Biomarkers of Ceramide Accumulation and Decline in Verbal Memory in Coronary Artery Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Background: Biomarkers in cognitively vulnerable populations like those with coronary artery disease (CAD) may inform earlier intervention in vascular neurodegeneration. Circulating ceramide C18:0 (CerC18:0) is associated with cognitive changes in early neurodegeneration and CAD progression.

Objective: To investigate whether plasma CerC18:0 accumulation is associated with longitudinal declines in verbal memory in CAD.

Methods: In addition to CerC18:0, we assessed its relative abundance to its precursors as ratios: to monohexosylceramide C18:0 (CerC18:0/MHxCer18:0), to sphingomyelin C18:0 (CerC18:0/SM18:0), and to sphingosine-1-phosphate (CerC18:0/S1P). In 60 CAD participants, we evaluated associations between baseline CerC18:0 and its ratios to changes in verbal memory, adjusted for potential confounders.

Results: Increased baseline CerC18:0 ($b$[SE]=−0.91[0.30], $p$=0.003), CerC18:0/MHxCer18:0 [(b[SE]=−0.90[0.40], $p$=0.03)], CerC18:0/SM18:0 (b[SE]=−1.11[0.36], $p$=0.004) correlated with steeper declines in verbal memory.
Conclusions: Aberrant CerC18:0 metabolism may be an early neurobiological change in vascular neurodegeneration. Future studies should measure enzymatic activity responsible for conversion of precursors into CerC18:0.
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Table of Contents

Acknowledgements ................................................................................................................................................ iv
List of Tables ......................................................................................................................................................... viii
List of Figures ......................................................................................................................................................... ix
List of Abbreviations ............................................................................................................................................ x

Chapter 1: Introduction ........................................................................................................................................ 1
  1.1. Statement of Problem .................................................................................................................................. 1
  1.2. Study Purpose and Objectives ....................................................................................................................... 2
  1.3. Hypotheses and Corresponding Rationale ..................................................................................................... 2
    1.3.1. Primary Hypothesis ................................................................................................................................. 2
    1.3.2. Secondary Hypothesis ............................................................................................................................ 3
    1.3.3. Tertiary Hypothesis ................................................................................................................................. 5
    1.3.4. Exploratory Hypothesis .......................................................................................................................... 5
  1.4. Review of literature ........................................................................................................................................ 6
    1.4.1. Coronary artery disease ......................................................................................................................... 6
    1.4.2. Coronary artery disease and cognitive decline ....................................................................................... 7
    1.4.3. Potential pharmacotherapy for vascular cognitive impairment in CAD ........................................... 8
    1.4.4. Biomarkers of vascular cognitive change in CAD .............................................................................. 11
    1.4.5. Ceramides as a potential mediator between CAD and cognitive decline ........................................ 12
    1.4.6. Ceramides in CAD and neurodegeneration ......................................................................................... 13

Chapter 2: Materials and Methods ....................................................................................................................... 16
  2.1. Study design .................................................................................................................................................. 16
  2.2. Study participants ......................................................................................................................................... 16
    2.2.1. Eligibility criteria ................................................................................................................................... 16
  2.3. Demographics and medical history ............................................................................................................. 18
  2.4. Neurocognitive battery and assessment of depressive symptoms ........................................................... 18
    2.4.1. Calculation of composite Z-scores ........................................................................................................ 19
    2.4.2. Possible vascular cognitive impairment, no dementia ........................................................................ 20
2.5. Biochemical assays .......................................................................................................................... 20
  2.5.1. Blood collection .......................................................................................................................... 20
  2.5.2. Measurement of plasma sphingolipids ....................................................................................... 20
2.6. Selection of covariates ....................................................................................................................... 21
  2.6.1. Age .............................................................................................................................................. 21
  2.6.2. Body-mass index ....................................................................................................................... 22
  2.6.3. Years of education .................................................................................................................... 22
2.7. Statistical analyses ............................................................................................................................ 22
  2.7.1. Data pre-processing .................................................................................................................. 22
  2.7.2. Characterization of study participants and comparison of sub-cohorts ..................................... 23
  2.7.3. Longitudinal analyses .............................................................................................................. 23
2.8. Calculation of sample size ................................................................................................................. 24

Chapter 3: Results ........................................................................................................................................ 26
  3.1. Recruitment of study participants ................................................................................................. 26
  3.2. Differences between study participants who returned and those who did not ...................... 27
  3.3. Associations between sociodemographic and clinical characteristics and change in verbal memory performance in all study participants ................................................................. 28
  3.4. Associations between sociodemographic and clinical characteristics and change in verbal memory performance in study participants with possible VCIND ............................................ 30
  3.5. Sociodemographic and clinical differences between those with VCIND and those with no possible VCIND ........................................................................................................................................ 31
  3.6. Trajectory of verbal memory performance throughout the study .............................................. 32
  3.7. Covariate multi-collinearity and normalization of plasma sphingolipid concentrations ......... 33
  3.8. Primary: Associations between baseline CerC18:0 and change in verbal memory performance ........................................................................................................................................ 38
  3.9. Secondary: Associations between CerC18:0/MHxCerC18:0 ratio and change in verbal memory ........................................................................................................................................ 38
  3.10. Tertiary: Associations between CerC18:0/SM18:0 ratio and change in verbal memory .................................................................................................................................................................... 39
  3.11. Exploratory analyses: Differences in associations between those with possible VCIND and those with no possible VCIND ........................................................................................................ 40
  3.11.1. Association between CerC18:0 and change in verbal memory performance ..................... 40
3.11.2. Association between CerC18:0/MHxCerC18:0 and change in verbal memory performance

3.11.3. Association between CerC18:0/SM18:0 and change in verbal memory performance

3.11.4. Association between CerC18:0/S1P and change in verbal memory performance

3.12. Summary of Results

Chapter 4: Discussion and Recommendations for Future Studies

4.1. Study Findings and Interpretations

4.1.1. Characterization of study participants and comparison of sub-cohorts

4.1.2. Primary Findings

4.1.3. Secondary Findings

4.1.4. Tertiary Findings

4.1.5. Exploratory Hypothesis

4.2. Pathological mechanisms of ceramides in neurodegeneration

4.3. Strengths

4.4. Limitations

4.4.1. Methodological limitations with recommendations for future studies

4.4.2. Mechanistic Limitations

4.5. Implications on drug development

4.6. Conclusions

References

Appendix 1: Approval from Research Ethics Board

Appendix 2: Informed Consent Form
List of Tables

Table 1: Comparison of study participants who returned and those who did not ..................28
Table 2: Associations with change in verbal memory in overall population .......................30
Table 3: Associations with change in verbal memory in those with possible VCIND .............31
Table 4: Comparison of those with possible VCIND and those without ............................32
Table 5: Change in verbal memory performance over course of the study ..........................33
Table 6: Multi-collinearity statistics of covariates ..........................................................33
Table 7: Raw and log-transformed sphingolipid concentrations .....................................34
Table 8: Association between CerC18:0 and change in verbal memory in overall population .................................................................38
Table 9: Association between CerC18:0/MHxCerC18:0 and change in verbal memory in overall population ........................................................................39
Table 10: Association between CerC18:0/SM18:0 and change in verbal memory in overall population ........................................................................39
Table 11: Association between CerC18:0/S1P and change in verbal memory in overall population ........................................................................40
Table 12: Association between CerC18:0 and change in verbal memory in terms of possible VCIND .........................................................................41
Table 13: Association between CerC18:0/MHxCerC18:0 and change in verbal memory in terms of possible VCIND .........................................................................41
Table 14: Association between CerC18:0/SM18:0 and change in verbal memory in terms of possible VCIND .........................................................................42
Table 15: Association between CerC18:0/S1P and change in verbal memory in terms of possible VCIND .........................................................................43
Table 16: Summary of Results .........................................................................................43
List of Figures

Figure 1: Ceramides biosynthesis pathways.................................................................4
Figure 2: Flow of participant recruitment.................................................................27
Figure 3: Trajectory of verbal memory performance throughout study......................32
Figure 4: Normal Q-Q plot of log-transformed CerC18:0........................................34
Figure 5: Normal Q-Q plot of log-transformed SM18:0...........................................35
Figure 6: Normal Q-Q plot of log-transformed MHxCerC18:0.................................35
Figure 7: Normal Q-Q plot of log-transformed S1P..................................................36
Figure 8: Normal Q-Q plot of log-transformed CerC18:0/MHxCerC18:0....................36
Figure 9: Normal Q-Q plot of log-transformed CerC18:0/SM18:0..............................37
Figure 10: Normal Q-Q plot of log-transformed CerC81:0/S1P.................................37
List of Abbreviations

AChEI  Acetylcholinesterase inhibitors
AD    Alzheimer’s Disease
APOE4  Apolipoprotein E4
ASM   Acid sphingomyelinase
BBB   Blood-brain-barrier
BEST  Biomarkers, EndpointS, and other Tools
BMI   Body-mass index
BVMT-R Brief Visuospatial Memory Test-Revised
df    Degrees of freedom
C18:0  Acyl chain length of 18 carbons
CABG  Coronary artery bypass grafting
CAD   Coronary artery disease
Cer   Ceramides
CRP   C reactive protein
CSF   Cerebrospinal fluid
CVLT-II California Verbal Learning Test – 2nd edition
DSM-IV Diagnostic and Statistical manual – Version 4
EDTA  Ethylenediaminetetraacetic acid
FIASMA Functional inhibitors of acid sphingomyelinase
HbA1C Haemoglobin A1C
HDL   High-density lipoproteins
HIV   Human immunodeficiency virus
IL-6  Interleukin-6
LDL   Low-density lipoproteins
MCI   Mild cognitive impairment
MDD   Major depressive disorder
MI    Myocardial infarction
MHxCer Monohexosylceramides
NSM   Neutral sphingomyelinase
NINDS-CSN National Institute of Neurological Disorders and Stroke-Canadian Stroke Network
PD    Parkinson’s Disease
PTCA  Percutaneous transluminal coronary angioplasty
S1P   Sphingosine-1-phosphate
SD    Standard deviation
SE    Standard error
SM    Sphingomyelin
sMMSE Standardized mini mental state examination
SCID  Structured clinical interview for depression
TNF-α Tumour necrosis factor alpha
TRI   Toronto Rehabilitation Institute
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>VCI</td>
<td>Vascular cognitive impairment</td>
</tr>
<tr>
<td>VCIND</td>
<td>Vascular cognitive impairment, no dementia</td>
</tr>
<tr>
<td>VD</td>
<td>Vascular dementia</td>
</tr>
<tr>
<td>VIF</td>
<td>Variance inflation factor</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1. Statement of Problem

Half of Canadians with vascular cognitive impairment, no dementia (VCIND) progress to dementia within 5 years (Wentzel et al., 2001), which results in high institutionalization rates and poor quality of life. Brain damage in those with dementia may be too extensive to be reversed by therapeutic intervention, implicating that intervention at earlier pre-symptomatic stages of dementia may improve clinical outcomes (Becker, Greig, & Giacobini, 2008). In addition, given that there are no recommended drugs to prevent this progressive vascular neurodegeneration, early identification and active control of cardiovascular risk factors are currently considered the only ways to modify the course of this disease (Rockwood, 2002; Ikeda, 2003).

As a result, there is significant interest in the development of biological markers or biomarkers to detect disease signatures in cognitively vulnerable populations. One such population is patients with coronary artery disease (CAD) as those with CAD experience increased brain atrophy (Koschack & Irle, 2005; Barekatain et al., 2014), increased white matter lesions (Debette et al., 2007), and an increased risk of memory impairment (Vinkers et al., 2005). Consequently, this clinical population is approximately 2 times more likely to develop incipient neurodegenerative disorders such as VCIND (Roberts et al., 2010), which may progress to dementia (van Oijen et al., 2007). Because of this increased risk of neurodegeneration, those with CAD are an ideal prodromal population to identify biomarkers of early cognitive changes and identify potential therapeutic targets, which may improve our understanding of the
relationship between CAD and neurodegeneration (Buratti et al., 2015).

1.2. **Study Purpose and Objectives**

The purpose of the present study is to evaluate the association between baseline concentration of ceramides and verbal memory performance over time in a CAD population. This information will help us determine whether ceramides are a clinically important correlate of cognitive decline in a population that is at-risk of developing vascular dementia (VD) in the future. Furthermore, this research may inform us whether there are relevant targets in the biosynthesis pathways of sphingolipids for drug discovery initiatives.

1.3. **Hypotheses and Corresponding Rationale**

1.3.1. **Primary Hypothesis**

*Primary hypothesis:* Greater plasma concentrations of ceramides with an acyl chain length of 18 carbons (CerC18:0) at baseline will predict greater decline in verbal memory performance over time in CAD participants.

