Associations of circulating second messenger
glycerophosphatidylcholines with cardiovascular disease
risk factors in adolescents

by

Simon Czajkowski

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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2015

Abstract

Circulating glycerophosphatidylcholines and their second messenger metabolites (smGPCs) are phospholipids that modulate risk for cardiovascular disease (CVD). CVD is a slow-progressing disease culminating by acute vascular events or chronic vascular conditions in middle-to-late adulthood, but its pre-clinical stages may already occur during adolescence. We investigated whether circulating smGPCs are associated with CVD risk factors – excess visceral fat, elevated blood pressure, insulin resistance, and low-grade inflammation – in a population-based sample of adolescents as part of the Saguenay Youth Study. Several of the smGPCs were associated with multiple CVD-risk factors and may serve as novel biomarkers of early CVD development; the most significant of these were PC(16:0/2:0) and LPC(14:1/0:0). Mediation tests revealed that PC(16:0/2:0) mediated the directed relationship between VF and blood pressure or C-reactive protein, and LPC(14:1/0:0) mediated the directed relationship between VF and fasting insulin. Elucidating pathways regulating circulating levels of these smGPCs may provide new pharmaceutical targets of CVD.
To my godson, Jacek,

for reminding me

what it's like

to see the world from a child's eyes,

and to Anne-Marie,

for bringing me joy and inspiring me.
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<th>Definition</th>
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<tbody>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>cIMT</td>
<td>carotid intima-media thickness</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computer tomography</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>GPC</td>
<td>glycerophosphatidylcholine</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>IHD</td>
<td>ischemic heart disease</td>
</tr>
<tr>
<td>LC-ESI-MS</td>
<td>liquid chromatography-electrospray ionization-mass spectroscopy</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LPC</td>
<td>lysophosphatidylcholine</td>
</tr>
<tr>
<td>MetS</td>
<td>metabolic syndrome</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>oxidized low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>smGPC</td>
<td>second messenger glycerophosphatidylcholine</td>
</tr>
<tr>
<td>TBF</td>
<td>total body fat</td>
</tr>
<tr>
<td>TIA</td>
<td>transient ischemic attack</td>
</tr>
<tr>
<td>TRLs</td>
<td>triacylglycerol-rich lipoproteins</td>
</tr>
<tr>
<td>VF</td>
<td>visceral fat</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein cholesterol</td>
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</tbody>
</table>
Chapter 1

1 Introduction (Part I): Cardiovascular disease

1.1 Cardiovascular disease prevalence and outcomes

Cardiovascular disease (CVD) is a set of diseases and injuries affecting the heart and vascular system throughout the body and within the brain. CVDs include ischemic heart diseases and cerebrovascular diseases.

Although the age-adjusted death rate due to CVD has declined over the past several decades\(^1\), CVD is still the leading cause of death, disability, and lost productivity in adults worldwide\(^2\). Heart disease and stroke are two of the three leading causes of death, together accounting for 29 percent and 34.4 percent of all deaths in Canada and the USA, respectively\(^3,4\). Approximately half of all CVD deaths are caused by ischemic heart disease, while the rest of major diagnoses that contribute to CVD mortality include stroke, heart attack, and heart failure\(^3,4\). The economic burden of CVD is also remarkable. The estimated health care and lost productivity costs of heart disease and stroke yearly exceed $20.9 billion in Canada\(^5\), while the estimated annual direct and indirect costs of total CVD in the USA exceed $400 billion\(^6\).

Atherosclerosis – the thickening of arterial walls by plaque formation – is a major underlying mechanism of CVD. It is a slow developing process that begins in childhood and adolescence, and culminates in adulthood by acute vascular events, such as myocardial infarction and stroke, and by chronic vascular conditions, such as chronic heart failure and certain types of dementia. It is estimated that 90 percent of CVD is preventable\(^7\). Decreasing risk factors through dietary, pharmacological, and other life-style interventions are ways of preventing atherosclerosis progression and CVD development. Since the pathogenesis of CVD begins in childhood and adolescence, methods of identifying subclinical atherosclerosis in addition to conventional risk factor assessment may help identify otherwise asymptotic individuals at such a risk for CVD who should be targeted for early intervention and secondary prevention.

1.2 Cardiovascular disease pathophysiology

The development of atherosclerosis occurs in several stages. In the initial stages, atherogenic lipoproteins rich in apolipoprotein B (such as low density lipoprotein cholesterol –
LDL enter the subendothelial space of blood vessels. Following deposition in the vascular subendothelium, LDL undergoes oxidation. Oxidation of LDL promotes a local inflammatory response marked by the release of inflammatory cytokines and by the expression of several vascular cell-adhesion molecules, including vascular cell-adhesion molecule-1, intracellular adhesion molecule-1, and P-selectin, which in turn recruit monocytes to the arterial wall. Upon entering the vascular subendothelium, monocytes differentiate into macrophages, a process that is characterized by an increase in the expression of scavenger receptors capable of binding oxidized-LDL (ox-LDL). Ox-LDL enters macrophages via the scavenger receptors and concentrates in lysosomes where cholesterol esters belonging to ox-LDL are hydrolyzed to fatty acids and free cholesterol. The enzyme acyl CoA:cholesterol acyltransferase then catalyzes the conversion of free cholesterol to cholesteryl ester, which is the preferred form of cholesterol storage, and which is characteristic of foam cells (i.e. fat-laden macrophages forming atherosclerotic plaques) (Figure 1). Any cholesterol that is not esterified leaves the macrophages by passive or facilitated diffusion, or is actively removed by the membrane-bound ATP-binding cassette (ABC) A1 protein. Lecithin cholesterol acyl transferase activity, which is ordered towards the incorporation of cholesterol into high-density lipoprotein cholesterol – HDL, governs the diffusion gradient for free cholesterol from the macrophages. Deficiencies in cholesterol removal from foam cells can likewise contribute to the development of atherosclerosis. Following the formation of mature atherosclerotic lesions, macrophages are important determinants of atherothrombotic events related to the rupturing of atherosclerotic plaques and causing thrombosis. Macrophages within lesions secrete metalloproteinases that break down the connective tissue of plaques, thereby increasing the likelihood of plaque rupture. This is supported by the finding that ruptured plaques usually contain high numbers of macrophages. In addition, plaque macrophages secrete prothrombotic tissue factors that promote thrombus formation after the rupture or erosion of lesions.
Figure 1.1: Atherosclerotic lesion formation.
Atherosclerosis develops in many stages: (i) LDL is deposited in the subendothelium and is oxidized; (ii) LDL oxidation promotes local inflammation and expression of vascular-cell adhesion molecules, such as VCAM-1 and ICAM-1, and chemokines, such as MCP1, which results in the recruitment of monocytes; (iii) M-CSF stimulates monocytes to differentiate into macrophages upon entering the subendothelium; (iv) macrophages accumulate ox-LDL via the SR-A and SR-B1; (v) free cholesterol within macrophages is catalyzed by ACAT1 into cholesterol esters, while NCEH catalyzes the opposing reaction; (vi) free cholesterol leaves macrophages via ABCA1; (vii) LCAT drives the diffusion gradient of cholesterol by esterification of free cholesterol for incorporation into HDL. ABCA1, ATP binding cassette A1; ACAT, acyl CoA:cholesterol acyltransferase; ACAT1, acyl CoA:cholesterol acyltransferase 1; apo A-I, apolipoprotein A-I; ICAM-1, intercellular adhesion molecule-1; LCAT, lecithin-cholesterol acyltransferase; M-CSF, macrophage colony stimulating factor; MCP1, monocyte chemoattractant protein-1; MSRA, macrophage scavenger receptor A; NCEH, neutral cholesteryl ester hydrolase; Ox-LDL, oxidized LDL; SR-A, scavenger receptor A; SR-B1, scavenger receptor B1; VCAM-1, vascular cell adhesion molecule-1; VLA4, very late antigen-4.


Inflammation plays an important role in all stages of atherosclerosis development, as well as in the thrombotic complications that may result. Inflammation in the endothelium leads to an increase in the expression of adhesion molecules that bind leukocytes in the early
atheroma\textsuperscript{18}. Following adhesion of leukocytes to the endothelium, pro-inflammatory cytokines like monocyte chemoattractant protein-1 provide a chemical signal for leukocyte migration to the vascular subendothelial space\textsuperscript{19,20}. Macrophages residing in the subendothelial space express scavenger receptors that mediate ox-LDL intake, while monocyte chemoattractant protein-1 and macrophage colony-stimulating factor facilitate the further differentiation of macrophages from circulating monocytes\textsuperscript{21,22}. T-lymphocytes joining macrophages in the subendothelial space secrete various inflammatory cytokines that stimulate macrophages, vascular endothelial cells, and smooth muscle cells\textsuperscript{23}. As inflammation progresses, activated leukocytes further stimulate smooth muscle cell replication and migration to the extracellular matrix, which contributes to the more advanced atherosclerotic lesion phenotype\textsuperscript{24}. In the presence of inflammation, macrophages within lesions express enzymes that degrade the elastin and collagen of the plaque’s protective cap, thereby making it vulnerable to rupture. Furthermore, activated T-lymphocytes within lesions can inhibit smooth muscle cells from synthesizing new collagen, preventing the reinforcement of the plaque’s cap\textsuperscript{25,26}. Finally, inflammatory mediators manage the expression of procoagulant tissue factor by macrophages within lesions\textsuperscript{26}, which promotes thrombosis after plaque rupture, leading to the many acute outcomes of CVD.

Given that inflammation plays an integral part of the initiation, progression, and culmination of atherosclerosis, it is possible to predict the likelihood of related future CVD outcomes by measuring chronic low-grade inflammation. C-reactive protein (CRP) is an acute phase reactant that rises in response to underlying inflammatory activity and is a widely-used clinical measure of low-grade inflammation, as well as a circulating marker of CVD risk\textsuperscript{27}. Besides being a measure of inflammation, CRP is a critical component of the immune system: it dissociates pentameric CRP to activate leukocyte-endothelial interactions under pro-inflammatory conditions\textsuperscript{28} and it opsonizes bacteria and dying cells, allowing for their clearance by phagocytes via the complement system\textsuperscript{29}. CRP belongs to the pentraxin protein family\textsuperscript{30} and is primarily expressed in hepatocytes in response to rising IL-6 levels, and to a lesser extent, IL-1\textbeta and tumor necrosis factor \alpha levels\textsuperscript{31} (Figure 1.2).
Many studies demonstrate that baseline circulating levels of CRP in healthy men and women are predictive of future CVD. The Emerging Risk Factors Collaboration is the largest study showing an association between low-grade inflammation and future CVD outcomes. It is a meta-analysis of 54 long-term prospective population-based studies of 160,309 individuals without a history of vascular disease. Most notably, the results showed that risk ratios for coronary heart disease and ischemic stroke were higher by 1.63 and 1.44, respectively, per 1-S.D. log \(_e\) CRP concentration (when adjusting for age, sex, and within person variation in risk factor levels), and 1.37 and 1.27, respectively (when additionally adjusting for conventional risk factors — i.e., systolic blood pressure [SBP], smoking, history of diabetes, body mass index [BMI], serum concentrations of triacylglycerols, non-HDL cholesterol, HDL, and alcohol consumption).

Clinically, “high sensitivity” CRP testing is used alongside measures of LDL to detect individuals at risk of future CVD. These blood tests are done alongside each other because each test measures a different component of the disease process. It is said that individuals with circulating CRP levels (a) under 1 mg/L are at overall low CVD risk, (b) between 1 and 3 mg/L...
are at moderate CVD risk, and (c) above 3 mg/L are at high CVD risk (Figure 1.3). It is recommended that lone measures of CRP above 10 mg/L be excluded due to the probability of them reflecting a response to acute infection and, therefore, not having the same predictive value for CVD risk as baseline measures.


