

The relationship between Very Long Chain Plasma Ceramides
and Anxiety in Coronary Artery Disease

by

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Abstract

Anxiety is a highly prevalent comorbidity in coronary artery disease (CAD) and confers increased risk of subsequent cardiac events and mortality. However, biological mechanisms of this relationship are not well understood. Ceramides are sphingolipids involved in inflammatory signaling and cell viability in the periphery and nervous system, and are implicated in pathophysiological mechanisms associated with anxiety. This study aimed to investigate relationships between plasma ceramide concentrations and anxiety symptomology as assessed by the Spielberger State-Trait Anxiety Inventory trait subscale (STAI-T) in CAD patients with linear regressions. High performance liquid chromatography coupled electrospray ionization tandem mass spectrometry was used to assay sphingolipid species. Plasma C22:0 ceramide ($\beta=-0.232$, $p=0.018$) concentrations and 8 other species of sphingolipids (SM18:0, SM20:1, C18:0, C20:0, C18:1, DHC22:0, LacC22:0, LacC24:1) were negatively correlated with STAI-T score when controlling for gender, BMI, and CES-D. Findings suggest specific sphingolipids to be potential markers for anxiety severity in CAD.

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List of Abbreviations

Amu	Atomic mass units
aSMase	acid sphingomyelinase
BCL-2	B-Cell Lymphoma 2
BMI	Body mass index
BP	Blood pressure
C1P	Ceramide-1-Phosphate
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CDase	Ceramidase
CerS	Ceramide synthase
CERT	Ceramide transferase
CES-D	Center for epidemiological studies depression scale
CHF	Congestive heart failure
CRP	C-reactive protein
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
DHC	Dihydroceramide
dhS1P	Dihydro Sphingosine-1-Phosphate
ER	Endoplasmic reticulum
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
FFA	Free fatty acids

GAD	Generalized anxiety disorder
GAD-7	Generalized anxiety disorder 7-item scale
HADS	Hospital Anxiety and Depression Scale
HADS-A	Hospital Anxiety and Depression Scale anxiety scale
HADS-D	Hospital Anxiety and Depression Scale depression scale
HDL	High density lipoprotein
HPLC	High performance liquid chromatography coupled
HR	Heart rate
IFN	Interferon
IL	Interleukin
LacC	Lactosylceramide
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MHxC	Monohexosylceramide
MI	Myocardial infarction
MMSE	Mini mental status examination
MoCA	Montreal cognitive assessment
MOMP	Mitochondrial outer membrane permeabilization
nSMase	neutral sphingomyelinase
PCO	Phosphocholine
PTCA	Percutaneous transluminal coronary angioplasty
PTSD	Post traumatic stress disorder
PUFA	Polyunsaturated fatty acid

RA	Rheumatoid arthritis
S1P	Sphingosine-1-Phosphate
SM	Sphingomyelin
SMase	Sphingomyelinase
SPT	Serine palmitoyltransferase
STAI	Spielberger State-Trait Anxiety Inventory
STAI-S	Spielberger State-Trait Anxiety Inventory State Subscale
STAI-T	Spielberger State-Trait Anxiety Inventory Trait Subscale
Th	T-helper
TH	Tyrosine hydroxylase
TIA	Transient ischemic attack
TNF	Tumour necrosis factor
Treg	T-regulatory
VCAM	Vascular cell adhesion molecule
WT	Wild type

1. Introduction

1.1. Statement of Problem

Coronary artery disease (CAD) is a tremendous public health burden and cause of mortality in the developed world (WHO, 2011). Furthermore, it is exacerbated by myriad other comorbidities. In particular, psychiatric comorbidities have gained a good deal of attention with respect to their high rate of comorbidity with CAD and vascular disease, as well as their effect on health outcomes in the CAD population. Anxiety specifically, has been found not only to be highly prevalent in the CAD population (Todaro *et al.*, 2007; Lavie & Milani, 2004), but to also be correlated with the presentation of a number of physiological risk factors (Lavie & Milani, 2004; Martens *et al.*, 2008), and with an increased rate of subsequent cardiac events and death (Frasure-Smith & Lespérance, 2008; Shibeshi *et al.*, 2007). A good deal of research has implicated inflammatory processes – some of the same processes involved in the progression of CAD and vascular disease – in psychiatric illness and in the etiology of anxiety specifically (Pistavos *et al.*, 2006; Zimmerman *et al.*, 2012; O'Donovan *et al.*, 2010; Guo *et al.*, 2012). Not only might this help explain the degree of comorbidity between heart disease and psychiatric illness, it presents novel opportunities to study the pathophysiology of psychiatric symptoms in cardiac patients. However, many of the inflammatory markers that have been predominantly focused on have proven to be markers generalized to a multitude of immune and psychiatric conditions (Swardfager *et al.*, 2010; Weizman *et al.*, 1999, 1996; Spitzer *et al.*, 2010; Hounie *et al.*, 2008), and do not provide insight into the downstream mechanisms by which neuropsychiatric symptoms are manifested.

Sphingolipids are ubiquitous endogenous lipids that have recently been shown to play a role in inflammatory signaling (Wheeler *et al.*, 2009; Barth *et al.*, 2011), in addition to both cardiac illness (Ichi *et al.*, 2006), and neuropsychiatric disease – including depression and anxiety (Gracia-Gracia *et al.*, 2011; Hammad *et al.*, 2013). The diverse array of sphingolipid species – with diverse roles in cellular viability, growth, and apoptosis – combined with the emerging specificity of new lipidomic analyses, provides not only an opportunity to ascertain novel and more specific downstream markers and mediators, but also an opportunity to further elucidate the etiology of neuropsychiatric symptoms in CAD. In particular, 2 very long-chain ceramide species (C22:0 and C24:0) have emerged as potential markers of a number of pathophysiological mechanisms associated with anxiety, and with mood disorders highly comorbid with anxiety (Filippov *et al.*, 2012; Gracia-Gracia *et al.*, 2011; Mielke *et al.*, 2010)

1.2. Purpose of Study and Objective

The aim of the present study is to bridge the independent lines of research on sphingolipid species and their relationship with anxiety, inflammation, and CAD. The study will investigate specific species, namely C22:0 and C24:0, and their peripheral concentrations as potential correlates of anxiety severity in a CAD population. As mentioned, not only may this research contribute to the search for novel biomarkers of anxiety severity, but also help add to the picture of pathophysiological mechanisms and mediators for neuropsychiatric illness – otherwise defined as behavioural and psychiatric conditions of a believed neurobiological basis (Sachdev and Mohan, 2013; Sachdev, 2005).

1.3. Statement of Research Hypotheses and Rationale for Hypotheses

Primary Hypotheses: Log-transformed plasma concentrations of both C22:0 and C24:0 ceramide will correlate positively with trait anxiety as assessed by the Spielberger State-Trait Anxiety Inventory trait subscale (STAI-T) in CAD patients.

Secondary Hypotheses: Using established cutoffs for the STAI-T and the Hospital Anxiety and Depression Scale anxiety subscale (HADS-A), anxious groups will have significantly higher mean log-transformed C22:0 and C24:0 plasma concentrations.

Rationale: Animal models of anxiety have suggested elevated levels of sphingolipids to correlate with or precipitate increased anxiety like behaviour and modifications in catecholamine synthesis (Jang *et al.*, 2011, 2008; Ono *et al.*, 2008). *In vitro* studies have similarly indicated that ceramide synthesis is essential in excitotoxic and neurodegenerative processes closely linked to anxiety in the current understanding of its pathophysiology (Novgorodov *et al.*, 2011). And in clinical samples, elevated concentrations of ceramide were found in more anxious groups whether based on self-report anxiety scale cut-offs (Demirkan *et al.*, 2012), or on clinical diagnosis of a particular anxiety disorder (Hammad *et al.*, 2013). Furthermore very long chain plasma ceramides C22:0 and C24:0 have been previously shown to predict structural and functional changes, right hippocampal atrophy, and memory impairment over 1 year (Mielke *et al.*, 2010) as well as shown elevated concentrations in a depressed population (Gracia-Gracia *et al.*, 2011). In a post mortem study, C24:0 concentrations in the brain were elevated in samples with more widespread evidence of neurodegeneration (Filippov *et al.*, 2012).

Exploratory Analyses and Rationale: Given the widespread diversity in sphingolipid species implicated in neurobiological mechanisms linked to anxiety (Novgorodov *et al.*, 2011; Wheeler *et al.*, 2009), anxiety like behaviour in animal models (Jang *et al.*, 2008; Ono *et al.*, 2008), and in clinical presentation of anxiety (Demirkan *et al.*, 2012; Hammad *et al.*, 2013), this study aims to undertake a similar regression analysis approach as in the primary analysis to the entire panel of sphingolipid species assayed and to look at correlations with a variety of measures of anxiety as well as depression and cognition.

1.4. Review of the Literature

1.4.1. Coronary Artery Disease

CAD is the leading cause of death in the developed world (WHO, 2011). It is the leading cause of mortality in North America and United States (Lloyd-Jones *et al.*, 2009), and is responsible for nearly a third of all deaths in Canada (Statscan, 2010).

CAD is characterized by the narrowing of the blood vessels supplying oxygenated blood to the heart. This narrowing is caused by atherosclerosis, which is comprised of inflammation, calcification, and lipid deposition in the arterial walls. Plaques or atherosclerotic lesions are formed under the vascular endothelium, composed of macrophages, lipid deposits, and a rich glycoprotein and collagen extracellular matrix (Fuster *et al.*, 1992). Plaque progression involves platelet recruitment to the endothelial surface, subsequent platelet secretion of vascular adhesion molecules,

platelet-induced upregulation of leukocyte migration, and differentiation into the plaque, which in turn further secretes inflammatory cytokines (Massberg *et al.*, 2002; Smith *et al.*, 1995). Smooth muscle cells are concurrently recruited and a fibrous cap forms over the plaque. This cascade of events manifests into an acute coronary event, or syndrome, with the erosion of the overlying fibrous cap. This produces a thrombus that occludes the artery as well as exposes the blood to its pro-coagulant and cellular-adhesive molecules, further exacerbating the migration of cells to the region and blockade of the vessel (Hansson, 2005).

The downstream clinical consequences of atherosclerosis and thrombus formation include angina, myocardial infarction – myocardial cell death – and ischemic death (Falk, 1985; Fuster *et al.*, 1990). In order to prevent this eventuality or to treat an existing occlusion, patients at risk of myocardial infarction and ischemic death may undergo revascularization procedures including percutaneous transluminal coronary angioplasty (PTCA, or stenting) or a coronary artery bypass graft (CABG).

1.4.2. Anxiety and CAD

Anxiety is a prevalent comorbidity in CAD. While some conservative estimates found it to be present in approximately a quarter of CAD patients at a given time point (Lavie & Milani, 2004), others have found presentation of at least 1 anxiety disorder in a cardiac population at assessment to be 36%, and lifetime presentation of 45.3% (Todaro *et al.*, 2007). This is significantly higher than the 5.1% estimated lifetime prevalence in the general population (Wittchen *et al.*, 2002) the estimated

8% prevalence in primary care patients (Maier *et al.*, 2000), and even the estimated 28.8% lifetime presentation in an adult population (Kessler *et al.*, 2005).

Prognostically, anxiety is also implicated as a predictor of CAD and its outcomes. Prospective meta-analytic findings showed a pooled hazard ratio of 1.26 for the effect of anxiety on incident CAD, and 1.43 and 1.48 for nonfatal MI and ischemic death, respectively (Roest *et al.*, 2010). Additionally, anxiety is linked to a number of behavioural modifications, such as reduced physical activity, cigarette smoking, poor diet, and excess alcohol consumption (Antonogeorgo *et al.*, 2012) that are key risk factors for the development of CAD (Kubzansky *et al.*, 1998).