*Rationale:* Aberrant CerC18:0 metabolism is consistently associated with both cardiovascular and neurodegenerative diseases. Plasma levels of CerC18:0 are elevated in coronary plaques, a characteristic pathology of CAD (Uchida et al., 2017). Evidence from numerous studies also implicates CerC18:0 in the biochemical profile of various neurodegenerative diseases. In adults at-risk of Alzheimer’s disease (AD), cerebrospinal fluid (CSF) CerC18:0 was associated with higher CSF β-amyloid and higher CSF tau levels, which are hallmark pathologic markers of AD (Mielke et al., 2014). Notably, these associations were stronger among individuals aged 54 years and older, an age range during which the prevalence of CAD increases. In a cognitively
asymptomatic population, higher levels of serum CerC18:0 were associated with an increased risk of incident memory impairment over 9 years of follow-up (Mielke, Bandaru, Haughey, et al., 2010). In combination with its biological role as a pro-apoptotic signalling molecule (Obeid, Linardic, Karolak, & Hannun, 1993), CerC18:0 may be involved in the etiopathology of CAD and neurodegeneration. Collectively, this evidence coupled with the abundance of CerC18:0 in brain tissue (Filippov et al., 2012) demonstrates the potential and feasibility of CerC18:0 as a biomarker of the pre-symptomatic phases of cognitive impairment due to vascular causes.

Compelling evidence suggests that changes in verbal memory may be one of the earliest events in cognitive decline caused by vascular pathology. A previous study elucidated the neuropsychological profiles of patient populations along the spectrum of vascular cognitive impairment (VCI): healthy elders, those at-risk of cerebrovascular disease, those with VCIND, and those with VD. Those with VCIND had significantly worse verbal memory compared to the healthy participants, while performing within normative ranges in other cognitive domains (Garrett et al., 2004). Moreover, our laboratory previously reported that plasma CerC18:0 concentrations significantly correlated with verbal memory performance over 6 months of cardiac rehabilitation (CR) (Saleem et al., 2017). However, it remains to be seen whether this association can be observed over a longer period of time in which cognitive deterioration may become more apparent.

1.3.2. **Secondary Hypothesis**

*Secondary hypothesis:* Greater plasma ratios of CerC18:0 relative to monohexosylceramide C18:0 (CerC18:0/MHxCerC18:0) will predict greater decline in verbal memory performance over time in CAD participants.
**Rationale:** Given that the majority of sphingolipids are synthesized within the body (Lee et al., 2004; Jana & Pahan, 2007), CerC18:0 abnormalities are likely the result of enzymatic imbalances, which can be represented empirically by ratios. Ratios of sphingolipids have been reported to be stronger predictors of cognitive decline than individual sphingolipids (Mielke et al., 2011), but this has not been investigated in the context of VCI.

Briefly, ceramides are generated through four biosynthesis pathways (Kitatani, Idkowiak-Baldys, & Hannun, 2008) (Figure 1). The salvage pathway involves the catabolism of monohexosylceramides (MHxCer) such as galactosylceramides and glucosylceramides to generate ceramides. In the catabolic pathway, sphingomyelinase breaks sphingomyelin (SM) down into ceramides. The *de novo* pathway synthesizes ceramides from palmitoyl coenzyme A and serine. Ceramides may also be formed from sphingosine-1-phosphate (S1P) through the recycling pathway. This relationship should be particularly apparent with MHxCerC18:0 as the pre-cursor as the salvage pathway accounts for 50-90% of sphingolipid generation (Kitatani et al., 2008); thus dysregulation of this pathway may have greater cognitive impact.
Figure 1: The four primary biochemical pathways through which ceramides are synthesized.

1.3.3. Tertiary Hypothesis

*Tertiary hypotheses:* Greater plasma ratios of CerC18:0 relative to sphingomyelin C18:0 (CerC18:0/SM18:0) and sphingosine-1-phosphate (CerC18:0/S1P) will predict greater decline in verbal memory performance over time in CAD participants.

*Rationale:* Elevated peripheral inflammation may induce CerC18:0 biosynthesis through the catabolic pathway (Zeidan & Hannun, 2010). Since levels of pro-inflammatory cytokines are frequently observed to be elevated in those with CAD, a significant association between CerC18:0/SM18:0 and verbal memory performance may suggest the importance of peripheral inflammation in perpetuating vascular cognitive decline.

S1P has been touted to be neuroprotective in AD (Couttas et al., 2014; Ceccom et al., 2014), while involvement of CerC18:0 in pro-apoptotic pathways suggests that it contributes to neurodegeneration. Collectively, the antagonizing biochemical functions of these sphingolipids suggest that a ratio of CerC18:0/S1P may be sensitive to changes in verbal memory performance in CAD participants.

1.3.4. Exploratory Hypothesis

*Exploratory Hypothesis:* Inverse associations between baseline CerC18:0 ratios and change in verbal memory performance will be significantly different between those with possible VCIND and those without possible VCIND.
Rationale: These changes may be particularly important in those with VCIND as this is an intermediate stage between normal cognition and VCI. Moreover, therapeutic intervention or CR may be more beneficial in early prodromal states as a more preventative approach (Intzandt et al., 2015). Our exploratory hypothesis focuses on study participants with possible VCIND, because the neuropathology may be too advanced by the time the stricter neurocognitive criteria of VCIND is met (Gorelick et al., 2011). Moreover, evidence shows that cognitive decline is more rapid following onset of detectable deficits (Stern et al., 1994).

1.4. Review of literature

1.4.1. Coronary artery disease

CAD is a leading contributor to global mortality as it is globally responsible for an estimated 32% of deaths (Mortality & Causes of Death, 2015). Characterized by significant narrowing of one or more major coronary arteries (defined as ≥50% of diameter), CAD substantially reduces the flow of oxygen-rich blood to the heart. These blockages, otherwise known as coronary stenosis, are formed through the process of atherosclerosis, in which leukocytes, lipid deposits, and collagen-rich extracellular matrix form plaques, ultimately thickening the arterial wall. In more severe cases, CAD can precipitate myocardial infarction (MI), angina, and even death (Nabel & Braunwald, 2012).

Lifestyle choices and vascular risk factors such as smoking, hyperlipidemia, diabetes, hypertension, obesity, and peripheral inflammation contribute to the incidence and progression of CAD (Jousilahti, Vartiainen, Tuomilehto, & Puska, 1999; Canto et al., 2011; Rimm et al., 1995). Fortunately, pharmacological interventions (e.g. statins) and non-pharmacological interventions (i.e. dietary modifications, physical exercise during CR) are effective in managing CAD progression (Task Force et al., 2013; Heran et al., 2011). However, in CAD patients with
severe arterial occlusions or those at high-risk of MI, revascularization procedures such as coronary artery bypass grafting (CABG) or percutaneous transluminal coronary angioplasty (PTCA) may be required.

1.4.2. Coronary artery disease and cognitive decline

Epidemiologically, CAD has long been associated with increased likelihood of developing dementia in later life. In a longitudinal study, a history of CAD in cognitively normal elderly adults was associated with significantly greater rates of cognitive decline (Zheng et al., 2012). A recent systematic meta-analysis of 24,801 persons across 10 prospective studies quantified this association, reporting that those with CAD have 45% increased odds of developing cognitive impairment or dementia at follow-up (Deckers et al., 2017). An autopsy study elucidated this relationship at the pathological level. The authors reported that extent of CAD correlated significantly with AD-related neuropathology like neurofibrillary tangles (Beeri et al., 2006). However, most studies do not distinguish between cognitive decline secondary to vascular risk factors or AD-related neurodegeneration. While their perpetuating interactions are well-recognized, it is important to make a distinction between these different etiologies as it may improve our ability to accurately predict the prognosis of a person with dementia and provide unique targets for therapeutic interventions. In response to this need, the concept of VCI has emerged.

However, it is clinically difficult to study the development of both diseases as they rarely emerge concurrently in the same patient. This is likely because VCI follows a chronic disease model (White et al., 2002; Roman et al., 2004). Briefly, a disease that follows this progression model begins latently, presenting with molecular damage and no changes in functions or behaviour. This is followed by a prodromal stage with greater molecular damage and mild
changes in function. Ultimately, the syndrome progresses to dementia, which is typically irreversible (Katzman, 1976). Since CAD is generally diagnosed before onset of dementia, many patients may experience profound molecular-level changes and express mild cognitive impairment attributable to these cardiovascular risk factors, but do not satisfy neuropsychological criteria for dementia. This is otherwise known as VCIND.

Specifically, compelling evidence suggests that changes in verbal memory may be one of the earliest events in cognitive decline caused by vascular pathology. A previous study elucidated the neuropsychological profiles of patient populations along the spectrum of cognitive decline due to cardiac risks: healthy elders, those at-risk of cerebrovascular disease, those with VCIND, and those with VD. Those with VCIND had significantly worse verbal memory compared to the healthy participants, while performing within normative ranges in other cognitive domains (Garrett et al., 2004). As such, this study will investigate longitudinal changes in verbal memory in CAD participants.

1.4.3. **Potential pharmacotherapy for vascular cognitive impairment in CAD**

No pharmacotherapy has been successfully developed for VCI. Acetylcholinesterase inhibitors (AChEI) originally indicated for AD, have been proposed to slow progression from VCI to dementia, based on findings that demonstrate cerebrovascular damage affects cholinergic tracts (Moretti et al., 2008; Humpel, 2011). However, a meta-analysis of randomized controlled trials reported that AChEIs only improve cognition by half of the magnitude observed in previous AD trials (Russ & Morling, 2012) and is further obscured by an increased incidence of adverse events (Rojas-Fernandez, 2013). Memantine, an N-methyl-D-aspartate antagonist, is another drug that is approved for moderate-severe AD. Like the AChEIs, memantine demonstrated
minimal, clinically insignificant improvement in cognition scores in those with VCI (McShane, Areosa Sastre, & Minakaran, 2006).

It comes with little surprise when clinical consensus from the fourth Canadian Consensus Conference on the Diagnosis and Treatment of Dementia recommended against using AChEIs in VCI patients, unless there is comorbid AD (Gauthier et al., 2012). In contrast, the American Heart Association and Canadian Stroke Best Practices recommend that AChEIs could be considered for use in those with VCI in light of limited therapeutic options, but notes that this is only supported by intermediate grade evidence (Eskes et al., 2015; Gorelick et al., 2011).

Other therapies have also been investigated in the context of VCI. The potential of a serotonin 5-HT$_2$ receptor antagonist, naftidrofuryl, was evaluated, because of its ability to increase availability of oxygen and energetic mediators in cerebral tissue through vasodilation (James, Newbury, & Woolard, 1978). Despite its theoretical benefits, naftidrofuryl has yet to be shown to be an effective treatment in clinical trials (Lu, Song, Hao, Wu, & McCleery, 2011). Another class of drugs, calcium channel blockers, have been investigated as a potential treatment of VCI, because their ability to restrict calcium influx into neural cells decreases likelihood of excitotoxicity, which is hypothesized to contribute to neuropathologies like AD (Harkany et al., 2000). For instance, nomidipine crosses the blood-brain-barrier (BBB) and decreases calcium ion influx into cells; thus, modulating synaptic excitability and neurotransmission. Clinically, however, its use is controversial as its short-term cognitive benefits do not justify its long-term use in dementia patients (Lopez-Arrieta & Birks, 2002). Similar results were observed with nilvadipine, leading to conclusions stating that there is insufficient clinical trial evidence to support its use (Hanyu et al., 2007). Likewise, other calcium channel blockers like amlodipine
have been investigated in this regard, but its inability to cross the BBB prevents further investigation (Paris et al., 2011).

In those with mild cognitive impairment, current evidence suggests that peripheral management of cardiovascular risk factors may be effective prophylaxis (Langa & Levine, 2014). Peripheral endothelial dysfunction is a primary concern, because it may reflect impairment in cerebral blood perfusion and contribute to the pathogenesis of AD and VD (Zuliani et al., 2008). As such, existing cardiovascular medications such as β-blockers and angiotensin-converting-enzyme inhibitors that improve endothelial function may potentially be effective in improving endothelial function (Peller et al., 2015).

The failure to develop effective treatments for VCI is likely multi-factorial. First, the clinical concept of VCI is in its infancy; it has only been recently recognized as a syndrome that can be caused by multiple cerebrovascular diseases. As such, therapies may need to be designed to target specific etiologic mechanisms (Smith et al., 2017). This stagnancy in drug development is compounded by a lack of reliable biomarkers. Currently, neuroimaging biomarkers are preferred to detect cerebrovascular damage, but they are expensive, unable to detect early microstructural cerebrovascular damage (Baykara et al., 2016), and ultimately may not correlate with cognition as in the case of white matter hyperintensities (Schmidt et al., 2012). An alternative physiological medium is blood. Although blood-based biomarkers may be difficult to develop due to the uncertain link between peripheral markers and central cognitive processes, a reliable blood-based biomarker would cheap, non-invasive, and more easily incorporated into clinical care for those in the early stages of VCI (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group," 1998).