1.3 Cardiovascular disease risk factors

CVD is associated with various risk factors that can be classified into non-modifiable and modifiable risk factors. Age, sex, race, and a genetic predisposition group among the non-modifiable risk factors. Advancing age is the most powerful predictor of CVD. Men are at greater risk of heart disease than pre-menopausal women; however, after menopause, women’s risk is similar to that of men. African and Asian ancestry is associated with higher risk of developing CVD, and having a first-degree relative who has had coronary heart disease or stroke before age 55 years, for a male relative, and 65 years, for a female relative, also increases risk of developing CVD (www.world-heart-federation.org/cardiovascular-health/cardiovascular-disease-risk-factors). The classical modifiable risk factors of CVD are obesity (especially abdominal obesity), elevated blood pressure, dyslipidemia, hyperglycemia and insulin resistance, low-grade inflammation, and a pro-thrombotic state, all of which are described in more detail below:

Obesity – Obesity is a major and independent risk factor for CVD. Overweight and obesity have become highly prevalent not only in adults but also in children and adolescents. In Canada and the USA, around 60% of adults and 30% of adolescents are overweight or obese. Findings from the Behavioural Risk Factor Surveillance System (a task force organized by the Centers for Disease Control and Prevention) demonstrate that the prevalence of obesity in US adults increased by 61% from 1991 to 2000. During the past decade, however, the prevalence rates of childhood obesity have plateaued in developed countries, but continued to...
increase in developing countries\textsuperscript{42}. This plateau in childhood obesity rates in developed
countries may be related to public health efforts aimed at curbing obesity and corresponding
societal adjustments\textsuperscript{42}.

Obesity can be defined as an excess of body fat and can be assessed in several ways. Classification by body mass index (BMI) is a common, albeit indirect, way of measuring body fat in adults. Clinically, individuals with BMI of 25-29 kg/m\textsuperscript{2} are considered overweight, whereas individuals with BMIs $\geq$30 kg/m\textsuperscript{2} are considered obese\textsuperscript{43}. Another clinical method of estimating abdominal body fat is by measurement of waist circumference, since abdominal fat - it is most strongly associated with CVD risk\textsuperscript{36,43}. The National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) defines abdominal obesity as a waist circumference of 102 cm or more in men and 88 cm or more in women\textsuperscript{36}. Risk for developing CVD, as well as type 2 diabetes and hypertension (which are additional risk factors for CVD), increases with increasing BMI, both in men and women with normal and obese waist circumference\textsuperscript{43}. Notably, an obese waist circumference is associated with higher disease risk throughout most BMI categories, compared to a normal waist circumference, attesting to the fact that abdominal obesity captures additional disease risk than BMI alone\textsuperscript{43}.

Numerous epidemiological studies confirm the association between obesity and CVD. For example, the Chicago Heart Association Detection Project in Industry study, a prospective, population-based study of 17,643 men and women aged 31 to 64 years, examined from 1967 to 1973, and followed for a mean 32 years, found that obesity in middle age increases the risk for coronary heart disease (CHD), CVD, and diabetes –related hospitalization and mortality later in life\textsuperscript{44}. The findings showed that the blood pressure and total cholesterol-independent odds ratio for CHD mortality for obese participants compared to participants of normal weight was 1.43 in a low CVD risk category and 2.07 in a moderate risk category. The low-risk category consisted of non-smokers who had normal blood pressure and cholesterol, and who were not taking any antihypertensive or lipid-lowering medication, while the moderate risk category consisted of non-smokers who had pre-hypertension or cholesterol levels between 5.2 and 6.2 mmol/L, but who were not taking any antihypertensive or lipid-lowering medication. For CHD hospitalization, the corresponding results were 4.25 in the low risk category and 2.04 in the moderate risk category. Similar BMI-related odds ratios were observed for CVD mortality and hospitalization. Importantly, obesity was noted to be an independent CVD risk factor – being obese with or without other risk factors such as smoking, high blood pressure, and high cholesterol, conferred increased risk of CVD mortality\textsuperscript{44}. Another prospective study examining the association between
obesity and future CVD in 21,414 men – the Physician’s Health Study – found that overweight men (BMIs 25-29.9 kg/m²) had a 1.32 relative-risk for total stroke, a 1.35 risk for ischemic stroke, and a 1.25 risk for haemorrhagic stroke compared to men with BMI under 25 kg/m², while obese men (BMI ≥30 kg/m²) had the relative-risks of 1.91, 1.87, and 1.92, respectively, compared to men with BMI under 25 kg/m². In this study, each 1-unit increase in BMI was associated with a 4% increase in risk for ischemic stroke, and a 6% increase in risk for haemorrhagic stroke.45

_Elevated blood pressure_ – It is well established that individuals with elevated blood pressure are at an increased risk of CVD. Data from a meta-analysis of one million adults in 61 prospective studies shows that ischemic heart disease and stroke–related mortality increases progressively and linearly with blood pressure in each decade of age relative to the usual blood pressure at the start of that decade (Figure 1.4)46.
Figure 1.4: CVD-related mortality increase with blood pressure.
Stroke mortality rate (above), and ischemic heart disease (IHD) mortality rate (below), in each decade of age compared to the usual blood pressure at the start of that age decade.

In addition, longitudinal observations from the Framingham Heart Study show that the 8-year age-adjusted rate of incident cardiovascular events per 100 study participants significantly
increases in both borderline and definitive hypertension, in both men and women\textsuperscript{47}, and that ‘high-normal’ blood pressure (i.e. SBP/diastolic blood pressure [DBP]: 130-139/85-89 mmHg) is associated with more than a two-fold increase in relative risk for CVD, compared to ‘optimal’ blood pressure (i.e. SBP/DBP below 120/80 mmHg), in both sexes\textsuperscript{48}.

\textit{Dyslipidemia} – An important risk factor for CVD is atherogenic dyslipidemia, which is characterized by an increase in triacylglycerols, a decrease in HDL, and an increase in LDL and very-low density lipoprotein –cholesterol (VLDL) particiles\textsuperscript{49}. Elevated levels of LDL and VLDL together account for the increase in total apolipoprotein B commonly observed in atherogenic dyslipidemia.

LDL particles, especially very small LDL particles that can permeate the arterial wall more easily, are implicated in the developmental mechanisms of atherosclerosis and elevated levels are important predictors of CVD\textsuperscript{50,51}. A greater availability of LDL means that more LDL can be deposited underneath the vascular endothelium under pro-inflammatory conditions, undergo oxidation, and contribute to the formation of foam cells and atherosclerotic lesions\textsuperscript{52}.

In contrast, HDL participates in the ‘reverse’ transport of cholesterol from macrophage foam cells (\textit{Figure 1.1}). With decreased levels of HDL, this mechanism may be attenuated and disposal of cholesterol from the atherosclerotic lesions to the liver may be decreased, thereby promoting atherosclerosis\textsuperscript{49,53}. Examples of other theories about how HDL is anti-atherogenic relate to it having anti-inflammatory properties and protecting against LDL oxidation\textsuperscript{53}.

Finally, it is established that triacylglycerol-rich lipoproteins (TRLs) are atherogenic. TRLs are a class of a mixture of lipoproteins with high triacylglycerol content. Levels of TRLs rise with circulating triacylglycerols, and triacylglycerols are a marker for TRLs\textsuperscript{54}. Various animal, genetic, epidemiological studies implicate TRLs in CVD\textsuperscript{54–56}. TRLs undergo remodelling in peripheral tissue where TRL remnant particles rich in apolipoprotein E and cholesterol, but poor in triacylglycerols, are produced\textsuperscript{54}. The TRL remnant particles contribute to atherosclerotic plaque formation either directly by penetrating the arterial wall, or potentially indirectly by releasing pro-inflammatory molecules, such as certain free-fatty acids, during the remodelling of TRLs\textsuperscript{54}.

\textit{Hyperglycemia and insulin resistance} – Diabetes-level hyperglycemia is a major and an independent risk factor for CVD\textsuperscript{36}. Although pre-diabetes hyperglycemia (commonly assessed by impaired fasting glucose and/or impaired glucose tolerance) already confers increased risk for CVD\textsuperscript{57}, CVD risk rises sharply with the onset of diabetes-level hyperglycemia. Several meta-
analyses have demonstrated that the risk for developing coronary heart disease increases 2- to 3-fold with diabetes. It is uncertain whether hyperglycemia per se is atherogenic. Nevertheless, a longitudinal study of 1,229 patients with type 1 diabetes found a reduction in intima-media thickness of carotid arteries 6 years after the initial assessment, in patients who received intensive diabetes treatment, compared to patients who received conventional diabetes treatment, suggesting that in fact hyperglycemia is in itself atherogenic (Table 1.2).

**Table 1.1: Reduction of intima-media thickness with intensive diabetes treatment.** Least-squares mean change in the intima-media thickness of the common carotid artery and of the combined common and internal carotid arteries from year 1 to year 6 of the Epidemiology of Diabetes Interventions and Complications Study, according to the treatment assignment in the diabetes control and complications trial*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Change in Intima–Media Thickness of Common Carotid Artery</th>
<th>Change in Combined Intima–Media Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least-Squares Mean (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>Conventional treatment</td>
<td>0.046 (0.023 to 0.068)</td>
<td>0.007</td>
</tr>
<tr>
<td>Intensive treatment</td>
<td>0.032 (0.010 to 0.055)</td>
<td></td>
</tr>
<tr>
<td>Difference between groups</td>
<td>0.013 (0.003 to 0.024)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* The change in the intima-media thickness was used as the outcome to fit a general linear model, adjusted for sex, age, ultrasonography equipment used, and the year 1 thickness. CI denotes confidence interval.


Several mechanisms have been proposed whereby hyperglycemia might promote atherosclerosis. These relate to glycosylation-induced changes in the normal function of proteins that interact with macrophages, endothelial cells and smooth muscle cells, and increase oxidative stress and protein kinase C activation, thus, resulting in changed growth factor expression. It is unclear whether any of these mechanisms are directly or predominantly responsible in the pathogenesis of atherosclerosis, however, they appear to play a role and may in fact be interrelated.

Insulin resistance and compensatory hyperinsulinemia are considered the primary defect in type 2 diabetes and is thought to precede β-cell failure and overt hyperglycemia even by several decades. It is possible that insulin resistance per se may be directly atherogenic and
contribute to the development of CVD long before the onset of hyperglycemia. Several studies have found hyperinsulinemia or insulin resistance to be associated with CVD\textsuperscript{66}. Worth special mentioning is a prospective, population-based study of 2,493 Danish men and women, aged 41 to 72 years, which investigated the risk associated with insulin resistance independently of the metabolic syndrome following the International Diabetes Foundation’s and the National Cholesterol Education Program’s definitions, the former of which includes hyperglycemia (i.e. fasting circulating glucose \( \geq 5.6 \text{ mmol/L} \) or anti-diabetic drug use) as a necessary component for diagnosis\textsuperscript{67}. After a median follow-up of 9.4 years, the findings showed that insulin resistance, measured by the homeostasis model assessment (i.e. fasting glucose x fasting insulin / 22.5), was an independent predictor for incident CVD – the relative risk of experiencing incident CVD was 1.67\textsuperscript{67}.

Low-grade inflammation – As described in Chapter 2.1, inflammation plays an integral role in all stages of atherogenesis. It is therefore conceived that atherogenesis necessitates a chronic state of low-grade inflammation. The potential of circulating CPR levels to predict major cardiovascular events suggests that more advanced and less stable atherosclerotic plaques form at a higher state of inflammation than less advanced and more stable plaques\textsuperscript{27,68}. This notion is supported by the findings that macrophages play an important role in the degradation of connective tissue of plaques, thereby, increasing the likelihood of plaque rupture, and that ruptured plaques typically contain high numbers of macrophages\textsuperscript{15,16}. In addition, inflammation is often present in obesity suggesting that it may represent developmental link between obesity and atherosclerosis. It is well known that adipose tissue is an endocrine organ that secretes many bioactive molecules (Figure 1.5)\textsuperscript{69,70}, some of which are pro-inflammatory and may therefore contribute to the development and/or progression of CVD.