Understandably then, anxiety in CAD patients has been found to be predictive of greater risk of subsequent cardiac events. In a sample of 804 patients, anxiety predicted the occurrence a major acute coronary event over 2-year follow up (Frasure-Smith & Lespérance, 2008). Other studies have shown similar predictive relationships of anxiety on subsequent MI as well as mortality (Shibeshi *et al.*, 2007). Furthermore, anxiety has been associated with increased prevalence of cardiac risk factors such as reduced heart rate variability (Martens *et al.*, 2008), increased LDL/HDL ratio, BMI, body fat (Lavie & Milani, 2004), and even reduced adherence in cardiac rehabilitation populations (Mcgrady *et al.*, 2009; Yohannes *et al.*, 2007).

While meeting clinical criteria for an anxiety disorder is suggested to play a role in the prognosis of CAD and its progression, it is important to stress the significance of subclinical presentation of anxiety as well. Individuals with subclinical but chronic anxiety are also suggested to be at increased risk of cardiovascular disease (Kubzansky *et al.*, 1998). Additionally, given the robust findings on the role of depression in CAD - its development, and progression -, the overlap of symptomology, and the comorbidity of anxiety and depression, one must consider that depression may confound the relationship between anxiety and CAD. However, a number of studies still indicate anxiety to be an independent predictor of incident CAD (Janszky *et al.*, 2010; Nabi *et al.*, 2010; Denollet *et al.*, 2009, 2008; Kubzansky *et al.*, 2006).

1.4.3. Shared Pathophysiology: Somatic correlates and Inflammation

Anxiety and stress are frequently measured in tandem, discussed interchangeably, and are conceptualized either as corresponding conditions, or disparate dimensions of personality. However defined, stress and anxiety have long been linked to cardiovascular disease (CVD) and have long been acknowledged to present with a number of somatic symptoms associated with cardiac disease.

As previously mentioned, anxiety is associated with reduced heart rate variability (Kubzansky *et al.*, 1998; Miu *et al.*, 2009). It is also linked to increased sympathetic tone, characterized by symptoms such as increased resting heart rate, baroreflex dysfunction, and variability in ventricular repolarization (Rozanski *et al.*, 2005; Carpeggiani *et al.*, 2005; Fleet *et al.*, 2005). Anxiety for example, has further been

implicated in reduced vagal tone (Miu *et al.*, 2009). Reductions in vagal, parasympathetic innervation, allows a dominant sympathetic tone, which predisposes towards ventricular arrhythmia and cardiac death (Gorman and Sloan, 2000).

1.4.4. Inflammation and CAD

Inflammatory immune activity is a key component in the development and progression of CVD. LDL deposition in the intima of the artery initiates an inflammatory response (Skålén *et al.*, 2002; Leitinger *et al.*, 2003) including the upregulation of inflammatory genes (Dai *et al.*, 2004). Increased expression of cellular adhesion molecules such as VCAM-1 by overlying endothelial cells preferentially recruit monocytes and lymphocytes that express the corresponding receptors (Cybulsky and Gimbrone, 1991). These leukocytes then migrate via endothelial junctions into the intima where the inflamed tissue induces monocyte differentiation to macrophages by way of the macrophage colony-stimulating factor (Smith *et al.*, 1995). Toll-like receptors and other upregulated pattern-recognition receptors then contribute to macrophage LDL uptake, cellular activation, and resulting secretion of inflammatory cytokines (Janeway *et al.*, 2002). Inflammatory markers, such as inflammatory cytokines, have been implicated in CVD development, and are important markers of its progression (Hansson, 2005). Activated T-cells in the plaque secrete Interferon (IFN)- γ which plays a role in macrophage activation and production of inflammatory Tumour Necrosis Factor (TNF)- α and Interleukin (IL)-1 (Szabo *et al.*, 2001; Frostegård *et al.*, 1999; Uyemura *et al.*, 1996). IL-6 and C-Reactive Protein (CRP) are two other downstream

inflammatory cytokines in this inflammatory cascade, are frequently detected at elevated levels in the periphery in atherosclerosis, and can aid in clinical diagnosis (Liuzzo *et al.*, 1994; Biasucci *et al.*, 1996; Lindahl *et al.*, 2000).

1.4.5. Inflammation, Mood, and Anxiety

An ever-growing body of literature has been pointing to the involvement of inflammation in a range of psychiatric disorders. While the bulk of the research has focused on depression, basic and clinical studies have continued to implicate inflammation in a multitude of anxiety disorders and subclinical symptomology – not surprisingly given the ample overlap in symptoms and substantial comorbidity between depression and anxiety.

Support for this neuropsychimmune relationship exists in the form of both epidemiological findings as well as more strictly pathophysiological studies. A Danish nationwide study following up 3.56 million people, for example, indicated that both prior hospital contact due to an autoimmune disease and any hospitalization resulting from infection were associated with a 45% and 62% increased risk for later diagnosis with a mood disorder, respectively (Benros *et al.*, 2013).

With respect to the biological mediators and/or markers of this relationship, a number of studies lend support to the correlation between inflammatory markers and anxiety specifically. As part of the ATTICA study, Pistavos *et al.*, (2006) found anxiety severity, as assessed by the STAI, to be positively correlated with TNF- α , IL-

6, CRP, and fibrinogen. In posttraumatic anxiety, serum IL-1 β was found to be associated with anxiety severity (Zimmerman *et al.*, 2012). In a group comparison between clinically anxious and non-anxious adults, O'Donovan *et al.* (2010) found significantly higher levels of IL-6 in anxious patients, even when controlling for depression. They also found a correlation in the entire sample between severity of anxiety and IL-6 concentration. Patients with a primary PTSD diagnosis were found to have elevated serum concentrations of 6 different cytokines, including TNF- α and IL-6 (Guo *et al.*, 2012). Hoge *et al.*, (2009), found PTSD and panic disorder (PD) to have similarly elevated generalized proinflammatory state when compared to controls in an even larger spectrum of inflammatory cytokines.

Beyond a general inflammatory state, findings suggest a more widespread immune dysregulation. The immune response used to be conceptualized as a balance between the T-helper (Th)-1 and Th2 arms, anti-inflammatory and inflammatory, respectively. However, not only are the roles of the Th cells more complex than this distinction suggests, but Th3, T-regulatory (Treg), and Th-17 subsets have entered the fold and have their own distinct roles in the complex interplay between immune cells. Given this diversity in T cell subsets, Vieira *et al.*, (2010) studied *in vitro* activation of T cells in patients with generalized anxiety disorder (GAD) compared with controls. They found an overall reduction in cytokines, including anti-inflammatory cytokines, produced by all subsets other than the Th-17s in cell cultures from the GAD group. IL-17 and TNF- α concentrations, both secreted by Th17 cells, were found to be significantly higher in cell cultures of the anxious

patients. In a clinical sample of patients with rheumatoid arthritis (RA), IL-17 was similarly found to be significantly higher in anxious patients and correlated with severity of anxiety (Liu *et al.*, 2012).

Whether inflammation is an effector mechanism or an epiphenomenon in anxiety is of debate. While the relationship is likely to be more complex and bidirectional than currently disputed, there are a number of findings suggesting inflammation to precipitate anxiety. Haji *et al.* (2012) showed TNF- α administration in mice to induce anxious behaviour. Furthermore, they also showed etanercept, a TNF- α antagonist, to reduce anxious behaviour. Anti-inflammatory COX inhibitors have similarly been shown to reduce anxiety in mouse models (Kumar *et al.*, 2010). In humans, immune challenge via lipopolysaccharide (LPS) injection has been found to elicit anxiety symptoms (Reichenberg *et al.*, 2001). Clinically, TNF- α antagonist infliximab has also been shown to reduce anxiety in patients with ankylosing spondylitis (Ertenli *et al.*, 2010).

This relationship between inflammation and anxiety has also been supported by studies in the population of interest. In cardiac patients, studies have shown a correlation between anxiety severity and CRP concentrations in GAD (Bankier *et al.*, 2008), anxiety disorders not otherwise specified (Bankier *et al.*, 2009), and PTSD patients (von Känel *et al.*, 2010).

This emerging body of knowledge on inflammation and anxiety – in addition to numerous other psychiatric conditions – is further supporting the case for general immune involvement in brain based disease, and for the lack of specificity of many of the inflammatory markers that have been studied to any one given psychiatric disorder.

1.4.6. Lipidomics and sphingolipids: structure, synthesis, apoptosis, and inflammatory signaling

High throughput lipidomic analysis has been one of the novel and exciting approaches to better understanding the rich diversity in both form and function of lipid species. Given the role many lipid species play in immune related signaling cascades, these approaches have the potential to uncover more specific markers and mediators in neuropsychimmunology, and in psychiatric disease.

1.4.6.1. Sphingolipid structure and synthesis

Sphingolipids are a class of highly diverse and complex polyunsaturated fatty acids (PUFAs) that play a role as inflammatory mediators and signal transducers as well as mediators of lipid membrane conformation. Ceramides in particular, are sphingolipids composed of sphingosine, an 18-carbon amino alcohol and an amide linked fatty acid chain of variable lengths (Gault *et al.*, 2010). There are three prominent pathways of synthesis: the *de novo* pathway involves the acylation of sphingosine; the sphingomyelinase (SMase) pathway involves the hydrolysis of sphingomyelin (SM) into its two constituent components, ceramide and phosphocholine (PCO) (Gault *et al.*, 2010); and the salvage pathway involves the

catabolism of more complex sphingolipids followed by the reacylation of sphingosine (Mullen *et al.*, 2011; Kitatani *et al.*, 2008).

SM can be found on the outer leaflet of the plasma membrane, in intracellular organelles (Barenholz *et al.*, 1980; Kolesnick and Golde, 1994; Hannun *et al.*, 1994), and constitute 25% of the phospholipid content of LDL in circulating human blood (Chapman, 1986). It is metabolized by acid SMase (aSMase) in the lysosomal compartment and in plasma lipoproteins, secretory aSMase in the extracellular space (Schissel *et al.*, 1998; Schissel *et al.*, 1996), or by neutral SMase (nSMase), which is predominantly bound to the golgi or plasma membrane (Marchesini *et al.*, 2004). *de novo* synthesis is largely confined to the intracellular space and is initiated by the rate limiting enzyme serine palmitoyltransferase (SPT) which is involved in the synthesis of ceramide in the endoplasmic reticulum (ER) (Gault 2010; Mandon *et al.*, 1992). Ceramide can then be transported to the golgi apparatus via vesicular transport or ceramide transferase (CERT) protein (Hanada *et al.*, 2003). At this point it can be further conjugated to form glucosylceramide or galactosylceramide - glycosphingolipids that serve as precursors to more complex groups such as monohexosylceramide and lactosylceramide (Tettamanti, 2004)– or have a phosphate or PCO attached to form ceramide-1-phosphate (C1P) or SM respectively (Gault *et al.*, 2010; Chalfant and Spiegel, 2005).

Amongst the other bioactive sphingolipid products is sphingosine-1-phosphate (S1P), which is produced through the enzymatic attachment of a phosphate to a

sphingosine base by sphingosine kinase, and is involved in intracellular signaling and calcium loading (Taha *et al.*, 2006; Baumruker *et al.*, 2005; Olivera and Spiegel, 2001).