Furthermore, considering that dementia symptoms may not become clinically evident until
neuropathology becomes irreversible, regulatory agencies like the Food and Drug Administration are starting to accept the role of biomarkers as surrogate clinical trial outcomes in drug development for dementia (The Food and Drug Administration, 2018).

1.4.4. Biomarkers of vascular cognitive change in CAD

Since VCI does not become clinically apparent until later stages despite early molecular damage, prognostic biomarkers that are indicative of pathologic biological processes may help to predict clinical progression as suggested by the Biomarkers, EndpointS, and other Tools (BEST) guidelines (Group, 2016). However, identifying robust biomarkers of VCI is difficult due to its heterogeneous nature as well as the presence of vascular risk factors in patients presenting with AD-like characteristics. As a result, there is no standardized blood-based biomarker in the literature ready for clinical use.

The BBB is a selectively permeable barrier that separates the brain’s circulation from the rest of the body’s. BBB dysfunction may contribute to the pathogenesis of VCI as its integrity is clearly compromised in animal models that exhibit symptoms of VD like rats with chronic carotid hypoperfusion (Ueno, Tomimoto, Akiguchi, Wakita, & Sakamoto, 2002; Ueno et al., 2016). This observation has been extrapolated to humans using the CSF/serum albumin quotient, which is indicative of clinical BBB leakage because liver-made albumin does not normally appear in the CSF. Thus, a greater quotient indicates more severe BBB compromise. Indeed, in a meta-analysis of clinical studies, the CSF/serum albumin quotient is particularly high in those with VCI, compared to AD (Farrall & Wardlaw, 2009). However, CSF/serum albumin quotient is elevated in both VCI and AD compared to control, making differentiation in the presence of AD difficult.
This increased BBB permeability allows the measurement of blood-based biomarkers to be reflective of their levels in the brain. In particular, peripheral inflammatory markers have been extensively studied as it is a key contributor to the progression of cardiovascular and cerebrovascular disease (Rosenberg, 2009). In the vasculature, interleukin-6 (IL-6) is secreted as a pro-inflammatory cytokine, which in turn, triggers the production of C reactive protein (CRP) from the liver (Yoshida et al., 2010). Elevated levels of CRP and IL-6 are both associated with increased risk of incident VD (Ravaglia et al., 2007; Engelhart et al., 2004). In a neuroimaging study, CRP is found to be associated with decline in white matter microstructural integrity (Wersching et al., 2010). Moreover, sulfatide, a prominent glycosphospholipid in oligodendrocyte-produced myelin sheaths, has been reported to be severely elevated in the CSF of those with VCI-SSVD in relation to controls and those with AD, suggesting white matter degradation (Fredman et al., 1992).

In 2006, the US National Institute for Neurological Disorders and Stroke and the Canadian Stroke Network convened and harmonized expert recommendations for biomarkers in VCI (Hachinski et al., 2006). While multiple biomarkers for VCI have been proposed, they concluded that none were ready for standardization and widespread clinical application.

1.4.5. Ceramides as a potential mediator between CAD and cognitive decline

Sphingolipids are characterized by an 18-carbon amino-alcohol backbone, known as sphingosine. Modification of this fundamental structure results in various sub-classes of sphingolipids. Sphingomyelins are produced by the attachment of a phosphocholine headgroup and are the most abundant sphingolipids in humans (Gault, Obeid, & Hannun, 2010). A more complex sphingolipid sub-class is the glycosphingolipids, which can be further sub-divided into galactosphingolipids and glucosphingolipids depending on the sugar residue attached to the
head group. Ceramides have a sphingosine base attached to a fatty acid of varying chain length via an amide linkage. As ceramides are our sub-class of interest due to their role in apoptotic signalling, the rest of this biochemical review will focus on them.

Ceramides are generated through four main biosynthesis pathways (Kitatani et al., 2008) (Figure 1). The salvage pathway involves the catabolism of monohexosylceramides such as galactosylceramides and glucosylceramides to generate ceramides. In the catabolic pathway, sphingomyelinase breaks down sphingomyelin into ceramides. The de novo pathway synthesizes ceramides from palmitoyl coenzyme A and serine. Ceramides may also be formed from sphingosine through the recycling pathway.

Initially thought to be an inert component of cellular membranes, sphingolipids are now widely recognized to be involved in essential cellular signalling processes. For instance, the hydrolysis of sphingomyelins by sphingomyelinases results in the production of ceramides, which function as second messengers to modulate pro-apoptotic pathways (Hannun & Bell, 1989) and govern cellular responses to stress (Hannun, 1996). Given their involvement in fundamental pathways that determine cellular fate, dysregulation of ceramide metabolism has been implicated in the pathophysiology of various diseases including diabetes (Galadari, Rahman, Pallichankandy, Galadari, & Thayyullathil, 2013), cancer (Morad & Cabot, 2013), atherosclerosis (Bismuth, Lin, Yao, & Chen, 2008), and neurodegenerative disorders (Mencarelli & Martinez-Martinez, 2013).

1.4.6. Ceramides in CAD and neurodegeneration

Although the biochemical mechanisms by which aberrant sphingolipid metabolism can cause structural brain lesions and precipitate cognitive decline in CAD are unclear, their presence as a frequently observed pathophysiological feature in both CAD and cognitive decline suggests that
they may be a potential mediator between the two diseases. Ubiquitous in membranes, ceramides have been found to independently escalate the progression of both diseases, but whether they are a mediator of one to another is a question that has yet to be answered.

Ceramides propagate worsening of atherosclerosis. For instance, in those with CAD, ceramides levels were found to be higher in those with unstable coronary plaques relative to those with stable angina (Uchida et al., 2017). During plaque formation, low-density lipoproteins (LDL) transport sphingomyelins to the arterial wall, where sphingomyelinases cleave them to yield ceramides. This accumulation of ceramides causes LDL aggregation, which induces foam cell formation and consequently, atherogenic formation (Xu & Tabas, 1991). This mechanism is sustained by chronic inflammation that is often observed in those with CAD (Hansson, 2005), to which tumour necrosis factor alpha (TNF-α) is a prominent contributor (Hannun, 1996). Of interest, a specific ceramide species with an acyl chain length of 18 carbons (CerC18:0) is associated with significantly increased risk of cardiovascular death in stable CAD patients and in those with acute coronary syndromes (Laaksonen et al., 2016).

Evidence from numerous studies implicates the role of CerC18:0 in the profile of biochemical perturbations in various neurodegenerative states. In adults genetically at-risk of AD, CerC18:0 in cerebrospinal fluid was associated with build-up of β-amyloid and tau proteins (Mielke et al., 2014). Notably, these associations were stronger in individuals aged 54 years and older, an age range that is similar to that of individuals with CAD. In subjects infected with the human immunodeficiency virus (HIV), CerC18:0 in cerebrospinal fluid was a strong predictor of performance in multiple cognitive domains (Mielke, Bandaru, McArthur, Chu, & Haughey, 2010). Even in a cognitively asymptomatic population, higher levels of CerC18:0 predicted an increased risk of incident verbal memory impairment (defined as <1.5 standard deviation (SD)
below standard norms) over 9 years of follow-up (Mielke, Bandaru, Haughey, et al., 2010). These sphingolipid abnormalities in neurodegenerative processes are likely the result of enzymatic imbalance as the majority of sphingolipids are synthesized within the body (Lee et al., 2004; Jana & Pahan, 2007).
2.1. **Study design**

This observational, longitudinal study investigated associations between plasma sphingolipid concentrations at baseline and verbal memory performance over time. This study was approved by research ethics boards at both the Sunnybrook Health Sciences Centre and Toronto Rehabilitation Institute (TRI) in the University Health Network. The results from this observational study are reported in accordance to the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) guidelines (von Elm et al., 2007).

2.2. **Study participants**

All study participants were recruited from a CR program at the Rumsey Centre of University Health Network - TRI and provided written, informed consent to participate before study enrolment and assessment for inclusion and exclusion criteria.

2.2.1. **Eligibility criteria**

Patients were included into the study if they met all of the following inclusion criteria:

- Between age 45-80
- Spoke and understood English
  - To complete assessments
- Diagnosis of CAD based on at least one of the following:
  - Coronary angiographic evidence of ≥50% blockage in ≥1 major coronary artery
  - Previous hospitalization for acute myocardial infarction
  - Had undergone a prior revascularization procedure (PTCA or CABG)
- Previous diagnosis of ischemic heart disease
- Stable CAD defined as no hospitalizations for cardiac events such as an acute MI, unstable angina or coronary revascularization procedure within 4 weeks of baseline assessment
- Statin medication use
  - Because statins may lower plasma sphingolipid concentrations (Bergheanu et al., 2008; Tarasov et al., 2014).

Conversely, patients were excluded from the study if they met any of the following criteria that could affect interpretation of outcomes:

- Significant acute medical illness (including active cancer, anemia, autoimmune condition, drug overdose, hypothyroidism, sepsis, severely impaired kidney, liver, or lung function, uncontrolled diabetes mellitus)
- A history of a neurodegenerative disease (dementia, Parkinson’s, Huntington’s chorea)
- Co-morbid psychiatric diagnosis of conditions that may impair cognitive function (e.g. schizophrenia, bipolar disorder)
- Surgery planned within 12 months
- Significant cognitive impairment
  - The Standardized Mini Mental Status Examination (sMMSE) was used to screen for this and consequently, those with a sMMSE score <24 were excluded (Molloy & Standish, 1997).
2.3. **Demographics and medical history**

Demographic information, comprehensive medical history, co-morbidities, concomitant medications, and vascular risk factors (hypertension, smoking, hypercholesterolemia, obesity, and diabetes) were collected from participant interviews. Anthropometric data such as height, weight, body-mass index (BMI) were obtained from patient charts at TRI or assessed by trained researchers. VO$_{2\text{peak}}$ (an indicator of cardiopulmonary fitness) was collected from electronic medical records from TRI. Lipid profiles including total cholesterol, triglycerides, low-density lipoproteins (LDL), high-density lipoproteins (HDL) were measured using standard clinical assays.

2.4. **Neurocognitive battery and assessment of depressive symptoms**

Study participants were assessed for their cognitive performance at a baseline visit (in the beginning of CR), 3-month visit, 6-month visit, and post 12-month visit. The time interval between the baseline and post 12-month visit was variable; this will be addressed in Section 2.8. A 30-min standardized battery of tests was used to assess cognitive performance due to vascular causes, as recommended by the National Institute of Neurological Disorders and Stroke-Canadian Stroke Network (NINDS-CSN) (Hachinski et al., 2006). A trained researcher administered the battery at a standardized time (0930 hr ±30 min) and participants refrained from eating or drinking any caffeine-containing beverages for at least 4 h before testing. As the primary outcome, verbal memory was assessed using the California Verbal Learning Test 2nd Edition (CVLT-II). The CVLT-II yields multiple measures of verbal memory function, including verbal learning (recall of a word list over 5 learning trials), short-delay free recall (recall of a word list after an interfering list), and long-delay free recall (recall of a word list after 20 min) (Hachinski et al., 2006). Its use in this population is appropriate, because it is able
to distinguish elderly adults with MCI from cognitively normal elderly individuals as it assesses learning across multiple trials (Rabin et al., 2009).

VCIND patients also have deficits in other cognitive domains along a continuum of severity. Measures of executive function included the Trail-Making Test Part B (Gaudino, Geisler, & Squires, 1995) and Stroop Color-Word Interference Test. Performance in visuospatial memory was assessed using the Brief Visuospatial Test-Revised (BVMT-R), which presents a measure of visual learning and delayed recall (Benedict, 1996). The domain of processing speed was assessed using the Trail-Making Test Part A (Gaudino et al., 1995) and the Digit Symbol-Coding task, a measure of complex attention and psychomotor speed from the Wechsler Adult Intelligence Scale 3rd Edition (Joy, Kaplan, & Fein, 2004).

The Structured Clinical Interview for Depression (SCID) was used to diagnose depression at baseline. It was administered by a trained researcher under the supervision of the study psychiatrist, to determine if participants met the Diagnostic and Statistical Manual, Version 4 (DSM-IV) criteria for minor or major depressive disorder.

2.4.1. Calculation of composite Z-scores

Z-scores were determined from age, gender and education-matched normative scores (Delis, 2000). Z-scores from tests that involve the same cognitive domain were summed into a composite Z-score to provide a more stable representation of performance in that particular domain and to avoid multiple comparisons (Weuve et al., 2004; Harrison et al., 2007). For verbal memory, three Z-scores from the CVLT-II: learning, short-delay free recall, and long-delay free recall were summed into a composite score. For executive function, the Z-scores from the Trails-Making Test Part B and Stroop Colour-Word Interference Test were summed into a
composite score. For visuospatial memory, the Z-scores from visuospatial learning and delayed recall from the BVMT-R were summed. For processing speed, the Z-scores from Trails-Making Test A and the Digit Symbol-Coding task were summed. Once the component scores were summed together, the composite scores were standardized according to the following equation:

\[
\text{Standardized scores} = \frac{\text{Individual} - \text{mean}}{\text{S.D.}}
\]

2.4.2. Possible vascular cognitive impairment, no dementia

In this study, participants with possible VCIND were defined as those with composite Z-scores of ≤1 in either the verbal memory or executive function domain, as described previously (Suridjan et al., 2017).