Pro-thrombotic state – a pro-thrombotic state, which is another risk factor for CVD\textsuperscript{36}. It is possible that a pro-thrombotic state confers risk by increasing the likelihood of more severe thrombosis after the rupture of atherosclerotic plaque. A pro-thrombotic state is mainly characterized by elevated levels of circulating fibrinogen and plasminogen activator inhibitor-1 (PAI-1)\textsuperscript{36}. Adipose tissue is known to synthesize and secrete PAI-1 (Figure 1.5), and visceral adipose tissue, which is more metabolically harmful than adipose tissue found elsewhere in the body (discussed in more detail later), is known to produce more PAI-1 than subcutaneous adipose tissue\textsuperscript{71}. 
The classical risk factors for CVD described above, namely abdominal obesity, elevated blood pressure, dyslipidemia, hyperglycemia and insulin resistance, low-grade inflammation, and a pro-thrombotic state, tend to cluster together in the same individuals\textsuperscript{36}. This constellation of risk factors has been recognized as the metabolic syndrome (MetS). Although these components can be measured in practice, the National Cholesterol Education Program Adult Treatment Panel III (ATP III) report's criteria for clinical diagnosis defines the MetS as the presence of at least 3 of the following 5 characteristics in the same individual: increased abdominal obesity (measured by waist circumference), elevated blood pressure, atherogenic dyslipidemia (defined as high triacylglycerides and low HDL), and elevated glucose fasting levels (Table 1.3)\textsuperscript{36,72,73}. Individuals with the MetS are at an increased risk for CVD, with MetS predicting approximately 25% of all incident CVD cases\textsuperscript{36}. Globally, the prevalence of MetS is reaching epidemic proportions. In Canada, for example, it has been estimated that >25% of adults suffer from the syndrome\textsuperscript{74}. Similar numbers have been reported in the USA\textsuperscript{75}, and with more recent data showing a persistent increase of prevalence of MetS\textsuperscript{76}. Given the adverse cardiovascular consequences of MetS, perhaps most alarming is the fact that the syndrome, typically regarded as a middle- to late-adulthood disorder, is now present in adolescence with close to 10% of all 12-19-year olds being already affected\textsuperscript{77,78}.

![Figure 1.5: Adipose tissue as an endocrine organ – examples of secreted bioactive molecules.](image-url)
The National Cholesterol Education Program Adult Treatment Panel III report recommends measuring waist circumference as a mean of assessing the obesity component of the MetS. This is because abdominal obesity is more closely associated cardiovascular and overall health risks than total body fat. Abdominal fat is stored in two major compartments: subcutaneous and visceral. Although visceral fat (VF) represents a relatively small fraction of total body fat (approximately 5-10%), it is considered central to obesity-induced cardio-
metabolic abnormalities. Findings from the Framingham Heart Study show that visceral adipose tissue is more strongly associated with most cardio-metabolic risk factors, including blood pressure, fasting plasma glucose, and triacylglycerols, and more inversely correlated with HDL than subcutaneous adipose tissue. Furthermore, the odds ratios for MetS per 1-S.D. increase in visceral adipose tissue were significantly higher than those for subcutaneous adipose tissue, in both women and men, and visceral adipose tissue, but not subcutaneous adipose tissue, contributed significantly to risk factor variation after adjustment for routine clinical measures of obesity, namely body mass index and waist circumference. Further evidence for the critical role of VF in cardio-metabolic abnormalities of obesity comes from human studies demonstrating that the surgical removal of abdominal subcutaneous fat by liposuction does not improve cardio-metabolic profiles, whereas the surgical removal of omental fat (a part of VF) does. Previous findings from our lab suggest that the adverse cardio-metabolic associations with VF are already present in adolescence, wherein males and females with high VF content (versus low VF content) demonstrate a more adverse profile of MetS components.

Current clinical methods of assessing obesity are limited because neither BMI nor waist circumference can quantify VF alone. BMI and waist circumference are composite measures reflecting not only the quantity of fat mass (both visceral and subcutaneous) but also the quantity of lean body mass (muscles and bones), and as such they may be misleading in classifying “cardio-metabolically adverse obesity.” It is recognized that some individuals who have normal BMI and normal waist circumference have an excessive amount of VF and high cardio-metabolic risk. Our ability to identify “viscerally obese” individuals is limited to the use of methods that are either invasive (including X-ray computed tomography) or expensive (magnetic resonance imaging).

1.4 Cardiovascular disease origins in childhood and adolescence

Atherosclerosis begins in childhood and youth and progresses into adulthood to result in clinically apparent morbidity and mortality. The earliest evidence of this comes from pathology studies of soldiers killed in the Korean War (1950 – 1953) and in the war in Vietnam (1955 – 1975). Enos and colleagues first published findings from autopsies of fallen soldiers from the Korean War (average age: 22.1 years; age range: 18 – 48 years), which showed that in 77.3 percent of the examined hearts there was evidence of coronary atherosclerosis. Eighteen years later, McNamara and colleagues performed angiography and dissection of hearts from 105 US
soldiers killed in Vietnam (average age: 22.1 years; age range: 18 – 37 years) and found evidence of atherosclerosis in 45 percent of cases, and gross evidence of severe coronary atherosclerosis in 5 percent of cases.

In the 1970s, several longitudinal studies had been established, namely the Muscatine Heart Study, the Bogalusa Heart Study, and the Cardiovascular Risk in Young Finns Study, which began to follow children and adolescents for known adult CVD risk factors. The participants of these studies had CVD risk factor variables measured initially early in life, while measurements of atherosclerotic lesions were subsequently obtained either during post-mortem autopsies, in those participants who had died of trauma, or non-invasively by high resolution B-mode ultrasound. Assessment of atherosclerotic lesions by ultrasound is done by measuring carotid intima-media thickness (cIMT). In children, increased cIMT is associated with several CVD risk factors, some of which have been shown to persist into adulthood, and in adults, it is well established that increased cIMT is associated with coronary artery disease and predictive of future CVD-related events.

Results based on multivariable models form the Muscatine Study in 346 and 379 women, aged 33 to 42 years, showed that total cholesterol measured in childhood (8 – 18 years) was a significant predictor of cIMT in adulthood in both sexes, while childhood BMI was additionally a significant predictor in women. In this study, cIMT was measured by ultrasound at twelve locations, and measurements were averaged for each participant. Findings from the Bogalusa Heart Study also support an association between CVD risk factors present in early life and the presence of atherosclerotic lesions later in life. Autopsies performed in 204 young persons (aged 2 to 39 years), 93 of whom had pre-mortem risk factor data available, revealed that multiple risk factors were associated with increased atherosclerosis in the aorta and coronary vessels. The risk factors assessed were BMI, SBP, serum triacylglycerols, and serum LDL. Participants with 0, 1, 2, and 3 or 4 risk factors had, respectively, 19.1 percent, 30.3 percent, 37.9 percent, and 35.0 percent of the intimal surface of the aorta covered with fatty streaks (p for trend = 0.001), which are the first grossly visible lesions in the development of atherosclerosis, while the corresponding percentages for coverage of the coronary arteries were 1.3, 2.5, 7.9, and 11.0, respectively, for fatty streaks (p for trend = 0.001), and 0.6, 0.7, 2.4, and 7.2, respectively, for fibrous plaque deposits (p for trend = 0.003) (Figure 1.6). Furthermore, results from all 204 autopsies showed that although the prevalence of fibrous plaque lesions in the aorta and coronary arteries increased with age, lesions were already present in childhood and youth, with approximately 20 percent of 2 to 15 year olds and 10 percent of 16 to 20 year olds.
olds showing evidence of lesions in the aorta, and approximately 10 percent of 2 to 15 year olds and 30 percent of 16 to 20 year olds showing evidence of lesions in coronary arteries (Figure 1.7)\textsuperscript{94}. Together, these findings clearly demonstrate that not only do CVD-risk factors present in childhood and youth predict the extent of atherosclerotic lesions later in life, but also, and perhaps more importantly, that atherosclerotic lesions are already seen early in life.

Figure 1.6: The effect of multiple risk factors on the extent of atherosclerosis in the aorta and coronary arteries in children and young adults. Values shown are the percentages of the intima surface covered with lesions in subjects with 0, 1, 2, and 3 or 4 risk factors. Risk factors were elevated values for body-mass index, systolic blood pressure, and serum triglyceride and LDL cholesterol concentrations, defined as values above the 75th percentile for the study group (specific for study period, race, sex, and age). There were 52 subjects with no risk factors, 20 with one, 14 with two, and 7 with three or four. The P value is based on the analysis of trend. A marked increase in the percentage of the intima surface is evident in the coronary vessels of subjects with multiple risk factors. Reproduced with permission from Berenson, G. et al. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. \textit{N. Engl. J. Med.} 314, 138–44 (1998), Copyright Massachusetts Medical Society.
Figure 1.7: The prevalence of fibrous-plaque lesions in the aorta and coronary arteries in 204 children and young adults, according to age. There is a consistent trend toward a greater prevalence of coronary-artery lesions with increasing age (P=0.001). Reproduced with permission from Berenson, G. et al. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. *N. Engl. J. Med.* 314, 138–44 (1998), Copyright Massachusetts Medical Society.

More recently, another cohort belonging to the Bogalusa Heart Study was examined for the associations between various CVD risk factors measured in childhood (4 – 17 years) and cIMT assessed by ultrasound in young adulthood (25 – 37 years, 39 percent men). Altogether, 486 participants were included in the study, and the main CVD risk factors measured were BMI, SBP, LDL, HDL, and triacylglycerols. Multivariable regression analyses revealed that childhood measures of LDL and BMI were significant predictors of cIMT in adulthood. The sex, age, and race –specific odds ratios for being in the top versus the lower three quartiles of cIMT thickness were 1.42 for LDL, and 1.25 for BMI (95 percent confidence intervals for both).

Lastly, findings from the Cardiovascular Risk in Young Finns Study largely conform to the findings from Bogalusa and other longitudinal studies, namely that childhood BMI and cholesterol (specifically LDL) are associated with cIMT in adulthood. In this Young Finns Study cohort, cIMT was measured by ultrasound in 2,229 young adults (aged 24 to 39 years) who were examined in childhood and adolescence an average 21 years earlier for several CVD risk factors, namely BMI, blood pressure, smoking, and serum total cholesterol, LDL, HDL, and triacylglycerides. In multivariable models adjusted for sex and age, adolescent LDL and BMI were significantly associated with adulthood cIMT (p=0.001 and p=0.007, respectively). In addition, SBP and smoking were significantly associated with cIMT (p<0.001 and p=0.02, respectively). Interestingly, when adjusting for LDL, BMI, SBP, and smoking measured in adulthood, adolescent LDL and SBP remained independently associated with cIMT (p=0.02 and
p=0.006, respectively), suggesting that exposure to these risk factors early in life may play an important role in the development of atherosclerosis progressing into adulthood.

Findings from the studies described above shed light on two very important themes: (a) that there is evidence of atherosclerotic lesions already in childhood and adolescence, and (b) that CVD risk factors are likewise already present early in life, and might cause changes in arteries that contribute to the development of atherosclerosis. Given that many of the risk factors for atherosclerosis can be modified by lifestyle changes or pharmacological treatment, detection of subclinical atherosclerosis at an early age may be of great importance for targeting high-risk individuals for early and more effective intervention.
Chapter 2

2 Introduction (Part II): Glycerophosphatidylcholines and cardiovascular disease

2.1 Glycerophosphatidylcholines definition: what they are and where they are in the body

Considering that atherosclerotic lesion formation begins in childhood and youth, early identification of individuals at risk of CVD is warranted. Recent advancements in spectrometric technologies (e.g., mass spectroscopy and nuclear magnetic resonance spectroscopy) are enabling the large-scale identification and study of various novel biological molecules. In particular, advancements in the large-scale profiling of lipid species (i.e., lipidomics) are beginning to contribute to our understanding of metabolic interconnections, and how lipids may modulate physiological functions. Lipidomics is beginning to show that novel, previously unsuspected lipid species, such as specific glycerophospholipids and their metabolites, may be involved in the pathophysiology of CVD. Furthermore, lipidomics may uncover species that can be used as biomarkers of CVD risk or progression.

Glycerophospholipids are major constituents of the biological membranes. They can be divided into 20 molecular classes based on the classification system developed by the LIPID MAPS consortium. The molecular classes are defined by distinct polar head groups attached to the glycerol backbone. The most abundant glycerophospholipids are glycerophosphatidylcholines (GPCs), which have choline as the polar head group. All GPCs consist of a glycerol backbone, which is bound to two fatty acid residues at the sn-1 and sn-2 positions, and to the choline at the sn-3 position by a phosphodiester linkage. Together, the glycerol backbone, the phosphate, and the choline make up the hydrophilic head of GPCs, which is exposed to the extracellular space or the cytosol in the outer or inner membrane leaflets, respectively, while the fatty acid residues make up the hydrophobic tails of GPCs, which line up against one another in the mid-section of membranes.
**Figure 2.1: Basic structure of glycerophosphatidylcholines.** Each GPC is made up of a hydrophilic head (having a choline, phosphate, and glycerol backbone), and either one or two hydrophobic tails attached to the glycerol backbone.