Ceramide synthases (CerS) are key enzymatic mediators of both the *de novo* and salvage pathway by catalyzing the acylation of the sphingosine base (Mullen *et al.*, 2012). There are 6 different CerS enzymes that have been identified (CerS1-6), each of which is involved in the synthesis of particular acyl-chain length ceramides (Mullen *et al.*, 2012; Laviad *et al.*, 2008). CerS2 for example is responsible for the synthesis of very long chain ceramides C20-C26 through the acylation of sphingosine (Mullen *et al.*, 2012). Alternatively, CerS1 and CerS5 are responsible for the bulk of C18 and C16 ceramide, respectively (Venkataraman *et al.*, 2002; Riebeling *et al.*, 2003; Laviad *et al.*, 2008). CerS2, which synthesizes this study's two primary species of interest in the scope of this study, saturated C22 and C24 (C22:0 and C24:0), has significantly elevated expression of mRNA as compared to other CerS's (Laviad *et al.*, 2008), and has the broadest tissue distribution (Riebeling *et al.*, 2003). Furthermore, S1P can act to inhibit CerS2 via an S1P receptor-like motif found on CerS2, an indication of the signaling function of sphingolipid species (Laviad *et al.*, 2008).

1.4.6.2. Apoptosis and mitochondrial dysfunction

Studies suggest ceramides play a significant role in apoptotic mechanisms and mitochondrial dysfunction. Furthermore, increased neuronal apoptosis and

associated mediators have been found in animal models of PTSD, upregulated pro-apoptotic genes have been detected in pharmacologically induced anxiety in mice, and anxiolytic medications have also been shown to reduce apoptosis and resultant neurodegeneration (Li *et al.*, 2013; Kajiyama *et al.*, 2010; Inta *et al.* 2012; Réus *et al.*, 2012).

The B-Cell Lymphoma 2 (BCL-2) proteins contribute to cellular integrity. While some are anti-apoptotic, others are key mediators of apoptosis via caspase activation and mitochondrial outer membrane permeabilization (MOMP), their activity also being associated with ceramide generation and metabolism (Siskind *et al.*, 2010). Cytochrome c release from the mitochondria is a key step in the apoptotic cascade, and BCL-2 is a potent inhibitor of its release (Ruvolo *et al.*, 1998, 1999). Ceramides have been shown to activate protein phosphatase 2 (Dobrowsky *et al.*, 1993, Wolff *et al.*, 1994), a ceramide activated protein phosphatase, which in turn dephosphorylates BCL-2 (Ruvolo *et al.*, 1998).

Examples of this relationship include findings showing long chain ceramide generation to be integral for cell death, which in turn is dependent on the presence of BCL-2 antagonist/killer (BAK) in a cell culture study (Siskind *et al.*, 2010).

However, in the same study ceramide synthesis was independent of the downstream MOMP and caspase activation due to the BCL-2 pro-apoptotic proteins. Furthermore, ceramides have also been directly linked to MOMP induction via the formation of ceramide channels *in vitro* in the absence of BCL-2 pro-apoptotic

proteins (Siskind *et al.*, 2004). Ceramides are also reactive oxygen species-dependent activators of the c-Jun N-terminal kinase pathway, which is involved in mitochondrial caspase release, generating additional cytotoxic reactive oxygen species, and eventually apoptosis (Qin *et al.*, 2009; Hartfield *et al.*, 1998; Goswami and Dawson, 2000). Mitochondrial dysfunction, BCL-2 gene mutations, and reductions in mitochondrial anti-apoptotic BCL-2 levels have been associated with anxiety like behaviour in mice (Einat *et al.*, 2005). These lines of evidence support the potential link between anxiety and ceramides due to the role of ceramides in this biological mechanism implicated in anxiety.

1.4.6.3. Inflammatory signaling

Sphingolipid species, and ceramides in particular, have been suggested to play an integral mediating role in inflammatory signaling. They have been implicated as downstream effectors of the apoptotic cascade initiated by inflammatory cytokines. For example, TNF- α treatment of primary hepatocytes was found to both induce an elevation in ceramide concentration and to induce apoptosis (Osawa *et al.*, 2005). TNF- α has also been found to induce sphingosine kinase activity and the production of S1P (Xia *et al.*, 1999). Similarly, in hippocampal slices, TNF- α was found to increase nSMase2 activity and ceramide production, while ceramide production was reduced by pretreatment with an nSMase2 inhibitor and not with an inhibitor of SPT (Wheeler *et al.*, 2009). Additionally, SMase was found to mediate TNF- α induced oxidative stress in neuroblastoma cells, as inhibition of SMase reduced subsequent oxidative stress (Barth *et al.*, 2011). These findings are just a few of those

implicating ceramides in inflammatory signaling and the regulation of their synthesis being closely tied to inflammatory cytokines. Furthermore, they may suggest the importance of the SM pathway over the salvage pathway or the *de novo* pathway for ceramide synthesis in brain tissue.

1.4.6.4. Sphingolipids and CAD

Disparate lines of research suggest that sphingolipid metabolism may play a role in both the development of CAD and in mood and anxiety disorders. Ceramides are suggested to play a role in atherogenesis, with findings indicating higher concentrations in low-density lipoprotein (LDL) of atherosclerotic plaques (Schissel *et al.*, 1996). Higher plasma concentrations of very long chain ceramides, C22:0 and C24:0, were also found to be associated with higher LDL concentration and other cardiac risk factors such as increased systolic blood pressure (BP), total cholesterol, triglycerides, and free fatty acids (FFA) (Ichi *et al.*, 2006). Additionally, SMase activity was found to be upregulated in cardiac failure patients (Dohner *et al.*, 2007).

SMases in the arterial walls can produce ceramides (Schissel *et al.*, 1996) that promote oxidation and aggregation of LDL (Schissel *et al.*, 1996; Xu and Tabas, 1991). This process supports foam cell formation by stimulating macrophage chemotaxis and LDL uptake (Yla-Herttuala *et al.*, 1989), a part of the cascade of inflammatory events previously discussed to be integral in the development and progression of CAD.

1.4.6.5. Sphingolipids and the brain

While research into sphingolipids' contributions to neurological and psychiatric disorders has been largely confined to cognitive impairment, dementia, and depression (Mielke *et al.*, 2010_{a,b,c}, 2011; Gulbins *et al.*, 2013; Gracia-Gracia *et al.*, 2011; Kornhuber *et al.*, 2005), there are indications that they may play a role in anxiety, understandably considering the high comorbidity of anxiety and depression. In an animal model of anxiety, early weaning of mice was associated with both the development of anxious behaviours and increased galactosylceramide concentrations in the basolateral amygdala, one of the chief areas implicated in anxiety disorders (Ono *et al.*, 2008). In a clinical sample, PTSD patients were found to have concurrently higher levels of pro-inflammatory cytokines, C18:0 ceramide, S1P, dhS1P, and aSMase activity (Hammad *et al.*, 2013). In a large lipidomic analysis conducted in Dutch families, increased plasma levels of SM 23:1 and reduced PCO 36:4 were associated with higher anxiety as measured by the Hospital Anxiety and Depression Scale (HADS) (Demirkan *et al.*, 2012).

On a more molecular level, increased NMDA receptor subunit NR1 localization and clustering on the membrane surface has been recorded in primary hippocampal neurons when treated with TNF- α , and was found to be dependent on nSMase activity and ceramide production (Wheeler *et al.*, 2009). NMDA receptors, glutamate receptors implicated in both synaptic plasticity and neurodegeneration, are suggested to be involved in both the increased glutamate signaling linked to the pathophysiology of anxiety disorders and the resultant excitotoxicity theorized to

precipitate depression and poorer cognitive outcomes. Consistent with the above mentioned, the TNF- α , nSMase, and ceramide dependent upregulation of NR1 subunits increased NMDA-evoked calcium bursts and excitatory transmission (Wheeler *et al.*, 2009). Additionally, the *in vivo* administration of glutamate to oligodendrocytes resulted in apoptosis, which could in turn be inhibited by the inhibition of ceramide synthesis (Novgorodov *et al.*, 2011). Jang *et al.* (2008) have similarly found that microinfusion of S1P into the cerebroventricle resulted in higher expression of NR1, oxidative stress as measured by iNOS, and neurodegeneration. In a later study, S1P microinfusion was also found to reduce expression of tyrosine hydroxylase (TH) – responsible for catecholamine synthesis – in the amygdala, as well as elicit anxiety like behaviour in rats (Jang *et al.*, 2011). These latter two findings are interesting given that S1P has long been suggested to play an anti-apoptotic role and oppose the effects of ceramide (Mandala *et al.*, 2004; Johnson *et al.*, 2003).

1.4.6.6. Sphingolipids and psychiatric medications

While not explicitly linked to anxiety, sphingolipid metabolism has been tied to the therapeutic effects of a number of psychiatric medications, antidepressants in particular. In a mouse model, therapeutic concentrations of both amitriptyline and fluoxetine have been shown to reduce aSMase activity and ceramide concentrations in the hippocampus (Gulbins *et al.*, 2013), one of the structures most highly scrutinized for its role in emotional regulation, depression, and anxiety. Kornhuber *et al.* (2008; 2010) found numerous antidepressant medications to be functional inhibitors of aSMase. Moreover, the same group found that functional inhibition

occurs within the same concentration range that patients achieve during antidepressant drug therapy (Mühle *et al.*, 2013).

2. Methods

2.1. Patient recruitment

Patients between the ages of 50-80 years old were recruited from Toronto Rehabilitation Institute's cardiac rehab program. Both male and female patients were recruited, and eligibility firstly required a primary diagnosis of CAD based on either an acute coronary incident (MI), a revascularization procedure (CABG, PTCA/stent) or angiographic evidence of $\geq 50\%$ blockage in at least 1 major coronary artery. Patients were screened for working knowledge of the English language, and were excluded if they were hospitalized in the prior 4 weeks for any indicators of unstable CAD such as angina, CHF, ventricular arrhythmia, revascularization, Canadian Cardiovascular Society class 4 angina, or an acute MI.

Diabetic patients were also excluded, due to the observed impact on sphingolipid metabolism (Haus *et al.*, 2008; Aerts *et al.*, 2007, 2011; Blachnio-Zabielska *et al.*, 2011). Patients were excluded on the basis of hypothyroidism, Parkinson's, dementia or Alzheimer's disease, autoimmune disease (i.e. Crohn's disease, rheumatoid arthritis, multiple sclerosis), Huntington's chorea, brain tumour, CVA, TIA, impaired liver or kidney function, active cancer, or any additional conditions likely to significantly impact cognitive, mood, inflammatory, or metabolic indices. Furthermore, patients with either significant cognitive impairment as assessed by the mini mental status examination (MMSE), or a current Axis I disorder other than depression, anxiety, a specific phobia, or nicotine dependence, were also excluded from participating.

Use of an antipsychotic or anticholinergic medication was also grounds for exclusion from the study given their effects on both cognitive and mood outcomes, as well as ceramide concentrations (Kornhuber *et al.*, 2008; Kölzer *et al.*, 2004; Albouz *et al.*, 1986). Conversely, statin use was a requirement for participation given the high proportion of patients (96%) enrolled in the cardiac rehab using this class of lipid lowering medication (Swardfager *et al.*, 2010).

2.2. Collection of demographics and medical history

After obtaining consent, cardiac medical history, cardiopulmonary and metabolic health indices (HR, BP, VO_{2Peak} , height, weight, and body fat percentage), BMI was calculated according to the standard [mass (kg)/height (m) ²] formula, and angiographic evidence of stenosis severity were collected from TRI's electronic and physical medical records.

2.3. Assessment

Patients were scheduled to come in for their first of 3 total assessments within a maximum of 2 weeks of their TRI intake class. A number of scales were administered assessing anxiety. The STAI trait subscale was used as the primary outcome measure for anxiety. STAI score has been shown to correlate with clinician rated GAD severity (Hopko *et al.*, 2000), and the trait subscale and its negative items in particular have been found to be strong predictors of anxiety disorder (Hishinuma *et al.*, 2001). Trait Anxiety has also previously been found to be a good predictor of cardiovascular morbidity and mortality (Szekely *et al.*, 2007).