2.5. Biochemical assays

2.5.1. Blood collection

On the same day as cognitive testing during the baseline visit, a trained clinician or researcher drew blood from fasting participants at 0900 h ± 1 h, in order to control for diurnal and dietary influences on concentration of ceramides and other sphingolipids. The blood was drawn into EDTA-containing vacutainer tubes and then centrifuged at 1000 g for 10 min at 4°C. The resultant plasma fraction was immediately isolated, aliquoted, and stored at –80°C until time of measurement.

2.5.2. Measurement of plasma sphingolipids

Sphingolipids were isolated from the plasma fraction using high-performance liquid chromatography coupled electrospray ionization tandem mass spectrometry. Briefly, high performance liquid chromatography (PerkinElmer, MA, USA) with a reverse phase C18 column
(Phenomenex, Torrance, CA, USA) was used for temporal resolution. The eluted sample was then injected into the ion source for detection and quantification of ceramides and sphingomyelins (m/z 264.4, 266.4 for ceramides and 184.4 for sphingomyelins respectively).

Data were collected and processed by Analyst 1.4.2 software package and MultiQuant software (AB Sciex Inc, Thornhill, Ontario, Canada). Ceramide concentrations were initially presented in counts per second (cps), but were converted to ng/ml by applying a standard curve. These eight-point calibration curves (0.1–1000 ng/mL) were constructed by plotting area under the curve for different sphingolipids for each calibration standard normalized to the internal standard. The final sphingolipid concentrations (ng/mL) were determined by fitting the identified species to standard curves based on acyl chain length. All samples were measured while blinded to the clinical characteristics of participants.

2.6. Selection of covariates

Potential confounders that were adjusted for in multivariate analyses were chosen a priori, because the relationship between ceramides and cognition has been explored previously in the literature. Potential multi-collinearity of these covariates was assessed using variance inflation factor (VIF) and tolerance statistics: covariates with a VIF $\geq 2.5$ and tolerance $\leq 0.4$ were considered collinear.

2.6.1. Age

Increasing age is correlated with ceramide concentrations (Mielke et al., 2015). Previous work by our group also reported mean age to be associated with baseline ceramide levels (Saleem et al., 2013).
2.6.2. **Body-mass index**

Higher BMI has been shown to be associated with increasing ceramide concentrations (Mielke et al., 2015). Notably, concentrations of sphingomyelins and ceramides were found to be increased in adults who were obese, defined by BMI (Hanamatsu et al., 2014). Furthermore, another study reported higher BMI to be correlated with worse verbal memory performance (De Wit et al., 2017).

2.6.3. **Years of education**

In epidemiologic studies, lower level of education has been demonstrated to be a risk factor for accelerated memory decline, independent of education bias in measurement of verbal memory (Schmand et al., 1997). A pivotal investigation by Mortimer and Borenstein postulated that attained education may even bolster one’s cognitive reserve and off-set the presence of dementia-related pathophysiology (Mortimer, Borenstein, Gosche, & Snowdon, 2005). Years of education are associated with better verbal memory performance (De Wit et al., 2017); thus it is pertinent for us to adjust for this confounder. Indeed, this was found to be true as more years of education at baseline visit was significantly associated with milder decline in verbal memory (Table 2).

2.7. **Statistical analyses**

2.7.1. **Data pre-processing**

Ratios of CerC18:0 relative to its precursors were used to explore accumulation of CerC18:0 (Figure 1). Ratios of CerC18:0/SM18:0, CerC18:0/MHxC18:0, CerC18:0/S1P were calculated using raw sphingolipid concentrations. The resulting CerC18:0 ratios and CerC18:0 were then log-transformed to obtain a normal distribution prior to analyses and presented as Q-Q plots.
Only sphingolipid ratios with identical acyl chain lengths were included, because bi-directional conversions between ceramides and its precursors primarily modify the head group and not the acyl chain length (Mielke, Bandaru, McArthur, et al., 2010).

2.7.2. Characterization of study participants and comparison of sub-cohorts

T-tests and χ² tests were conducted on SPSS software (IBM, Chicago, Illinois) to determine if there were any baseline sociodemographic or clinical difference between those who returned for the post 12-month visit and those who did not. Results were presented as corresponding test values, degrees of freedom, and p-values. Similar univariate comparisons were performed to determine whether there were any baseline sociodemographic or clinical differences between those with possible VCIND and those without.

To characterize our participant population, mixed regression models were used to evaluate bivariate relationships between sociodemographic features at baseline and change in verbal memory. These analyses were conducted in two populations: the overall study population and those with possible VCIND.

2.7.3. Longitudinal analyses

For the primary, secondary, and tertiary hypotheses, mixed regression models were used to evaluate the longitudinal relationship between baseline CerC18:0 concentrations, baseline ratios of CerC18:0 to its precursors, and changes in verbal memory over time. For the exploratory hypotheses, mixed regression models from the main analyses were analyzed with possible VCIND and CerC18:0 biomarker interaction as a covariate. This was to evaluate whether associations between baseline sphingolipid concentrations and change in verbal memory were significantly different between those with possible VCIND and those without. These analyses
were only conducted with the baseline CerC18:0 ratios, aligning with the primary, secondary, and tertiary hypotheses.

Mixed regression model analyses were conducted using the *PROC MIXED* procedure in SAS University Edition statistical software (SAS Institute Inc., North Carolina, USA) with a two-tailed significance level of $p \leq 0.05$. However, a two-tailed significance level is $p \leq 0.025$ is used in tertiary analyses after applying Bonferroni’s correction to account for multiple comparisons.

As discussed in **Section 2.7**, age, BMI, and years of education were included as potential confounders a priori based on previous literature, while the number of days since baseline was added as a fixed effect to build a spatial power covariance structure. Moreover, in the exploratory analysis, substantially smaller sample sizes of the sub-groups does not allow for the same number of covariates while maintaining adequate power. As such, the covariate with the highest $p$-value was omitted from the model in a backward manner.

The spatial power covariance structure was selected in order to adjust for the time intervals between visits, which were unique to each participant. In general, in this structure, adjacent times have the highest correlation, while increasing distance between time points results in a systematically decreasing correlation (Science). Results were presented as $b$, which quantifies the SD change in verbal memory Z-score per 1 unit increase in the independent variable and standard error (SE).

**2.8. Calculation of sample size**

Given that validated calculations of sample size for mixed models are limited (Guo, Logan, Glueck, & Muller, 2013), sample size calculation for our mixed model analysis was based on that of a general linear multivariate model (Helms, 1992). The sample size was calculated using
IBM SPSS SamplePower 3.0 (Chicago, IL, USA). Assuming a mean SD of 0.60 in log ceramide concentrations based on previous findings in MCI (Mielke et al, 2010) and a mean SD of 3.41 in verbal memory domain Z-score (our data in 115 undergoing CR showed a mean CVLT-II composite Z-score of 1.50±3.41), a sample size of 58 provided 80% power for a detection of a change in slope (b) of 2 units with a two-sided α of 0.05. This allows for us to conservatively adjust for up to 3 confounding covariates, in addition to the predictor of interest and days since baseline in our spatial covariance structure.
Chapter 3: Results

3.1. Recruitment of study participants

Between February 2012 and November 2015, 1563 patients at TRI were screened for evidence of CAD before starting the CR program. Of the 933 who had angiographic evidence of CAD, 555 agreed to be contacted for research. After explaining the study and asking for informed consent, 300 agreed to participate and gave written informed consent. From those who agreed to participate, 159 were excluded based on the exclusion criteria. In addition, 21 participants were not assessed due to various reasons including, but not limited to: missed study visits, withdrawal of consent, vacation, and conflicts with work schedule.

In total, 120 participants enrolled into this study and completed the baseline visit. By the end of the 6-month visit, 101 participants remained in the study. Between August 2016 and September 2017, these 101 participants were contacted to return for a post 12-month visit. Of those 101, 60 participants completed the extension visit. 41 were not assessed due to various reasons including, but not limited to: vacation, conflict with work schedule, concurrent health issues, lack of transport to study site, lack of interest, and loss to follow up. In summary, a flow diagram illustrating the flow of participant recruitment is presented in Figure 2, in accordance to the CONsolidated Standards Of Reporting Trials (CONSORT) guidance (Schulz, Altman, Moher, & Group, 2010).
3.2. Differences between study participants who returned and those who did not

A significant portion (40.6%) of study participants did not return for the post 12-month visit after completing the 6-month visit. As such, an evaluation of sociodemographic and clinical differences between those who returned and those who did not was conducted in order to assess potential selection bias. A greater proportion of the non-returning cohort had the presence of an
apoipoprotein E4 (APOE4) allele. There were no other significant inter-group differences in terms of the characteristics (Table 1).

Table 1: Comparison of clinical and sociodemographic characteristics between study participants who returned for the post 12-month visit (n=60) and those who did not (n=41).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Returned for post-12 month visit (n=60)</th>
<th>Did not return for post-12 month visit (n=41)</th>
<th>Test values (df), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sociodemographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.5 (6.0)</td>
<td>64.7 (6.6)</td>
<td>0.14 (99), 0.89</td>
</tr>
<tr>
<td>Female</td>
<td>10 (16.7)</td>
<td>6 (14.6)</td>
<td>0.08 (2), 0.96</td>
</tr>
<tr>
<td>BMI</td>
<td>29.4 (5.6)</td>
<td>28.8 (4.6)</td>
<td>-0.52 (99), 0.60</td>
</tr>
<tr>
<td>Education (years)</td>
<td>16.6 (3.2)</td>
<td>15.9 (3.1)</td>
<td>-1.17 (99), 0.25</td>
</tr>
<tr>
<td>Married</td>
<td>49 (81.7)</td>
<td>32 (78.0)</td>
<td>1.83 (2), 0.40</td>
</tr>
<tr>
<td>Has a smoking history</td>
<td>32 (53.3)</td>
<td>28 (68.3)</td>
<td>4.51 (2), 0.11</td>
</tr>
<tr>
<td>Has a APOE4 allele</td>
<td>11 (18.3)</td>
<td>12 (29.3)</td>
<td>8.17 (2), 0.02*</td>
</tr>
<tr>
<td><strong>CAD Severity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative stenosis (%)</td>
<td>152.28 (71.36)</td>
<td>141.97 (60.46)</td>
<td>-0.68 (79), 0.50</td>
</tr>
<tr>
<td><strong>Fitness Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂peak (ml/kg/min)</td>
<td>20.67 (5.76)</td>
<td>21.71 (5.66)</td>
<td>0.88 (98), 0.38</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>8 (13.3)</td>
<td>5 (12.2)</td>
<td>2.67 (2), 0.26</td>
</tr>
<tr>
<td>Diabetic</td>
<td>10 (16.7)</td>
<td>6 (14.6)</td>
<td>0.08 (2), 0.96</td>
</tr>
<tr>
<td><strong>Cognitive Domain Z-Scores</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verbal memory (SD)</td>
<td>0.13 (0.86)</td>
<td>0.04 (1.06)</td>
<td>-0.44 (98), 0.66</td>
</tr>
<tr>
<td>Visuospatial memory (SD)</td>
<td>0.01 (0.89)</td>
<td>-0.06 (1.06)</td>
<td>-0.39 (99), 0.70</td>
</tr>
<tr>
<td>Processing speed (SD)</td>
<td>0.19 (0.98)</td>
<td>-0.11 (0.90)</td>
<td>-1.53 (99), 0.13</td>
</tr>
<tr>
<td>Executive function (SD)</td>
<td>-0.01 (1.04)</td>
<td>0.08 (0.83)</td>
<td>0.45 (98), 0.66</td>
</tr>
</tbody>
</table>

Abbreviations: APOE4, apolipoprotein E4; BMI, body-mass index; CAD, coronary artery disease; df, degrees of freedom; SD, standard deviation.

3.3. Associations between sociodemographic and clinical characteristics and change in verbal memory performance in all study participants

In total, 60 study participants returned for the post 12-month visit; thus, completing all four assessments. Study participants were followed a median of 3.5 years from their baseline visit.
(inter-quartile range: 1.1 years). The majority of participants were male (n=49, 83.1%) and Caucasian (n=47, 79.7%) with an average age of 65±6 years.