Often considered a single species, GPCs are in fact a group consisting of many species distinguished by the linkages of their \( sn-1 \) and \( sn-2 \) fatty acid residues to the glycerol backbone, and by their \( sn-1 \) and \( sn-2 \) fatty acid residue lengths and degrees of unsaturation. As shown in **Figure 2.2**, a fatty acid residue bound to the glycerol backbone at the \( sn-1 \) position by an ester linkage is called an acyl residue; a fatty acid residue bound by an ether linkage is called an alkyl residue; and a fatty acid residue bound by a vinyl linkage is called an alkenyl residue. At the \( sn-2 \) position, it is most common for the fatty acid residue to be bound by an ester linkage making it an acyl residue.

GPCs can be processed by cytosolic phospholipase \( A_2 \) and calcium-independent phospholipase \( A_2 \) enzymes via Land’s cycle into smaller bioactive messengers\(^{106} \). Two classes of second messengers are produced by processing of GPCs by phospholipase \( A_2 \): (a) oxygenated derivatives of free fatty acids released from the \( sn-2 \) position, and (b) bioactive metabolites that retain the phosphoglyceride backbone, termed second messenger...
glycerophosphatidylcholines (smGPCs); both can be released either into the extracellular or cytosolic space, or reincorporated into the plasma membrane. The smGPCs that are directly generated by the enzymatic actions of phospholipase A$_2$ are lysophosphatidylcholines, lyso-platelet-activating factors, and lyso-plasmalogens. These classes of smGPCs can then be further processed into acyl-platelet-activating factors, platelet-activating factors, and plasmenyl platelet-activating factors (Figure 2.2).

![Figure 2.2: Land's cycle – generation of second messenger glycerophosphatidylcholines (GPCs). GPCs in the membrane undergo constant remodeling via the Land’s Cycle. Membrane GPCs can be metabolized by phospholipase A$_2$ into lysophosphatidylcholines, which can then be further processed into other smGPCs, including platelet-activating factors. The individual identities of GPCs and smGPCs differ based on the lengths and degrees of unsaturation of the fatty acid residues at the sn-1 and sn-2 positions on the glycerol backbone, as well as on the types of linkages that bind the fatty acid residues to the glycerol backbone (acyl, ether, or plasmenyl linkages).](image)

The GPC nomenclature that we will be using from hereinafter is based on the nomenclature adopted by the LIPID MAPS consortium$^{107}$. The structures of the fatty acid residues are indicated within parentheses in the ‘head group (sn-1/sn-2)’ format. The ‘PC’
abbreviation specifies the phosphocholine head group of GPCs (the ‘LPC’ abbreviation specifies all lyso- species) whereas the first and third numbers in parentheses specify the fatty acid residue lengths at the sn-1 and sn-2 positions, respectively, and the second and fourth numbers in parentheses specify the fatty acid residue degrees of unsaturation at the sn-1 and sn-2 positions, respectively (e.g. PC[12:1/6:0] – Figure 2.3A). Fatty acid residue linkage types are specified by letter prefixes that are located before the sn-1 and sn-2 number designations in parentheses. The absence of a prefix implies the presence of an ester linkage, while the “O-” prefix represents an ether linkage, and the “P-” prefix represents a vinyl linkage (Figure 2.3A-C, respectively). Certain times, especially when the lipidomics method that is employed in a study cannot elucidate individual fatty acid residues, a ‘bulk’ notation may be used wherein sn-1 and sn-2 fatty acid residue lengths and degrees of unsaturation are summed together (e.g. PC[18:0] – Figure 2.3D). The main limitation of using the ‘bulk’ notation relates to the fact that any single GPC presented in that format may correspond to multiple species with both sn-1 and sn-2 fatty acid residues elucidated, and with potentially varying physiological functions. For example, PC(18:0) (Figure 2.3D) can correspond to PC(16:0/2:0) or LPC(18:0/0:0).

Figure 2.3: Standard glycerophosphatidylcholine notation (following Fahy E, et al. 107). (A) Example of glycerophosphatidylcholine notation; (B) example of platelet-activating factor notation; (C) example of plasmalogen notation; (D) example of ‘bulk’ (i.e. PC[18:0]) vs. ‘expanded’ (i.e. PC[18:0/0:0]) notation of a lysophosphatidylcholine, wherein the number of
carbons and degrees of unsaturation of individual fatty acid residues are summed vs. annotated separately, respectively.

GPCs and their second messengers in the circulation are found in lipoproteins, or travel in isolation or bound to other proteins, such as albumin\textsuperscript{108}. Considering that second messenger GPCs constitute a class of heterogeneous species with distinct functionalities, measuring second messenger GPCs individually rather than together in HDL and LDL, for example, may provide new insights about lipid derangements associated with various human diseases. Several studies have already uncovered significant associations between smGPCs and CVD outcomes (reviewed in the coming Chapter 2.3). Circulating second messenger GPCs that fall within the 450-600 Da mass range were included in the present study (Chapter 5).

### 2.2 Glycerophosphatidylcholines and cardiovascular disease

Recent lipidomics studies have uncovered new associations between CVD and circulating GPCs. Four recent prospective studies about associations between circulating GPCs and CVD-related outcomes, namely coronary artery disease (CAD) mortality, myocardial infarction, and ischemic stroke, are presented and summarized below.

The first study, by Sigruener and colleagues\textsuperscript{101}, investigated the relationships between CAD mortality, as well as total mortality, and plasma GPCs (including lysophosphatidylcholines), glycerophosphatidylethanolamines, sphingomyelins, and ceramides\textsuperscript{101}. The lipid species were quantified by electrospray ionization tandem mass spectroscopy and derived from 3,316 participants (average age: 62.7 years; 69.7% males) of the Ludwigshafen Risk and Cardiovascular Health Study. Overall, n=178 lipid species were detected, including 38 GPCs, 15 lysophosphatidylcholines (LPCs), and 30 ether-linked GPCs. The study cohort included 2,583 coronary artery disease positive participants (1,368 of whom had survived a myocardial infarction prior to enrolment) and 733 controls, with 768 participants succumbing to total mortality and 484 to CAD mortality during a median follow-up of 8 years. As shown in Figure 2.4A-C, 29 GPCs, including 3 LPCs and 7 ether-linked GPCs, were found to be significantly associated with total and/or CAD mortality. All identified GPCs were associated with total and CAD mortality in the same direction. Sixteen GPCs, including all 7 ether-linked GPCs, were positively associated with mortality (Figure 2.4A, C). The non-ether-linked GPCs that were positively associated with mortality tended to have shorter fatty acid residues and lower degrees of unsaturation. The remaining 13 GPCs, including 3 LPCs, were significantly associated with a protective effect against mortality (Figure 2.4A, B). In contrast to the GPCs positively
associated with mortality, the GPCs that were protective tended to have longer fatty acid residues and higher degrees of unsaturation (Figure 2.4A).

Figure 2.4: Associations between total and coronary artery disease mortality and plasma glycerophosphatidylcholine species. (A) Associations with glycerophosphatidylcholine species; (B) associations with lysophosphatidylcholine species; (C) associations with ether-linked glycerophosphatidylcholine species. EDTA plasma concentrations were determined by ESI-MS/MS. Species with significant association to CAD and total mortality are shown. Models were adjusted for age, gender, smoking, LDL, HDL, diabetes and hypertension. Bars represent the hazard ratio -1. Positive association with CAD is shown in grey and positive association with total mortality in black. Negative association with CAD is shown by a dashed white bar and negative association with total mortality in white. Species are named according to the number of carbon atoms and degree of desaturation. Besides their association the mean concentration and standard deviation are shown. PC and LPC species were annotated based on assumption of even numbered carbon chains only. Reproduced from Sigruener, A. et al. Glycerophospholipid and sphingolipid species and mortality: The Ludwigshafen risk and cardiovascular health (LURIC) study. PLoS One 9, (2014) with permission.
In the second study, Ganna and colleagues used a mass-spectroscopy-based non-targeted approach (which differs from targeted approaches in that the identities of the studied lipids are not pre-selected) to investigate associations between circulating lipid and non-lipid metabolites and incident CAD. In the discovery phase of the study, 1,028 participants (average age: 71.0 years; 100% male) of the ULSAM study, free of CAD events at baseline, were followed for a median of 10.0 years, during which 131 incident CAD events were observed. Altogether, there were 32 unique plasma metabolites significantly associated with CAD in the discovery cohort, including 10 (and possibly 14) GPCs and their second messenger metabolites: LPC(18:2/0:0), LPC(18:1/0:0), LPC(20:0/0:0), LPC(22:5/0:0), LPC(20:5/0:0), PC(34:1), LPC(18:3/0:0), PC(O-35:5), PC(31:1) (or PE(34:1)), PC(32:2) (or PE(35:2)), PC(36:1) (or PE(39:1)), LPC(20:2/0:0), LPC(20:4/0:0), and PC(32:1). The authors then sought to replicate their findings from the ULSAM cohort in another longitudinal cohort – the TwinGene cohort. The thirty-two metabolites previously associated with incident CAD were screened in sera of 1,670 participants (average age: 64.7 years; 48% male) of the TwinGene study who were followed for a median of 3.9 years, during which 282 incident CAD events were observed. Five of the 32 metabolites showed consistent direction and significant association with incident CAD. Among those 5 metabolites was LPC(18:2), which remained inversely and significantly associated with incident CAD after meta-analysis of TwinGene and ULSAM findings, and after adjustment for main cardiovascular risk factors, namely age, sex (only in TwinGene), SBP, BMI, current smoking, hypertension (assessed as being treated with antihypertensive medication), LDL, HDL, triacylglycerols, and type 2 diabetes at baseline. Considering that LPC(18:2) was the metabolite with the strongest association (inverse) with incident CAD in the discovery ULSAM cohort and in older participants from the replication TwinGene cohort, the authors extended their analysis to four additional LPC species in order to evaluate common patterns and pathways. From those species, LPC(18:1) was inversely and significantly associated with incident CAD independently of the main cardiovascular risk factors in both the ULSAM and TwinGene cohorts. LPC(18:1) and LPC(18:2) (along with the other two metabolites that remained significantly associated with CAD after adjustment for the cardiovascular risk factors) were then evaluated for association with LDL, HDL, triacylglycerols, BMI, and SBP in the ULSAM, TwinGene, and PIVUS cohorts. The PIVUS study is another longitudinal study from which serum samples of 970 participants (average age: 70.2 years; 50% male) were included in this final part of the study. Both LPC(18:1) and LPC(18:2) showed similar patterns of association across all three study cohorts: positive associations with HDL and LDL, and negative associations with BMI. In addition, when LPC(18:1) and LPC(18:2) were probed for associations with markers of oxidative stress, inflammation, and subclinical CVD in the PIVUS cohort, both
LPCs were associated with lower serum levels of inflammation markers and less subclinical CVD, while their associations with indices of oxidative stress were varied. Worth special mentioning are the particularly strong inverse associations between LPC(18:2) and CRP (a marker of inflammation), PAI-1, fibrinogen, and left-ventricular mass index (all indices of subclinical CVD) that were observed independently of sex, SBP, BMI, current smoking, hypertension, LDL, HDL, triacylglycerols, and type 2 diabetes at baseline. In summary, Ganna and colleagues identified 4 metabolites as markers of lower incident CAD, including LPC(18:1) and LPC(18:2), which were also associated inversely with several cardiovascular risk factors and indices of inflammation, oxidative stress, and subclinical CVD.