The Hospital Anxiety and Depression Scale anxiety subscale (HADS-A) and the Generalized Anxiety Disorder 7-Item Scale (GAD-7) were used as additional measures of anxiety. The HADS is a 14 item scale with integrated anxiety and depression subscales shown to reliably differentiate between anxiety and depression (Zigmonds & Snaith, 1983; Bjelland *et al.*, 2002). HADS scores have also been linked to prognosis in patients with stable CAD (Frasure-Smith & Lespérance, 2008). The HADS will be administered due to its widespread use and efficacy in the cardiac population (Martin *et al.*, 2003).

On the GAD7, total scores range from 0-21 with four severity ranges - minimal or no anxiety (0-4), mild (5-9), moderate (10-14), and severe (15-21) (Löwe *et al.*, 2008). It has a proposed cut off value of 10 for GAD with a sensitivity of 89% and a specificity of 82%, has good test-retest reliability, and has high internal consistency (Spitzer *et al.*, 2006). It is moderately effective at screening three other common anxiety disorders – panic disorder (sensitivity 74%, specificity 81%), social anxiety disorder (sensitivity 72%, specificity 80%), and post-traumatic stress disorder (sensitivity 66%, specificity 81%). It's been used in in the elderly in public housing (Simning *et al.*, 2011), in primary care (Ying *et al.*, 2010; Kroenke *et al.*, 2007), and in patients with atrial fibrillation (Spertus *et al.*, 2011).

Depression was assessed using the Center for Epidemiological Studies Depression scale (CES-D) as has been previously used in this population (Swardfager *et al.*,

2010, 2011_{a,b}). The Montréal Cognitive Assessment (MoCA) was also used as an additional measure of cognition amended onto the protocol for exploratory analysis, and has been recommended for use to assess global cognition in patients at risk of or experiencing vascular cognitive impairment (Hachinski *et al.*, 2006).

2.4. Assay

2.4.1. Blood collection

Fasting blood was drawn into EDTA containing vacutainer tubes immediately prior to the patient assessments. The majority of assessments were scheduled for 0900 h \pm 30 min. The standardized time and fasting blood allows for relative control of diurnal and dietary influence on lipid concentrations. Blood was then centrifuged at 1000 x g for 10 min, and plasma was immediately isolated and stored at -80°C until analyzed.

2.4.2. Lipidomic analysis

Sphingolipid, phospholipid, and sterol species analyses were performed using high performance liquid chromatography coupled electrospray ionization tandem mass spectrometry (HPLC ESI-MS/MS). Samples were injected into an ESI (i.e., Turbo Ion Spray module) Sciex API 3,000 triple stage quadrupole MS/MS from Sciex Inc. (Thornhill, Ontario, Canada), operated in the positive mode, using a Harvard Apparatus pump at 15 μ l/min for detection of sterols and lipid peroxidases. The MS/MS was set to scan between 300 to 2,000 atomic mass units (amu) per second at a step of 0.1amu. A Q1 mass scan followed by precursor ion scanning or neutral loss scanning of a purified standard was used to identify each species of cholesterol, cholesterol esters, long-chain triglycerides, and lipid peroxides. Samples were

injected into the ESI/MS/MS for 3 minutes and the mass counts accumulated as well as the sum of the total counts under each peak were used to determine each species. HPLC and subsequent MS/MS were used to detect and quantify sphingomyelins, ceramides, and tocopherol. Samples were injected into a PerkinElmer HPLC equipped with a Phenomenex, Luna 100 x 2 mm, 5 μ m, C18 column coupled with guard column containing identical packing material (Phenomenex, Torrance, CA) using a PAL autosampler. A mobile phase consisting of 85% methanol, 15% H₂O, and 5 mM ammonium formate is first injected for 0.5 min into the LC column for pre-equilibration. The column is then eluted with the second mobile phase consisting of 99% methanol, 1% formic acid, and 5 mM ammonium formate at the flow rate of 100.0 μ l /min. The eluted sample is then injected into the ion source, for detection and quantification (m/z 264.4, 266.4 for ceramides and 184.4 for sphingomyelins, respectively). Data was collected and processed by Analyst 1.4.2 software package. Ceramide concentrations were initially presented in counts per second (cps) and a standard curve was applied to derive concentration in ng/ml.

2.5. Sample size calculation

Sample size was calculated based on existing findings in a pilot sample. In cross-sectional analysis using linear regression, log-transformed plasma C22:0 ($N = 30$, $\beta = 0.316$, $p = 0.092$) and C24:0 ($N = 30$, $\beta = 0.34$, $p = 0.068$) cps showed a positive trend with trait anxiety. Based on the pilot data, a sample size of 24 was found to provide 80% power. Accounting for depression, gender, and BMI as covariates the sample size was increased to 54.

2.6. Statistical analysis

Ceramide concentrations were log-transformed to account for non-normal distribution. Linear regression analyses were performed for C22:0 and C24:0 as predictors of STAI-T score. BMI, gender, and depression as measured by the CES-D were entered as covariates in the model. Descriptive statistics and bivariate correlation analysis were also performed to determine patient characteristics and possible correlations between demographic and anthropomorphic data with ceramide concentrations. To account for multiple comparisons, a p-value of ≤ 0.025 was considered significant for the primary analyses.

2.7. Exploratory hypotheses and analyses

In addition to correlating anxiety severity with ceramide concentrations, group differences between anxious and non-anxious groups were investigated based on multiple anxiety scale cutoff points. In assessing clinically significant GAD, optimal HADS and STAI-T cutoff scores of ≥ 8 , and ≥ 45 have been suggested in CAD, although STAI-T had low specificity at that cutoff (Bunevicius *et al.*, 2013). Similarly Devier *et al.* (2009) and Glozman *et al.* (2004) have previously suggested STAI-T cutoffs of >30 for moderate to high anxiety.

While C22:0 and C24:0 were the primary potential predictors investigated, it must be acknowledged that multiple sphingolipid, sterol, and phospholipid species have been suggested to play a role in cell signaling and function implicated in neuropsychiatric disorders. As such, further linear regression analyses was

performed on the spectrum of sphingolipid species assayed. The results were presented in the form of a heat map with a gradient of colours.

3. Results

3.1. Demographics and clinical characteristics

Patient characteristics, including demographics, anthropomorphic data, and cardiac diagnoses are presented in [Table I] along with means and standard deviations for continuous variables, and percentages for dichotomous variables. Mean anxiety and depression scores and standard deviations are also presented therein.

Table I. Patient characteristics (N=54)					
	Mean±SD or %	Association with C22:0		Association with C24:0	
		F or r	p-value (≤0.05*)	F or r	p-value (≤0.05*)
Demographics					
Age (yrs)	65.3±6.9	0.050	0.720	0.049	0.723
Sex (% male)	81.5%	0.030	0.863	0.371	0.545
Ethnicity (% Caucasian)	88.9%	2.501	0.054	2.129	0.091
Marital status(% married)	74.1%	0.807	0.527	0.191	0.942
Employment (% employed)	51.9%	0.298	0.587	1.368	0.248
Years of education	16.1±3.4	-0.081	0.561	-0.034	0.806
Cardiopulmonary Fitness Parameters					
Body Mass Index (kg/m ²)	27.9±3.6	0.209	0.130	0.082	0.556
Body Fat %	29.4±7.6	0.122	0.381	0.064	0.644
VO ₂ peak (ml/kg/min)	20.8±5.2	0.282	0.039*	0.105	0.450
Cardiac History					
Ischemic Heart Disease (%)	16.7%	0.643	0.426	0.121	0.730
Myocardial Infarction (%)	38.9%	0.180	0.673	0.669	0.417
Coronary artery bypass graft surgery (%)	35.2%	1.131	0.292	1.157	0.287
PTCA-Stent (%)	59.3%	0.729	0.397	0.001	0.981
Angina (%)	7.4%	3.033	0.087	2.236	0.141
Hypertension (%)	40.7%	0.275	0.603	2.231	0.141
Comorbidities					
Depression - Assessed by SCID (%)	14.8%	0.001	0.979	0.885	0.351

Anxiety – Assessed by SCID (%) (N=30)	3.2%	1.086	0.351	0.097	0.908
Medications					
B-blockers (%)	83.3%	2.429	0.124	0.502	0.482
Diuretics (%)	14.8%	0.215	0.645	0.106	0.746
Anti-hypertensives (%)	72.2%	0.897	0.348	0.147	0.703
Ca ²⁺ channel blockers (%)	7.4%	1.255	0.268	0.218	0.643
Platelet Inhibitor (%)	96.3%	1.517	0.224	0.040	0.842
Anxiolytics (%)	11.1%	0.349	0.557	1.068	0.306
Antidepressants (%)	1.9%	1.036	0.313	0.340	0.562
Psychometrics					
STAI-T	32.9±9.2	-0.291	0.033*	-0.181	0.191
STAI-S	29.1±8.3	0.000	0.998	0.136	0.325
CES-D	6.8±6.6	-0.199	0.149	-0.162	0.241
GAD-7	3.2±3.7(N=43)	-0.297	0.053	-0.281	0.067
HADS-A	4.5±3.3(N=43)	-0.231	0.136	-0.192	0.218
HADS-D	2.5±2.5(N=43)	-0.209	0.178	-0.329	0.031*
MMSE	29.1±1.0	0.135	0.325	-0.108	0.434
MoCA	26.1±2.2 (N=43)	-0.235	0.129	-0.311	0.042*

3.2. Independent and dependent variable distributions

Ceramide concentrations were log-transformed to normalize distribution. Mean log-C22:0 and C24:0 concentrations at baseline were 2.91±0.22 [range=2.12-3.26] and 3.37±0.30 [range=2.49-4.08], respectively as indicated in **Figure 1**. Mean STAI-T score at baseline was 32.87±9.23 [range=20-55][**Figure 2**].

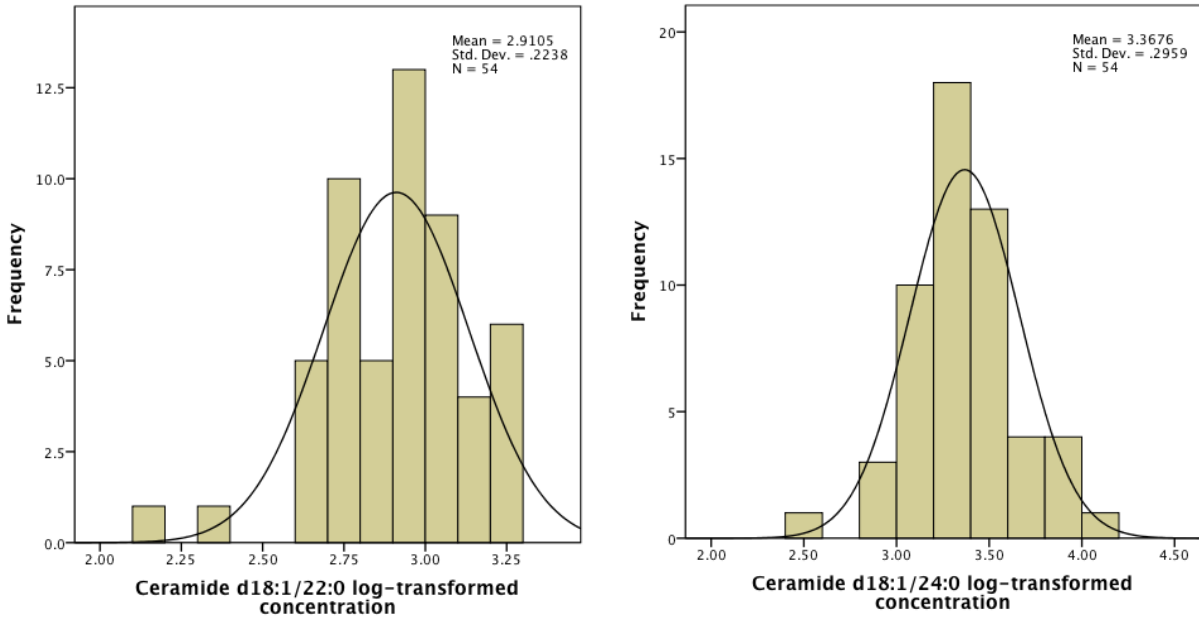


Figure 1. Distribution of log-transformed plasma C22:0 and C24:0 concentrations.