In bivariate longitudinal associations, greater years of education (b[SE]=0.05[0.02], p=0.04) and greater VO$_2$peak at baseline (b[SE]=0.03[0.01], p=0.01) was associated with a less decline in verbal memory. Conversely, having depression (b[SE]=-0.54[0.24], p=0.03) was associated with steeper decline in verbal memory performance over time. There were no other significant associations between participant characteristics and verbal memory (Table 2).
### Table 2: Baseline clinical and demographic characteristics in association with verbal memory Z-score over a median of 3.53 years in all study participants (n=60)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD) or N(%)</th>
<th>Association with verbal memory (b [SE], p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sociodemographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.5 (6.0)</td>
<td>0.004288 [0.01], 0.74</td>
</tr>
<tr>
<td>Female</td>
<td>10 (16.7)</td>
<td>-0.24 [0.21], 0.26</td>
</tr>
<tr>
<td>BMI</td>
<td>29.4 (5.6)</td>
<td>-0.01 [0.01], 0.49</td>
</tr>
<tr>
<td>Education (years)</td>
<td>16.6 (3.2)</td>
<td>0.05 [0.02], 0.04*</td>
</tr>
<tr>
<td>Married</td>
<td>49 (81.7)</td>
<td>-0.04 [0.20], 0.83</td>
</tr>
<tr>
<td>Has a smoking history</td>
<td>32 (53.3)</td>
<td>0.05 [0.15], 0.76</td>
</tr>
<tr>
<td>Has a APOE4 allele</td>
<td>11 (18.3)</td>
<td>-0.05 [0.20], 0.81</td>
</tr>
<tr>
<td><strong>CAD Severity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative stenosis (%)</td>
<td>152.28 (71.36)</td>
<td>0.002 [0.001], 0.05</td>
</tr>
<tr>
<td><strong>Lipid Profile and HbA1c levels</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>1.59 (0.63)</td>
<td>-0.07 [0.12], 0.59</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.28 (0.34)</td>
<td>-0.16 [0.23], 0.48</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>3.45 (0.83)</td>
<td>-0.13 [0.09], 0.17</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.23 (0.62)</td>
<td>-0.23 [0.12], 0.07</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>0.06 (0.01)</td>
<td>-17.13 [11.77], 0.15</td>
</tr>
<tr>
<td><strong>Fitness Parameter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO_{2peak} (ml/kg/min)</td>
<td>20.67 (5.76)</td>
<td>0.03 [0.01], 0.01*</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>8 (13.3)</td>
<td>-0.54 [0.24], 0.03*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>10 (16.7)</td>
<td>-0.05 [0.21], 0.81</td>
</tr>
</tbody>
</table>

Abbreviations: APOE4, apolipoprotein E4; BMI, body-mass index; CAD, coronary artery disease; df, degrees of freedom; HbA1C, hemoglobin A1C; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

3.4. Associations between sociodemographic and clinical characteristics and change in verbal memory performance in study participants with possible VCIND

In total, 14 (23.3%) study participants had possible VCIND at baseline. In those with possible VCIND, having diabetes at baseline was associated with less decline in verbal memory performance (b[SE]=0.68[0.24], p=0.01). There were no other significant associations between participant characteristics and verbal memory (Table 3).
Table 3: Baseline clinical and demographic characteristics in association with verbal memory z-score over a median of 3.96 years in participants with possible VCIND (n=14).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD) or N (%)</th>
<th>Association with verbal memory (b [SE], p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sociodemographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>62.5 (5.6)</td>
<td>0.02[0.02], 0.44</td>
</tr>
<tr>
<td>Female</td>
<td>3 (21.4)</td>
<td>0.23[0.32], 0.49</td>
</tr>
<tr>
<td>BMI</td>
<td>29.0 (3.5)</td>
<td>0.0008[0.0009], 0.38</td>
</tr>
<tr>
<td>Years of education</td>
<td>16.1 (3.6)</td>
<td>-0.02[0.04], 0.59</td>
</tr>
<tr>
<td>Married</td>
<td>13 (92.9)</td>
<td>-0.58[0.48], 0.25</td>
</tr>
<tr>
<td>Has a smoking history</td>
<td>7 (50.0)</td>
<td>-0.12[0.26], 0.64</td>
</tr>
<tr>
<td>APOE4 allele</td>
<td>2 (14.2)</td>
<td>-0.39[0.35], 0.29</td>
</tr>
<tr>
<td><strong>CAD Severity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative stenosis</td>
<td>117.8 (65.4)</td>
<td>-0.003[0.002], 0.29</td>
</tr>
<tr>
<td><strong>Lipid Profile and Hb1AC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>1.7 (0.9)</td>
<td>-0.07[0.21], 0.74</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 (0.3)</td>
<td>-0.05[0.30], 0.86</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>3.6(1.1)</td>
<td>0.08[0.14], 0.60</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4(0.7)</td>
<td>0.06[0.17], 0.71</td>
</tr>
<tr>
<td>Hemoglobin A1C</td>
<td>0.06(0.01)</td>
<td>-0.20[27.26], 0.46</td>
</tr>
<tr>
<td><strong>Fitness Parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max VO2</td>
<td>18.2(5.1)</td>
<td>-0.03[0.02], 0.32</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>3(21.4)</td>
<td>-0.45[0.32], 0.18</td>
</tr>
<tr>
<td>Diabetic</td>
<td>5(35.7)</td>
<td>0.68[0.24], 0.01*</td>
</tr>
</tbody>
</table>

Abbreviations: APOE4, apolipoprotein E4; BMI, body-mass index; CAD, coronary artery disease; df, degrees of freedom; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

3.5. Sociodemographic and clinical differences between those with possible VCIND and those with no possible VCIND

A greater proportion of those with possible VCIND were diabetic at baseline ($\chi^2$(df)=4.77(1), p=0.03), compared to those with no possible VCIND. In terms of performance on the cognitive battery, those with possible VCIND performed significantly worse in all cognitive domains.

There were no other significant sociodemographic or clinical differences between these two sub-groups (Table 4).
Table 4: Comparison of baseline clinical and demographic characteristics between those with possible VCIND and those with no possible VCIND.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Possible VCIND (n=14)</th>
<th>No possible VCIND (n=46)</th>
<th>Test Value (df), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sociodemographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>62.5 (5.8)</td>
<td>65.1 (6.0)</td>
<td>1.45 (58), 0.15</td>
</tr>
<tr>
<td>Female</td>
<td>3 (21.4)</td>
<td>7 (15.2)</td>
<td>0.30 (1), 0.59</td>
</tr>
<tr>
<td>BMI</td>
<td>29.0 (3.7)</td>
<td>29.5 (6.0)</td>
<td>0.27 (58), 0.79</td>
</tr>
<tr>
<td>Years of education</td>
<td>16.1 (3.7)</td>
<td>16.8 (3.1)</td>
<td>0.64 (58), 0.53</td>
</tr>
<tr>
<td>Married</td>
<td>13 (92.9)</td>
<td>36 (78.3)</td>
<td>1.53 (1), 0.22</td>
</tr>
<tr>
<td>Has a smoking history</td>
<td>7 (50.0)</td>
<td>21 (54.3)</td>
<td>0.08 (1), 0.78</td>
</tr>
<tr>
<td>Has a APOE4 allele</td>
<td>2 (14.3)</td>
<td>9 (19.6)</td>
<td>0.20 (1), 0.66</td>
</tr>
<tr>
<td><strong>CAD Severity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative stenosis</td>
<td>117.8 (69.0)</td>
<td>161.6 (70.0)</td>
<td>1.76 (45), 0.09</td>
</tr>
<tr>
<td><strong>Fitness Parameter</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max VO₂ (ml/kg/min)</td>
<td>18.20 (5.31)</td>
<td>21.43 (5.73)</td>
<td>1.87 (58), 0.07</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>3 (21.4)</td>
<td>5 (10.9)</td>
<td>1.04 (1), 0.31</td>
</tr>
<tr>
<td>Diabetic</td>
<td>5 (35.7)</td>
<td>5 (10.9)</td>
<td>4.77 (1), 0.03*</td>
</tr>
<tr>
<td><strong>Cognitive Domain Z-Scores</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verbal memory (SD)</td>
<td>-0.67 (0.92)</td>
<td>0.35 (0.70)</td>
<td>4.34 (57), 0.00*</td>
</tr>
<tr>
<td>Visuospatial memory (SD)</td>
<td>-0.58 (1.02)</td>
<td>0.19 (0.78)</td>
<td>3.04 (58), 0.00*</td>
</tr>
<tr>
<td>Processing speed (SD)</td>
<td>-0.31 (0.92)</td>
<td>0.34 (0.96)</td>
<td>2.21 (58), 0.03*</td>
</tr>
<tr>
<td>Executive function (SD)</td>
<td>-1.16 (0.80)</td>
<td>0.34 (0.83)</td>
<td>5.98 (58), 0.00*</td>
</tr>
</tbody>
</table>

Abbreviations: APOE4, apolipoprotein E4; BMI, body mass index; CAD, coronary artery disease; df, degrees of freedom; SD, standard deviation; VCIND, vascular cognitive impairment, no dementia.

3.6 Trajectory of verbal memory performance throughout the study

Across all study participants, verbal memory performance did not change significantly over time (b=-0.0001, p=0.32). This was consistent even when separate mixed regression models were conducted for those with possible VCIND (b[SE]=0.00024[0.00020], p=0.24) and those with no possible VCIND (b[SE]=0.00005[0.0001], p=0.70).
Table 5: Composite Z-scores of verbal memory performance in standard deviations over the course of the study.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Baseline</th>
<th>3-month visit</th>
<th>6-month visit</th>
<th>Post 12-month visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>0.11</td>
<td>-0.03</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Possible VCIND</td>
<td>-0.67</td>
<td>-0.59</td>
<td>-0.55</td>
<td>-0.79</td>
</tr>
<tr>
<td>No possible VCIND</td>
<td>0.33</td>
<td>0.14</td>
<td>0.26</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Abbreviations: VCIND, vascular cognitive impairment, no dementia.

Figure 3: Trajectory of average composite Z-score in verbal memory domain in participant cohorts.

3.7. Covariate multi-collinearity and normalization of plasma sphingolipid concentrations

The covariates were not collinear as seen in Table 6. Therefore, they were included in all subsequent multivariate models.

Table 6: Multi-collinearity statistics of a priori covariates in mixed regression models

<table>
<thead>
<tr>
<th></th>
<th>Tolerance</th>
<th>VIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.98</td>
<td>1.02</td>
</tr>
<tr>
<td>BMI</td>
<td>0.95</td>
<td>1.05</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.98</td>
<td>1.03</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body-mass index; VIF, variance inflation factor.
Raw and log-transformed concentrations of sphingolipids are presented below (Table 7).

CerC18:0, SM18:0, MHxCerC18:0, and S1P plasma concentrations were log-transformed to obtain a normal distribution for mixed regression analyses, so that their concentrations on a notionally common scale. The normal distributions of the log-transformed sphingolipid concentrations are presented in Figures 4 to 7. CerC18:0 ratios were calculated using the log-transformed sphingolipid ratios.

**Table 7:** Overview of plasma sphingolipid concentrations at baseline and their log-transformed values.

<table>
<thead>
<tr>
<th>Species (ng/mL)</th>
<th>Mean (S.D.)</th>
<th>Range (min.-max.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CerC18:0</td>
<td>7.60 (5.14)</td>
<td>1.45-30.89</td>
</tr>
<tr>
<td>SM18:0</td>
<td>123505 (27762)</td>
<td>65300-205000</td>
</tr>
<tr>
<td>MHxCerC18:0</td>
<td>8.33 (4.66)</td>
<td>1.84-28.86</td>
</tr>
<tr>
<td>S1P</td>
<td>197.70 (178.16)</td>
<td>16.32-878.95</td>
</tr>
<tr>
<td>Log CerC18:0</td>
<td>0.80 (0.27)</td>
<td>0.16-1.49</td>
</tr>
<tr>
<td>Log SM18:0</td>
<td>5.08 (0.10)</td>
<td>4.81-5.31</td>
</tr>
<tr>
<td>Log MHxCerC18:0</td>
<td>0.87 (0.22)</td>
<td>0.27-1.46</td>
</tr>
<tr>
<td>Log S1P</td>
<td>2.13 (0.39)</td>
<td>1.21-2.94</td>
</tr>
</tbody>
</table>

Abbreviations: C18, acyl chain of 18 carbons; Cer, ceramide; MHxCer, monohexosylceramide; S1P, sphingosine-1-phosphate; SD, standard deviation; SM, sphingomyelin; VCIND, vascular cognitive impairment, no dementia.