The last study herein presented is a study by Jove and colleagues who examined the plasma metabolome in transient ischemic attack (TIA) patients, with the aim to identify new biomarkers associated with stroke recurrence, temporal patterns of recurrence, and large-artery atherosclerosis, which is currently a main predictor of ischemic stroke recurrence. Patients with TIA who were tended to in the emergency department during the first 24 hours after the onset of symptoms were prospectively and consecutively recruited for the study and allocated to one of two cohorts. TIA was defined by the acute onset of focal cerebral or monocular symptoms lasting less than 24 hours and being attributable to brain ischemia. Stroke recurrence was defined as an endpoint in the study, and follow-up visits took place at 7 days, 3 months, and every 6 months afterwards, where imaging was used to confirm stroke recurrence in all suspected cases. A non-targeted metabolomics approach using liquid chromatography coupled to mass spectroscopy was performed on the first cohort of 131 participants (average age: 70.5 years; 64.9% male), which revealed a specific metabolomic signature in TIA participants with subsequent stroke recurrence. Univariate statistical analysis revealed that 94 ions significantly differentiated stroke recurrence from non-recurrence patients. LPC(16:0) was then identified using orthogonal approaches as a molecule being significantly decreased in participants who experienced stroke recurrence. The results also showed that participants with early stroke recurrence (<3 months) had a different metabolomic profile from participants with later stroke recurrence. The authors of the study then evaluated the metabolomic profile of patients with large-artery atherosclerosis, given its current utility as a predictor of stroke recurrence. The presence of large-artery atherosclerosis was defined as symptomatic carotid or intracranial stenosis of at least 50 percent. In sum, 73 metabolites were significantly different in participants with large-artery atherosclerosis compared to participants without large-artery atherosclerosis. LPC(22:6) was among those metabolites and was increased in participants with large-artery atherosclerosis. Validation in the second cohort of 162 participants (average age: 72.1; 58%
male) confirmed the metabolomic signature of stroke recurrence, the metabolomics-based differentiation of stroke recurrence temporal patterns, and the differences between large-artery atherosclerosis positive and negative participants. Finally, in a pooled analysis of both study cohorts, the authors applied receiver operating characteristic curves using metabolites present in at least 70 percent of the samples in the same group (i.e. in the stoke-recurrence and non-recurrence groups) to test the capacity of potential biomarkers. Results from this analysis identified LPC(20:4) as a potential biomarker, which significantly increased the predictive power of age, blood pressure, clinical features, duration of symptoms, and diabetes scale, and of large-artery atherosclerosis for stroke recurrence. In summary, the study by Jove and colleagues found plasma LPC(16:0), LPC(22:6), and other metabolites to be significantly associated with stroke recurrence, while LPC(20:4) was found to significantly increase the power of common predictors of stroke recurrence.

The studies summarized in this chapter all implicate GPCs and their second messengers in CVD-related outcomes. Sigruener et al.\textsuperscript{101} found non-ether-linked GPCs with long fatty acid residues and high degrees of unsaturation, as well as LPCs, to be associated with a protective effect against total and CAD mortality, while non-ether-linked GPCs with short fatty acid residues and lower degrees of unsaturation, as well as ether-linked GPCs, to be positively associated with total and CAD mortality. Ganna et al.\textsuperscript{109} found LPC(18:1) and LPC(18:2) to predict incident myocardial infarction and to be associated with several cardiovascular risk factors. And lastly, Jove et al.\textsuperscript{110} identified LPC(16:0), LPC(20:4), and LPC(22:6) as potential biomarkers of stroke recurrence. The common thread between the three studies summarized above is that LPCs are inversely associated with CVD outcomes.
Chapter 3

3 Rationale, hypothesis, and aims

3.1 Rationale

CVD is a slow-developing disease that begins in childhood and adolescence and which culminates in middle and late adulthood with acute vascular events and chronic vascular conditions. The early identification of individuals at risk of CVD is therefore of great importance. Advancements in mass spectroscopy and related spectrometric technologies enable the large-scale identification and study of novel biological molecules, some of which can be used as biomarkers of early CVD development.

3.2 Hypothesis

GPCs are the most abundant glycerophospholipids in the circulation, liver, and adipose tissue\textsuperscript{111}. GPCs and their signaling metabolites – smGPCs – are implicated in CVD (Chapter 2.3). Investigating the relationships between smGPCs with CVD risk factors may improve our understanding of CVD development and aid in the discovery of novel biomarkers used for the identification of at risk individuals who can be targeted for early intervention.

3.3 Aims

(1) To determine the clustering patterns and correlations between circulating smGPCs.

(2) To identify circulating smGPCs associated with the main CVD risk factors – visceral adiposity, elevated blood pressure, fasting insulin (as a measure of insulin resistance), and CRP (as a measure of low-grade inflammation) – in a population-based sample of adolescents.
Chapter 4

4 Methods and materials

4.1 Study sample

A total of 1,029 adolescents (496 males and 533 females, aged 12 to 18 years) were studied. The participants were recruited from the genetic founder population of the Saguenay Lac St. Jean region of Quebec, Canada, as part of the Saguenay Youth Study (SYS)\textsuperscript{112,113}. The SYS is a cross-sectional, population-based study of adolescents and their middle-aged parents. It is aimed at investigating the aetiology, early stages, and trans-generational trajectories of common cardio-metabolic and brain diseases. The SYS is a family-based study of 486 families studied. All adolescents were recruited through local high schools. In 2001, there were 17,494 French-speaking students in 31 high schools (27 public and 4 private). There are four school boards in this region, all of which participated in this research. Consent was obtained in accordance with the research ethics committees of the Chicoutimi Hospital (Chicoutimi, QC, Canada) and the Hospital for Sick Children (Toronto, ON, Canada).

One advantage of studying a cohort of adolescents is the potential of uncovering initial stages of disease and not having to deal with likely confounding effects of previous or ongoing medical intervention. In addition, the discovery of any potential abnormalities in the initial stages of development may lead to the discovery of more effective means of treatment and early prevention.

4.2 Measurements

4.2.1 Body adiposity

Body adiposity was assessed as a quantity of visceral fat (VF) and total body fat (TBF). VF was measured by magnetic resonance imaging (MRI). Currently, MRI is the only non-invasive (i.e., without radiation like computer tomography, CT) method that can differentiate between VF and subcutaneous abdominal fat in population-based studies of children and adolescents\textsuperscript{114,115}. T1 relaxation time of adipose tissue is relatively short, and thus, fat on T1-weighted MRI images is bright and easy to segment by semi-automated or automated techniques\textsuperscript{116}. Therefore, heavily T1-weighted, spin-echo (repetition time/echo time 200 ms/20 ms), axial 10-mm thick (with in-plane resolution 1.56x1.56 mm\(^2\)) images were acquired using a
Phillips 1.0-T magnetic resonance scanner and a single slice at the level of the umbilicus was used to quantify VF, as we described previously.\textsuperscript{86}

As VF rather than fat elsewhere in the body is associated with adverse cardiovascular health\textsuperscript{79–81}, we also assessed TBF for the purpose of secondary statistical analyses. TBF was assessed with bioelectrical impedance (4000B bio-impedance spectrum analyzer, Xitron Technologies, Inc., San Diego, CA). Participants were asked to refrain from caffeine, alcohol, and vigorous activity 24 hours before the test. The actual measurement was made after a 20-min stabilization period during which the participants rested in a supine position.

4.2.2 Blood pressure

Beat-by-beat brachial blood pressure was measured using Finometer\textsuperscript{TM} (FMS Finapres, Amsterdam, The Netherlands) during a 52-min cardiovascular protocol designed to mimic daily life activities. The Finometer is a device that monitors continuously finger blood flow and, by the reconstruction and level-correction of the blood-flow waveform, it derives beat-by-beat brachial systolic and diastolic BPs (as well as a number of other hemodynamic parameters). The Finometer is a reliable device for tracking blood pressure in adults and children older than six years\textsuperscript{117,118}. The 52-min cardiovascular protocol included a posture test consisting of three periods of during which the participants rested in a supine position for 10 min, stood for 10 min, and sat for 10 min, a mental stress test involving a 30 sec explanation segment administered 5 min before a 2 min sequence of 23 math problems of increasing difficulty. The mental stress sequence was followed by a 10 min period of resting in a sitting position. Following standard recommendation for blood pressure measurement\textsuperscript{119}, blood pressure analyzed in the present study were averages over 5-min segment of the protocol after the participants were seated at rest for 5 min (i.e., the last 5 min of the sitting section of the posture test). In the present study, we chose to study systolic blood pressure (SBP) and not diastolic blood pressure (DBP) as the main outcome variable, as (i) systolic rather than diastolic hypertension is predominant among obese children\textsuperscript{120} and young adults\textsuperscript{121}; (ii) population variance in SBP vastly exceeds that in DBP\textsuperscript{122}.

4.2.3 Blood measurements

All blood samples were drawn between 8AM and 10AM following overnight fasting. Blood was collected in silicone-coated gold BD Hemogard\textsuperscript{TM} tubes (#367986, BD, ON, Canada). After clotting, serum was separated by centrifugation at 1300 x g for 15 min at room temperature and stored in 1mL aliquots at -80°C.
4.2.3.1 Insulin resistance, and low-grade inflammation

Insulin resistance and compensatory hyperinsulinemia are thought to precede β-cell failure and overt hyperglycemia even by several decades\textsuperscript{64,65}. Insulin resistance was evaluated as fasting serum concentration of insulin\textsuperscript{123}.

Low-grade inflammation was assessed as serum concentration of C-reactive protein (CRP)\textsuperscript{27}. CRP is an acute-phase pentameric protein synthesized in the liver and released into circulation in response to circulating inflammatory cytokines. It is considered to be a clinical marker of low-grade inflammation and a risk factor for CVD. The \textit{Beckman Coulter AU5800 analyzer} (Beckman Coulter, Inc. March 2012) was used to measure CRP levels. This was performed by process of turbidimetry using a “high sensitive” immunoassay. The \textit{Beckman Coulter AU System} procedure measures CRP levels by the rate of decrease in light intensity transmitted through the sample, which results from an increase in complexes formed between the CRP of the patient and rabbit anti-CRP antibodies coated on latex particles.

4.2.3.2 Circulating second messenger glycerophosphatidylcholines

We used front-end separation by liquid chromatography coupled to mass spectrometry (i.e., liquid chromatography, electrospray ionization mass spectrometry [LC-ESI-MS]). We identified and quantified a total of 81 serum smGPs within the 440-640 Da mass range. LC-ESI-MS allows for the structural elucidation of linkages through which fatty acyl residues are attached to the glycerol backbone and the degree of their saturation; as such, different lipid species of identical molecular mass can be distinguished.

The smGPC species were extracted using a modified Bligh and Dyer procedure\textsuperscript{124}. To facilitate lipid identification and provide a standard curve for a comparison of retention across different runs, denatured standards were spiked at the time of mass spectroscopy analysis (Blanchard et al., 2015, submitted to \textit{Bioinformatics}). For LC-ESI-MS, 5 µl of extracted lipid samples were loaded successively onto an Agilent 96-well sampling plate, covered with a pre-slit well-cap and thermostated at 4°C. The analytes were loaded onto a pre-column (200 µm×50 mm) using an Agilent 1100 autosampler and then eluted through a PicoTip emitter (75 µm×50 mm; New Objective, Woburn, MA) interfaced with a 5500 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via ESI. Both columns were packed with reverse phase C4 beads (5 µm, 120Å, Waters, Milford, MA). LC-ESI-MS analysis of the smGPC species was performed in positive ion mode with a diagnostic precursor and neutral loss scan. Chain lengths of the smGPC species were further identified by tandem mass-spectrometry\textsuperscript{125,126}.
The assignment of acyl, alkyl and alkenyl branches based on spectra analysis was also confirmed for key species following acid hydrolysis/acidic vapour separation in a separate series of extractions in MRM mode. Collected mass spectra were quantified with Analyst software (version 1.5.0) and relative abundance was expressed as the peak area normalized to the internal standard calculated per ml (in arbitrary units)\textsuperscript{127}.

For the purpose of secondary analyses (circulating smGPCs may be bound to lipoproteins\textsuperscript{108} and lipoprotein metabolism may be influenced by total cholesterol and triacylglycerols), the lipidomic profiling of circulating smGPCs (described above) was complemented by traditional measures of lipid metabolism, namely serum HDL, LDL, total cholesterol and triacylglycerols (LDL was calculated using the Friedewald equation, as LDL = total cholesterol – HDL – [triacylglycerides/5])\textsuperscript{128}.

4.3 Statistical methods

The aim of the present study was to examine whether any of the identified 81 smGPCs are associated with classical CVD risk factors – excess abdominal fat, elevated blood pressure, insulin resistance and low-grade inflammation – during adolescence.

For all study variables, a preliminary analysis was performed assessing the normality assumption, upon which the statistical inference about correlation analysis and mixed linear model estimates relies. Variables exhibiting a violation of normality distribution were transformed using logarithm with base 10, which sufficiently improved the goodness of fit. Since CRP levels above 10 mg/L can be indicative of an acute infection and are beyond the established levels of cardiovascular risk\textsuperscript{27}, all values above 10 mg/L were considered clinically abnormal and excluded from further study, as suggested previously\textsuperscript{27}. Statistical outliers (i.e. data points falling 3 standard deviations above or below the mean of each variable) were removed. All preliminary, quality control, and descriptive statistical procedures were employed in JMP (11.0).