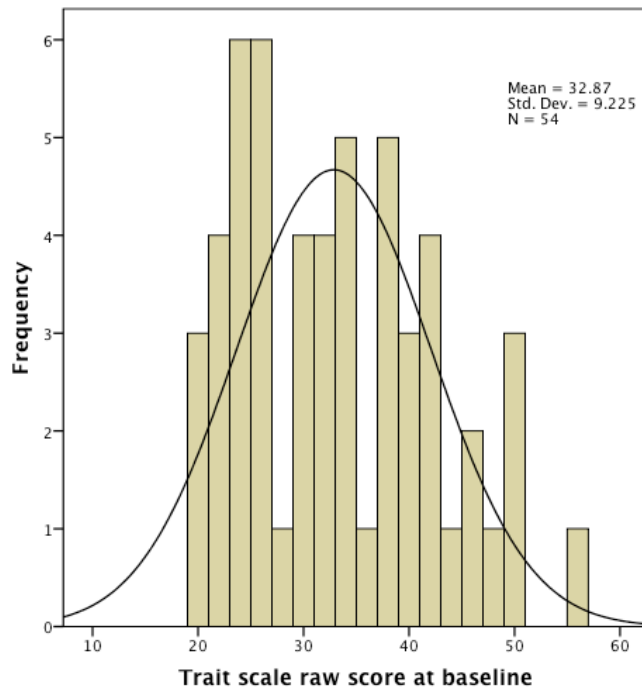


Figure 2. Distribution of STAI-T scores at baseline (N=54).

3.3. Primary Hypotheses

Log-transformed plasma C22:0 concentrations were significantly but negatively correlated with STAI-T scores when controlling for gender, BMI, and CES-D scores ($\beta = -0.232, p = 0.018$) [Figure 3]. However, there was no significant correlation between **log-transformed plasma C24:0 concentrations** and STAI-T scores while controlling for the same covariates ($\beta = -0.097, p = 0.320$) [Figure 4]. In models for both C22:0 and C24:0, BMI ($\beta = 0.291, p = 0.004$; $\beta = 0.240, p = 0.018$) and CES-D score ($\beta = 0.597, p < 0.001$; $\beta = 0.641, p < 0.001$) remained the strongest correlates of STAI-T score, while gender was not significantly correlated in either model ($\beta = -0.004, p = 0.963$; $\beta = -0.005, p = 0.957$).

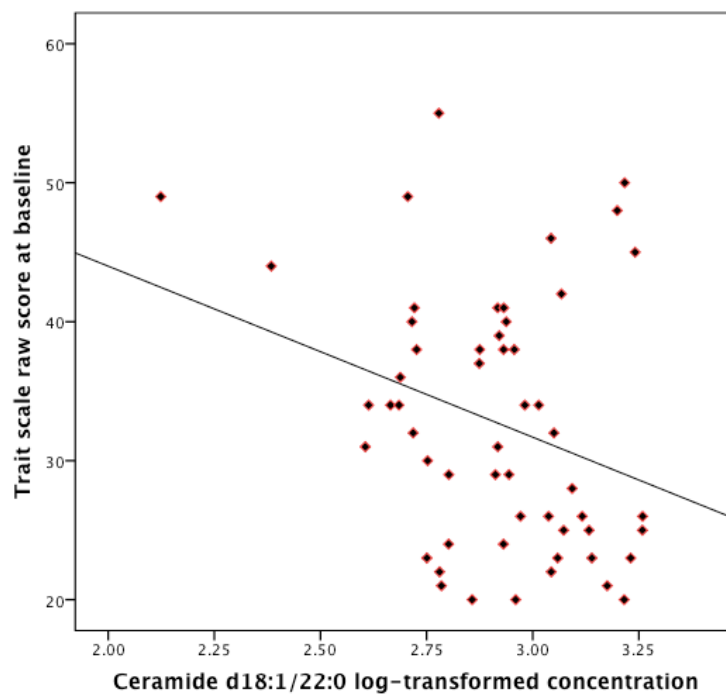


Figure 3. Scatter plot of log-transformed C22:0 ceramide concentrations by STAI-T score ($r^2=0.577, \beta = -0.232, p = 0.018$).

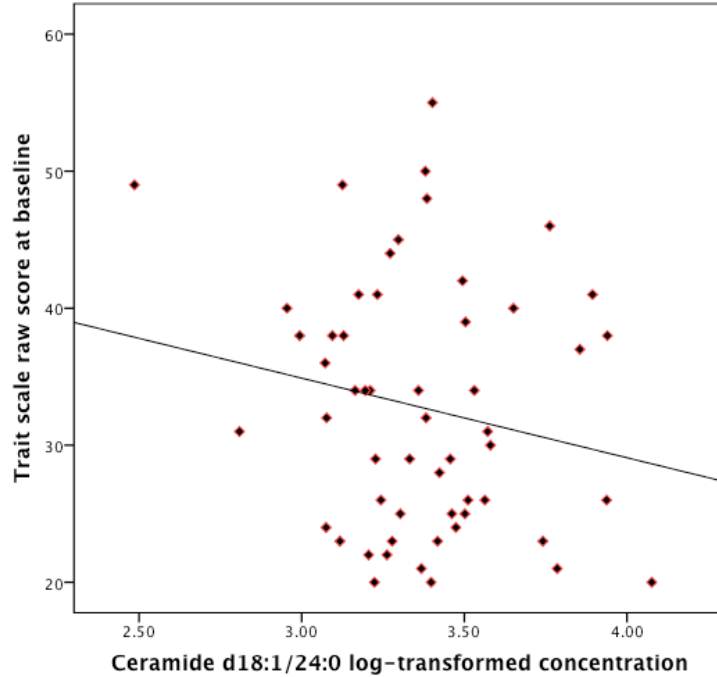


Figure 4. Scatter plot of log-transformed C24:0 ceramide concentrations by STAI-T score ($r^2=0.534$, $\beta = -0.097$, $p = 0.320$).

3.4. Post-Hoc Analyses

The C22:0 based model was repeated substituting the least significant predictor, gender, with VO_2 peak as a covariate, based on the significant correlation observed in bivariate analysis. The model including VO_2 peak ($F = 19.157$, $p < 0.001$) as a covariate remained significant in its predictive value of variance in STAI-T scores. However, VO_2 peak ($\beta = -0.042$, $p = 0.667$) was not a significant predictor in the model. C22:0 log-transformed plasma concentration continued to be a significant predictor of STAI-T scores in the covariate adjusted model controlling for VO_2 peak ($\beta = -0.223$, $p = 0.026$).

Two of the patients had C22:0 concentrations which were greater than two standard deviations from the mean, making them statistical outliers. The two data points were removed and the primary analysis repeated to address the possible influence of the outliers. The model remained significant, and C22:0 remained significantly associated with STAI-T score ($\beta = -0.203$, $p = 0.044$).

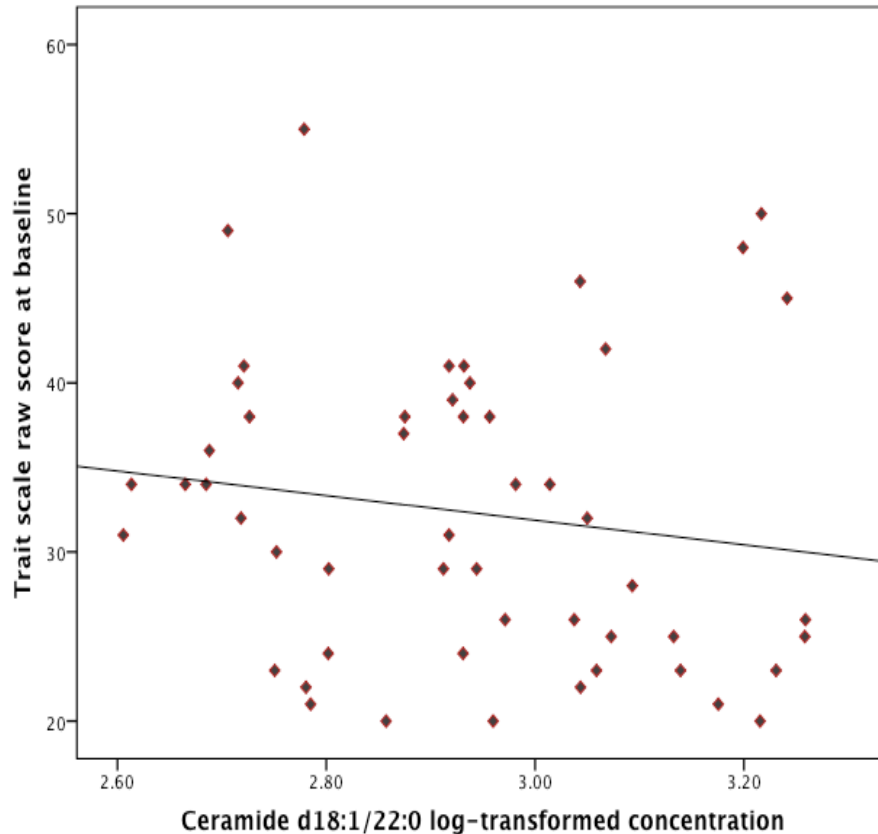


Figure 5. Scatter plot of log-transformed C22:0 ceramide concentrations by STAI-T score excluding statistical outliers ($r^2=0.536$, $\beta = -0.203$, $p = 0.044$).

3.5. Group comparisons

Using an analysis of covariance, with gender, BMI, and CES-D score as covariates, mean log-transformed C22:0 concentrations were not significantly different

between anxiety groups based on the HADS-A cutoff of ≥ 8 ($N = 43$, $F = 0.573$, $p = 0.454$). Similarly, concentrations were not significantly different between anxiety groups based on the ≥ 45 cutoff on the STAI-T ($N = 54$, $F = 0.131$, $p = 0.719$). However, using the proposed cutoff of >30 on the STAI-T yielded significantly different mean log-transformed C22:0 concentrations between anxious and non-anxious groups ($N = 54$, $F = 8.112$, $p = 0.006$)[**Figure 6**].