![Normal Q-Q Plot of Baseline Ceramides standardized d18:1/18:0 log-transformed](image)

**Figure 4:** Normal Q-Q plot of log-transformed CerC18:0
Figure 5: Normal Q-Q plot of log-transformed SM18:0

Figure 6: Normal Q-Q plot of log-transformed MHxCerC18:0
Figure 7: Normal Q-Q plot of log-transformed S1P

Figure 8: Normal Q-Q plot of log-transformed CerC18:0/MHxCerC18:0
Figure 9: Normal Q-Q plot of log-transformed CerC18:0/SM18:0

Figure 10: Normal Q-Q plot of log-transformed CerC18:0/S1P
3.8. **Primary: Associations between baseline CerC18:0 and change in verbal memory performance**

Longitudinally, each log-unit increase in baseline plasma CerC18:0 was significantly associated with a 0.91 SD decline in verbal memory performance \( (b[SE]=-0.91[0.30], \ p=0.003) \) after adjustment for age, BMI, years of education, and days since baseline visit by including them as fixed effects (Table 8).

### Table 8: Association between plasma CerC18:0 at baseline and change in verbal memory performance over the course of the study in participants with CAD (n=60), adjusted for age, BMI, years of education, and days since baseline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.95 [-3.09, 1.19]</td>
<td>1.07</td>
<td>55</td>
<td>-0.89</td>
<td>0.38</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0002 [-0.0004, 0.00008]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.29</td>
<td>0.20</td>
</tr>
<tr>
<td>Age</td>
<td>0.02 [-0.01, 0.05]</td>
<td>0.01</td>
<td>55</td>
<td>1.45</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.01 [-0.03, 0.02]</td>
<td>0.01</td>
<td>55</td>
<td>-0.36</td>
<td>0.72</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [-0.00555, 0.009]</td>
<td>0.02</td>
<td>55</td>
<td>1.77</td>
<td>0.08</td>
</tr>
<tr>
<td>CerC18:0</td>
<td>-0.91 [-1.51, 0.32]</td>
<td>0.30</td>
<td>55</td>
<td>-3.08</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body-mass index; C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; SE, standard error.

3.9. **Secondary: Associations between CerC18:0/MHxCerC18:0 ratio and change in verbal memory**

Baseline plasma MHxCerC18:0 concentration was not associated with change in verbal memory over time \( (b[SE]=-0.46[0.38], \ p=0.22) \), after adjusting for age, BMI, years of education and days since baseline visit. In contrast, baseline plasma CerC18:0/MHxCerC18:0 was significantly associated with change in verbal memory performance \( (b[SE]=-0.90[0.40], \ p=0.03) \), after adjusting for age, BMI, years of education and days since baseline visit. Each log-unit increase in the ratio was associated with a 0.90 SD decrease in verbal memory performance (Table 9).
Table 9: Association between plasma CerC18:0/MHxCerC18:0 at baseline and change in verbal memory performance over the course of the study in participants with CAD (n=60), adjusted for age, BMI, years of education, and days since baseline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.29 [-3.53, 0.94]</td>
<td>1.12</td>
<td>55</td>
<td>-1.16</td>
<td>0.25</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0002 [-0.0004, 0.0001]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.27</td>
<td>0.20</td>
</tr>
<tr>
<td>Age</td>
<td>0.01 [-0.02, 0.04]</td>
<td>0.01</td>
<td>55</td>
<td>0.78</td>
<td>0.44</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.004 [-0.03, 0.02]</td>
<td>0.01</td>
<td>55</td>
<td>-0.30</td>
<td>0.76</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.05 [0.002, 0.10]</td>
<td>0.02</td>
<td>55</td>
<td>2.10</td>
<td>0.04*</td>
</tr>
<tr>
<td>CerC18:0/MHxCerC18:0</td>
<td>-0.90 [-1.70, -0.09]</td>
<td>0.40</td>
<td>55</td>
<td>-2.23</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body-mass index; C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; MHxCer, monohexosylceramide; SE, standard error.

3.10. Tertiary: Associations between CerC18:0/SM18:0 ratio and change in verbal memory

Plasma concentrations of SM18:0 were not associated with change in verbal memory over time (b[SE]=-0.90[0.84], p=0.29), after adjusting for age, BMI, years of education, and days since baseline. However, baseline CerC18:0/SM18:0 was significantly associated with change in verbal memory performance (b[SE]=-1.11[0.36], p=0.004) in a mixed regression model adjusted for age, BMI, years of education, and days since baseline. Each log-unit increase in the CerC18:0/SM18:0 ratio correlated with a 1.11 SD decline in verbal memory performance (Table 10).

Table 10: Association between plasma CerC18:0/SM18:0 at baseline and change in verbal memory performance over the course of the study in participants with CAD, adjusted for age, BMI, years of education, and days since baseline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-6.30 [-10.54, -2.06]</td>
<td>2.11</td>
<td>55</td>
<td>-2.98</td>
<td>0.004</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.00016 [-0.0004, 0.0009]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.27</td>
<td>0.20</td>
</tr>
<tr>
<td>Age</td>
<td>0.02 [-0.01, 0.05]</td>
<td>0.01</td>
<td>55</td>
<td>1.41</td>
<td>0.16</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.01 [-0.04, 0.02]</td>
<td>0.01</td>
<td>55</td>
<td>-0.62</td>
<td>0.54</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [-0.01, 0.09]</td>
<td>0.02</td>
<td>55</td>
<td>1.75</td>
<td>0.09</td>
</tr>
<tr>
<td>CerC18:0/SM18:0</td>
<td>-1.11 [-1.84, -0.38]</td>
<td>0.36</td>
<td>55</td>
<td>-3.05</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body-mass index; C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; SE, standard error; SM, sphingomyelin.
Plasma concentrations of S1P were not associated with decrease in verbal memory performance (b[SE]=−0.41[0.20], p=0.05) over time, after adjusting for age, BMI, years of education, and days since baseline. Likewise, CerC18:0/S1P was not associated with change in verbal memory over time, after adjustment for the same covariates (b[SE]=0.01[0.21], p=0.97; Table 11).

Table 11: Association between plasma CerC18:0/S1P at baseline and change in verbal memory performance over the course of the study in participants with CAD, adjusted for age, BMI, years of education, and days since baseline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.04 [-3.41, 1.32]</td>
<td>1.18</td>
<td>52</td>
<td>-0.88</td>
<td>0.38</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0002 [-0.0004, 0.0001]</td>
<td>0.0001</td>
<td>168</td>
<td>-1.13</td>
<td>0.26</td>
</tr>
<tr>
<td>Age</td>
<td>0.01 [-0.02, 0.03]</td>
<td>0.01</td>
<td>52</td>
<td>0.52</td>
<td>0.60</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.001 [-0.03, 0.03]</td>
<td>0.01</td>
<td>52</td>
<td>-0.10</td>
<td>0.92</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.05 [-0.001, 0.10]</td>
<td>0.02</td>
<td>52</td>
<td>1.98</td>
<td>0.05</td>
</tr>
<tr>
<td>CerC18:0/S1P</td>
<td>0.01 [-0.41, 0.42]</td>
<td>0.21</td>
<td>52</td>
<td>0.04</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body-mass index; C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; S1P, sphingosine-1-phosphate; SE, standard error.

3.11. Exploratory analyses: Differences in associations between those with possible VCIND and those with no possible VCIND

Analyses were repeated with an interaction term of possible VCIND and baseline CerC18:0 ratio in mixed models.

3.11.1. Association between CerC18:0 and change in verbal memory performance

Although presence of VCIND was significantly associated with decline in verbal memory, it did not significantly affect the association between baseline CerC18:0 and change in verbal memory performance (b[SE]=0.10[0.56], p=0.86), after adjusting for age, years of education, days since baseline, possible VCIND, and baseline CerC18:0 (Table 12).
Table 12: Association between a possible VCIND and CerC18:0 interaction at baseline and change in verbal memory performance in CAD participants (n=60), adjusted for age, years of education, days since baseline, possible VCIND, and baseline CerC18:0.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.03 [-1.64, 1.58]</td>
<td>0.80</td>
<td>54</td>
<td>-0.04</td>
<td>0.97</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0001 [-0.0003, 0.0001]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.14</td>
<td>0.26</td>
</tr>
<tr>
<td>Age</td>
<td>0.01 [-0.02, 0.03]</td>
<td>0.01</td>
<td>54</td>
<td>0.48</td>
<td>0.63</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [-0.005, 0.08]</td>
<td>0.02</td>
<td>54</td>
<td>1.77</td>
<td>0.08</td>
</tr>
<tr>
<td>Possible VCIND</td>
<td>-1.00 [-1.94, -0.06]</td>
<td>0.47</td>
<td>54</td>
<td>-2.13</td>
<td>0.04*</td>
</tr>
<tr>
<td>CerC18:0</td>
<td>0.10 [-1.01, 1.21]</td>
<td>0.56</td>
<td>54</td>
<td>0.18</td>
<td>0.86</td>
</tr>
<tr>
<td>VCIND*CerC18:0</td>
<td>0.10 [-1.01, 1.21]</td>
<td>0.56</td>
<td>54</td>
<td>0.18</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Abbreviations: C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; MHxCer, monohexosylceramide; SE, standard error; VCIND, vascular cognitive impairment, no dementia.

3.11.2. Association between CerC18:0/MHxCerC18:0 and change in verbal memory performance

Both possible VCIND and CerC18:0/MHxCerC18:0 are significantly associated with change in verbal memory performance. However, the presence of VCIND did not significantly affect the association between baseline CerC18:0/ MHxCerC18:0 and change in verbal memory performance (b[SE]=1.08[0.74], p=0.15), after adjusting for age, years of education, days since baseline, possible VCIND, and baseline CerC18:0/MHxCerC18:0 (Table 13).

Table 13: Association between a possible VCIND and CerC18:0/MHxCerC18:0 ratio interaction at baseline and change in verbal memory performance in CAD participants (n=60), adjusted for age, years of education, days since baseline, possible VCIND, and CerC18:0/MHxCerC18:0.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.34 [-2.01, 1.33]</td>
<td>0.83</td>
<td>54</td>
<td>-0.41</td>
<td>0.69</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0001 [-0.0003, 0.00009]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.16</td>
<td>0.25</td>
</tr>
<tr>
<td>Age</td>
<td>-0.002 [-0.02, 0.02]</td>
<td>0.01</td>
<td>54</td>
<td>-0.15</td>
<td>0.88</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [0.001, 0.08]</td>
<td>0.02</td>
<td>54</td>
<td>2.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Possible VCIND</td>
<td>-0.86 [-1.20, -0.53]</td>
<td>0.17</td>
<td>54</td>
<td>-5.19</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>CerC18:0/MHxCerC18:0</td>
<td>-1.10 [-1.92, -0.27]</td>
<td>0.41</td>
<td>54</td>
<td>-2.67</td>
<td>0.01*</td>
</tr>
<tr>
<td>VCIND*CerC18:0/MHxCerC18:0</td>
<td>1.08 [-0.39, 2.56]</td>
<td>0.74</td>
<td>54</td>
<td>1.47</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Abbreviations: C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; MHxCer, monohexosylceramide; SE, standard error; VCIND, vascular cognitive impairment, no dementia.
3.11.3. Association between CerC18:0/SM18:0 and change in verbal memory performance

Both possible VCIND and CerC18:0/SM18:0 were not significantly associated with change in verbal memory performance. Accordingly, the association between baseline CerC18:0/SM18:0 and change in verbal memory performance was not statistically significant between those with possible VCIND and those without (b[SE]= -0.28[0.67], p=0.68), after adjusting for age, years of education, days since baseline, possible VCIND, and baseline CerC18:0/SM18:0 (Table 14).

Table 14: Association between a possible VCIND and CerC18:0/SM18:0 ratio interaction and change in verbal memory performance over the course of the study in CAD participants (n=60), adjusted for age, years of education, days since baseline, possible VCIND, and CerC18:0/SM18:0.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-3.51 [-7.69, 0.67]</td>
<td>2.08</td>
<td>54</td>
<td>-1.68</td>
<td>0.10</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0001 [-0.0003, 0.0001]</td>
<td>0.0001</td>
<td>177</td>
<td>-1.10</td>
<td>0.27</td>
</tr>
<tr>
<td>Age</td>
<td>0.0003 [-0.02, 0.03]</td>
<td>0.01</td>
<td>54</td>
<td>0.28</td>
<td>0.78</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [-0.004, 0.08]</td>
<td>0.02</td>
<td>54</td>
<td>1.82</td>
<td>0.07</td>
</tr>
<tr>
<td>Possible VCIND</td>
<td>-2.08 [-7.80, 3.65]</td>
<td>2.86</td>
<td>54</td>
<td>0.73</td>
<td>0.47</td>
</tr>
<tr>
<td>CerC18:0/SM18:0</td>
<td>-0.70 [-1.46, 0.06]</td>
<td>0.38</td>
<td>54</td>
<td>0.73</td>
<td>0.47</td>
</tr>
<tr>
<td>VCIND*CerC18:0/SM18:0</td>
<td>-0.28 [-1.62, 1.06]</td>
<td>0.67</td>
<td>54</td>
<td>-0.41</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Abbreviations: C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; SE, standard error; SM, sphingomyelin; VCIND, vascular cognitive impairment, no dementia.

