba Research Ethics Board and St. Boniface General Hospital may review records related to the study for quality assurance purposes and to ensure safe practices are being followed.

VOLUNTARY PARTICIPATION/WITHDRAWAL FROM THE STUDY

Your decision to take part in this study is voluntary, as is your withdrawal. You may withdraw your participation and information from this study at any time by contacting research study coordinator David Kent @ 204-237-2985. If you choose not to participate in this study your upcoming surgical procedure will not be affected and you will still receive current standard care while you are in the hospital

MEDICAL CARE FOR INJURY RELATED TO THE STUDY

If you should become physically injured as a result of any research activity, you will be provided with any immediate necessary treatment. You are not waiving any of your legal rights by signing this consent form or releasing the investigator(s) from their legal and professional responsibilities.

QUESTIONS

You are free to ask any questions that you may have about this research and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the principal investigator Dr. Todd Duhamel @ 204-235-3589 or study coordinator David Kent @ 204-237-2985. For questions about your rights as a research participant, you may contact The University of Manitoba, Bannatyne Campus Research Ethics Board Office at (204) 789-3389.

STATEMENT OF CONSENT

1. I have understood this Information and Telephone Consent provided, and freely and voluntarily agree to take part in this research study.
2. I have received an explanation of the purpose and duration of the study, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to research personnel. My questions were answered to my satisfaction.
3. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.
4. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.
5. By giving a verbal, telephone consent, I am aware that none of my legal rights are being waived.

If Yes:

Name of Participant: _________________________ Date: ___________________
(day/month/year)

We will provide you a copy of this signed consent form through mail or electronic mail depending on your request. Otherwise we can provide a copy in your hospital chart available to you upon discharge from the hospital."

Address to send consent: ____

"Thank you very much for your participation in our research study"

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name: _________________________ Date:___________________
(day/month/year)

Signature: _______________________ Role in the study: _____________________