We employed mixed linear regression models to evaluate associations between the 81 smGPCs and each of the 4 primary outcome variables: VF (as an index of abdominal adiposity), SBP (as a measure of elevated blood pressure), fasting insulin (as an index of insulin resistance), and CRP (as an index of low-grade inflammation). All models were tested using the R kinship ‘coxme’ package (\textit{lmekin} function). The \textit{lmekin} function fits a linear mixed-effects model that uses the kinship coefficient to define the correlation of random effects (such as that due to relatedness within families), whereas the fixed effects are used to test for associations.
and adjust for potential confounders (such as age, sex or height). In the present study, age, sex and relatedness were included in all models and height was included in models that tested associations of smGPCs with VF and SBP. Height is known to correlate with blood pressure in children and adolescents, which has led to the recommendation that it be accounted for in blood pressure evaluations\textsuperscript{129,130}. All associations were reported as z-statistics, and a Bonferroni correction for multiple comparisons was applied where $\alpha=0.05/324$ (81 lipid species*4 outcomes)=$1.5\times10^{-4}$.

*Metaboanalyst 3.0* (http://www.metaboanalyst.ca), an online comprehensive tool for metabolomic data analysis, was used to determine the correlation matrix involving the 81 identified smGPCs. smGPC concentrations adjusted for sex, age, and relatedness were uploaded onto the online platform. To produce the matrix, Pearson r correlations were used and the software replaced any missing values (approximate 2% of the dataset) with the mean concentration of the smGPC corresponding to each missing data point. The data was additionally mean-centered and divided by the standard deviation of each variable for scaling. The smGPCs were ordered along the x and y axes based on hierarchical clustering, which is a clustering method that seeks to build a hierarchy of clusters based on the similarities between individual clusters or observations they contain. In the present study, between-cluster similarities were assessed by the Pearson r correlations.

Further, smGPCs may be bound to lipoproteins in the circulation\textsuperscript{108} and total cholesterol and triacylglycerols may influence lipoprotein metabolism\textsuperscript{49}, we also assessed whether significant associations were dependent (or not) on circulating concentrations of HDL cholesterol, LDL cholesterol, total cholesterol, and triacylglycerols (by additional adjusting for each of these 4 variables).

Moreover, as visceral fat (VF) rather than fat present elsewhere in the body is associated with adverse cardiovascular health\textsuperscript{79–81}, we also assessed whether significant associations between smGPCs and VF were independent of total body fat (by additional adjusting for this variable).

Finally, since adipose tissue may be a source of circulating lipids and since obesity, especially visceral obesity, is closely related to blood pressure elevation, insulin resistance, and low-grade inflammation\textsuperscript{69,79–81}, we tested whether certain smGPCs (smGPCs associated with VF and multiple other cardio-metabolic risk factors) may mediate the relationship between VF and the other cardio-metabolic risk factors, such as SBP, fasting insulin or CRP. We therefore
employed a series of conditional mixed linear regression analyses (Models 1-3), wherein we tested the possible directed relationships between VF (as the causal factor), a smGPC associated with both VF and SBP, fasting insulin, or CRP (as a potential mediator), and SBP, fasting insulin, or CRP (as an outcome) (Figure 4.1A, B)\(^1\). In addition, Model 3 tested the significance of the effect of X on Y independently of M (Figure 4.1B). All models were implemented in the R kinship ‘coxme’ package (lmekin function) where sex, age, height, and relatedness were included as potential confounders:

\[
Model 1: Y \sim X + sex + age + height + relatedness
\]

\[
Model 2: M \sim X + sex + age + height + relatedness
\]

\[
Model 3: Y \sim X + M + sex + age + height + relatedness
\]

For each series of relationships, we then implemented Sobel's test in the R 'bda' package (mediation.test function) to determine the significance of mediation by the smGPC of the directed effect of VF on another CVD risk factor\(^2\). Sobel's test tests the null hypothesis \((H_0)\), which is that the potential mediator does not mediate the effect of the causal factor on the outcome (Figure 4.1B):

\[
H_0: \quad ab = 0
\]

and

\[
c = ab + c' = c'
\]
Figure 4.1: Mediation of the directed relationship between visceral fat (the causal factor) and systolic blood pressure, fasting insulin, or C-reactive protein (the outcome). (A) The directed relationship between visceral fat and systolic blood pressure, fasting insulin, or C-reactive protein (pathway c); (B) the directed relationships between visceral fat and each a smGPC (a potential mediator) (pathway a) and systolic blood pressure, fasting insulin, or C-reactive protein, independently of a smGPC (pathway c'), as well as, the directed relationship between a smGPC and systolic blood pressure, fasting insulin, or C-reactive protein (pathway b).
Chapter 5

5 Results

5.1 Basic characteristics of the studied adolescents

Descriptive statistics used to characterize the study population included means and standard deviations for continuous variables and percentiles for categorical variables (presented in Table 5.1). The study sample consisted of 496 males and 533 females who were on average 15.0-year old, 163.1-cm tall, weighted 57.9 kg, and most of them were in the third Tanner stage of puberty development (Table 5.1). Based on the Centers for Disease Control and Prevention’s sex- and age-specific BMI weight status categories and corresponding percentiles (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html), 80.1% of the participants were of healthy weight (had BMI equal to or greater than the 5th percentile BMI, but lower than the 85th percentile BMI, relative to the population of children in the United States), 4.9% were underweight (had BMI lower than the 5th percentile BMI), 10.0% were overweight (had BMI ≥ 85th percentile BMI, but < 95th percentile BMI) (Table 5.1).
### Table 5.1. Basic characteristics of the studied adolescents.

Total number of participants: N=1,029

<table>
<thead>
<tr>
<th>Study characteristic</th>
<th>Mean ± S.D. (n)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>15.02 ± 1.84 (1,029)</td>
</tr>
<tr>
<td>Tanner scale (stage 1-5)</td>
<td>3.74 ± 0.87 (1,026)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.10 ± 9.57 (1,026)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.92 ± 13.66 (1,015)</td>
</tr>
<tr>
<td>BMI</td>
<td>21.66 ± 4.10 (1,016)</td>
</tr>
</tbody>
</table>

**BMI category**

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>4.92%</td>
</tr>
<tr>
<td>Healthy weight</td>
<td>80.12%</td>
</tr>
<tr>
<td>Overweight</td>
<td>10.04%</td>
</tr>
<tr>
<td>Obese</td>
<td>4.92%</td>
</tr>
</tbody>
</table>

| Total body fat (kg) | 12.67 ± 8.59 (978) |
| Visceral fat (cm³)  | 21.71 ± 17.54 (1,000) |

| Fasting insulin (pmol/L) | 63.82 ± 29.01 (964) |
| HDL cholesterol (mmol/L) | 1.41 ± 0.32 (974) |
| LDL cholesterol (mmol/L) | 2.58 ± 0.70 (1008) |
| Total cholesterol (mmol/L) | 4.15 ± 0.77 (995) |
| Triglycerides (mmol/L)   | 1.01 ± 0.51 (975) |
| C-reactive protein (mg/L) | 1.01 ± 1.48 (991) |

| Systolic blood pressure (mmHg) | 121.51 ± 13.02 (1,000) |
| Diastolic blood pressure (mmHg) | 77.43 ± 9.53 (995) |

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A total of 81 circulating smGPCs (mass range: 440-640 Da) were identified and quantified. They contained 7 classes of smGPCs. More specifically, 31 were LPCs, 22 were platelet-activating factors, 6 were diacylglycerophosphatidylcholines, 6 were acyl-platelet-activating factors, 6 were lyso-platelet-activating factors, 1 was a lyso-plasmalogen, 1 was a plasmenyl-platelet-activating factor, and 8 were smGPCs with un-determined subclass (Supplementary Table 5.1).
5.2 Clustering patterns and correlations between smGPCs

Based on hierarchical clustering the smGPCs were separated into three main clusters (clusters ‘1-3’) and nine sub-clusters (clusters ‘1A-3D’) (Figure 5.1). The most smGPCs (n=19) grouped into cluster ‘2B’, n=12 of which were LPCs, while the least smGPCs (n=5) grouped into each clusters ‘1A’ and ‘3B’. Overall, smGPC classes were typically distributed among different clusters (Figure 5.1). Cluster ‘1B’ contained the most highly correlated smGPC pair (PC[O-18:5/2:0] and PC[12:0/6:0]; r=0.91), while cluster ‘3C’ contained the most highly anti-correlated smGPC pair (PC[O-20:0/2:0] and LPC[20:1/0:0]; r=-0.37).

Hereinafter, all associations between the smGPCs and individual CVD-risk factors will be presented in figures wherein the smGPCs will be grouped into the clusters as shown in Figure 5.1.

5.3 Circulating smGPCs and adiposity

Overall, there were more inverse than positive associations with VF (Figure 5.2A). The inverse associations of the smGPCs with VF included 24 nominally significant associations (p<0.05), respectively; from these, 7 associations, respectively, were significant after Bonferroni correction for multiple comparisons (p<1.5x10^-4) (Figure 5.2A). Cluster ‘3D’ contained the greatest number of inversely and significantly associated smGPCs with VF (Figure 5.2A).

The positive associations of the smGPCs with VF included 13 nominally significant associations, respectively; from these 3 associations were significant after Bonferroni correction for multiple comparisons (Figure 5.2A). Cluster ‘1B’ contained the greatest number of positive associations between the smGPCs and VF (Figure 5.2A).

PC(16:0/2:0), an acyl-platelet-activating factor, showed the strongest inverse association with VF (β=-0.62, S.E.=0.071, z=-8.66, p=2.3x10^-18), while LPC(14:1/0:0), a LPC, showed the strongest positive association with VF (β=0.49, S.E.=0.091, z=5.36, p=8.3x10^-8) (Figure 5.2A). After additional adjustments of these VF associations for total body fat, PC(16:0/2:0) and LPC(14:1/0:0) remained strongly associated with VF (β=-0.39, S.E.=0.12, z=-3.43, p=6.1x10^-4 and β=0.57, S.E.=0.14, z=4.00, p=6.5x10^-5, respectively).

Finally, we tested the potentially confounding effects of HDL cholesterol, LDL cholesterol, triacylglycerols, and total cholesterol in the associations between VF and both PC(16:0/2:0) and LPC(14:1/0:0). The results showed that VF remained significantly associated
with both PC(16:0/2:0) and LPC(14:1/0:0) after additional adjusting for HDL ($\beta=-0.63$, S.E.=0.078, $z=-8.09$, $p=5.6\times10^{-16}$ and $\beta=0.49$, S.E.=0.097, $z=5.08$, $p=3.9\times10^{-7}$, respectively), LDL cholesterol ($\beta=-0.56$, S.E.=0.074, $z=-7.55$, $p=4.3\times10^{-14}$ and $\beta=0.41$, S.E.=0.094, $z=4.34$, $p=1.4\times10^{-5}$), and total cholesterol ($\beta=-0.58$, S.E.=0.073, $z=-7.87$, $p=3.4\times10^{-15}$ and $\beta=0.42$, S.E.=0.093, $z=4.50$, $p=6.7\times10^{-5}$) (Supplementary Table 5.2). VF remained significantly associated with PC(16:0/2:0), but not with LPC(14:1/0:0) after additional adjusting for triacylglycerols ($\beta=-0.59$, S.E.=0.079, $z=-7.45$, $p=9.4\times10^{-14}$ and $\beta=0.15$, S.E.=0.095, $z=1.61$, $p=0.11$) (Supplementary Table 5.2).

5.4 Circulating smGPCs and blood pressure

Compared with abdominal adiposity, the associations of smGPCs with SBP were fewer and of a lesser strength (Figure 5.2B), but they showed similarities in that there were more inverse than positive associations (Figure 5.2B).

The inverse associations of the smGPCs with SBP included 12 nominally significant associations; from these, 2 associations were significant after Bonferroni correction for multiple comparisons (Figure 5.2B). Although cluster ‘3B’ contained the greatest number of inversely associated smGPCs with SBP, cluster ‘3D’ also contained many inversely and significantly associated smGPCs. Most notably, PC(16:0/2:0), which was strongly associated with VF, was also inversely and significantly associated with SBP ($\beta=-0.0073$, S.E.=0.0017, $z=-4.37$, $p=1.3\times10^{-5}$; Figure 5.2B).

There were only two nominally significant positive associations and these associations did not remain statistically significant after Bonferroni correction for multiple comparisons (Figure 5.2B).