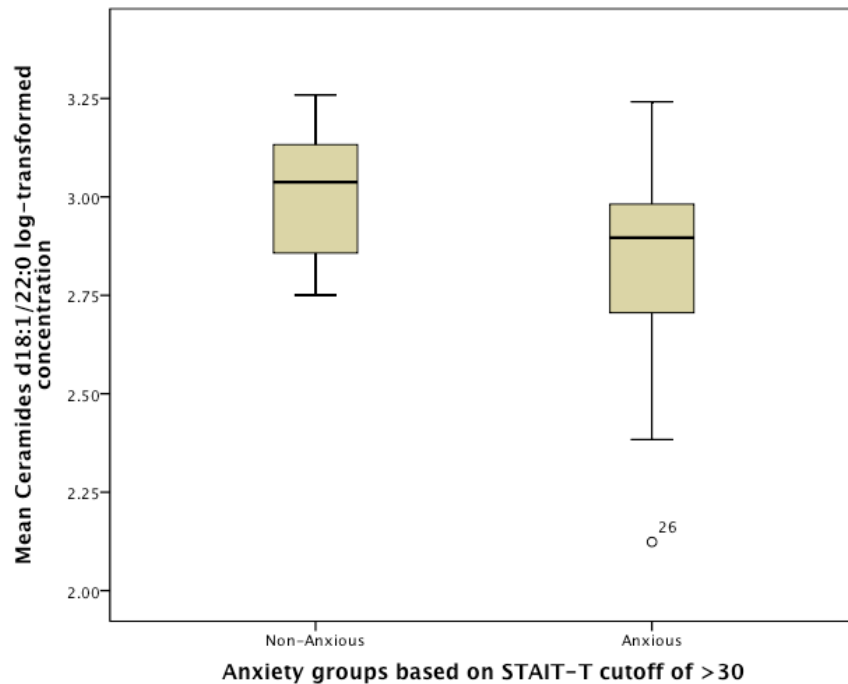


Figure 6. Mean log-transformed C22:0 concentrations in anxious and non-anxious groups based on a STAI-T cutoff of >30 .

3.6. Exploratory heat map analyses of sphingolipid panel

Further regression analyses showed log-transformed concentrations of an additional 10 species of sphingolipids to be negatively correlated with STAI-T score

when controlling for gender, BMI, and CES-D (SM18:0, SM20:1, C18:0, C20:0, C26:0, C20:1, DHC22:0, LacC22:0, LacC24:0, LacC24:1)[Table II]. CES-D score was negatively correlated with log-transformed concentrations of 3 sphingolipid species when controlling for age, gender, and BMI (SM24:0, C26:0, MHxC24:0) [Table II]. With respect to cognitive measures, MMSE score positively correlated with log-transformed C18:0 concentration, while in 43 patients for whom MoCA score was collected, 3 sphingolipid species negatively correlated with cognitive score when controlling for gender, BMI, and CES-D (C24:0, DHC24:0, MHxC18:1) [Table II].

Table II. Heat map of sphingolipid panel and psychometric correlates

$p < 0.005$		$p < 0.01$	$p \leq 0.05$	$0.05 < p > 0.05$	$p \leq 0.05$	$p < 0.01$	$p < 0.005$
Negative Associations				Positive Associations			
Sphingolipid species		STAI-T	STAI-S	CES-D ^a	MMSE	MoCA ^b	
Sphingomyelins	SM16:0						
	SM18:0						
	SM20:0						
	SM22:0						
	SM24:0						
	SM16:1						
	SM18:1						
	SM20:1						
	SM22:1						
	SM24:1						
Ceramides	C16:0						
	C18:0						
	C20:0						
	C22:0						
	C24:0						
	C26:0						
	C16:1						
	C18:1						
	C20:1*						
	C22:1						
	C24:1						
	C26:1*						
Dihydroceramides	DHC22:0						
	DHC24:0						
Monohexosylceramides	MHxC16:0						
	MHxC18:0						
	MHxC20:0						
	MHxC22:0						
	MHxC24:0						
	MHxC16:1						
	MHxC18:1						
	MHxC22:1						
MHxC24:1							
Galceramides	GalC20:0						
	GalC22:0*						
	GalC24:0						
Lactosylceramides	LacC16:0						
	LacC18:0						
	LacC20:0						
	LacC22:0						
	LacC24:0						
	LacC22:1						
LacC24:1							

^a Age, gender, and BMI run as covariates

^b N = 43 in this samples; covariates were gender, BMI, and CES-D

* Standardized concentration multiplied by 100 before log-transforming to prevent negative log-transformed values

4. Discussion

4.1. Summary

To our knowledge, the present study is the first to investigate the correlation between sphingolipid species and anxiety symptomology in CAD patients.

Sphingolipids are ubiquitous endogenous molecules that have long been associated with cardiovascular disease, inflammatory signaling, and modulation of cellular proliferation and apoptosis. This class of lipids, and ceramides in particular, have been garnering increasing attention with respect to their roles in neuropsychiatric symptoms and pathophysiology. Based on current literature, it is hypothesized that the plasma concentration of two particular species of ceramide, C22:0 and C24:0, would be positively correlated with anxiety severity based on the self-report STAI-T. While C22:0 in particular proved to be significantly correlated with STAI-T score, the direction of the relationship seen is in stark contrast to the hypotheses laid out. However, the direction of the relationship was consistent across multiple sphingolipid species, and with alternative psychometric measures [Table II].

4.2. Interpretation

4.2.1. Methodological considerations and possible limitations

This finding may be attributed to a number of potential factors. Firstly, the findings might be spurious ones due to error anywhere along the trajectory of the study, the multiple comparisons, or the coincidental product of an idiosyncratic sample population. For example, this might include sample contamination or degradation, inconsistency in the lipidomic assay, or significant variability in the outcome

measure. However, given the absence of any detection issues in the ESI-MS/MS assay, the application of a standard curve to determine concentration, the statistical modifications to address distribution, and the correlation in concentration amongst diverse species of sphingolipids, a technical, assay specific issue is not suspected to be responsible. Additionally, as previously mentioned, members of the research group have found very long chain ceramide concentrations to be predictive of cognitive decline (Mielke *et al.*, 2010). Given the negative correlation between increased log-transformed plasma C24:0 concentrations and global cognition as assessed by the MoCA in the study sample (albeit in a subset of the total sample; N=43), there is additional support for the legitimacy of the relationship seen with anxiety. However, based on previous observations by members of our group, ceramide concentrations were not supported to be strong cross-sectional markers, but to be of utility in longitudinal outcomes (Mielke *et al.*, 2010). As such, the current study may warrant longitudinal follow-up. Furthermore, the current study's sample size may have left it underpowered for the statistical analyses which was intended to be carried out. More covariates may have been necessary, and more significant covariates may have been overlooked.

A number of limitations also may obstruct the ability to draw a direct relationship between ceramides and anxiety. With respect to the primary dependent variable and anxiety measure used, the STAI-T includes a number of items that identify depressive symptoms. This may largely obscure the associations present in this sample. Although separate models using the CES-D as the dependent variable may

have partially addressed this issue, an item analysis of the STAI-T and associations with the ceramide concentrations would clarify what cluster of symptoms (somatic vs. cognitive; anxious vs. depressive) are associated with plasma ceramide concentrations.

Further consideration must also be given to the patient sample, patient characteristics, and study design of the present work. As previously discussed, the patient sample is composed of a non-psychiatric population enrolled in cardiac rehabilitation. The present patient population had a significantly lower mean STAI-T score in relation to the pilot sample (40.4 ± 12.4) and was composed predominantly of subclinical, or clinically insignificant, levels of anxiety. As a result the absence of clinically distinct anxious and control groups presents a considerable limitation in assessing the relationship with anxiety in this patient sample.

Lastly, from a mechanistic perspective, the analysis was limited by the absence of additional biological markers largely implicated in the metabolic pathways of sphingolipids and ceramides. In particular, as discussed above, ceramides are involved in inflammatory signaling, and their production can be induced by, and is closely tied to, the presence of other inflammatory signaling molecules such as the inflammatory cytokines (Osawa *et al.*, 2005; Barth *et al.*, 2011). Given the association between inflammatory cytokines and anxiety (Pistavos *et al.*, 2006; Zimmerman *et al.*, 2012; O'Donovan *et al.*, 2010), not having assayed concentrations of inflammatory cytokines presents a limitation. The analyses conducted cannot

account for the role inflammatory cytokines may play in the relationship, whether ceramides are collinear and potentially mediating an inflammatory signal, or whether cytokines themselves remain better predictors.

4.2.2. Mechanistic considerations

The literature on sphingolipid metabolism, characterization, and diversity of biological function, is quite rich and quite complex. There is much left to be elucidated in sphingolipid research. However, the existing literature presents a number of alternative biological and mechanistic considerations with which to approach the findings.

Firstly, one might consider localization of the species in question and in turn the alternative sphingolipid metabolic pathways. Extracellular ceramide concentrations might not reflect membrane bound ceramide concentrations, utilizing different SMases, CDases, and metabolic pathways (Hannun *et al.*, 2011; Tani *et al.*, 2007). *de novo* synthesis of ceramide, which occurs intracellularly, was suggested to be necessary for caspase activation and apoptosis (Siskind *et al.*, 2010). While membrane bound, intracellular ceramide concentrations, and specific metabolic pathways have thus been implicated in apoptotic mechanisms and excitotoxicity, the plasma assays used do not differentiate between metabolic pathways and cellular localization of the species assayed. Secretory SMase is likely responsible for the bulk of extracellular ceramide concentrations, and these are the concentrations the assay used is likely most sensitive to detect, while not necessarily giving us a complete picture of the cellular and membrane bound compositions.

Alternatively, the findings might suggest that increased peripheral concentrations of the significant predictor (C22:0) are associated with coinciding variations in concentrations of other species in the sphingolipid metabolic network. The trend towards higher ceramide concentrations in less anxious patients might be due to an increased metabolism of ceramide to S1P. Further metabolism of sphingomyelinase-generated ceramide to S1P was found to be pivotal in inhibiting ceramide induced hippocampal neuron excitability in hippocampal slices (Norman *et al.*, 2010). Alternatively one might conceive that ceramide synthesis is suppressed in more anxious individuals through a negative feedback mechanism. If considering the increased glutamate signaling implicated in anxiety, and the role ceramides and S1P have been suggested to play in NMDA receptor signaling (Wheeler *et al.*, 2009; Jang *et al.*, 2008), the increased excitatory signaling and receptor trafficking associated with ceramide synthesis may trigger a negative feedback loop of further ceramide synthesis.

However, as Hannun *et al.* (2011) point out in their mini-review, the sphingolipid metabolic network is a highly complex and dynamic system, where no individual arm, metabolite, substrate, product, or enzyme operates in isolation. This reality supports the need for a more robust lipidomic analysis, where fluctuations in multiple species concentrations are assessed in tandem, rather than having individual species studied in isolation with respect to their role in disease or behaviour. This approach is akin to the development of allostatic load models and

would require significantly more advanced statistical approaches, some of which are currently being investigated by members of our group.

Adding to the confusing state of the literature and lack of consensus on the neurodegenerative role of ceramides, CerS1 knockout mice and the rampant neurodegeneration observed as compared to WT mice suggest C18:0 and C18:1 to actually play a role in maintaining neuronal integrity as opposed to contributing to cellular death (Zhao *et al.*, 2011). Consistent with this anti-apoptotic role, CerS2 knockout mice have not only been found to have reduced C22-C24 concentrations both peripherally and centrally, but also to show white and grey matter loss as well as impairment of motor function (Imgrund *et al.*, 2009). These findings, taken together, suggest that C18 and C22-C24 may actually play an anti-apoptotic role and contribute to maintaining neuronal function. Although in conflict with existing findings suggesting C22:0 and C24:0 to be associated with hippocampal atrophy (Mielke *et al.*, 2010), another study in Alzheimer's patients indicated increases in C24:0 but not C22:0 in patients vs. controls and with increasing disease severity (Grosch *et al.*, 2012; Cutler *et al.*, 2004). These findings suggest that C22:0 and C24:0, despite both being very long chain ceramides and generated by the same CerS, may have disparate and potentially opposing functions.