3.11.4. Association between CerC18:0/S1P and change in verbal memory performance

Both possible VCIND and CerC18:0/S1P were not significantly associated with change in verbal memory performance. Similarly, the presence of VCIND did not significantly affect the association between baseline CerC18:0/S1P and change in verbal memory performance (b[SE]=0.65[0.55], p=0.25), after adjusting for age, years of education, days since baseline, possible VCIND, and baseline CerC18:0/S1P (Table 15).
Table 15: Association between plasma CerC18:0/S1P concentrations at baseline and change in verbal memory performance over the course of the study in CAD participants (n=60), adjusted for age, years of education, days since baseline, possible VCIND, and CerC18:0/S1P.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate (b) [95% CI]</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.15 [-2.08, 1.79]</td>
<td>0.96</td>
<td>51</td>
<td>-0.15</td>
<td>0.88</td>
</tr>
<tr>
<td>Days since baseline</td>
<td>-0.0001 [-0.0003, 0.0001]</td>
<td>0.0001</td>
<td>168</td>
<td>-0.95</td>
<td>0.34</td>
</tr>
<tr>
<td>Age</td>
<td>-0.005 [-0.03, 0.02]</td>
<td>0.01</td>
<td>51</td>
<td>-0.44</td>
<td>0.66</td>
</tr>
<tr>
<td>Years of education</td>
<td>0.04 [-0.0008, 0.08]</td>
<td>0.02</td>
<td>51</td>
<td>1.97</td>
<td>0.05</td>
</tr>
<tr>
<td>VCIND</td>
<td>-0.02 [-1.30, 1.26]</td>
<td>0.64</td>
<td>51</td>
<td>-0.03</td>
<td>0.98</td>
</tr>
<tr>
<td>CerC18:0:S1P</td>
<td>-0.12 [-0.51, 0.26]</td>
<td>0.19</td>
<td>51</td>
<td>-0.64</td>
<td>0.52</td>
</tr>
<tr>
<td>VCIND*CerC18:0:S1P</td>
<td>0.68 [-0.20, 1.55]</td>
<td>0.44</td>
<td>51</td>
<td>1.55</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Abbreviations: C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; CI, confidence interval; df, degrees of freedom; S1P, sphingosine-1-phosphate; SE, standard error; VCIND, vascular cognitive impairment, no dementia.

3.12. Summary of Results

Overall, higher plasma CerC18:0 concentrations as well as increased baseline ratios of CerC18:0/MHxCerC18:0 and CerC18:0/SM18:0 were significantly correlated with steeper decline in verbal memory performance over time. However, in exploratory analyses, none of these associations were mediated by the presence of VCIND. A summary of the results is presented in Table 16.

Table 16: Associations between baseline CerC18:0 biomarkers and change in verbal memory in the overall CAD population (n=60), adjusted for age, BMI, and years of education.

<table>
<thead>
<tr>
<th>b [SE], p-value</th>
<th>Without interaction</th>
<th>With interaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CerC18:0</td>
<td>-0.91 [0.30], 0.003*</td>
<td>0.10 [0.56], 0.86</td>
</tr>
<tr>
<td>CerC18:0/MHxCerC18:0</td>
<td>-0.90 [0.40], 0.03*</td>
<td>1.08 [0.74], 0.15</td>
</tr>
<tr>
<td>CerC18:0/SM18:0</td>
<td>-1.11 [0.36], 0.004*</td>
<td>-0.28 [0.67], 0.68</td>
</tr>
<tr>
<td>CerC18:0/S1P</td>
<td>0.01 [0.21], 0.97</td>
<td>0.68 [0.44], 0.13</td>
</tr>
</tbody>
</table>

*this column presents results of an interaction term of baseline CerC18:0 biomarker and possible VCIND

Abbreviations: C18:0, acyl chain length of 18 carbons; CAD, coronary artery disease; Cer, ceramide; MHxCer, monohexosylceramide; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SE, standard error; VCIND, vascular cognitive impairment, no dementia.
Chapter 4: Discussion and Recommendations for Future Studies

4.1. Study Findings and Interpretations

The present findings demonstrate a relationship between markers of aberrant CerC18:0 metabolism and cognitive decline, corroborating our previous finding that baseline plasma CerC18:0 is a robust marker of verbal memory performance in a cohort of CAD patients (Saleem et al., 2017). We investigated longitudinal relationships between the equilibrium of baseline CerC18:0 biosynthesis and change in verbal memory performance. In both the catabolic and salvage pathways, an increased baseline ratio of CerC18:0 to its respective precursor was significantly correlated with worse verbal memory performance over time. However, in exploratory analyses of these CerC18:0 ratios, none of these associations appeared to be mediated by the presence of VCIND. Collectively, our results show that ratios of CerC18:0 to its certain precursors may be sensitive marker of cognitive change in the early, prodromal stages of VCI.

4.1.1. Characterization of study participants and comparison of sub-cohorts

Considering the high rate of attrition in participant retention between the 6-month visit and post 12-month visit, a comparison of sociodemographic and clinical characteristics was warranted between study participants who returned for a post 12-month visit and those who did not. We found that a greater portion of those that did not return had an APOE4 allele. Given that presence of an APOE4 allele is associated with worse cognitive function (Weir, 1990), which in turn is associated with increased risk of dropping out of CR, this suggests that those who did not return would have worse cognitive performance. However, we did not find any significant differences in cognitive performance between those who returned and those who didn’t.
In the overall study population, greater years of education attained and greater VO\textsubscript{2peak} at baseline was associated with a smaller decline in verbal memory. This was expected as education is a well-characterized predictor of verbal memory performance (Schmand et al., 1997; Mortimer et al., 2005). To account for its confounding effect, years of education were included as \textit{a priori} covariate in subsequent analyses. The significant relationship between VO\textsubscript{2peak} and verbal memory performance is not unexpected either. Previous studies conducted by our group and others have found cardiopulmonary fitness to be predictive of verbal memory, alluding to the importance of physical exercise in modulating cognitive performance in older adults (Zhu et al., 2014; Saleem et al., 2017; Hayes, Forman, & Verfaellie, 2016). However, due to limited sample size, our models were not adequately powered to adjust for VO\textsubscript{2peak}.

In those with possible VCIND, the presence of diabetes mellitus at baseline was significantly associated with less decline in verbal memory performance, which is contrary to expectations (Kelly et al., 2016; Yau, Kluger, Borod, & Convit, 2014). A plausible explanation may be found in the insulin-sensitizing medications that diabetic patients take chronically. For instance, duration of metformin use was inversely correlated with risk of cognitive impairment in the Singapore Longitudinal Aging Study (Ng et al., 2014). Findings reported by Abbatecola and her colleagues corroborate this hypothesis as duration of metformin/rosiglitazone use stabilized cognitive decline in individuals with diabetes mellitus and mild cognitive impairment (Abbatecola et al., 2010). However, given our limited sample size (n=14), the significance of this association is likely by chance. Conversely, when comparing cross-sectional profiles of those with possible VCIND and those with no possible VCIND, a greater portion of those with possible VCIND also had diabetes mellitus compared those with no possible VCIND. As discussed, this aligns with evidence in the literature as those with diabetes mellitus may
experience rates of cognitive decline that are up to 2 times faster compared to those without (Biessels, Staekenborg, Brunner, Brayne, & Scheltens, 2006). The chronic hyperglycemia and reactive hyperinsulinemia associated with diabetes mellitus may cause the accumulation of oxidative stress products and resultant microvascular damage (Kalmijn, Feskens, Launer, Stijnen, & Kromhout, 1995; Gispen & Biessels, 2000).

4.1.2. Primary Findings

In our primary findings, baseline CerC18:0 concentrations were associated with change in verbal memory over a median of 3.5 years. This finding corroborates our previous report that CerC18:0 is associated with general cognitive decline over 6 months (Saleem et al., 2017), providing valuable evidence that CerC18:0 may be a robust biomarker of verbal memory change in those with CAD. As such, we postulate that inter-participant differences in CerC18:0 concentrations are associated with rate of cognitive change over time. Moreover, given that sphingolipids ratios may be more predictive of cognitive decline than individual sphingolipids (Mielke et al., 2011), CerC18:0 ratios were used as preliminary markers of shifts in enzymatic activity.

4.1.3. Secondary Findings

We report a novel inverse association between baseline CerC18:0/MHxCer18:0 ratio and change in verbal memory performance over time. In the salvage pathway, galactocerebrosidase and glucosylcerebrosidase catalyze the conversion from monohexosylceramides into ceramides (Figure 1). Given that the salvage pathway accounts for 50-90% of sphingolipid generation (Kitatani et al., 2008), dysregulation of these enzymes may have significant clinical impact. This is no more evident than in individuals with Krabbe disease; a disease characterized by infantile-
onset neurologic deterioration due to deficient galactocerebrosidase activity, ranging from 5-10% of normal levels (Suzuki, 2003). Interestingly, in adults diagnosed with late-onset Krabbe disease, a case series demonstrated a clinical profile similar to that of cerebrovascular disease, reporting progressive cognitive impairment and white matter hyperintensities not unlike those associated with VCI (Malandrini et al., 2013). Furthermore, galactocerebrosidase gene expression increases with severity of cognitive impairment in temporal regions; functional brain regions that are involved in tasks involving verbal memory (Katsel, Li, & Haroutunian, 2007; Swardfager et al., 2018).

As monohexosylceramides encompass both glucosylceramides and galactosylceramides, the ratio of C18:0/MHxCer18:0 may also reflect glucerebrosidase activity. In Parkinson’s disease (PD) patients with cognitive impairment, plasma levels of CerC18:0 were higher than those in PD patients without cognitive impairment. The authors suggest that this may be a result of a mutation in the glucerebrosidase gene, which is a common genetic risk factor for sporadic PD (Mielke et al., 2013). This was inconclusive, because genotyping was not done in that study. However, evidence from another study supports this hypothesis, demonstrating that glucerebrosidase mutations in PD patients are associated with more severe impairment in executive function (Mata et al., 2016). Furthermore, expression of UDP-glucose ceramide glucosyltransferase, an enzyme that catalyzes the synthesis of glycosphingolipids, was decreased in all stages of cognitive decline along the AD spectrum (Katsel et al., 2007).

As evident in sporadic PD and Krabbe disease, severe dysregulation of the salvage pathway has significant clinical consequences, but the extent of perturbation in those pathologies likely
exceeds that observed in our study population. Despite this, our findings suggest that chronic, mild dysregulation may have a cumulative, clinical impact.

4.1.4. Tertiary Findings

In our tertiary hypotheses, an increased ratio of CerC18:0/SM18:0 suggests increased activity of sphingomyelinase in the catabolic pathway, which catabolizes SM18:0 into CerC18:0. In support of this, plasma levels of secretory acid sphingomyelinase and ceramides have been found to be elevated in CAD patients (Pan et al., 2014). This increase in ceramides causes aggregation of LDL, which leads to formation of atherosclerotic plaques (Tabas, Williams, & Boren, 2007). However, the role of sphingomyelinase in cognition remains less clear. A study investigating gene expression in the brain, found that sphingomyelinase gene expression did not differ between cohorts along the cognitive impairment spectrum (Katsel et al., 2007). In contrast, a recent paper reported that an induced deficiency of neutral sphingomyelinase-2 improved cognition in an animal model of AD (Dinkins et al., 2016). As such, the influence of sphingomyelinase activity on cognitive performance in CAD patients remains unclear and warrants further investigation.

Surprisingly, elevated plasma S1P was associated with worse verbal memory performance over time. This was surprising, because numerous studies report S1P to be neuroprotective, particularly in AD pathology (Couttas et al., 2014; Ceccom et al., 2014). While there is evidence to suggest a neuroprotective role of S1P, other studies report that accumulation of S1P can be neurotoxic (Hagen et al., 2009; Mitroi et al., 2016). Alternatively, the elevation of S1P, driven by upregulation of sphingosine kinase, may be a compensatory mechanism to certain stimuli. This has been suggested to be a physiological response to inflammatory cytokines like TNF-α.
and interleukins (Spiegel & Milstien, 2003), which may be a potential mechanism through which ceramides is related to neurodegeneration. However, this has yet to be explored in clinical studies. Overall, these discrepancies may implicate the importance of S1P balance as suggested by Karunakaran and colleagues (Karunakaran & van Echten-Deckert, 2017). An alternative explanation may be that peripheral S1P levels in our participants may be more representative of CAD pathology than neurodegenerative change, due to its proximity to the anatomical location of CAD. In coronary arteries, S1P signalling induces adhesion molecule expression and subsequent atherogenesis (Xia et al., 1998), implicating its involvement in CAD pathogenesis. Furthermore, peripheral levels of S1P are elevated in CAD patients and predict occurrence and severity of coronary stenosis (Deutschman et al., 2003; Sattler et al., 2010). Taken together, we propose that greater CAD severity due to elevated peripheral S1P levels is associated with more severe cognitive impairment.