After additional adjusting for potentially confounding effects of HDL cholesterol, LDL cholesterol, total cholesterol and triacylglycerols in the association between SBP and the VF-associated PC(16:0/2:0), SBP remained significantly associated with PC(16:0/2:0) (HDL cholesterol: $\beta=-0.0070$, S.E.=0.0017, $z=-4.06$, $p=4.9\times10^{-5}$; LDL cholesterol: $\beta=-0.0068$, S.E.=0.0017, $z=-4.07$, $p=4.7\times10^{-5}$; total cholesterol: $\beta=-0.0071$, S.E.=0.0017, $z=-4.23$, $p=2.4\times10^{-5}$ and triacylglycerols; $\beta=-0.0069$, S.E.=0.0017, $z=-4.06$, $p=4.9\times10^{-5}$, Supplementary Table 5.2).
5.5 Circulating smGPCs and fasting insulin

In contrast to VF and SBP, fasting insulin showed a different pattern of associations with the smGPCs in that there were more positive than negative associations (Figure 5.2C).

The positive associations included 15 nominally significant associations; from these, 5 associations remained statistically significant after Bonferroni correction for multiple comparisons (Figure 5.2C). Clusters ‘3A’ and ‘3C’ contained the highest number of positively associated smGPCs (Figure 5.2C). LPC(14:1/0:0), which showed a positive and significant association with VF, also showed a positive and significant association with fasting insulin ($\beta=1.29$, S.E.=$0.13$, z=10.15, $p=1.7\times10^{-24}$, Figure 5.2C).

There were only five nominally significant inverse associations and none of them remained significant after Bonferroni correction for multiple comparisons (Figure 5.2C).

Finally, the association between the VF-associated LPC(14:1/0:0) and fasting insulin remained positively and significantly associated with fasting insulin after additional adjusting for HDL cholesterol ($\beta=1.31$, S.E.=$0.13$, z=10.30, $p=3.5\times10^{-25}$), LDL ($\beta=1.23$, S.E.=$0.13$, z=9.59, $p=4.4\times10^{-22}$), triacylglycerols ($\beta=0.98$, S.E.=$0.13$, z=7.46, $p=6.8\times10^{-13}$), and total cholesterol ($\beta=1.23$, S.E.=$0.13$, z=9.60, $p=8.0\times10^{-22}$) (Supplementary Table 5.2).

5.6 Circulating smGPCs and CRP as an index of low-grade inflammation

Circulating CRP, compared to the other CVD risk factors examined above, demonstrated the greatest number of associations, as well as, associations of the greatest magnitudes. The pattern of associations showed similarities with the patterns observed for adiposity and blood pressure, in that there were more inverse than positive associations (Figure 5.2D).

The inverse associations with the smGPCs included 43 nominally significant associations; from these, 30 associations remained statistically significant after Bonferroni correction for multiple comparisons (Figure 5.2D). Similar to the findings with adiposity, many of the smGPCs showing inverse and significant associations belonged to cluster ‘3D’ (Figure 5.2D). Clusters ‘2C’, ‘3A’, and ‘2B’ also contained many of the smGPCs that were inversely and significantly associated with CRP (Figure 5.2D).
PC(16:0/2:0), which was inversely and significantly associated with both VF and SBP, was also inversely and significantly associated with CRP ($\beta$=-0.49, S.E.=0.042, $z$=-11.45, $p=1.2\times10^{-30}$) (Figure 5.2D).

The positive associations of smGPCs with CRP included 8 nominally significant associations, none of which remained statistically significant after Bonferroni correction for multiple comparisons (Figure 5.2D).

The VF-associated PC(16:0/2:0) remained inversely and significantly associated with CRP after additional adjusting for HDL cholesterol ($\beta$=-0.49, S.E.=0.044, $z$=-11.01, $p=1.7\times10^{-28}$), LDL cholesterol ($\beta$=-0.47, S.E.=0.044, $z$=-10.80, $p=1.7\times10^{-27}$), total cholesterol ($\beta$=-0.47, S.E.=0.044, $z$=-10.84, $p=2.2\times10^{-27}$), and triacylglycerols ($\beta$=-0.49, S.E.=0.046, $z$=-10.71, $p=9.1\times10^{-27}$, Supplementary Table 5.2).

5.7 Mediation by the VF-associated PC(16:0/2:0) and LPC(14:1/0:0)

For the two smGPCs – PC(16:0/2:0) and LPC(14:1/0:0) – that were associated with VF as well as other CVD-risk factors, we found that they significantly mediated the directed relationship between VF (the causal factor) and the relevant CVD risk factor (the outcome) in all three of the tested relationships ($\alpha=0.05$) (Supplementary Table 5.3). Mediation analyses revealed that PC(16:0/2:0) may mediate the directed relationships between VF (as a causal factor) and either SBP or CRP (as an outcome, $p=4.3\times10^{-4}$ and $p=5.0\times10^{-10}$, respectively), and LPC(14:1/0:0) may mediate the directed relationships between VF (as a causal factor) and fasting insulin (as an outcome, $p=3.5\times10^{-4}$) (Supplementary Table 5.3). In more detail, for example, PC(16:0/2:0) was found to mediate significantly the directed relationship between VF and SBP. We first established that the directed relationship between VF and PC(16:0/2:0), and the relationship between PC(16:0/2:0) and SBP, were significant ($\beta$=-0.62, S.E.=0.073, $z$=-8.47, $p=2.5\times10^{-17}$ and $\beta$=-2.40, S.E.=0.62, $z$=-3.87, $p=1.1\times10^{-4}$, respectively) (Supplementary Table 5.3). We then established the significance of the predicted effect of VF on SBP alone, and subsequently, the predicted effect of VF on SBP, independently of PC(16:0/2:0) ($\beta$=4.82, S.E.=1.40, $z$=3.43, $p=5.9\times10^{-4}$ and $\beta$=3.35, S.E.=1.44, $z$=2.32, $p=2.0\times10^{-2}$, respectively). Sobel’s test then revealed the significant mediation by PC(16:0/2:0) between VF and SBP ($p=4.3\times10^{-4}$) (Supplementary Table 5.3).
Figure 5.1. Matrix showing correlations of the studied smGPCs. Pair-wise Pearson r correlations of 81 smGPCs. Ordering of the smGPCs on the x and y axes was based on hierarchical clustering. Missing values within species were replaced by the median value of each species, and the data was scaled wherein all smGPCs were mean-centered and divided by their respective standard deviations. Pearson correlation r were performed and visualized in MetaboAnalyst 3.0 (http://www.metaboanalyst.ca).
Figure 5.2. Mixed linear regressions of 81 smGPCs with CVD risk factors. (A) Associations of smGPCs and visceral fat measured by magnetic resonance imaging; (B) associations of smGPCs and systolic blood pressure measured beat-by-beat by Finometer<sup>TM</sup>; (C) associations of smGPCs and serum fasting insulin; (D) associations of smGPCs with C-reactive protein. All models were implemented in the R kinship package (<i>lmekin</i> function). Associations were reported as z-statistics, and a Bonferroni correction for multiple comparisons was applied where $\alpha=0.05/81^4$ lipid species$=1.5\times10^{-4}$. The number of significantly associated (inversely and positively) smGPCs with each CVD risk factor annotated as 'N'.
<table>
<thead>
<tr>
<th>smGPC Identity</th>
<th>smGPC builder notation identity</th>
<th>smGPC molecular mass (Da)</th>
<th>smGPC sub-class</th>
<th>smGPC correlation matrix number</th>
<th>Relative abundance (log2 PAI, Inf, x 104)</th>
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<tbody>
<tr>
<td>PG1 (31-1)</td>
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<td>543.4</td>
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<td>PG4 (31-2)</td>
<td>PG4 (31-2)</td>
<td>596.4</td>
<td>Unidentified</td>
<td>6</td>
<td>13.12</td>
</tr>
<tr>
<td>PG5 (31-1)</td>
<td>PG5 (31-1)</td>
<td>620.4</td>
<td>Dicataphosphophytylcholine</td>
<td>92</td>
<td>15.41</td>
</tr>
<tr>
<td>PG7 (31-1)</td>
<td>PG7 (31-1)</td>
<td>638.6</td>
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<td>15.82</td>
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<tr>
<td>PG2 (31-1)</td>
<td>PG2 (31-1)</td>
<td>528.6</td>
<td>Unidentified</td>
<td>40</td>
<td>16.07</td>
</tr>
<tr>
<td>PG3 (31-1)</td>
<td>PG3 (31-1)</td>
<td>620.4</td>
<td>Unidentified</td>
<td>23</td>
<td>15.99</td>
</tr>
<tr>
<td>LPC (20-10)</td>
<td>LPC (20-10)</td>
<td>622.4</td>
<td>Lyso phosphatidylcholine</td>
<td>6</td>
<td>15.39</td>
</tr>
<tr>
<td>PG3 (31-1)</td>
<td>PG3 (31-1)</td>
<td>584.3</td>
<td>Acyl phosphatidylcholine</td>
<td>2</td>
<td>15.76</td>
</tr>
<tr>
<td>PG4 (31-2)</td>
<td>PG4 (31-2)</td>
<td>500.4</td>
<td>Platelet-activating factor</td>
<td>1</td>
<td>13.81</td>
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<tr>
<td>PG5 (31-1)</td>
<td>PG5 (31-1)</td>
<td>622.4</td>
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<td>5</td>
<td>15.04</td>
</tr>
<tr>
<td>LPC (19-10)</td>
<td>LPC (19-10)</td>
<td>524.6</td>
<td>Lyso phosphatidylcholine</td>
<td>41</td>
<td>24.45</td>
</tr>
<tr>
<td>PG5 (31-1)</td>
<td>PG5 (31-1)</td>
<td>606.5</td>
<td>Unidentified</td>
<td>10</td>
<td>17.03</td>
</tr>
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</table>
**Supplementary Table 5.2.** Associations of the visceral fat-specific PC(16:0/2:0) and LPC(14:1/0:0) with CVD risk factors adjusted for HDL, LDL, triacylglycerides, and total cholesterol.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visceral fat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(16:0/2:0)</td>
<td>( \beta = -0.52 ) S.E = 0.071 z-score = -8.66 p-value = 2.3x10^{-18}</td>
<td>( \beta = -0.63 ) S.E = 0.078 z-score = -8.09 p-value = 5.6x10^{-18}</td>
<td>( \beta = -0.56 ) S.E = 0.074 z-score = -7.55 p-value = 4.3x10^{-14}</td>
</tr>
<tr>
<td>LPC(14:1/0:0)</td>
<td>( \beta = 0.49 ) S.E = 0.091 z-score = 5.36 p-value = 8.3x10^{-6}</td>
<td>( \beta = 0.49 ) S.E = 0.097 z-score = 5.08 p-value = 3.9x10^{-7}</td>
<td>( \beta = 0.41 ) S.E = 0.094 z-score = 4.34 p-value = 1.4x10^{-6}</td>
</tr>
<tr>
<td><strong>Blood pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(16:0/2:0)</td>
<td>( \beta = -0.0073 ) S.E = 0.0017 z-score = -4.37 p-value = 1.3x10^{-6}</td>
<td>( \beta = -0.0070 ) S.E = 0.0017 z-score = -4.06 p-value = 4.9x10^{-6}</td>
<td>( \beta = -0.0068 ) S.E = 0.0017 z-score = -4.07 p-value = 4.7x10^{-6}</td>
</tr>
<tr>
<td><strong>Fasting insulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPC(14:1/0:0)</td>
<td>( \beta = 1.29 ) S.E = 0.13 z-score = 10.15 p-value = 1.7x10^{-24}</td>
<td>( \beta = 1.31 ) S.E = 0.13 z-score = 10.30 p-value = 3.5x10^{-25}</td>
<td>( \beta = 1.23 ) S.E = 0.13 z-score = 9.59 p-value = 4.4x10^{-22}</td>
</tr>
<tr>
<td><strong>Low-grade inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(16:0/2:0)</td>
<td>( \beta = -0.49 ) S.E = 0.042 z-score = -11.45 p-value = 1.2x10^{-30}</td>
<td>( \beta = -0.49 ) S.E = 0.044 z-score = -11.01 p-value = 1.7x10^{-28}</td>
<td>( \beta = -0.47 ) S.E = 0.044 z-score = -10.80 p-value = 1.7x10^{-27}</td>
</tr>
</tbody>
</table>

**Model 1:** PC(16:0/2:0) or LPC(14:1/0:0) ~ CVD risk factor + sex + age + height (only when CVD risk factor is visceral fat or blood pressure) + relatedness

**Model 2:** PC(16:0/2:0) or LPC(14:1/0:0) ~ CVD risk factor + sex + age + height (only when CVD risk factor is visceral fat or blood pressure) + relatedness + HDL-cholesterol

**Model 3:** PC(16:0/2:0) or LPC(14:1/0:0) ~ CVD risk factor + sex + age + height (only when CVD risk factor is visceral fat or blood pressure) + relatedness + LDL-cholesterol

**Model 4:** PC(16:0/2:0) or LPC(14:1/0:0) ~ CVD risk factor + sex + age + height (only when CVD risk factor is visceral fat or blood pressure) + relatedness + triacylglycerols

**Model 5:** PC(16:0/2:0) or LPC(14:1/0:0) ~ CVD risk factor + sex + age + height (only when CVD risk factor is visceral fat or blood pressure) + relatedness + total cholesterol
**Supplementary Table 5.3. Mediation of the visceral-fat specific PC(16:0/2:0) and LPC(14:1/0:0) of the directed relationships of visceral fat and each blood pressure, fasting insulin, and C-reactive protein.**

<table>
<thead>
<tr>
<th></th>
<th>Coefficient estimate</th>
<th>Standard Error</th>
<th>z-score</th>
<th>p-value</th>
<th>Sobel's test p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. M: PC(16:0/2:0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: Visceral fat</td>
<td>a</td>
<td>-0.62</td>
<td>0.073</td>
<td>-8.47</td>
<td>2.5x10⁻¹⁷</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-2.40</td>
<td>0.62</td>
<td>-3.87</td>
<td>1.1x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>4.82</td>
<td>1.41</td>
<td>3.43</td>
<td>5.9x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>c'</td>
<td>3.35</td>
<td>1.45</td>
<td>2.32</td>
<td>2.0x10⁻²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y: Systolic blood pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>0.38</td>
<td>0.097</td>
<td>3.92</td>
<td>8.9x10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.062</td>
<td>0.0071</td>
<td>8.72</td>
<td>2.8x10⁻¹⁸</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>0.27</td>
<td>0.022</td>
<td>12.30</td>
<td>9.1x10⁻¹⁶</td>
</tr>
<tr>
<td></td>
<td>c'</td>
<td>0.24</td>
<td>0.021</td>
<td>11.56</td>
<td>6.6x10⁻¹⁵</td>
</tr>
<tr>
<td>2. M: LPC(14:1/0:0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: Visceral fat</td>
<td>a</td>
<td>0.63</td>
<td>0.072</td>
<td>-8.72</td>
<td>2.8x10⁻¹⁸</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>-0.18</td>
<td>0.021</td>
<td>-8.59</td>
<td>8.7x10⁻¹⁸</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>0.63</td>
<td>0.048</td>
<td>13.22</td>
<td>6.7x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>c'</td>
<td>0.52</td>
<td>0.048</td>
<td>10.75</td>
<td>5.9x10⁻⁷</td>
</tr>
<tr>
<td>3. M: PC(16:0/2:0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: Visceral fat</td>
<td>a</td>
<td>0.63</td>
<td>0.072</td>
<td>-8.72</td>
<td>2.8x10⁻¹⁸</td>
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<td>0.021</td>
<td>-8.59</td>
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<td>0.63</td>
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<td>6.7x10⁻⁴</td>
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<tr>
<td></td>
<td>c'</td>
<td>0.52</td>
<td>0.048</td>
<td>10.75</td>
<td>5.9x10⁻⁷</td>
</tr>
</tbody>
</table>

*a:* the directed effect of the causal factor (visceral fat in all studied cases) on the potential mediator (PC[16:0/2:0] or LPC[14:1/0:0])

*b:* the directed effect of the potential mediator on the outcome (systolic blood pressure, fasting insulin, or C-reactive protein)

*c:* the directed effect of the causal factor on the outcome

*c':* the directed effect of the causal variable on the outcome independent of the potential mediator

†Sobel's test tests the significance of mediation by the potential mediator of the directed effect of the causal factor on the outcome
Chapter 6

6 Discussion

To the best of my knowledge, the smGPCs examined in the present study represent the largest set of smGPCs thus far identified and studied in the context of CVD. The results of the present study, conducted in a population-based sample of 1,029 adolescents, demonstrate that a large number of circulating smGPCs are associated with individual CVD-risk factors, with some of these associations being positive and other inverse (Figure 5.2A-D). The inverse associations may represent potentially ‘protective’ CVD effects of smGPCs whereas the positive associations may indicate potentially ‘adverse’ CVD effects of smGPCs. We also found that several smGPCs were associated with multiple CVD-risk factors – the most significant of these were PC(16:0/2:0) and LPC(14:1/0:0). PC(16:0/2:0) was associated inversely with visceral adiposity, blood pressure and low-grade inflammation, and LPC(14:1/0:0) was associated positively with visceral adiposity and insulin resistance. Mediation analyses suggested that PC(16:0/2:0) may play a mediating role between visceral adiposity and both blood pressure and low-grade inflammation, and LPC(14:1/0:0) may play a mediating role between visceral adiposity and insulin resistance. These ‘mediation’ results are consistent with the possibility that adipose tissue may be a source of circulating lipids (such as smGPCs), and obesity (especially visceral obesity) is closely related to blood pressure elevation, insulin resistance, and low-grade inflammation.

In the present study, we found a large number of significant inverse associations with visceral adiposity, blood pressure and low-grade inflammation, major risk factors of CVD (Chapter 6). Consistent with these results, the studies by Siguener et al. and Ganna et al. (Chapter 2.3) identified LPC(18:2) as inversely associated with total and CAD mortality, and incident CAD, respectively. In addition, Ganna et al. found that LPC(18:2) was associated with specific CVD risk factors: inversely with BMI and CRP. This particular LPC was examined in the present study as LPC(18:2/0:0). Findings from the present study show similarities with the findings by Siguener et al. and Ganna et al. in that LPC(18:2/0:0) was inversely and significantly associated with several of the studied CVD risk factors, namely VF (nominally) (Figure 5.2A) and CRP (after Bonferroni correction for multiple comparisons) (Figure 5.2D).

The other studies that have previously examined circulating smGPCs for their association with CVD-risk factors include a study by Pietiläinen and colleagues, who studied
14 monozygotic twin pairs discordant for obesity, and 10 monozygotic twin pairs concordant for obesity, and showed that concentrations of LPCs (a subset of smGPCs studied in the present study) were higher in obese versus non-obese monozygotic co-twins. They also found two smGPCs inversely associated with whole-body insulin resistance: PC(0:0/20:4) and LPC(16:0/0:0). Only the latter smGPC was assessed in the present study and was not significantly associated with serum fasting insulin. The reason for this possible discrepancy is not clear at present but it may be related to the sample ascertainment (a twin sample of young adults ascertained for obesity vs. a population-based sample of adolescents not ascertained for any disease).

By and large, other studies have supported a ‘protective’ role of smGPCs against CVD. For example, LPCs and lyso-platelet-activating factor GPCs have been found to be inversely associated with BMI, (independently of sex, age, SBP, 2h post glucose load, and smoking status) in a in a large population-based cohort of adults from the San Antonio Family Heart Study (n = 1,076)\(^{134}\). Furthermore, a small cohort study of 30 participants revealed lower total and species-specific LPC levels in obese healthy and type 2 diabetes mellitus individuals and, compared to lean healthy individuals\(^{135}\). Finally, a recent study by Heimer and colleagues\(^{136}\) found that certain LPCs, as well as sums of saturated, unsaturated, monosaturated, and polyunsaturated LPCs, were inversely associated with circulating CRP in participants with BMI >30 kg/m\(^2\). Similar associations were observed with BMI, and circulating LPCs were significantly lower in obese versus normal-weight participants. Since Heimer et al.\(^{136}\) used ‘bulk’ notation where the sn-1 and sn-2 chain lengths and degrees of unsaturation of the studied LPCs were summed, it cannot be said with certainty that any given LPC within their dataset corresponds to a LPC within the present study’s dataset, since we have employed ‘expanded’ notation that allows for the elucidation of length and degree of unsaturation of each sn constituent. Nevertheless, comparisons can be made by summing the sn chain lengths and degrees of unsaturation of the lipid species examined in the present study. Of the 8 LPCs that were found to be inversely and significantly associated with CRP by Heimer et al.\(^{136}\) in adults, 6 LPCs were also inversely and significantly associated with CRP in the present study of adolescents. Most notably, PC(18:0), which may correspond to PC(16:0/2:0), was found to be inversely associated with CRP in both studies, and additionally with blood pressure and VF specifically in the present study. Finally, identities of individual fatty acyl residues (defined by their length and degree of unsaturation) and their linkages to the glycerol backbone at the sn-1 and sn-2 positions were not assessed in the study of Heimerl and colleagues\(^{136}\). Such detailed assessments were made for 90 percent of the smGPCs assessed in the present study. They showed that certain species
containing the same number carbons (i.e., the sum of carbons across both fatty acyl residues) may demonstrate very different relationships with cardio-metabolic risk factors. For example, PC(18:0) assessed as a single species in\textsuperscript{136} was present as two different species in the present study: PC(16:0/0:0) and LPC(18:0/0:0). Importantly, these two species showed different associations with certain CVD risk factors: PC(16:0/0:0) was inversely and significantly associated with VF and blood pressure, whereas LPC(18:0/0:0) was not associated with any of these risk factors (Figure 5.2A, B). Such heterogeneity may be a confounding factor in studies that do not assess the identities and types of linkages of fatty acyl residues.

Another key finding in the present study is the identification of two smGPCs, namely, PC(16:0/2:0) and LPC(14:1/0:0), which demonstrated inverse and positive associations with VF independently of total body fat, respectively. VF is more strongly associated with most other cardio-metabolic risk factors than subcutaneous fat\textsuperscript{79–81}; however, the current methods of measuring VF (e.g. X-ray computed tomography or magnetic resonance imaging) are not suited for being routine clinical measures of obesity. PC(16:0/2:0) and LPC(14:1/0:0) may serve as biomarkers of VF and potentially constitute a simple blood test that would identify viscerally obese individuals at increased risk for CVD. More detailed characterization of these two smGPCs, in particular, is warranted.

Importantly, the two VF-specific smGPCs identified in the present study – PC(16:0/2:0) and LPC(14:1/0:0) – were also associated with several other CVD risk factors. PC(16:0/2:0) was inversely associated not only with VF but also with blood pressure and low-grade inflammation (Figure 5.2B, D), while LPC(14:1/0:0) positively associated not only with VF but also with fasting insulin, as a measure of insulin resistance (Figure 5.2C). All of these associations were significant after Bonferroni correction for multiple comparisons, and most remained significant after additional adjustment for standard clinical measures of lipid metabolism – HDL-cholesterol, LDL-cholesterol, total cholesterol and triacylglycerols (Supplementary Table 5.2). The potential mediatory effects of PC(16:0/2:0) and LPC(14:1/0:0) between VF (as the causal factor) and each other associated CVD risk factor (as outcomes) were then investigated. PC(16:0/2:0) significantly mediated the directed relationships between VF and each blood pressure and CRP, while LPC(14:1/0:0) significantly mediated the directed relationships between VF and fasting insulin (Supplementary Table 5.3). Future studies should aim to determine whether the pharmacological targeting of the two VF-specific smGPCs identified in the present study mitigates at least some of the morbidity conferred by visceral obesity.
Finally, many of the smGPCs assessed in the present study grouped into hierarchal clusters that demonstrated consistent patterns of association with certain CVD-risk factors (Figure 5.1). For example, many of the smGPCs inversely associated with increased VF, CRP, and blood pressure grouped into cluster ‘3D’. These findings suggest shared regulatory pathways relevant to smGPC metabolism and their link to specific CVD-risk factors, which ought to be investigated in future studies.

The present study has several strengths. (1) It was conducted in adolescents and as such it may discover biomarkers of pre-clinical CVD, as adolescence is a developmental period when the initial stages of CVD emerge. Other major strength is the complete elucidation of lengths and degrees of unsaturation of both sn fatty acid residues of the studied smGPCs, as this information adds specificity to observed associations and thus enhances the potential for biologically meaningful discoveries.

A potential limitation of the present study is that it employed a cross-sectional design, which permits for time-invariant unobserved individual differences to confound the findings. Future studies should aim to replicate the findings of the present study using longitudinal designs, which have more power than cross-sectional studies.

In summary, the results of the present study suggest that specific circulating smGPCs are associated with multiple CVD risk factors during adolescence. Circulating smGPCs may serve as novel biomarkers of pre-clinical CVD.
References