Should C22:0 and C18:0 contribute to neuronal integrity, the presented results may in fact fit in the context of other reported findings. Referring to the exploratory analyses [**Table II**], it can be seen that C18:0 is significantly positively correlated

with cognition as measured by the MMSE, negatively correlated with STAI-T score, and that in a smaller cohort of the total sample (N=43) that C24:0 is negatively correlated with cognition as measured by the MoCA. Taken together these findings further support the diverse functional roles ceramides of differing acyl-chain length may play, and become more readily reconcilable with the context of the literature. In addition to these findings, Laviad *et al.*'s (2008) findings that S1P inhibits CerS2 activity may help explain Jang *et al.*'s (2008; 2011) findings that S1P microinfusion was associated with neurodegeneration, increased oxidative stress, iNOS, reduced TH expression, and increased anxiety like behaviour in animal models. Although S1P is classically considered protective or anti-apoptotic (Maceyka *et al.*, 2005; Johnson *et al.*, 2003; Mandala *et al.*, 2000), taken together these findings suggest that very long chain ceramides generated by CerS2 may in fact confer a neuroprotective effect, which is in turn inhibited by S1P.

Furthermore, while previous findings indicate a correlation between central and peripheral concentrations of ceramide (Alessenko *et al.*, 2005), the results and assays are not species specific showing corresponding variability of concentration for various specific species of sphingolipids. The relationship between peripheral and central ceramide concentrations is significantly more complex when considering specific species. For example, CerS2 mRNA is most highly expressed in peripheral organs – kidney and liver – and tends to inversely relate to CerS1 and CerS3 mRNA expression, which appeared to be the primary CerS species in the brain (Laviad *et al.*, 2008). Although this consideration may seem irrelevant due to the

current research primarily being concerned with the evaluation of peripheral biological markers of neuropsychiatric symptoms, in the scope of these findings it must be acknowledged that the peripheral markers do not necessarily mirror what is occurring centrally.

4.3. Future Studies and Replication

Given the novel nature of this particular study, and the unanticipated direction of the relationship uncovered, replication studies are warranted in order to confirm or add validity to the findings discussed herein. Due to the often-conflicting findings on sphingolipid metabolism, particular species, and particular biological functions, it is apparent that more robust research is necessary to clarify the roles each species play in diverse biological functions. For example, given the implication of S1P in anxiety behaviour and pathophysiological correlates of anxiety, the additional assay of S1P concentration and the calculation of its ratio to ceramide species concentration would be of additional utility. In the case of the current study, neuroimaging work assessing structural and functional correlates of peripheral ceramide concentration as well as anxiety symptomology would be an invaluable addition. Better determination of enzymatic activity, the cellular localization of specific species, and the metabolic pathway involved in generating specific species would make for critical additional indices of the complex relationship sphingolipids have with mood and behaviour. However, despite the nature of the findings relative to the hypotheses, this is the first study of sphingolipid involvement in anxiety in a cardiac population with such a wide array of sphingolipids investigated. It is

anticipated that it leads to further more in depth analysis and better understanding of the unique and significant role of sphingolipids and ceramides in neuropsychiatric function.

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List of Publications and Abstracts

Meeting/Conference Abstracts

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Mielke M, Haughey N, Oh P, Lanctôt K (2012) Higher ceramide C22:0 concentrations are associated with anxiety in coronary artery disease patients. *Visions in Pharmacology 2012*. Department of Pharmacology, University of Toronto (Toronto, ON).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Mielke M, Haughey N, Oh P, Lanctôt K (2012) Higher ceramide C22:0 concentrations are associated with anxiety in coronary artery disease patients. *Harvey Stancer Research Day 2012*. Department of Psychiatry, University of Toronto (Toronto, ON).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Mielke M, Haughey N, Oh P, Lanctôt K (2012) The relationship between very long chain plasma ceramides and anxiety in coronary artery disease. *Toronto Rehabilitation Institute Research Day 2012* (Toronto, ON).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Mielke M, Haughey N, Oh P, Lanctôt K (2012) The relationship between very long chain plasma ceramides and anxiety in coronary artery disease. *Society of Biological Psychiatry – 68th Annual Scientific Meeting 2013* (San Francisco, USA).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Nestor N, Black S, Oh P, Lanctôt K (2012) Perceived stress predicts reduced hippocampal volume in patients with coronary artery disease. *Visions in Pharmacology 2013*. Department of Pharmacology, University of Toronto (Toronto, ON).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Nestor N, Black S, Oh P, Lanctôt K (2012) Perceived stress predicts reduced hippocampal volume in patients with coronary artery disease. *Harvey Stancer Research Day 2013*. Department of Psychiatry, University of Toronto (Toronto, ON).

Rovinski, R, Herrmann N, Saleem M, Swardfager W, Nestor N, Black S, Oh P, Lanctôt K (2012) Perceived stress predicts reduced hippocampal volume in patients with coronary artery disease. *Canadian College of Neuropsychopharmacology Annual Meeting 2013* (Toronto, ON).

List of Awards and Sources of Funding

Scholarships

2011/2012 – University of Toronto Fellowship

2012/2013 – University of Toronto Fellowship

Travel Awards

2013 – Sunnybrook Trainee Travel Award

Appendices

Appendix A: Research Ethics Board Approval

To: Dr. Krista Lanctôt
Psychiatry
Room FG05

From: Dr. Philip Hébert

Date: November 11, 2011

Subject: **The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease**

Project Identification Number: 279-2011

Approval Date: November 11, 2011

Expiry Date: November 11, 2012

The Research Ethics Board of Sunnybrook Health Sciences Centre has conducted a Delegated Board review of the research protocol referenced above and approved the involvement of human subjects on the above captioned date. The quorum for approval did not involve any member associated with this project.

The approval of this study includes the following documents:

- Protocol dated August 15, 2011
- Informed Consent Form dated November 3, 2011
- The following scales/tools received October 18, 2011
 - o CES-D
 - o STAI-S
 - o STAI-T
 - o SCID
 - o CVLT-II Standard Form
 - o BVMT-R
 - o Digit Symbol – Coding
 - o Stroop Neuropsychological Screening Test (Victoria)
 - o Trails Making Test – Part A and B
 - o CVLT-II Delayed Recall Trials
 - o BVMT-R Delayed Recall Trials
 - o FAS and Animal Naming Test
 - o Word Fluency
 - o AES-C
 - o SRRS
 - o Pittsburgh Sleep Quality Index

The Research Ethics Board of Sunnybrook Health Sciences Centre Operates in Compliance with the Tri-Council Policy Statement 2nd edition, ICH GCP Guidelines, Part C Division 5 of the Food and Drug Regulations, Part 4 of the Natural Health Products Regulations, and Part 3 of the Medical Devices Regulations. All Health Canada regulated trials at Sunnybrook are conducted by a Qualified Investigator.

Fully affiliated with the University of Toronto

All correspondence with the REB must include the assigned Project Identification Number. The REB requires immediate notification of all internal serious adverse events and significant deviations. Study continuation beyond one year requires submission of a renewal form prior to the expiry date or a study completion report must be received to close the file with the REB.

All REB approved studies may be subject to review by the Sunnybrook Quality Assurance and Education Program and, as Principal Investigator, you are responsible for the ethical conduct of this study. Approval by the Sunnybrook REB entails compliance with current legislation outlined in the Ontario Personal Health Information Protection Act (PHIPA) and all policies and guidelines established by Sunnybrook. All applicable contracts and agreements must be submitted to Sunnybrook Legal Services before this research may be initiated.



Philip C. Hébert, MD PhD FCFPC
Chair, Research Ethics Board

OR

Miriam Shuchman, MD
Vice-Chair, Research Ethics Board

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January 30, 2012

Dr. Krista Lancot
Sunnybrook Health Sciences Centre
Psychopharmacology/Psychiatry
2075 Bayview Avenue
North York, Ontario
M4N 3M5

Dear Dr. Lancot:

RE: TRI REB #: 11-058

The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease

The Toronto Rehabilitation Institute Research Ethics Board has reviewed the above-named submission. Any concerns and requested revisions have been addressed to the satisfaction of the REB. The protocol version 1.1, dated January 2012 is approved for use for the next 12 months. If the study is expected to continue beyond the expiry date, you are responsible for ensuring the study receives re-approval. The REB must also be notified of the completion or termination of this study and a final report provided.

Also approved are the following documents:

- Information and Consent Form version 1.2, dated January 23, 2012
- Budget received December 2, 2011
- Acknowledgement of receipt of reimbursement for participation in study, received December 2, 2011
- Data Collection Forms received December 2, 2011
 - o Mini Mental State Examination (MMSE)
 - o Montreal Cognitive Assessment (MOCA)
 - o Digit Symbol – Coding
 - o Trails Making Test, Part A and B
 - o Brief Visuospatial Memory Test – Revised (BCMT-R)
 - o Brief Visuospatial Memory Test – Revised (BCMT-R) Delayed Recalls Trials
 - o Centre for Epidemiological Studies – Depression Scale (CES-D)
 - o State – Trait Anxiety Inventory – State (STAI-S)
 - o State – Trait Anxiety Inventory – Trait (STAI-T)
 - o Social Readjustment Rating Scale (SRRS)
 - o Pittsburgh Sleep Quality Index
 - o Structured Clinical Interview For the DSM – IV – Depression Module (SCID)
 - o California Verbal Learning Test II (CVLT-II) – Standard Form
 - o California Verbal Learning Test II (CVLT-II) – Delayed Recalls Trials
 - o Stroop Neuropsychological Screening Test (Victoria)

.../ 2

TRI REB conforms with the *Tri-Council Policy Statement (TCPS2): Ethical Conduct for Research Involving Humans* and Ontario Privacy Legislation *PHIPA*
Toronto Rehab is a teaching and research hospital fully affiliated with the University of Toronto.

Page 2
January 30, 2012
Dr. Krista Lancot
TRI REB #: 11-058


- FAS and Animal Naming Test
- Word Fluency
- Apathy Evaluation Scale – Self
- Daily Food Diary

If, during the course of the research, there are any serious adverse events, changes in the approved protocol or consent form or any new information that must be considered with respect to the study, these should be brought to the immediate attention of the Board.

Best wishes for the successful completion of your project.

Yours sincerely,

Paul Oh MD, MSc, FRCPC, FACP
Chair, Research Ethics Board
Toronto Rehabilitation Institute


Ann Heesters BEd, BA, MA, PhD (ABD)
Vice Chair, Research Ethics Board
Toronto Rehabilitation Institute

January 30, 2012
Date of Initial REB Approval

January 30, 2013
Expiry Date of REB Approval

TRI REB conforms with the *Tri-Council Policy Statement (TCPS2): Ethical Conduct for Research Involving Humans* and Ontario Privacy Legislation *PHIPA*



University Health Network
Research Ethics Board
10th Floor, Room 1056
700 University Ave
Toronto, Ontario, M5G 1Z5
Phone: (416) 581-7849

Notification of REB Continued Approval

Date: January 15th, 2013

To: Dr. Krista Lanctot
Sunnybrook Health Sciences Centre
2075 Bayview Ave., Rm FG-08
Toronto, Ontario
M4N 3M5

Re: 11-058-DE
The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease

REB Review Type:	Expedited
REB Initial Approval Date:	January 30th, 2012
REB Annual Approval Date:	January 7th, 2013
REB Expiry Date:	January 30th, 2014

The UHN Research Ethics Board operates in compliance with the Tri-Council Policy Statement, ICH Good Clinical Practice Guidelines, Ontario Personal Health Information Protection Act (2004), Part 4 of the Natural Health Product Regulations and Part C, Division 5 of the Food and Drug Regulations of Health Canada.

Best wishes on the successful completion of your project.

Sincerely,

Daeniell Miller
Research Ethics Coordinator

For: Ann Heesters
Co-Chair, University Health Network Research Ethics Board

Toronto Rehabilitation Institute, University Centre
550 University Ave., Toronto, ON M5G 2A2

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Appendix B: Informed Consent Form

Supported by a grant from the Canadian Institute of Health Research



Sunnybrook Health
Sciences Centre
2075 Bayview Ave. Suite FG05
Toronto, Ontario, M4N 3M5

University of Toronto
Faculty of Medicine
1 King's College Circle,
Toronto, Ontario, M5S 1A8

Toronto Rehabilitation Institute
Cardiac Rehabilitation Program
347 Rumsey Road,
Toronto, Ontario, M4G 1R7

The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease

Subject Information and Consent

Investigators:	Dr. K.L. Lanctôt, PhD	Sunnybrook Health Sciences Centre
	Dr. P. Oh, MD FRCPC	Toronto Rehabilitation Institute
	Dr. N. Herrmann, MD FRCPC	Sunnybrook Health Sciences Centre

INFORMED CONSENT:

You are being invited to participate in a research study conducted at the Toronto Rehabilitation Institute and Sunnybrook Health Sciences Centre under the supervision of the above investigators. A research study is a way of gathering information on a treatment, procedure or medical device or to answer a question about something that is not well understood. Participation is completely voluntary and you are free to withdraw from the study at any time. A description of this study follows.

This form explains the purpose of this research study, provides information about the study procedures, possible risks and benefits, and the rights of participants. Please read this form carefully and ask any questions you may have. Please ask the study staff or one of the investigators to clarify anything you do not understand or would like to know more about. Make sure all your questions are answered to your satisfaction before deciding whether to participate in this research study.

INTRODUCTION

You are being asked to consider participating in this study because you have coronary artery disease (CAD) and because you are taking part in the Toronto Rehabilitation Institute's Cardiac Rehabilitation Program. As a greater proportion of Canadians reach older ages, there is a need to maintain cognitive function later in life. The knowledge from this study will help us to better understand memory decline in patients with coronary artery disease.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to investigate how certain substances in the blood can affect thinking. It has recently been discovered that certain byproducts of fat breakdown involved in the development of CAD, called **ceramides**, can harm brain cells. This study is being conducted to determine if there is a relationship between the levels of ceramides in the blood and memory decline. In addition, relationships between ceramides and other aspects of brain function, such as thinking speed and the ability to plan and sort information will be explored.

WHAT WILL HAPPEN DURING THIS STUDY?

If you choose to participate in this study, we will notify your TRI physician and your TRI-Cardiac rehab team of your involvement. This study will not interfere with any of the usual care received in rehab or from your family physician.

Baseline Visit:

If you agree to participate in this study, we would ask to review information that you have provided to the rehab team including demographic data (age, gender and diagnoses), what medications you are using, and the results of your exercise tests in the past year. If you agree to participate, you will be asked to undergo an assessment with a trained researcher that will take about 2 hours. This will include assessments of memory and thinking speed, and a screening interview for depression or substance abuse. We are assessing depressive symptoms as it is not uncommon for CAD rehab patients to show signs of depression. You will be asked to complete a few simple depression questionnaires assessing your mood and anxiety. For the cognitive scales you will be asked to complete a few verbal and visual tasks and reproduce a few simple shapes on paper. With your permission, we would notify your Toronto Rehabilitation team if the results of this interview suggest you might benefit from the resources that are already in place to assist subjects showing signs of depression or cognitive impairment. These resources include the opportunity to make appointments with a psychologist on staff at the Toronto Rehab. *At this baseline visit* approximately 2½ tablespoons of blood will be drawn.

If the results from the interview or blood sample show clinical abnormalities, with your permission, we will contact your physician at TRI.

Visit 2 (3 months) and Visit 3 (6 months):

After the initial baseline visit, you would return for 2 in-clinic visits, each lasting approximately 2 hours. Visits 2 and 3 will take place 3 months and 6 months after your initial visit. At each visit you will be asked to complete a number of paper and pencil assessment questionnaires. If you choose to participate in this research study, it will be necessary to collect some fasting blood samples for analysis. At visits 2 and 3 another approximately 2½ tablespoons of blood will be drawn. All blood samples will be identified by a unique number only (not your name). All samples will be analyzed for only these markers needed for the study and then destroyed once the assay is complete.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

It is anticipated that about 129 people recruited from the Toronto Rehabilitation Institute will participate in the study conducted with Sunnybrook Health Science Centre. The length of this

study for participants is 6 months. The entire study is expected to take about 3 years to complete and the results should be known in 3½ years.

WHAT ARE THE RESPONSIBILITIES OF STUDY PARTICIPANTS?

If you decide to participate in this study you will be asked to do the following:

Attend 3 visits at Sunnybrook Health Sciences Centre (2075 Bayview Avenue, Room EG04). Each visit will last approximately 2 hours. You will be asked to complete a number of questionnaires, as well as give a blood sample at each visit.

WHAT ARE THE RISKS OR HARMS OF PARTICIPATING IN THIS STUDY?

There are no medical risks to you from participating in this study, as this is an observational study and does not involve a medical intervention but taking part in this study may make you feel uncomfortable. Blood draw: As with any blood test, you may experience slight discomfort or bruising.

Cognitive testing: You may experience mental stress as a result of memory or timed tasks.

WHAT ARE THE BENEFITS OF PARTICIPATING IN THIS STUDY?

You may or may not benefit directly from participation in this study. Your participation may or may not help other people with coronary artery disease in the future. Knowledge gained from this study may be helpful to subjects in the future in the management of depressive symptoms or cognitive changes resulting from heart disease. As mentioned, the results may suggest that you would benefit from existing Toronto Rehabilitation Institute resources. The study results will be published, and if you wish, we will be happy to forward to you a copy of any publication(s) that may arise from this work.

CAN PARTICIPATION IN THIS STUDY END EARLY?

You can choose to end your participation at any time. If you withdraw voluntarily from the study, the information about you that was collected before you left the study will still be used. No new information about you will be collected without your permission.

WHAT ARE THE COSTS OF PARTICIPATING IN THIS STUDY?

Participation in this study will not involve any additional costs to you.

ARE STUDY PARTICIPANTS PAID TO PARTICIPATE IN THIS STUDY?

You will not be paid to participate in this study. However you will be reimbursed \$23.00 for parking expenses each time you visit Sunnybrook for the purposes of this study.

DO THE INVESTIGATORS HAVE ANY CONFLICTS OF INTEREST?

There are no conflicts of interest to declare related to this study.

WHAT ARE THE RIGHTS OF PARTICIPANTS IN A RESEARCH STUDY?

All participants in a research study have the following rights:

1. You have the right to have this form and all information concerning this study explained to you and if you wish translated into your preferred language.
2. Participating in this study is your choice (voluntary). You have the right to choose not to participate, or to stop participating in this study at any time without having to provide a reason. If you choose to withdraw, your choice will not have any effect on your current or future medical treatment.
3. You have the right to receive all significant information that could help you make a decision about participating in this study. You also have the right to ask questions about this study and your rights as a research participant, and to have them answered to your satisfaction, before you make any decision. You also have the right to ask questions and to receive answers throughout this study. If you have any questions about this study you may contact the person in charge of this study (Principal Investigator) Dr. Lanctôt, Department of Psychiatry at [416-480 6100 x2241](tel:416-480-6100). If you have questions about your rights as a research participant or any ethical issues related to this study that you wish to discuss with someone not directly involved with the study, you may call Dr. Philip C. Hébert, Chair of the Sunnybrook Research Ethics Board at (416) 480-4276.
4. You have the right to have any information about you and your health that is collected, used or disclosed for this research study to be handled in a confidential manner.

If you decide to participate in this study, the investigator(s) and study staff will look at your personal health information and collect only the information they need for this study. “Personal health information” is health information about you that could identify you because it includes information such as your;

- name,
- address,
- telephone number,
- date of birth,
- new and existing medical records, or
- the types, dates and results of various tests and procedures.

The following people may come to the hospital to look at your personal health information to check that the information collected for the study is correct and to make sure the study followed the required laws and guidelines:

Representatives of the Sunnybrook Research Ethics Board, a group of people who oversee the ethical conduct of research studies at Sunnybrook.

Access to your personal health information will take place under the supervision of the Principal Investigator. In addition, any study data about you that is sent outside of the hospital will have a code and will not contain your name or address, or any information that

directly identifies you. "Study data" is information about you that is collected for the research study, but that does not directly identify you. Study data that is sent outside of the hospital will be used for the research purposes explained in this consent form.

The investigator(s), study staff and the other people listed above will keep the information they see or receive about you confidential, to the extent permitted by applicable laws. Even though the risk of identifying you from the study data is very small, it can never be completely eliminated.

When the results of this study are published, your identity will not be disclosed. The Principal Investigator will keep any personal information about you in a secure and confidential location for 25 years and then destroyed as required by Sunnybrook policy.

1. By signing this consent form, you do not give up any of your legal rights.
2. You have the right to receive a copy of this signed and dated informed consent form before participating in this study. You have the right to be told about any new information that might reasonably affect your willingness to continue to participate in this study as soon as the information becomes available to the study staff.
3. You have the right to access, review and request changes to your personal health information.
4. You have the right to be informed of the results of this study once the entire study is complete.

Contacts:

If you have any questions about this study or for more information you may contact the Study Co-ordinator, Russanthy Velummailum (416-480-6100 x3185), Dr. Krista Lanctôt (416-480-6100 x2241) or Dr. Paul Oh (416-597-3422 x5263).

Should you have any questions about your rights as a research subject, you may contact the Vice Chair of the UHN Rehabilitation Medicine and Sciences Research Ethics Board at (416) 597-3422 x3081 or the Sunnybrook Health Sciences Centre Research Ethics Board at (416) 480-4276. DOCUMENTATION OF INFORMED CONSENT

Full Study Title: **The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease**

Name of Participant: _____

Participant/Substitute decision-maker

By signing this form, I confirm that:

- This research study has been fully explained to me and all of my questions answered to my satisfaction
- I understand the requirements of participating in this research study
- I have been informed of the risks and benefits, if any, of participating in this research study
- I have been informed of any alternatives to participating in this research study
- I have been informed of the rights of research participants
- I have read each page of this form
- I authorize access to my personal health information, medical record and research study data as explained in this form
- I have agreed to participate in this study or agree to allow the person I am responsible for to participate in this study

Name of participant/Substitute decision-maker (print)	Signature	Date
_____	_____	_____

Person obtaining consent

By signing this form, I confirm that:

- This study and its purpose has been explained to the participant named above
- All questions asked by the participant have been answered
- I will give a copy of this signed and dated document to the participant

Name of Person obtaining consent (print)	Signature	Date
_____	_____	_____

Statement of Investigator

I acknowledge my responsibility for the care and well being of the above participant, to respect the rights and wishes of the participant as described in this informed consent document, and to conduct this study according to all applicable laws, regulations and guidelines relating to the ethical and legal conduct of research.

Name of Investigator (print)	Signature	Date
_____	_____	_____

Full Study Title: **The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease**

Name of Participant: _____

Participant/Substitute decision-maker

By signing this form, I confirm that:

- This research study has been fully explained to me and all of my questions answered to my satisfaction
- I understand the requirements of participating in this research study
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- I have been informed of any alternatives to participating in this research study
- I have been informed of the rights of research participants
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- I authorize access to my personal health information, medical record and research study data as explained in this form
- I have agreed to participate in this study or agree to allow the person I am responsible for to participate in this study

Name of participant/Substitute decision-maker (print)

Signature

Date

Person obtaining consent

By signing this form, I confirm that:

- This study and its purpose has been explained to the participant named above
- All questions asked by the participant have been answered
- I will give a copy of this signed and dated document to the participant

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Date

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Date