4.1.5. Exploratory Hypothesis

Despite evidence that suggest cognition in those with VCIND declines at a greater rate than those without VCIND (Stern et al., 1994), associations between CerC18:0 ratios and verbal memory did not remain significant in those with VCIND at baseline. The reason for this is unclear, but it is likely be attributed to our limited sample size as only 14 participants had possible VCIND at baseline. As such, stratification by neuropsychological criteria warrants further investigation in a larger, more balanced analysis of patients with possible VCIND and those with no possible VCIND.

Although our findings were statistically significant, the inconsistency in our findings brings attention to a prevailing need to integrate multiple biomarkers into predictive biomarker models,
if prognostic biomarkers are to be clinically useful, rather than investigating the predictive ability of a single biomarker. More advanced statistical methods like partial least discriminant analysis and random forest analysis are becoming more accessible for the general scientific community and is the logical progression in computational methods to discover and validate biomarker models such as in recent efforts to validate blood-based amyloid markers for AD (Nakamura et al., 2018).

4.2. Pathological mechanisms of ceramides in neurodegeneration

Ceramides contribute to progressive and irreversible cognitive deterioration by inducing the premature death of neurons and oligodendrocytes in multiple ways (Mencarelli & Martinez-Martinez, 2013). These terminally differentiated neural cells are particularly vulnerable to damage due to their low antioxidative capacity; thus they are not easily replaced (Thorburne & Juurlink, 1996; Juurlink, Thorburne, & Hertz, 1998). One extensively-studied mechanism is through the excessive apoptosis: increased level of ceramides induces release of TNF-α, which triggers cell death (Obeid et al., 1993), while their depletion hinders apoptotic processes (Bose et al., 1995). They can directly promote apoptosis by increasing permeability of mitochondrial membranes (Falluel-Morel et al., 2004), which subsequently releases pro-apoptotic factors such as cytochrome C and oxidative stress markers (Stoica, Movsesyan, Lea, & Faden, 2003; France-Lanord, Brugg, Michel, Agid, & Ruberg, 1997). Alternatively, ceramide may indirectly induce apoptosis by amplifying apoptotic signals. Ceramide can cluster key death signalling molecules (e.g. CD95 or TNF-α receptors) through spatial reorganization of the plasma membrane and formation of lipid microdomains (Gulbins, 2003).
While ceramides induces apoptosis, it is also known to inhibit pro-survival pathways such as the Akt pathway, which supplies cells with glucose and essential nutrients. In an aforementioned study, ceramides decreased the viability of neural cells by inhibiting the pro-survival PI3-K/Akt pathway through activation of protein phosphatase 2A (Scarlati et al., 2004). Interestingly, the addition of S1P directly counteracts ceramide-induced apoptosis by decreasing oxidative stress and upregulation of the anti-apoptotic protein, Bcl-2 (Czubowicz & Strosznajder, 2014). As such, the balance of ceramide and S1P is proposed to be an important determinant of cellular fate (Cuvillier et al., 1996).

CerC18:0 may induce senescence. For instance, CerC18:0 downregulates the expression of telomerase, which usually elongates the end of existing chromosomes to prevent senescence. Telomerase is constitutively expressed in the hippocampus, a brain region which is frequently associated with verbal memory performance (Bonner-Jackson, Mahmoud, Miller, & Banks, 2015). Moreover, the hippocampus is continuously supplied with neural stem and progenitor cells. As such, accumulation of CerC18:0 may increase neuronal vulnerability in the brain.

In considering these detrimental effects, ceramides have been suggested as a novel therapeutic target (Canals, Perry, Jenkins, & Hannun, 2011). However, it is important to be mindful that ceramides are also crucial in the proper functioning of the central nervous system (Furuya, Mitoma, Makino, & Hirabayashi, 1998). Therefore, an emphasis on maintaining sphingolipid equilibrium rather than decreasing levels indiscriminately is necessary. Further investigation on the role of these catalyzing enzymes is warranted as their activity is most proximal to this balance. Overall, a plethora of evidence and the role of ceramides in multiple biochemical pathways suggest that there are likely multiple mechanisms by which this occurs.
4.3. **Strengths**

This study has numerous strengths. The varying time intervals between study visits were adjusted for using a spatial covariance structure in mixed regression models. This ensures a more precise adjustment as opposed to assuming that inter-visit time intervals are equal between individuals. In addition, we also investigated the possible perturbations of three CerC18:0 biosynthesis pathways to ensure a comprehensive analysis of CerC18:0 metabolism. Although metabolites specific for the *de novo* pathway were not assessed, ceramide synthesis through this pathway accounts for only a minority of brain sphingolipids. Furthermore, we adjusted for pertinent covariates, particularly years of education. Our findings remained significant after adjustment for education, demonstrating robustness.

4.4. **Limitations**

4.4.1. **Methodological limitations with recommendations for future studies**

Our main analysis did not adjust for cardiopulmonary fitness, because our sample size did not permit the addition of another covariate into the model. Although exercise in CR has been shown to improve certain cognitive domains (Gunstad et al., 2005), the effect of aerobic exercise on cognition is inconsistent (Young, Angevaren, Rusted, & Tabet, 2015). Nonetheless, future studies should adjust for baseline cardiopulmonary fitness if sample size is adequate. Secondly, the independent influence of CAD on plasma ceramides levels could not be elucidated as we did not have a healthy control group. However, this population of CAD participants with perturbed lipid metabolism and normal cognitive function is the ideal population to investigate our hypotheses as they are representative of the latent, presymptomatic stage of VCI. Our analyses should be investigated in a larger population in validation studies. Another limitation is the technical inability of mass spectroscopy to isolate
glucosylceramides from galactosylceramides (Savica et al., 2016). As such, more extensive techniques should be used to separate them in future studies to see if there are clinically impactful differences between these two related isomers.

4.4.2. Mechanistic Limitations

A key limitation in the interpretation of our findings is that peripheral levels of ceramides may not be indicative of its levels in the brain, the area in which its downstream effects are most relevant. Synthetic ceramides (e.g. CerC2:0 and CerC6:0) can cross the BBB (de la Monte et al., 2010), but the BBB permeability of long-chained ceramides like CerC18:0 has yet to be investigated. In light of this, matched CSF and serum sphingolipid concentrations have been found to be correlated in patients with HIV (Bandaru et al., 2009). Ultimately, even if peripheral concentrations of long-chained ceramides do not correlate with those in the central nervous system, they may still be indicative of pathophysiological changes in the brain. Since ceramides function as secondary messengers, their downstream effectors (e.g. TNF-α) may cross the BBB and elicit neurotoxic effects (de la Monte, 2012). Lastly, the use of CerC18:0 ratios in this study are exploratory and do not directly support the discussed implications on enzymatic activity. Future investigations should consider the use of biochemical assays such as those to measure sphingomyelinase (Taki & Chatterjee, 1995), galactocerebrosidase (Martino et al., 2009), and ceramide synthase activity (Kim, Qiao, Toop, Morris, & Don, 2012) as a more accurate and direct measurement of enzymatic activity.

4.5. Implications on drug development

Considering the established role of ceramides in apoptosis and cellular survival, the present findings invite further research into whether these sphingolipid pathways should be considered
for drug development in vascular neurodegeneration. Although ceramides are a direct inducer of these pathologic effects, it is a poor drug target given its unpredictability as a protein (Makley & Gestwicki, 2013). Instead, targeting the enzymes that catalyze its synthesis may be a more feasible strategy due to their specificity of action, crucially neutral and acidic sphingomyelinases.

Functional inhibitors of acid sphingomyelinase (FIASMA) block ASM activity by preventing it from attaching to inner lysosomal membranes and results in its proteolytic inactivation. Most FIASMA such as desipramine, dextromethorphan, and maprotiline are already approved for clinical use. However, given that these FIASMA are part of many other drug classes, their effects on their originally intended drug targets need to be minimized (Kornhuber et al., 2010).

The abundance of neutral sphingomyelinase (NSM) in the hippocampus (Hofmann, Tomiuk, Wolff, & Stoffel, 2000) and its effect on nerve growth factor-induced neurite outgrowth, synaptogenesis (Brann et al., 1999), and synaptic plasticity (Wheeler et al., 2009) due to downstream ceramide production has inspired efforts to modulate NSM activity in order preserve neuronal function. Recent high-throughput screening at Johns Hopkins found that cambinol decreased TNF-induced and ILB-induced ceramide production; thus, it may have neuroprotective properties (Figuer-Losada et al., 2015). However, caution should be exercised, because the detrimental effect of NSM modulation has also been reported. The same group at Johns Hopkins reported that inhibition of NSM-2 disrupted spatial memory performance in rats through an AMPA receptor-mediated mechanism (Tabatatze et al., 2010). While these results demonstrate the importance of sphingolipid balance in memory function, these discrepant findings highlight a need for better characterization of these enzymes and their cognitive impact.
4.6. Conclusions

In summary, higher baseline CerC18:0 concentrations, higher CerC18:0/MHxCerC18:0, and higher CerC18:0/SM18:0 ratios were significantly associated with steeper decline in verbal memory performance in CAD participants. These ratios may indicate perturbations within the catabolic pathway and salvage pathway of ceramides biosynthesis, respectively. However, when we compared these relationships between those with possible VCIND and those who without, they were not significantly different between these two groups. This is likely due our limited sample size. Taken together, our present findings suggest that altered sphingolipid metabolism may be among the earliest neurobiological changes associated with vascular neurodegeneration and invite further research to determine whether its metabolic pathways may be a clinically relevant target for drug discovery. Future studies should measure the enzymes responsible for conversion of sphingolipid precursors into CerC18:0 to directly assess enzymatic activity.
References


*Neurol Sci, 34*(1), 79-83. doi: 10.1007/s10072-012-0956-6


62


Rojas-Fernandez, C. H. (2013). Little evidence that cholinesterase inhibitors prevent progression of mild cognitive impairment to dementia, but they are associated with adverse effects. Evid Based Ment Health, 16(2), 39. doi: 10.1136/eb-2012-101087


Appendix 1: Approval from Research Ethics Board
Research Ethics Board (REB)  
RENEWAL FORM

The Renewal Form is an application for continuing ethics approval and must be submitted for review and approval prior to the study's expiry date. Ethics approval expires each subsequent year from the day REB approval was initially granted unless otherwise indicated by the Sunnybrook REB. Failure to submit this form prior to the expiry date signifies that the study does not have REB approval and all research activities must be suspended. Conducting research without REB approval may result in a notice of non-compliance involving corrective action, up to and including, termination of the research study.

Principal Investigator (PI): Dr. Krista Lanctôt

REB Project Identification Number (PIN): 279-2011

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

1. Date of initial Sunnybrook REB approval (dd/mmm/yyyy).
   11/Nov/2011

2. Type of REB review requested. (Final decision rests with the REB Chair.)
   - [ ] Delegated Review
   - [x] Full Board Review

3. Is this an Industry-Sponsored/Supported study?
   - [ ] YES (If YES, complete the table below.)
   - [x] NO (If NO, proceed to question 4.)

<table>
<thead>
<tr>
<th>Invoicing Information for Industry-Sponsored/Supported Studies</th>
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<td>A fee of $500 Cdn is invoiced for all Industry-Sponsored/Supported Studies applying for continuing ethics approval.</td>
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4. Is this study open for enrollment at Sunnybrook? [x] YES [ ] NO

If YES, attach a copy of the current Informed Consent Form(s).
Appendix 2: Informed Consent Form
The Heart-Mind Connection: Evaluating the Association between Ceramides and Cognitive Decline in Coronary Artery Disease

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), Visit 3 (6 months) and Visit 4 (post 12 months):  
After the initial baseline visit, you would return for 3 in-clinic visits, each lasting approximately 2 hours. Visits 2 and 3 will take place 3 months and 6 months after your initial visit. Visit 4 will take place at least 12 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. At visit 2 and 3 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 5 years. The entire study is expected to take about 5 years to complete and the results should be known in 5½ 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 4 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 visits 1, 2 and 3.

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. Lancôtôt, Department of Psychiatry at 416-480 6100 x2241. 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.
April 1, 2016

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.

5. By signing this consent form, you do not give up any of your legal rights.

6. 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.

7. You have the right to access, review and request changes to your personal health information.

8. 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, Janelle Bradley (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 or the Sunnybrook Health Sciences Centre Research Ethics Board. DOCUMENTATION OF INFORMED CONSENT

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

Ceramides and Cognitive Decline in CAD
April 1, 2016

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

_______________________  ___________________________  _________________________

Ceramides and Cognitive Decline in CAD
Informed Consent Checklist/Note

Consent version # / date: 

Consent signed and dated by subject prior to any study related procedures:  □ YES  □ NO

Date/Time signed: 

Was a copy of the consent given to the subject?  □ YES  □ NO

Did subject demonstrate comprehension of consent form contents?  □ YES  □ NO

Comments:

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

Consent obtained by: