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Searching for Novel Cardiometabolic Biomarkers: Method Development for Differential Approaches and Analysis of Lipids Involved in Lipoprotein Metabolism

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RATNANINGRUM DEWI KARUNA
M.Sc., Leiden University in the Netherlands

born January 11, 1977

citizen of Indonesia

accepted on the recommendation of
Prof. Dr. Renato Zenobi, examiner
Prof. Dr. Katharina M. Rentsch, co-examiner
Prof. Dr. Arnold von Eckardstein, co-examiner
Prof. Dr. Karl-Heinz Altmann, co-examiner

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SUMMARY

The current strategy for the prevention of cardiovascular diseases (CVD) is to stratify patients at risk for CVD using different algorithms. However, this has resulted in a high false-positive rate, for example, of 68% and 86% for high and intermediate CVD risks, respectively, in the Munster Heart Study (PROCAM). Novel biomarkers are hence needed to improve the reliability and the cost-benefit relationship of preventative measures. Since a biomarker should ideally be an indicator of normal and pathogenic processes as well as pharmacologic responses to a therapeutic intervention, the potential candidate new markers are those that are relevant to the pathophysiology of atherosclerosis. Several candidates have emerged, for example 27-hydroxycholesterol (27OHC), sphingosine-1-phosphate (S1P), sphingolipids and phospholipids.

Research over the last decade has revealed that HDL plays a pivotal role in the pathophysiology of atherosclerosis, mainly due to its role in removing the excess of cholesterol in peripheral cells via a reverse cholesterol transport pathway. However, HDL also displays pleiotropic effects on the cardiovascular system which are independent of the reverse cholesterol transport mechanism. 27OHC and S1P are of potential interest due to their roles in HDL metabolism and function.

Published LC-MS methods for the analysis of 27OHC still lacked sensitivity for the measurement in animal models (single mouse) and lipoprotein fractions. Due to this reason, an LC-MS method using atmospheric pressure photoionization (APPI) has been developed and validated. The method was more sensitive than the published LC-APCI-MS or GC-MS methods, enabling us to analyse as little as 15 µL sample volume with a lower limit of quantification (LLOQ) of 40 ng/mL or 4.9 pmol on column.

Subsequently, the role of 27OHC in relation to cholesterol metabolism was evaluated for the first time in patients with monogenic disorders affecting HDL metabolism. In most cases, the defects in HDL metabolism affected the 27OHC concentrations in HDL in a similar degree as the cholesterol concentrations. The most important exception was observed in samples from individuals with mutations in the lecithin:cholesteryl acyl transferase (LCAT) gene. A reduced LCAT activity appeared to impair the 27OHC esterification in the plasma compartment more pronouncedly than the cholesterol esterification. In addition, several defects in HDL metabolism led to a re-distribution of 27OHC into apoB-containing lipoproteins, which was not observed for cholesterol.

An LC-MS method was developed and validated for the analysis of S1P in plasma and lipoprotein fractions. The method was subsequently used to investigate the determinants of circulating S1P in patients with inborn errors of HDL metabolism. Although only ≤5% of HDL particles carry one S1P molecule, both mildly and severely lowered HDL concentrations limited the quantity of S1P in plasma. By contrast, high concentrations of HDL-cholesterol and apoA-I did not influence S1P levels in plasma. Since S1P exerts several anti-atherogenic functions of HDL, these findings are in agreement with the concept of a threshold concentration of HDL or apoA-I which is needed for atheroprotection.

Since there exist biochemical interactions among different lipids involved in cholesterol metabolism, profiling of lipids may reveal more key determinants in the pathogenesis of atherosclerosis. A sphingo- and phospholipid profiling project has been started, in which a method has been developed using a combination of normal phase HPLC separation and parent-ion scanning by a triple quadrupole mass spectrometer. Feasibility of the method was demonstrated in patients with stable coronary heart disease.

ZUSAMMENFASSUNG

Die aktuelle Strategie zur Prävention von kardiovaskulären Erkrankungen beruht auf der Risikostratifizierung der Patienten mittels verschiedener Algorithmen. Dies führt jedoch bei einer grossen Zahl von Personen zu einer Überschätzung des Risikos. So wird das Risiko für ein kardiovaskuläres Risiko zum Beispiel in der Münsteraner PROCAM Studie um 68% beziehungsweise 86% bei Patienten mit einem hohen oder intermediären Risiko überschätzt. Deshalb braucht es neue Biomarker, die eine bessere und zuverlässigere Einschätzung des Risikos ermöglichen. Idealerweise sollte ein Biomarker ein Indikator für normale oder pathologische Prozesse sowie das Ansprechen auf therapeutische Massnahmen darstellen. Deshalb leiten sich Kandidaten für neue Biomarker für die Erkennung von kardiovaskulären Erkrankungen aus der Pathophysiologie der Atherosklerose ab. Mögliche Kandidaten sind zum Beispiel 27-Hydroxycholesterin (27OHC), Sphingosin-1-phosphat (S1P), sowie allgemein die Sphingo- und Phospholipide.

Die Forschung in den letzten Jahren hat klar gezeigt, dass das HDL, vor allem durch seinen Transport von Cholesterin aus der Peripherie zur Leber (reverser Cholesterintransport), eine entscheidende Rolle in der Pathophysiologie der Atherosklerose spielt. HDL hat aber auch noch verschiedene pleiotrope Effekte auf das kardiovaskuläre System, welche unabhängig vom reversen Cholesterintransport sind. Durch ihre Rollen im HDL Stoffwechsel und seinen pleiotropen Funktionen sind 27OHC und S1P in dieser Beziehung von speziellem Interesse.

Publizierte Methoden zur Bestimmung von 27OHC waren zu wenig empfindlich für die Bestimmung in Tiermodellen (einzelne Mäuse) oder in der HDL-Fraktion von humanen Proben. Aus diesem Grund wurde eine LC-MS – Methode mit Atmosphärendruck Photoionisation (APPI) entwickelt und validiert. Die neue Methode ist viel empfindlicher als bisher publizierte LC-APCI-MS- oder GC-MS – Methoden und erlaubte uns, Probenvolumina von 15 µl mit einer Quantifizerierungsgrenze (LLOQ) von 40 ng/ml oder 4.9 pmol pro Injektion zu untersuchen.

Im Anschluss wurde die Rolle von 27OHC im Verhältnis zum Cholesterinstoffwechsel zum ersten Mal in Patienten mit monogenen Erkrankungen im HDL-Stoffwechsel untersucht. In den meisten Fällen waren die 27OHC - Konzentrationen in ähnlicher Weise verändert wie die Cholesterinkonzentration. Die wichtigste Ausnahme waren Individuen mit Mutationen im Gen für die Lecithin-Cholesterin-Acyltransferase (LCAT). Eine Reduktion der LCAT –

Aktivität führte zu einer grösseren Reduktion des veresterten 27OHC im Plasma als des veresterten Cholesterins. Zusätzlich führten verschiedene Gendefekte im HDL – Stoffwechsel zur einer Umverteilung von 27OHC in ApoB enthaltende Lipoproteine. Dieser Effekte wurde für das Cholesterin nicht beobachtet.

Es wurde ebenfalls eine LC-MS – Methode für die Bestimmung von S1P im Plasma und in den Lipoproteinfraktionen entwickelt und validiert. Diese Methode wurde anschliessend dazu verwendet, die Konzentration von S1P in Patienten mit angeborenen Defekten im HDL – Stoffwechsel zu untersuchen. Obwohl nur weniger als 5% der HDL – Partikel ein S1P – Molekül beinhalten, führten sowohl leichte als auch massive Erniedrigungen der HDL – Konzentration zu einer Beschränkung der S1P – Menge im Plasma. Da S1P verschiedene anti-atherogene Funktionen in HDL ausübt, stimmen diese Resultate mit dem Konzept, dass es für die Atheroprotektion durch HDL oder ApoA-I eine Grenzkonzentration gibt, überein.

Da es zwischen den verschiedenen Lipiden, die im Cholesterinstoffwechsel eine Rolle spielen, unterschiedliche biochemische Interaktionen gibt, können durch das Erstellen eines Lipidprofils mehr Schlüssesubstanzen in der Pathogenese der Atherosklerose erkannt werden. Ein Projekt zum Profiling von Sphingo- und Phospholipiden wurde initiiert und eine Methode entwickelt, die eine Kombination zwischen Normalphasen HPLC und Parent-ion Scan mittels eines Triple-Stage-Quadrupol - Gerätes darstellt. Durch das Profiling von Proben von Patienten mit stabiler koronarer Herzkrankheit konnte die Anwendbarkeit der Methode gezeigt werden.

1. INTRODUCTION

1.1. Biomarkers: Definition and Purposes

The term biomarker (biological marker) is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (1). Hence, the functions of biomarkers can shortly be classified into 3 aspects: as indicators of disease trait (or as risk factors or risk markers), of disease state (preclinical or clinical), and of disease rate (progression) (2). In accordance to these functions, biomarkers can serve as a diagnostic tool for the identification of those patients with a disease or abnormal condition, a tool for staging or classification of the extent of the disease, an indicator for the disease prognosis, and a tool for the prediction and monitoring of clinical responses to intervention. Biomarkers which are highly sensitive and specific indicators of disease pathways can serve as surrogate endpoints, in which they substitute the clinical endpoints in assessing the benefits and risks of a therapeutic intervention in randomized clinical trials. Biomarkers contribute knowledge for clinical pharmacology as well as provide bases for designing clinical trials, selection of lead compounds for phase III clinical trials, and guidance in dosing (1, 2).

To be of clinical value, a biomarker must be accurately and reproducibly measured, sensitive and specific, acceptable to the patient, and easy to interpret by clinicians for diagnostic or prognostic purposes (2). Ideally, a novel biomarker adds clinical value to the previously established markers or classical procedures.

1.2. The Need for Novel Cardiovascular Biomarkers

Cardiovascular diseases (CVD), which include coronary heart disease (including myocardial infarct / heart attack and angina pectoris / chest pain), stroke, hypertension, and heart failure, are the leading causes of disability and mortality in developed countries. An alarming increase of CVD in developing countries has also been reported, suggesting that the prevention and treatment of CVD is one of the most important public health issues worldwide (3).

CVD is a life course disease that begins with the evolution of risk factors that in turn contribute to the development of subclinical atherosclerosis (2). As a number of CVD events occur in asymptomatic patients, prevention includes not only secondary prevention in patients

who survive an event, but also primary prevention and hence the early identification and treatment of patients at significant risk. Global risk assessment is necessary for accurate risk prediction and for the development of appropriate treatment strategies (3).

The presence of several moderately expressed risk factors in a given individual – such as hypercholesterolemia (high LDL-cholesterol), hypertriglyceridemia, low HDL-cholesterol, hypertension, smoking, diabetes, age, male gender, and a positive family history of premature atherosclerosis - can produce a significant increase in CVD risk. Therefore at present, the most advanced strategy for CVD global risk assessment is to combine the information from several risk factors into algorithms or scores. This procedure allows the calculation of an individual's risk within the next 10 years (4).

An estimated global risk of >20% per 10 years in an asymptomatic patient is considered to be high. The affected patient is given advice to be treated as aggressively as a symptomatic patient. This implies lowering of LDL-cholesterol and systolic blood pressure. An estimated risk ranging between 10 and 20% in 10 years is considered as moderate, and treatment is also targeted to lower LDL-cholesterol and systolic blood pressure. An estimated risk <10% is considered as low. In this case, drug treatment recommendations are not offered to the majority of individuals (4).

In the Munster Heart Study (PROCAM), the finding of an estimated global risk of <10% has a negative predictive value of 97%. However, the positive predictive value of the high risk estimates (>20%) amounts to maximally 32%, implying a false positive rate as high as 68%. The intermediate risk of 10–20% has positive and negative predictive values of 14 and 86%, respectively (4). A better risk stratification is clearly needed to optimize the cost-benefit relationship of preventive measures. Novel biomarkers are one such tool to improve risk prediction and clinical assessment.

1.3. The Pathophysiology of Atherosclerosis and Lipoprotein Metabolism

Atherosclerosis is the major underlying cause for CVD events. Despite the old view of atherosclerosis resulting from merely a passive deposition of lipids in the arterial wall, many studies have recently shown that dynamic inflammation pivotally participates in all stages of atherosclerosis, from the initiation to the progression and destabilization of atherosclerotic plaques (5). Atherosclerosis begins when the endothelial cells of the blood vessels undergo inflammatory activation, leading to an increase in the expression of various leukocyte

adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 selectively binds certain leukocyte classes, such as monocyte and T lymphocyte. Leukocytes, recruited then by various chemo-attractant cytokines (chemokines), migrate through endothelial cell junctions into the arterial intima. Once resident in the intima, monocytes acquire the morphological characteristics of macrophages and eventually form the so-called foam cells, the cytosolic lipid droplets that appear foamy under the microscope. Foam cells are the hallmark of atherosclerotic lesions (5).

In vitro and animal studies as well as epidemiological studies have shown that cholesterol metabolism in macrophages is central to the development of atherosclerosis. Cholesterol is transported mainly by plasma lipoproteins in the form of cholesteryl esters. Macrophages ingest and digest low-density lipoprotein (LDL)-derived cholesterol by taking it up via unregulated scavenger receptors (figure 1). After entering macrophages, cholesteryl esters are hydrolyzed by an acid lipase and the free cholesterol is secreted from the cells and transported to the liver and steroidogenic organs. However, macrophages take up more cholesterol than they can excrete, thus accumulating cholesteryl ester-rich lipid droplets and forming the foam cells (6).

High-density lipoprotein (HDL)-mediated reverse cholesterol transport is the main pathway for the efflux of cholesterol from macrophages to the liver. As depicted in figure 1, reverse cholesterol transport involves several proteins that directly affect HDL synthesis, maturation, conversion and catabolism. Crucial proteins are: apolipoprotein A-I (apoA-I), the main protein of HDL which activates lecithin:cholesterol acyl transferase (LCAT), stimulates cholesteryl ester efflux and is a ligand for HDL binding sites; ATP-binding cassette transporter A1 (ABCA1) which mediates cholesterol and phospholipid efflux from macrophages and peripheral cells to lipid-poor apoA-I; LCAT which esterifies cholesterol and converts nascent HDL to mature HDL; cholesteryl ester transfer protein (CETP) which exchanges cholesteryl ester from HDL with triglycerides from apoB-containing lipoproteins and thereby re-generates lipid-poor apoA-I; scavenger receptor type B class 1 (SR-BI) which mediates selective uptake of cholesterol into the liver; hepatic lipase (HL) which hydrolyzes triglyceride and phospholipids in HDL and is a co-factor of SR-BI in selective uptake; and endothelial lipase (EL), an endothelium-derived lipase which preferentially hydrolyzes HDL-phospholipids, increases susceptibility of cholesteryl ester to SR-BI-mediated selective uptake (7, 8).

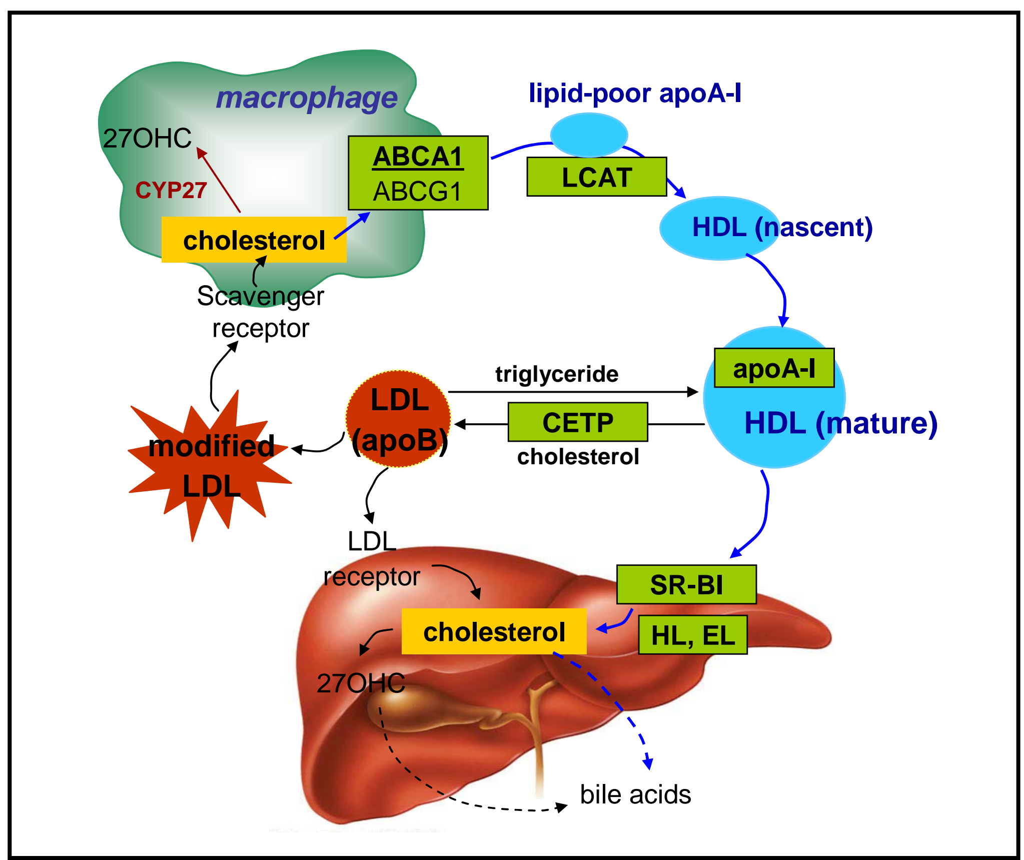

Figure 1. Schematic representation of HDL-mediated cholesterol efflux from macrophages to the liver (reverse cholesterol transport), as marked with the blue arrow (→). As shown from the cartoon, risk factors for CVD (atherosclerosis) include high LDL-cholesterol and low HDL-cholesterol levels.

Both genetic and environmental factors determine HDL-cholesterol levels in humans and the associated CVD risk. Genetic factors include both frequent polymorphisms with little effects on HDL-cholesterol levels and rare mutations with strong effects. The latter include mutations in the genes of apoA-I, ABCA1 and LCAT, which cause low HDL-cholesterol levels in heterozygotes (frequently below 5th percentile) and virtual absence of HDL in homozygotes or compound heterozygotes. By contrast, mutations in the genes of CETP, SR-BI, EL or HL cause elevations of HDL-cholesterol (9-11). The cardiovascular risk association of these mutations is controversially described. Nevertheless, the finding of elevated HDL-cholesterol levels and reduced cardiovascular risk in Japanese CETP mutation carriers (10-14) has stimulated the pharmaceutical industry to develop CETP inhibitors like torcetrapib, anacetrapib and dalcetrapib, which are currently under clinical evaluation (15).

1.4. Lipids Relevant to the Patophysiology of Atherosclerosis

1.4.1. 27-Hydroxycholesterol (27OHC)

27-Hydroxycholesterol (27OHC) is derived from cholesterol by oxygenation of its side-chain at the position C27 (figure 2), formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27). It is the most abundant oxysterol (oxidized cholesterol) in the circulation (16) and is part of the cholesterol catabolic pathway to transport excess cholesterol from macrophages to the liver. Secretion of 27OHC from macrophages has been proposed as an alternative mechanism to the HDL-mediated reverse cholesterol transport (17-19). In the liver, 27OHC is an important intermediary product of the so-called alternative bile acid synthesis pathway (20). Moreover, 27OHC is one of the activating ligands for the liver-X-receptors (LXR) which regulate the transcription of several genes involved in cholesterol metabolism (21, 22).

It has been shown that plasma levels of 27OHC correlate with the cholesterol content in atherosclerotic lesions and the severity of the disease (23-25). Consistent with this, patients with the rare disease of cerebrotendinous xanthomatosis because of mutations in the CYP27 gene, which cause deficiency of this enzyme, develop premature atherosclerosis despite having normal levels of plasma cholesterol (26). Together with CYP27, 27OHC has been proposed as a potentially anti-atherogenic molecule (16-18, 27-30).

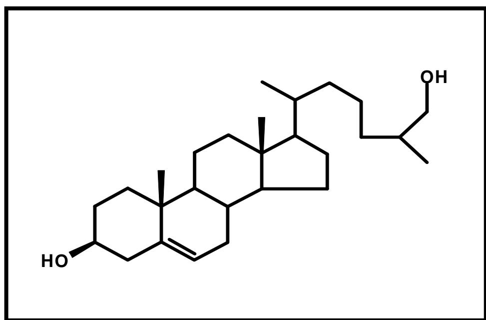

Figure 2. Structure of 27OHC

1.4.2. Sphingolipids and Phospholipids

Sphingolipids are a family of >300 lipid species which share a sphingoid backbone (figure 3). The structural diversity of sphingolipids in mammals includes variations in the backbone (sphingosine (d18:1), sphinganine (d18:0) or 4-hydroxysphinganine (t18:0)), fatty-acid tail (16 to 26 carbon-chain length, 0 to 1 double bonds), as well as in the head group

which then determine its sphingolipid class. Head groups include a hydroxyl residue (ceramides), phosphates (sphingosine-1-phosphate, ceramide-1-phosphate), phosphocholines (sphingomyelins) as well as the combination of glucose and galactose with sialic acid or sulphate (glycosphingolipids). The structural diversity of sphingolipids is widely appreciated and addressed on a number of "omics" websites, for example SphinGOMAP (www.sphingomap.org) (31, 32).

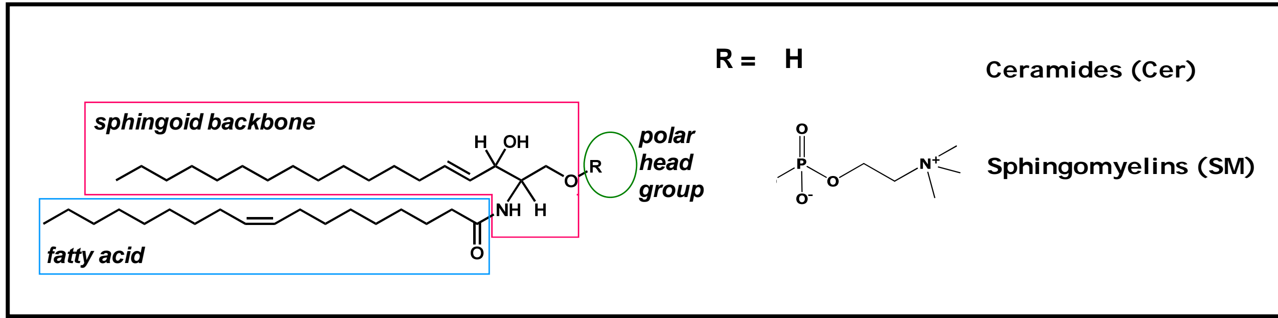

Figure 3. Sphingolipid structures and head group variation

Phospholipids, comprising >700 lipid species, are structurally derived from glycerol containing a phosphate group (hence: glycerophospholipids) as well as one (lyso-phospholipids) or two fatty-acid tails. As with sphingolipids, the variations in the head group determine their phospholipid classes (figure 4) as well as their functions in signal transduction. Phosphatidic acid is the key precursor of all mammalian phospholipids, as it is hydrolyzed and converted into diacyglycerol and cytidine diphosphate (CDP)-diacylglycerols, which are then required for the biosynthesis of phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI), phosphatidylglycerols (PG), diphosphatidylglycerols (cardiolipins) (33).

Sphingolipids and phospholipids are the essential building blocks of all cell membranes. The tendency of the hydrophobic moieties to self-associate (entropically driven by water), and the tendency of the hydrophilic moieties to interact with aqueous environments and with each other, are the physical bases of spontaneous membrane formation. This amphipatic property of lipids enables the cells to segregate their internal constituents from the external environment as well as the compartmentalization within the cells (34).

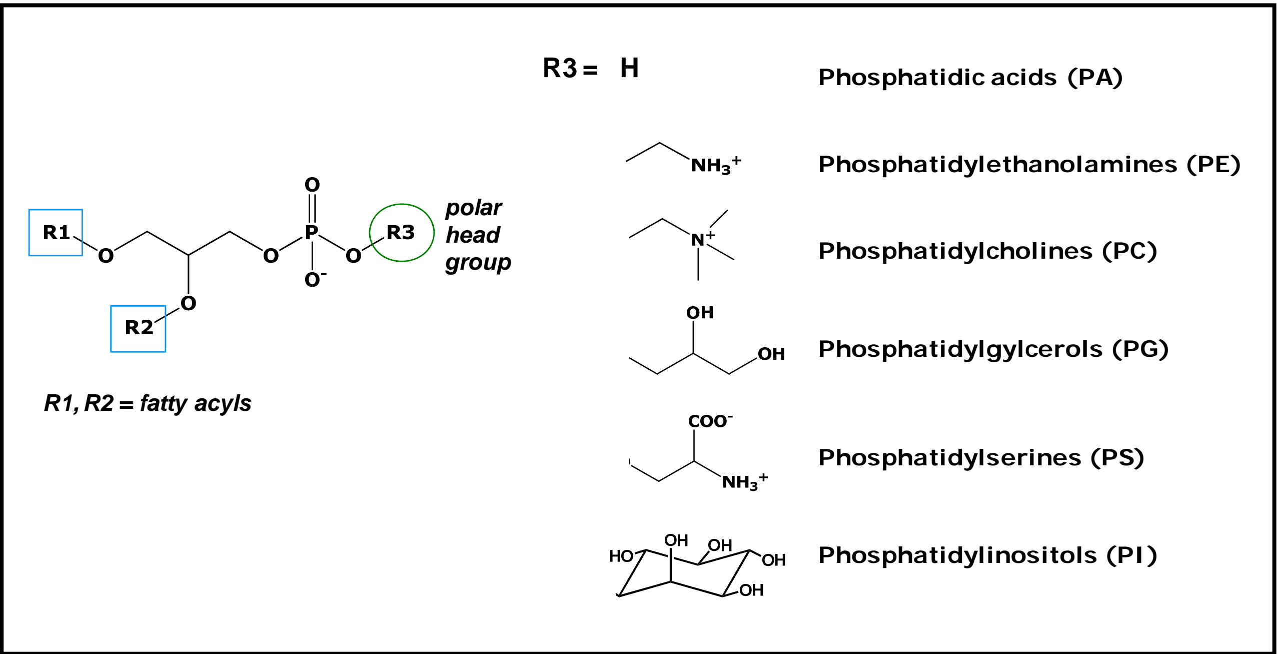

Figure 4. Phospholipid structures and head group variation

In addition to maintaining structural membrane integrity, lipids act as first and second messengers in mediating cell-cell recognition and interaction (34). The role of sphingolipids and phospholipids in the pathogenesis and progression of atherosclerosis is an area of active research (35-41). The phospholipid content in HDL has been shown to have an inverse correlation to the incidence of coronary heart diseases, as well as influence SR-BI-mediated cholesterol efflux and reverse cholesterol transport (38-41). Experimental data, mostly from animal studies, have suggested an association between sphingolipids (particularly sphingomyelins and ceramides) with the development and progression of atherosclerosis (37, 42-47). A few studies in humans have shown that plasma sphingomyelin levels were increased in patients with familial high HDL-cholesterol (48) and were associated with subclinical atherosclerosis (49) and clinical coronary artery diseases (50, 51). The ratio of sphingomyelins to the sum of sphingomyelins and phosphatidylcholines was higher in patients with coronary artery diseases. In fact, plasma sphingomyelin and its relative concentration to phospholipids (particularly phosphatidylcholines) have been suggested to be independent risk factors for atherosclerosis (50, 51). However, a recent large, multi-ethnic,

study showed a contrary finding. High plasma sphingomyelin levels were not associated with increased risk, but with a modest reduction of coronary heart disease events after 5 years of follow-up (52). Hence, further investigation is needed to reveal the roles and relationships of different sphingolipids and phospholipids in CVD events.

1.4.3. Sphingosine-1-phosphate (S1P)

The inverse association between HDL-cholesterol and the risk for cardiovascular diseases (53, 54) is usually attributed to the role of HDL in reverse cholesterol transport. However, HDL possesses additional pleiotropic functions independent of cholesterol metabolism which may contribute to the reduction of cardiovascular risk (55, 56). HDL carries the majority of sphingosine-1-phosphate (S1P) in plasma, which is an important signalling mediator (56-58).

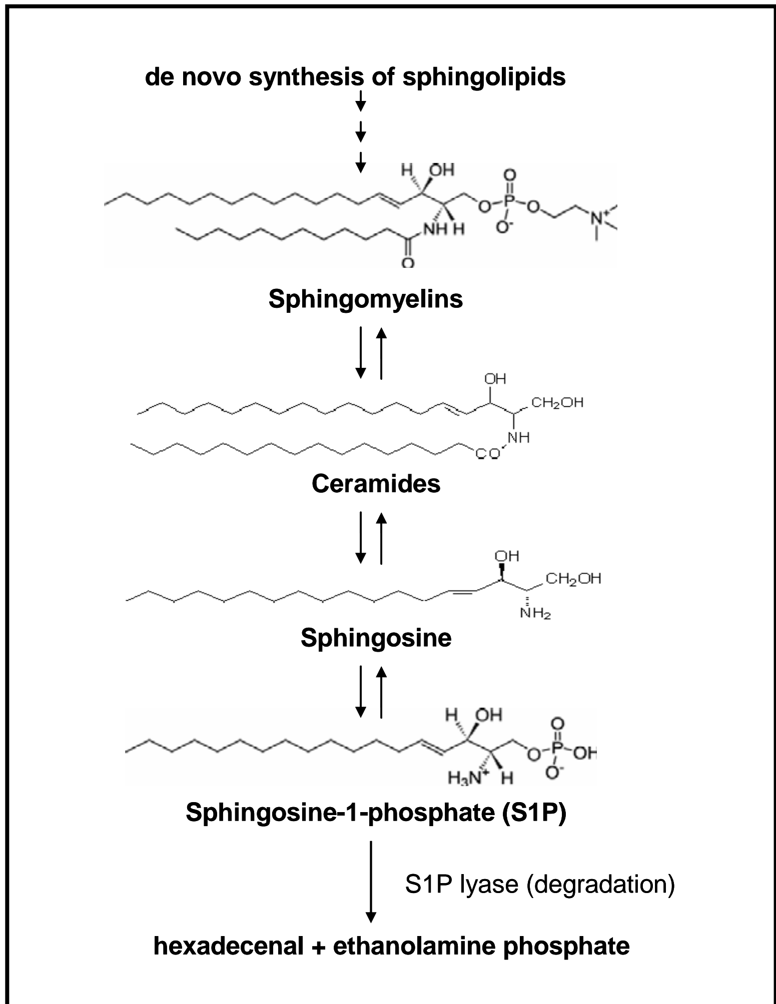

Figure 5. The biosynthesis of S1P

S1P is a bioactive lysosphingolipid that is produced primarily by the degradation of sphingomyelins into ceramides and sphingosine (figure 5) (59). *In vitro* and animal studies have shown that HDL-associated S1P inhibits the migration of vascular smooth muscle cells (VSMCs), promotes nitric-oxide mediated vasodilatation which might be beneficial in reducing the blood pressure, promotes endothelial cell growth and survival, inhibits endothelial cell expression of VCAM-1 and reduces monocyte and lymphocyte recruitment and migration to the arterial intima and hinders foam cell formation (56-60). All reported effects of HDL-associated S1P are potentially beneficial for the cardiovascular system (58).

1.5. Preview of the Work

Research over the last decade has revealed that HDL plays a pivotal role in the pathophysiology of atherosclerosis, mainly due to its role in removing excess of cholesterol from peripheral cells to the liver. However, HDL also displays pleiotropic effects on the cardiovascular system, which are independent of its reverse cholesterol transport mechanism. 27OHC and S1P are the two lipids which are of potential interest, due to their roles in HDL metabolism.

Published studies on 27OHC have indicated its role in an alternative pathway to the classical reverse cholesterol transport mechanism, via the conversion of cholesterol to its oxidized metabolite by the CYP27 enzyme (29, 61, 62). Hence, measuring circulating levels of 27OHC may reflect the activity of this alternative pathway.

27OHC has been analyzed using gas chromatography – mass spectrometry (GC-MS), which gives a good sensitivity albeit laborious sample pre-treatment. A method using liquid chromatography – mass spectrometry (LC-MS) with atmospheric pressure chemical ionization (APCI) has been developed in our institute (63). However, this method still lacked sensitivity for the measurement of 27OHC in animal studies (single mice) and lipoprotein fractions. For this reason, an LC-MS method using atmospheric pressure photoionization (APPI) was developed to improve the sensitivity. The method was validated as well. Subsequently, the role of 27OHC in relation to cholesterol catabolism was evaluated for the first time in patients having monogenic disorders of HDL metabolism.

S1P has been proposed as a potential contributor to the pleiotropic effects of HDL independently of reverse cholesterol transport. Although it has been shown to be an important signalling molecule, the determinants of S1P levels in plasma have not yet been investigated.

An LC-MS method was developed and validated for the analysis of S1P in plasma and lipoprotein fractions. The method was subsequently used to analyze S1P in patients with monogenic disorders affecting HDL metabolism.

A few *in vitro* and animal studies have indicated that some lipids belonging to the groups of sphingolipids and phospholipids may play important roles in the pathophysiology of atherosclerosis. Thorough investigations of all the different lipid classes and species in these lipid groups have not yet been reported. Moreover, the two lipid groups consist of more than 1000 lipid species with highly heterogeneous molecular structures. The advance of mass spectrometry has enabled the analysis of hundreds of lipid species within a single run, providing an opportunity to search for potential candidate markers in a high-throughput manner. To reach this, an LC-MS-based profiling method was needed to be developed for comprehensive analyses of sphingolipids and phospholipids in plasma.

To summarize, the aim of this work was to establish sensitive and reliable methods for the quantification of different lipids and to apply the methods in very well defined patient cohorts, namely patients with distinct monogenic disorders of HDL metabolism. Hence, this work aimed to set the stage for future evaluation of different lipid candidates in larger-scale clinical studies of atherosclerosis.

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2. A BRIEF OVERVIEW ON THE MASS SPECTROMETRIC ANALYSIS OF LIPIDS

The term "lipids" is generally referred to a broad group of naturally-occurring compounds which have in common a ready solubility in organic solvents, such as chloroform and alcohol. However, this description can be misleading since many of the compounds have amphiphilic properties and may have higher solubility in water than in organic solvents. An interesting definition is given by W.W. Christie (1), who defined lipids as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds". This includes a wide range of chemically unrelated groups such as sphingolipids, glycolipids, glycerophospholipids (phospholipids), eicosanoids, tocopherols, waxes, triacylglycerols, sterols (cholesterol, oxysterols) and fatty acids. For convenience, lipids are referred in this text either as groups, classes, or species as classified in table 1.

Table 1. Classification of lipids into groups*, classes and species. The species is lipid molecular species with distinct fatty-acid chain structures.

Lipids		
Group	**Class**	**Species**** (examples)
Fatty acids	Free fatty acids	Palmitic acid, stearic acid
	Eicosanoids	Arachidonic acid, prostaglandin
Glycerolipids	Triacylglycerols .	1,2-Dihexadecanoyl-3-(9Z-octadecenyl)-*sn*-glycerol
	Diacylglycerols	1-Palmitoyl-2-oleoyl-gylcerol
Glycerophospholipids	Phosphatidic acids	PA(18:0/0:0), PA(18:0/18:1)
	Phosphatidylglycerols	PG(16:0/0:0), PG(16:1/18:0)
	Cardiolipins	CL([18:0/16:1],[16:0/18:1])
	Phosphatidylethanolamines	PE(18:0/0:0), PE(18:0/16:0)
	Phosphatidylserines	PS(16:0/0:0), PS(16:0/18:1)
	Phosphatidylinositols	PI(18:1/0:0), PI(18:0/16:0)
	Phosphatidylcholines	PC(16:0/0:0), PC(18:0/18:1)
Sphingolipids	Long-chain bases	Sphingosine, sphingosine-1-phosphate
	Ceramides	Cer(d18:1/16:0)
	Sphingomyelins	SM(d18:1/18:0)
	Glycosphingolipids	GlcCer(d18:1/18:0)

Table 1. (continued)

Lipids		
Group	**Class**	**Species**** (example)
Sterols	Cholesterol	
	Bile acids	Cholic acid
Prenols	Isoprenoids	Diterpenoids
	Vitamine E	α-tocopherols
Saccharolipids	Lipid A	Lipid A-disaccharide-1-phosphate

* *Modified from ref. (2)*

** *For lipid nomenclatures, see ref. (3-4)*

Numerous reviews have been published on the mass spectrometric analyses of specific lipid groups with emphasis on structural elucidation (5-11), quantitative analysis (12-18) or lipid profiling (19-26). This chapter will give a brief overview about current developments in the mass spectrometric analysis of lipids in biological samples, with the focus on oxysterols (oxidized metabolites of cholesterol), sphingolipids, and phospholipids.

2.1. The Nature of the Problem

Each lipid group exists in nature as a mixture of closely related molecular lipid species. It is hence easier to divide lipid analyses according to two purposes: analyses of individual molecular species and analyses of specific lipid groups or classes (in which the total lipid species within the class are summed up).

Lipids generally consist of fatty acids which are linked by ester bonds to alcohols (such as to cholesterol) or by amide bonds to long-chain bases (such as in sphingolipids). The aliphatic chains are present in various carbon numbers (typically even numbers of 14 to 26 carbons), various degrees of unsaturation and different attached functional groups (such as hydroxyl group and sugars). In addition, variations may also be present at the core lipid structures or additional side chains, such as in oxysterols and bile acids. The analysis of lipid molecular species is thus a challenge. Nevertheless, complete structural elucidation using tandem mass spectrometry has been elegantly demonstrated, for example for oxysterols (27) and phospholipids (7, 28). (Semi)-quantification of lipid molecular species is usually sought in lipid profiling. One possibility to achieve this is by employing multiple reaction monitoring

(MRM) of each species after separation by reverse phase high performance liquid chromatography (HPLC), by which the apolar fatty acid chains are retained on the HPLC column (16-17). Upon different approaches, lipid groups such as sphingolipids and phospholipids are sub-grouped into classes based on their polar head groups. Profiling of one or a few lipid classes is then conducted by direct-infusion into the MS (29-32) or with prior separation of the different classes by normal phase HPLC (33-35). Quantification, however, is complicated by the fact that different numbers of carbons and double bonds in the fatty acid chains affect the ionization efficiency of each molecular species (36).

The analysis of total lipids within a specific lipid group often employs acid and/or alkaline hydrolysis to break the fatty acyl amide and ester. This has been applied, for example, to the quantification of oxysterols (37-39) and long-chain sphingoid bases (16).

2.2. Hyphenated Techniques for Targeted Lipid Analysis

For the analysis of simple lipids, for example fatty acids, gas chromatography – mass spectrometry (GC-MS) is currently still the "standard", well-established technique, although the sample pre-treatment is quite laborious due to the need of prior derivatization of the non-volatile lipids (40-44). In recent years, liquid chromatography – mass spectrometry (LC-MS) has become a more common technique for lipid analysis (20-21). Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) can be employed for the analysis of sphingolipids, phospholipids and sterol derivates (such as oxysterols) (27, 45-47). However, for sensitivity reasons, quantification of most sphingolipids and phospholipids utilizes ESI (11, 16-17), while oxysterols (without derivatization) are better ionized with APCI (38, 48). Recently, a new interface utilizing atmospheric pressure ionization (APPI) emerged (49-50) and has been shown to improve the sensitivity for the detection of oxysterols and other sterol derivates (37, 51-52).

2.2.1. Atmospheric Pressure Photoionization (APPI)

Photoionization (PI) itself is not a revolutionary technique and has been used in GC with photoionization detector (PID) and in ion-mobility spectrometry (IMS) (49). However, the hyphenation to LC-MS was first introduced by Robb *et al* only in 2000 (53).

APPI is based on the interaction between the photon beam produced by a discharge lamp (usually a Kr-filled UV lamp which has lower photon energy (10 eV) than the major components of air) and the vapor produced from the nebulization of solvent and analyte mixture. PI occurs in several steps. First, the analyte or solvent molecule (M) is excited due to the absorption of a photon ($E = h\nu$) (49-50):

$$M + h\nu \rightarrow M^*$$

When the ionization energy (IE) of the molecule is lower than the photon energy, an energetic electron is released (E_{e-} (max) = $h\nu - IE_M$), leading to a molecular ion with an unpaired electron (49-50):

$$M^* \rightarrow M^{\bullet+} + e^-$$

When the IE of the molecule is higher than the photon energy, de-excitation may occur (49-50):

photodissociation	:	$M^* \rightarrow A + B$
photon emission	:	$M^* \rightarrow M + h\nu$
collisional quenching	:	$M^* + C \rightarrow M + C^*$

Ideally, the energy of the ionizing photon needs to be higher than the IEs of the target analytes and lower than the IEs of the constituent air and solvents. When this is not the case, however, adding a relatively high amount (as compared to the analytes) of additional substances called dopant may help to increase the probability of analyte ion formation. Depending on the proton affinity (PA) and/or electron affinity (EA) of its photoions, the dopant (D) functions as an intermediate between the analyte and photon reaction via proton transfer mechanism (49-50):

proton transfer : $D^{\bullet+} + M \rightarrow [D-H]^{\bullet} + [M+H]^+$ if $PA_M > PA_{[D-H]}$.

The sensitivity that can be achieved using APPI hence directly depends on the reactant ion composition, which in turn depends on the presence of the analytes, the impurities, the solvents, the dopant, and the nebulizing gas as well as the components of the surrounding air (49-50, 54).

2.3. Lipid Profiling

Due to the advances of mass spectrometry and the need to understand the complexity of lipid biochemistry, lipid analysis has been focusing on lipidomics which can been defined as "the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" (55). Different mass spectrometric techniques have been employed for this comprehensive lipid analysis, both for the structural characterization and (semi)-quantification of the lipid species. One practical outcome of this practice is the discovery of novel lipid species by (tandem) mass spectrometry (56-59).

Among the numerous mass spectrometry-based methods that have been developed for lipid profiling, the most commonly used are: "shotgun lipidomics" (intra-source separation and single parent-ion or neutral-loss scans with a direct infusion), multiple parent-ion or neutral-loss scans (with and without data-dependent acquisition control), and multiple-reaction monitoring (MRM) after reverse phase chromatographic separation (19-21, 23).

2.3.1. Shotgun Lipidomics

In principal, shotgun lipidomics is an intra-source separation and selective ionization of lipids and their lithium adducts, as depicted in figure 1. Total lipid extracts are infused directly into the ESI source and lipid species are subsequently identified in a triple quadrupole mass analyzer using lipid class-specific parent-ion scans and neutral-loss scans. By varying the solvent composition and spraying conditions, it is possible to specifically enhance the ionization of certain lipid classes and, hence, to improve the dynamic range of lipid detection (60-61). This approach has been applied to profile lipid species of different phospholipid classes (29, 31, 62-71).

The major drawback of this technique is the ion suppression of the low abundance lipids and the contamination of the instrument with lithium ions (61).

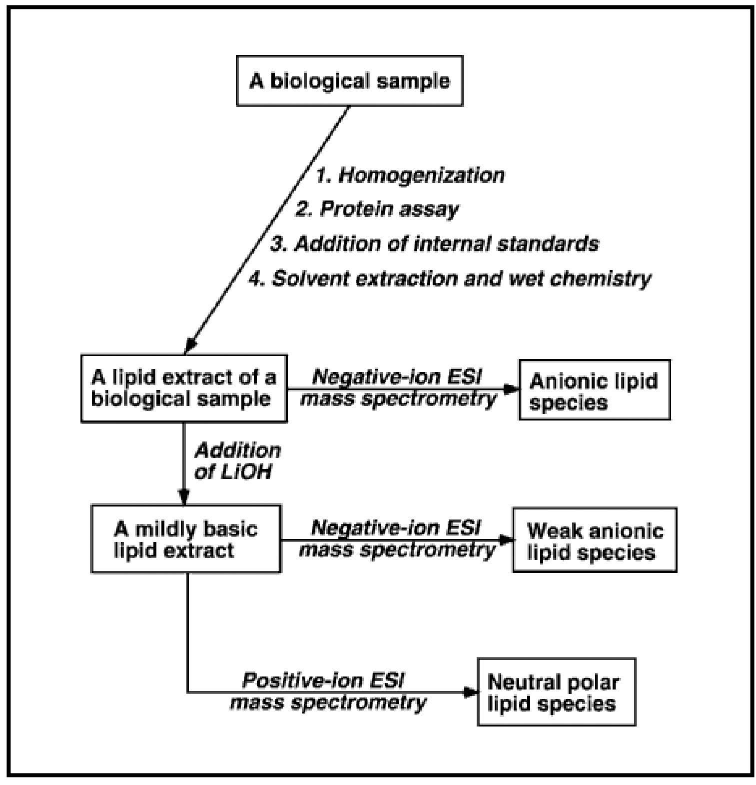

Figure 1. Intra-source separation and selective ionization of lipids (61)

2.3.2. Multiple Parent-ion Scans (MPIS)

Similar parent-ion scan experiments like shotgun lipidomics can also be performed on a Q-TOF instrument. In contrast to the triple quadrupole, in which each parent-ion spectrum can only be acquired sequentially in time, QTOF has the advantage that multiple parent-ion scans (MPIS) can be acquired at the same time and that the fragment ions are detected at a high accuracy. This MPIS is achieved by stepping the quadrupole across the desired parent mass (m/z) range and recording all the obtained MS/MS spectra. Data are generated to allow deconvolution or the ‘‘construction’’ of multiple parent-ion spectra (72).

An alternative to this approach has been developed utilizing data-dependent acquisition control. In this approach, a survey scan is first performed to detect parent-ions of interest in the sample. MS/MS is then performed only on targeted parent-ions. This method can be conducted on a QTOF or on hybrid FT-ICR or Orbitrap instruments; the latter gives the advantage of high accuracy and sensitivity for the detection of the fragment ions (73-74).

2.4. Quantitative Analysis and Validation

Validation of analytical methods is important in order to ensure that the analysis of samples gives reliable and reproducible results. The essential parameters required according to the official guides such as the FDA Guidance for Bioanalytical Method Validation (75) are selectivity, sensitivity, accuracy, precision, reproducibility, and stability. Other parameters are usually also determined during validation, for example: extraction efficiency, linear dynamic range, matrix suppression and dilution integrity for analyzing analyte with concentration above the upper limit of quantification (76).

As opposed to targeted analysis, results obtained from profiling-based methods are much more difficult to assess due to the much larger numbers of measured analytes as compared to the numbers of replicates, the inadequacy of the existing methods to provide selectivity and accurate quantification, and the still lack of standard protocols for validation (77-79). Particularly in lipid profiling, different techniques exist; each has its own limitation, which can compromise the reliability of quantitative analysis. For example, it is frequently impossible to obtain deuterated standards for each lipid species. Non endogenous lipids with odd numbers of carbon in the fatty acid chains hence are used as internal standards. However, different numbers of carbons and double bonds in the fatty acid chains affect the ionization efficiency of each molecular species (36), leading to inaccurate quantification. Although comparative profiling can be done to reveal lipids that show differences between sample clusters (22), these results can be difficult to reproduce in other studies (77). Moreover, due to the long carbon chains of the fatty acyls, deisotoping is needed to correct the overlapping isotope peaks with adjacent masses. Han and Gross et al addressed these two problems by employing correction factors for C13 isotopes and for the carbon number differences between internal standards and target analytes. However, the correction factor for the carbon number differences depends on the specific lipid class and must be determined experimentally. Furthermore, selectivity is still a problem as their quantification is complicated by the presence of isobaric lipid species (61, 80).

In the context of novel biomarker discovery, a candidate novel marker needs to undergo verification and clinical evaluation to determine its sensitivity and specificity in indicating abnormal conditions. Before this clinical verification, the analytical performance of the novel candidate needs to be assessed so that its accuracy and precision is demonstrated (validated) (81-82). Hence, similar to targeted analysis, several parameters need to be assessed in profiling-based method. One example to achieve this is by using various non-endogenous

lipid class-specific internal standards spiked to plasma matrix to assess the linearity, limit of detection, repeatability and recoveries of the method (83). However, this approach only provides an indication for the reliability of the endogenous analytes measurement, due to the differences in the abundance and ionization efficiency of different lipid species. As yet, the best option offered to accurately quantify lipids is by employing multiple reaction monitoring (MRM) and stable isotope dilution on targeted lipid species chosen from profiling approach, as this is amenable to standard quantitation and analytical quality controls (77, 79, 82, 84-85).

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3. DOPANT ASSISTED - ATMOSPHERIC PRESSURE PHOTOIONIZATION (DA-APPI) LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY FOR THE QUANTIFICATION OF 27-HYDROXYCHOLESTEROL IN PLASMA

R. Karuna, A. von Eckardstein and K.M. Rentsch

Institute of Clinical Chemistry, University Hospital Zurich, and Competence Center for Systems Physiology and Metabolic Diseases, Zurich

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Abstract

27-Hydroxycholesterol (27OHC) is of potential diagnostic interest due to its role in maintaining whole-body cholesterol homeostasis. DA-APPI (Dopant Assisted - Atmospheric Pressure Photoionization) has improved the sensitivity of 27OHC analysis, in comparison to the published LC-APCI-MS method, allowing quantification from a very low amount of sample (≤ 50 µL plasma). The method was validated for quantification from 50 µL and 15 µL plasma, with the limit of quantification (LOQ) of 10 and 40 ng/ml plasma, respectively. A further advantage is that no prior derivatization was needed, unlike the LC-ESI-MS or the standard GC-MS method. The method validation also resulted in good linearity and recovery of 91% to 106%. The within-day and between-day coefficient of variation were less than 15% while giving the accuracy of 90.9% to 113.4%. This report summarizes the effects of some critical parameters on the ionization of 27OHC using APPI, as well as the validation of the method. The sensitivity achieved with DA-APPI broadens the usefulness of the LC-MS method in clinical applications.

3.1. Introduction

27-Hydroxycholesterol (27OHC) is an oxygenated metabolite of cholesterol formed by the sterol 27-hydroxylase (CYP27), an enzyme which is widely distributed among tissues and organs, particularly in vascular endothelium and macrophages (1-3). The oxidation at the C27 of the cholesterol side-chain is part of the cholesterol catabolic pathway to transport excess cholesterol from peripheral cells to the liver, as the metabolite is then able to pass lipophilic membranes quicker, and is the first intermediate in the synthesis of the more hydrophilic bile acids (4). 27OHC is one of the oxysterols (oxidized cholesterols) that function as activating ligands for the liver-X-receptors (LXR), regulating the transcription of genes involved in cholesterol catabolism, transport and elimination (5). The importance of 27OHC is mostly attributed to its role in maintaining cholesterol homeostasis, since it provides an alternative route to the main HDL-dependent reverse cholesterol transport and represents a potential anti-atherogenic effect (1-4, 6-9). It has been shown that patients with cerebrotendinous xanthomatosis, a deficiency in the CYP27 caused by mutations in the coding gene, suffer from premature atherosclerosis despite having normal levels of plasma cholesterol (10). The plasma level of 27OHC also correlates with the cholesterol content in atherosclerotic lesion and the severity of the disease (11-14). Recently, there is a growing interest in the role of 27OHC in diseases with disturbed cholesterol homeostasis (atherosclerosis) such as neurodegenerative diseases (cognitive decline in elderly, Alzheimer's disease) and breast cancer (15-18).

Considering the potential diagnostic interest of 27OHC particularly for improving cardiovascular disease risk-assessment, the limited sample volume that might be obtained from animal models and plasma lipoprotein subfractions, the low endogenous levels of 27OHC and the presence of interferences such as cholesterol which is present at 10^3-10^6 higher endogenous level, an accurate, sensitive and selective analytical method is important.

For more than a decade, GC-MS has been a well-established method to quantify cholesterol and its oxidation products. The method requires prior derivatization of alcohol groups to trimethylsilyl (TMS) ethers (19). The very low injection volume of the GC-MS system (~2 μL) gives advantage in allowing preconcentration of the target analyte, contributing to the high sensitivity achieved by this method.

LC-MS has also been introduced for the analysis of cholesterol oxidation products, either utilizing an ESI or APCI interface depending on the aim of the analysis. Using ESI for the oxysterols needs both derivatization and downscaling to nanoscale to achieve a high

sensitivity. One of the successful applications is the conversion of the hydroxyl group of the steroid ring into an oxo group with cholesterol oxidase, hence gives broader options for derivatization reagents. Using this approach and subsequent derivatization with Girard P reagent, structurally rich tandem MS spectra with a sub-pg sensitivity has been achieved by Griffiths *et al* (20).

The oxysterols can be well ionized without any prior derivatization using APCI, which is advantageous over the ESI and the GC-MS methods. Hence, the sample preparation can be faster and less laborious. The $[M+H-H_2O]^+$ ion produced by the APCI is not structurally informative since stable and structure-specific tandem MS spectra cannot be obtained. Thus, the confirmation for which C position the cholesterol is oxygenated is obtained from the LC retention time in comparison with reference standards. This method has been previously developed in our lab and has been used to establish the normal reference values of 24S- and 27OHC in human plasma (21). Starting with 500 μL plasma and 5 times preconcentration prior to the LC-MS analysis, the method is able to achieve a detection limit of 25 ng/ml plasma (22). However, due to the need to analyze smaller amounts of plasma, we investigated the possibility to enhance the sensitivity of the method using APPI.

Photoionization itself is not a revolutionary technique and has been used in gas chromatography (PID detector) and in ion-mobility spectrometry (IMS) (23). However, the hyphenation to LC-MS was first introduced by Bruins *et al* only in 2000 (24). Since then, APPI has been believed to be able to compensate the drawbacks from the older ESI and APCI sources. In a few published applications, APPI has been reported to extend linear dynamic range (25), reduce or omit offline sample cleanups (26-27) and generally lower detection limit. Especially in the area of lipid analysis, APPI has been discussed as a promising technique (28-30).

The ionization process in APPI directly depends on the reactant ion composition, which in turn depends on the presence of the dopant, the solvent, the nebulizing gas, the auxiliary gas, and the impurities as well as the components of the surrounding air (31). In this report, some critical APPI parameters for improving the sensitivity of 27OHC have been investigated.

Since 27OHC in plasma is about 85-90% esterified with fatty acids, a hydrolysis step prior to the sample cleanup was conducted whenever the total amount of endogenous 27OHC was analyzed (19, 21). Subsequent sample preparation using a solid-phase extraction method was as described by Burkard *et al* (22). Validation results of the method are presented.

DA-APPI (dopant-assisted APPI) improved the sensitivity for the quantification of 27OHC, in comparison to the previous LC-APCI-MS method (22), allowing the quantification from as little as 50 µL plasma with the limit of quantification (LOQ) of 10 ng/ml plasma. The method was also validated for analyzing 15 µL plasma, with the LOQ of 40 ng/ml plasma. The sensitivity achieved broadens the usefulness of the LC-MS method in clinical applications.

3.2. Experimental

3.2.1. Chemicals and Reagents

27OHC was purchased from Medical Isotopes (Pelham, NH, USA). The deuterated internal standard, 27OHC-d_6 ([1,2,3,4,4,6-$^2H_6$] C^5-3β,27-diol), was obtained from Sugaris (Muenster, Germany). All chemicals were purchased from Fluka (Buchs, Switzerland). All solvents were of HPLC grade and obtained from SDS (Cedex, France). Blank plasma was obtained from healthy volunteers.

3.2.2. Sample Collection

Blood was collected in evacuated blood collection tubes containing EDTA (3 ml Vacutainer, 0.184 M K_3EDTA; Becton Dickinson, Basel, Switzerland). The sample was centrifuged at 2000x g for 10 min and stored at -20°C. Upon analysis, the plasma samples were thawed and 10 µL butylated hydroxytoluene (5 mg/ml solution in methanol), which served as an antioxidant, was added per milliliter plasma prior to hydrolysis and / or solid-phase extraction.

3.2.3. Sample Preparation

3.2.3.1. Hydrolysis of 27OHC Esters

The hydrolysis of the 27OHC esters was conducted whenever the total endogenous 27OHC was analyzed. To 15 µL undiluted plasma, 150 µL 1 M ethanolic sodium hydroxide was added, followed by 50 µL internal standard solution (100 ng/ml 27OHC-d_6 in methanol). The alkaline hydrolysis was performed for 2 h at 50°C in a water bath. The hydrolyzed plasma was subsequently neutralized to pH 7 with 50% phosphoric acid and 200 µL

phosphate buffer pH 7. The mixture was then centrifuged for 5 min at 1000x g and the supernatant was taken for the subsequent solid-phase extraction.

3.2.3.2. Solid-phase Extraction

The solid-phase extraction was performed using C_{18} cartridges (200 mg, Bond Elut; Varian, Zug, Switzerland). The column was preconditioned with 1 ml n-heptane/2-propanol (50:50 v/v), 1 ml methanol and 2 ml water. The sample was applied to the cartridge using only gravity (no vacuum was applied to the chamber of the solid-phase extraction manifold). The cartridge was then washed with 4 ml methanol/water (75/25 v/v) and briefly dried under vacuum. Elution of the analyte was done using 2 ml n-heptane/2-propanol (50:50 v/v) using only gravity. The eluted components were dried by evaporation at 30°C (Rotavapor; Büchi, Flawil, Switzerland). The residue was reconstituted in 50 µL methanol/HCOOH 0.1% (85:15) and 40 µL was injected to the LC-MS system.

3.2.3.3. Sample Preparation for Validation Purposes

For the determination of the precision, accuracy and the LOQ, non-hydrolyzed diluted plasma was used because spiking of hydrolyzed undiluted plasma with 27OHC was not possible (see "Results and Discussion"). Stored plasma was first thawed and butylated hydroxytoluene was added as described in the "Sample Collection" previously. Then, four parts of water were added to one part of plasma (yielding a dilution of 1:5). Different concentration of 27OHC standard solution was spiked to this diluted plasma as described in the section of "Method Validation" below. Aliquots of either 15 µL or 50 µL diluted plasma were then taken. Internal standard solution was subsequently added. Ethanol (100 µL) and phosphate buffer pH 7 (~ 200 µL) were added to increase the final volume and the subsequent solid phase extraction was then performed.

3.2.4. LC-MS system

The LC system consisted of a Rheos 2000 pump (Flux Instruments, Basel, Switzerland) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The MS system was the LCQ Deca XP Max™ (Thermo Fisher Scientific, Waltham, MA, USA) which

had a three-dimensional ion trap mass analyzer. The vaporizer temperature and the capillary temperature were set at 400°C and 190°C, respectively. The protonated-and-dehydrated ion $[M+H-H_2O]^+$, m/z 385.2, was monitored in single ion monitoring (SIM) mode with 3 amu scan-width. The duty cycle was ~350 milliseconds. Data acquisition was performed using Xcalibur® software (Xcalibur 2.0, Service Release 2). During the APPI ionization, it was observed that high sheath gas and auxiliary gas pressure was needed to achieve good sensitivity, despite the composition of the mobile phase which had 95% methanol.

The separation was performed using a reversed-phase LC column (Nucleosil HD, 250 x 4.6 mm, 3 μm). The eluent A was methanol and the eluent B was formic acid 0.1%. The elution of 27OHC was performed isocratically with 95% eluent A and 5% eluent B. However, the washing of other contaminants such as endogenous cholesterol required gradient elution to methanol 100%. Hence for the first 5 min, the mobile phase was methanol/HCOOH 0.1% (95:5) pumped at 800 μL/min. Within the subsequent 0.5 min, the mobile phase flow rate was decreased to 400 μL/min to ensure the separation of 27OHC from the co-eluting contaminants. After 12 min, the mobile phase composition was changed to 100% methanol for 10 min at 800 μL/min to wash some lipophilic contaminants from the column. The small particle size of the column (3 μm) did not permit a higher flow rate since the back pressure became very high. The re-equilibration of the column as well as further removal of contaminants followed, using methanol/HCOOH 0.1% (95:5) at 800 μL/min.

3.2.5. Method Development

3.2.5.1. Ionization of 27OHC using APPI

The optimal conditions needed for the ionization of 27OHC with APPI were investigated by direct infusion of the analyte. A solution of 27OHC (1 μg/ml in methanol) was introduced to the LC eluent with a syringe pump at 10 μL/min. The eluent was methanol/HCOOH 0.1% (95:5) or otherwise mentioned in the “Results and Discussion”. The eluent was introduced using an LC pump at 400 μL/min. When conducting the DA-APPI, toluene (dopant) was introduced to the mixture of eluent and sample before entering the ionization chamber, using a tee and a second LC pump (Shimadzu LC-9A pump, Shimadzu, Kyoto, Japan). The flow rate of the dopant was 100 μL/min or otherwise mentioned in the “Results and Discussion”.

The dual-source experiment was carried out by turning on and off the corona needle voltage or the UV lamp. The mass spectrometer used had both APCI and APPI sources built in one ionization chamber to be used together without the need to remove or replace any hardware. The MS parameters were optimized (tuned) for APCI and the corona needle, when it was used, was set as giving a 4 μA current.

3.2.5.2. Ion Suppression Test

The test for ion suppression was carried out according to the postcolumn infusion system used by King *et al* (32), with an additional tee for the introduction of the APPI dopant (toluene) to the system. A solution of 27OHC (1 μg/ml in methanol) was post-column infused through a zero dead volume tee to the LC eluent at 10 μL/min using a syringe pump equipped in the LCQ Deca XP Max™ system. Prior to entering the ionization chamber, a second tee was installed for the post-column infusion of toluene (100 μL/min) to the mixture of sample and LC eluent using a Shimadzu LC-9A pump (Shimadzu, Kyoto, Japan) 15 μL blank plasma were hydrolyzed and extracted as described previously and injected into the LC-MS system by the autosampler. All other LC-MS conditions were as described previously.

3.2.5.3. Assessing the Linearity in Plasma Matrix

Calibration curves were constructed to assess the linearity between the response and the spiked concentration of the analyte. From the 1 μg/ml and 10 μg/ml standard solution of 27OHC in methanol, different aliquots were taken to make standards in plasma. To the aliquots of 10, 20 and 50 μL of 1 μg/ml methanol standard solution, plasma was added until the final volume was 1 ml, hence yielding plasma standards of 10, 20 and 50 ng/ml. To the aliquots of 10 and 25 μL of 10 μg/ml methanol standard solution, plasma was added until the final volume was 1 ml, hence yielding plasma standards of 100 and 250 ng/ml. 100 μL of each plasma standard, as well as a non-spiked plasma to serve as a blank and for background subtraction, were pipetted in clean tubes. The deuterated internal standard was then added, the mixture was hydrolyzed and the solid-phase extraction was performed as described previously. Reconstitution following the evaporation step was either in 100 μL mobile phase or in 500 μL mobile phase (to obtain 5 times dilution). The whole procedure was conducted in triplicates for each different concentration.

The dilution curve was constructed between the response and the estimated endogenous 27OHC concentration. One part of plasma was added to 1 part of water, yielding a dilution of 1:2. Subsequently, this twice-diluted-plasma was diluted again 1:2. This was done consecutively, hence the final dilution made were 1:2, 1:4, 1:8, 1:16 and 1:32. From each dilution, aliquots of 50 µL and 100 µL were taken. These diluted plasma samples were then hydrolyzed, the solid-phase extraction was performed and the sample was reconstituted either in 50 µL (for the 50 µL aliquot) or 100 µL (for the 100 µL aliquot) mobile phase. The aliquoting and subsequent sample preparation were conducted in triplicates. The dilution curve was then constructed between the peak area and the calculated endogenous 27OHC concentration in each dilution.

The endogenous 27OHC concentration of the dilution curve was calculated from the ratio of the response between the spiked plasma (containing unknown plus known concentration) and the blank (containing unknown concentration). Two aliquots were taken from the undiluted plasma, one of them was spiked with 50 ng/ml 27OHC standard solution. These samples underwent the same sample preparation procedure and were analyzed at the same time as the diluted plasma above.

3.2.6. Method Validation

3.2.6.1. Linearity

The calibrator standards were prepared in methanol. Defined amounts of 27OHC and the deuterated internal standard (100 ng/ml) were dissolved in methanol, evaporated and reconstituted in 50 µL methanol/HCOOH 0.1% (85/15) prior to the LC-MS analysis. The concentration of 27OHC in the reconstituted calibrator standards ranged between 10 to 250 ng/ml. The calibration curve was plotted as the peak area ratio of 27OHC to the internal standard *vs.* concentration.

3.2.6.2. Recovery

Defined amounts of 27OHC were added to 15 µL plasma (samples) and to clean vials (standards). Six replicates were performed for each spiked concentration, both for the samples and the standards. The samples were hydrolyzed and extracted as described in the "Sample Preparation", whereas the standards were only evaporated, reconstituted and injected to the

LC-MS. The recovery was determined by comparing the average peak areas of the samples to the average peak areas of the standards.

3.2.6.3. Precision and Accuracy

As described in “Sample Preparation for Validation”, different concentrations of 27OHC standard solution in methanol were spiked to the non-hydrolyzed diluted plasma. Aliquots of 15 µL or 50 µL were then taken.

To the 50 µL aliquots of diluted plasma, 27OHC standard solution had been added to give the final concentration of 10 and 40 ng/ml, respectively. To the 15 µL aliquots of diluted plasma, higher concentrations of 27OHC standard solution had been added to give the final concentrations of 40, 125 and 200 ng/ml. Subsequently, 50 µL internal standard solution (100 ng/ml 27OHC-d_6 in methanol), 100 µL ethanol and 200 µL phosphate buffer pH 7 were added. The solid-phase extraction and the subsequent LC-MS analysis were performed as described previously.

Six replicates of each concentration were analyzed within one day to determine the within-day precision and accuracy. Six replicates of each concentration were analyzed on six different days to determine the between-days precision and accuracy.

The precision was expressed as the coefficient of variation of the assayed concentrations. The accuracy was assessed as the bias of the assayed concentration to the weight-in concentration.

In addition, the between-days precision was also confirmed in a hydrolyzed, undiluted plasma sample. After thawing and the addition of butylated hydroxytoluene, 15 µL plasma directly underwent the hydrolysis and solid phase extraction procedure as described in the “Sample Preparation” previously. This was done to confirm the precision of the measurement in real plasma samples.

3.2.6.4. Limit of Quantification (LOQ)

The LOQ was determined by assessing the results from the within-day precision. The LOQ was determined as the lowest spiked concentration with a calculated imprecision of less than 15%.

3.3. Results and Discussion

3.3.1. Method Development

3.3.1.1. Ionization of 27OHC using APPI

For trace bioanalysis, fine-tuning of all parameters to optimize the ionization efficiency is needed to achieve higher signal-to-noise ratios. Thus, the effects of different dopants, dopant flow rates, different solvents and the use of the dual-source on the ionization of 27OHC were investigated.

Figure 1 shows the mass spectra observed for 27OHC. Both APPI and DA-APPI (dopant-assisted APPI) of 27OHC gave the protonated-and-dehydrated molecular ion, $[M+H-H_2O]^+$, as the most prominent observed ion, indicating that a chain of ion-molecule reaction occurred following photoionization.

A direct photoionization of an analyte molecule is believed to be a statistically unlikely event, partly because the large excess of solvent molecules may undergo photoexcitation and deplete the limited photon flux (23-24). For a compound to be photoionized, it needs to have a gas-phase ionization potential (IP) lower than the photon energy emitted by the lamp (10eV for the Kr-filled lamp used in this experiment). Most LC solvents have higher IP, so that the number of primary ions produced by a discharge lamp is very low. However, the percentage of ionizable molecules in vaporized LC eluent can be raised significantly by the addition of a large quantity of dopant. The dopant photoions subsequently ionize the target analyte by proton transfer or charge exchange. Theoretically, there are many substances having lower IP than the photon energy of a discharge lamp. However, only a few have been tested with LC-APPI-MS and the majority of published applications make use of acetone (IP 9.70 eV) and, more favorably, toluene (IP 8.83 eV). Figure 2 shows that acetone and toluene enhanced the ionization efficiency of 27OHC 2 and 5 fold, respectively. Bruins *et al* suggested that, in the case when proton transfer is the main occurring ion-molecule reactions, toluene acts as dopant towards a wider range of compounds (those with both low and high proton affinity (PA) in comparison to acetone (24). The high ionization efficiency achieved during APCI and solvent-mediated DA-APPI suggested that 27OHC has high PA. Empirical evidence for the mechanisms leading the ionization of 27OHC is outside the scope of this paper though.

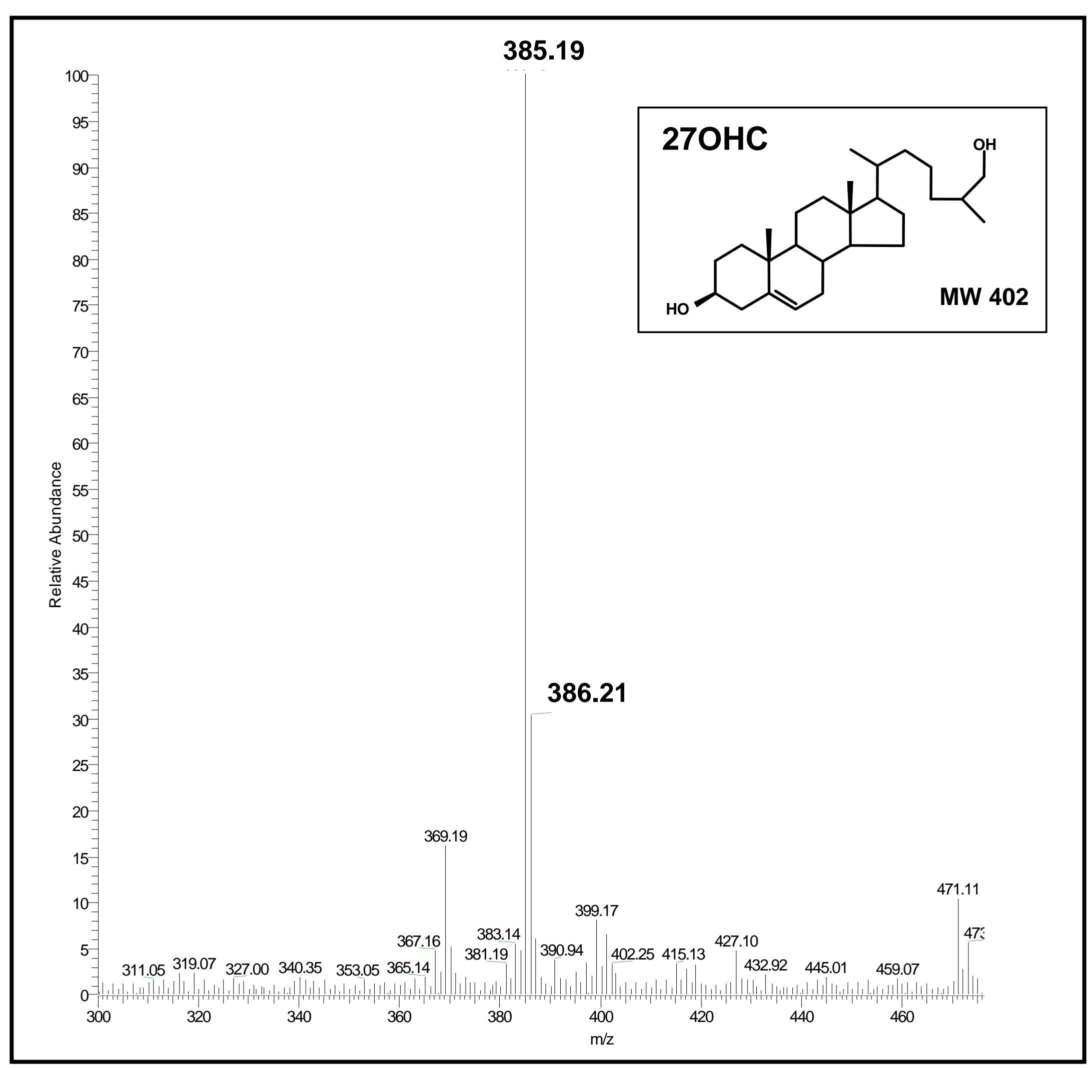

Figure 1. Full-scan mass spectrum of 27OHC showing prominent $[M+H-H_2O]^+$ ion (m/z = 385.2)

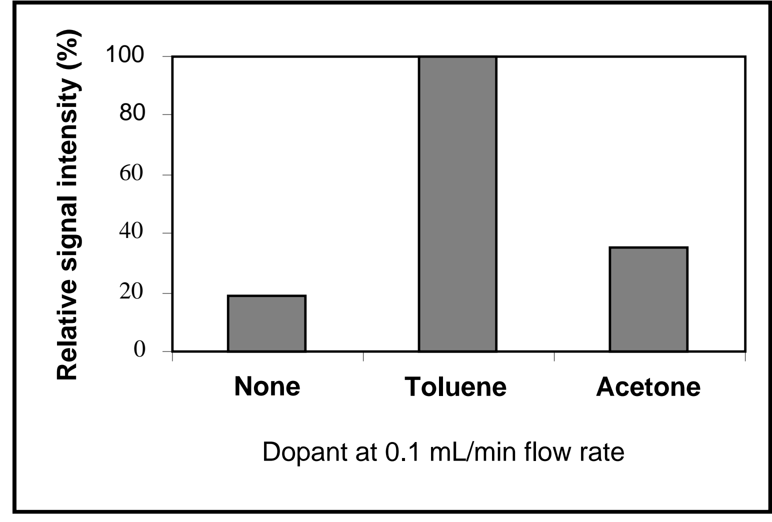

Figure 2. Effect of different dopants on the signal intensity of 27OHC

Figure 3 shows the increasing response of 27OHC with increasing toluene flow up to a certain rate (the eluent flow was kept constant). Above 0.05 ml/min toluene, the observed 27OHC signal reached a plateau. This result was qualitatively in agreement with the mechanistic study conducted by Blades *et al* (33). One of the possible explanations proposed by Blades *et al* is the loss of ions due to the recombination of oppositely-charged species, whose rate is in quadratic dependence on the charged-particle density. Within the field-free region of the APPI source, the separation of oppositely-charged species does not immediately follow the ionization process; hence, neutralization via recombination may occur. Raising the lamp current increases the primary ion generation, but this cannot overcome the apparent limit in the signal trend. In a separate experiment, Blades *et al* also noted that a significant gain in the primary ion production can actually still be obtained by raising the dopant flow rate even above 0.1 ml/min (33). Thus, the asymptotic intensity of the analyte signal with the increasing dopant flow was not due to the limitation in the primary ion production.

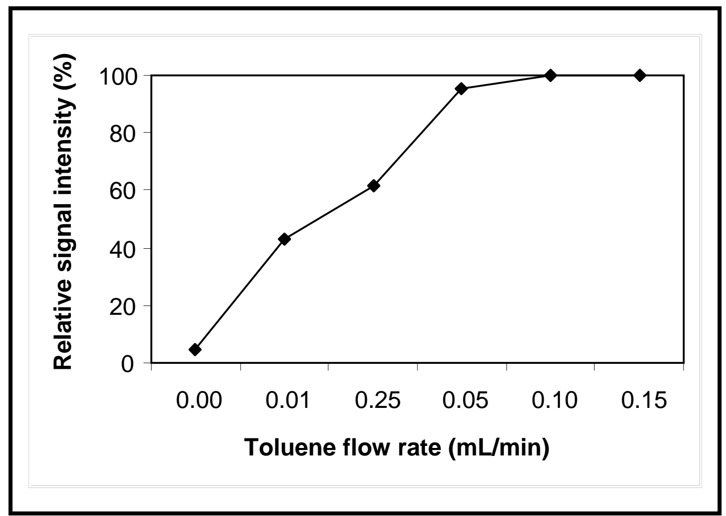

Figure 3. Effect of toluene flow rate on the signal intensity of 27OHC

The composition of the LC eluent has an impact not only on the chromatographic separation, but also on the ionization efficiency of 27OHC. It has been described previously that the LC eluent as well as impurities present in the solvent might influence the ionization of a target analyte (23-24, 31).

The published LC-APCI-MS method for 27OHC utilized a reversed-phase column for LC separation(22). However, the sample preparation of 27OHC in published GC-MS methods utilized the hydrophilic silica material for solid-phase extraction(4), suggesting that the separation of 27OHC could be conducted also in normal-phase HPLC. Figure 4 shows the

effect of several common reversed-phase and normal-phase HPLC solvents to the sensitivity of the DA-APPI of 27OHC. Methanol was found to assist the ionization of 27OHC as the signal intensity observed was profoundly enhanced. This phenomenon is generally attributed to the interaction between methanol and a toluene radical cation forming a cluster. Kauppila *et al* described the formation of trimer and possibly higher order methanol clusters. Individual monomers of the solvent have a lower PA than the benzyl radical, so the proton transfer between the toluene radical cation and the solvent molecules cannot take place. However, the solvent cluster has a higher PA than its individual monomer and its proton transfer with toluene photoion is then thermodynamically possible. The protonated solvent species can then subsequently ionize the target analyte molecules (23, 31).

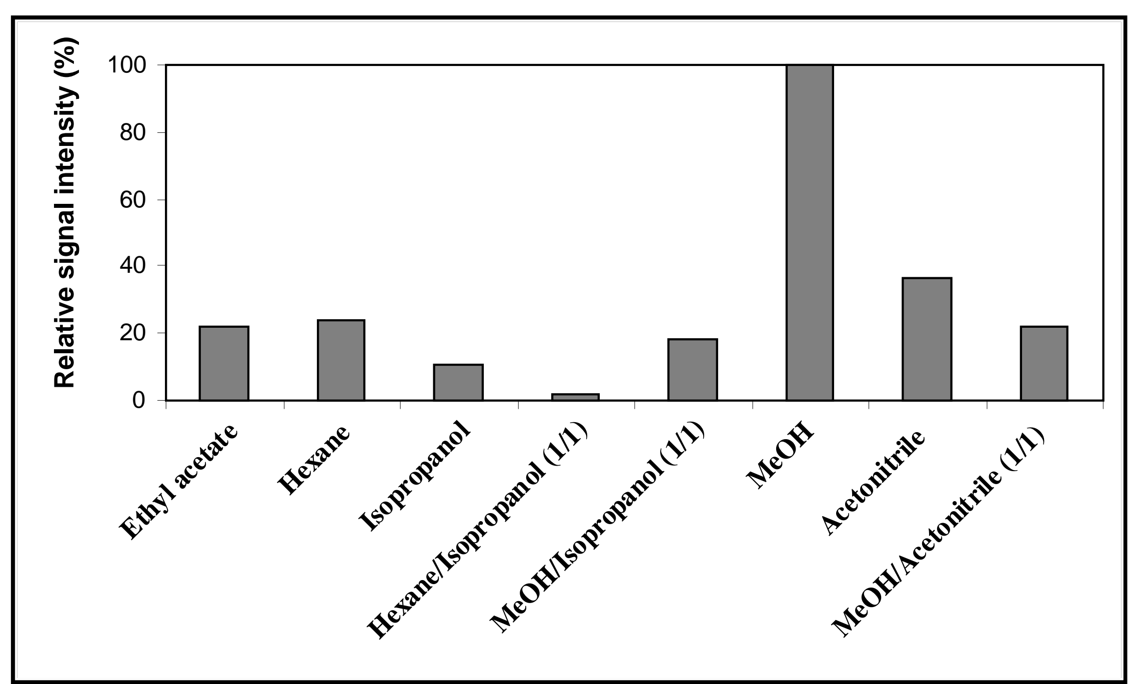

Figure 4. Effect of different solvents on the signal intensity of 27OHC

As shown in Figure 4, using acetonitrile or the combination of methanol and acetonitrile severely decreased the ionization efficiency of 27OHC. Acetonitrile or the combination of methanol and acetonitrile were actually favorable for the reversed-phase HPLC eluent, as it gave better peak shapes, more selective separation and lower back pressure. However, the LC-APPI-MS method developed in this paper used methanol/HCOOH 0.1% (95/5) due to the sensitivity achieved. Using HCOOH 0.1% in the eluent did not compromise the sensitivity of the method (data not shown).

27OHC is a relatively non-polar compound and lacks acidic or basic groups to be well ionized by ESI. On the other hand, APCI can give good sensitivity, although it is still not high enough for our purposes. Since both APCI and DA-APPI give the same type of observed 27OHC ion ($[M+H-H_2O]^+$), we have investigated the possibility to improve the sensitivity by using both ionization sources together. A few successful experiments using a dual-source have been reported by Syage *et al* (34). Excess of electrons from the corona needle might interfere with the ion-molecule reactions during APPI though, resulting in the competition or recombination of ions. From Figure 5, it can be seen that the DA-APPI process was depleted when combined with APCI for the analysis of 27OHC. The DA-APPI alone increased the signal intensity of 27OHC by a factor of 2.5, as compared to the dual-source approach.

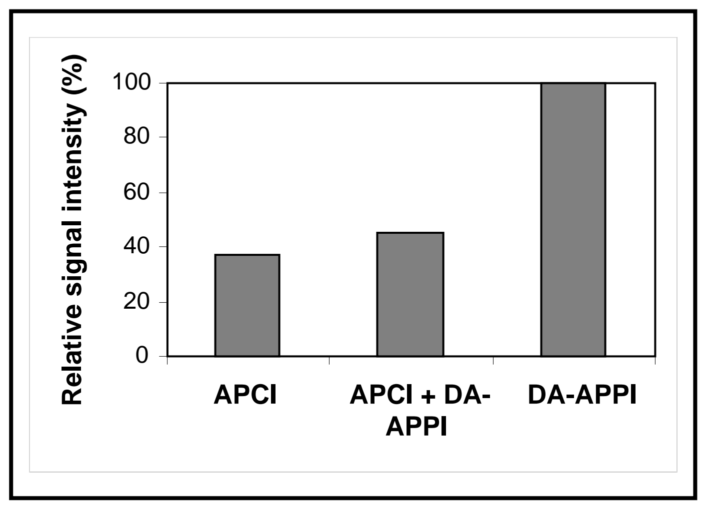

Figure 5. Effect of different ionization modes on the signal intensity of 27OHC

3.3.1.2. Matrix Substitution for Calibrator Standard

One of the difficulties in developing methods for the purpose of biomarker studies is the unavailability of analyte-free matrix. The plasma concentration of total 27OHC in healthy volunteers is in the range of 95-300 ng/ml(22). A non-linear response and large random variation have been observed during the addition of 27OHC standard solution to 100 µL of plasma (Figure 6a) or even diluted plasma (Figure 6b), which seemed like approaching plateau as the spiked concentration was increased. When the same plasma was simply diluted, a linear relationship could be determined between the dilution factor and the signal (Figure 7).

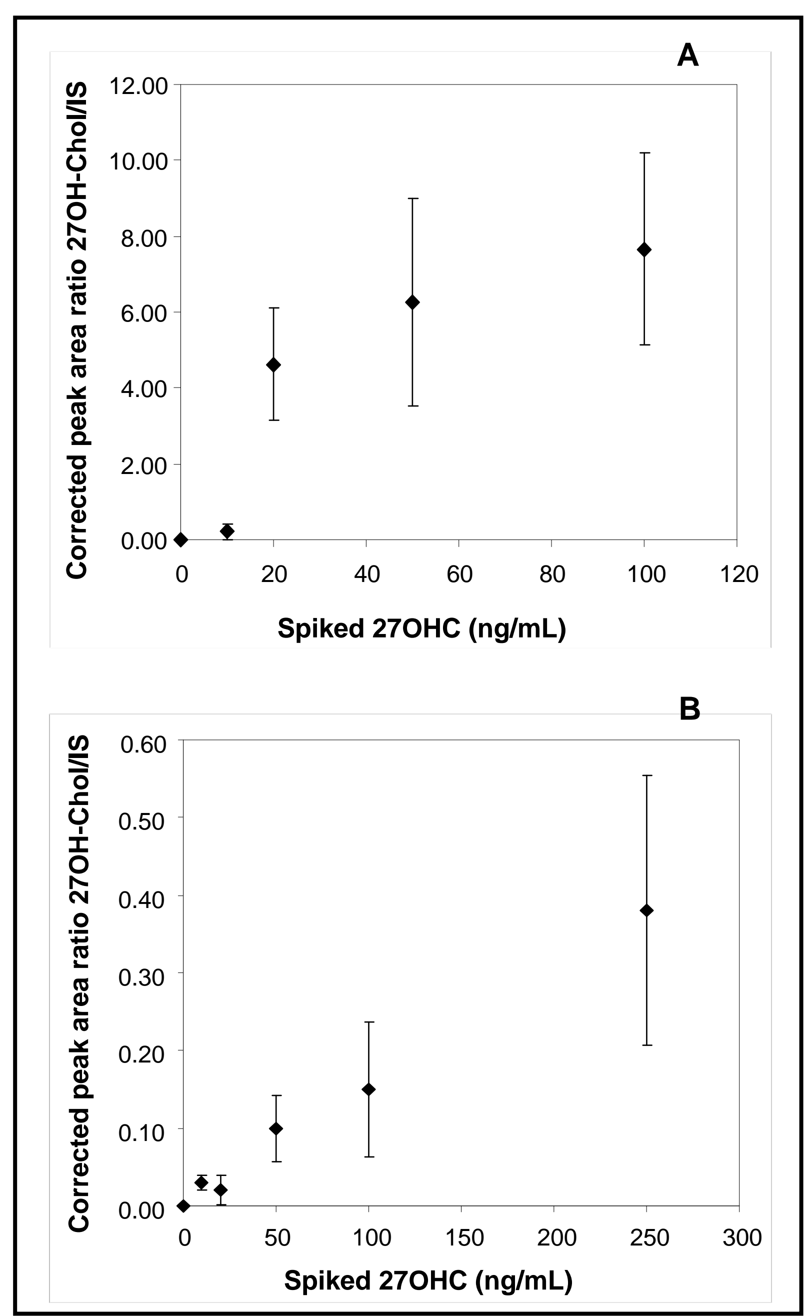

Figure 6. The addition of 27OHC standard solution to 100 μL plasma (A) and 5-times diluted 100 μL plasma (B). Corrected peak area ratios were calculated by subtracting peak area ratio of the spiked plasma from the blank plasma. Peak area ratio is the response ratio of 27OHC to its deuterated internal standard.

Figure 6a, 6b and 7 altogether indicate several factors contributing to the variability in the 27OHC response. First, spiking the plasma on the top of a high endogenous level of the analyte, to construct a calibration curve, resulted in the non-linearity and large variation of the

analyte response. The second problem was the limited linear dynamic range of the method which did not allow spiking an even diluted plasma matrix. Therefore, we decided to substitute the matrix plasma with methanol for our calibrator standards so that less variability is introduced during the analysis. The recovery of spiked 27OHC from plasma (see section "Recovery" below) gave a result of near 100% (91% for 40 ng/mL and 98% for 100 ng/mL) enabling this matrix substitution. Furthermore, the ion suppression test confirmed that there was no suppression of the 27OHC response (t_r ~10-11 min) when a methanolic 27OHC solution infused continuously during the injection of a plasma matrix sample (Figure 8).

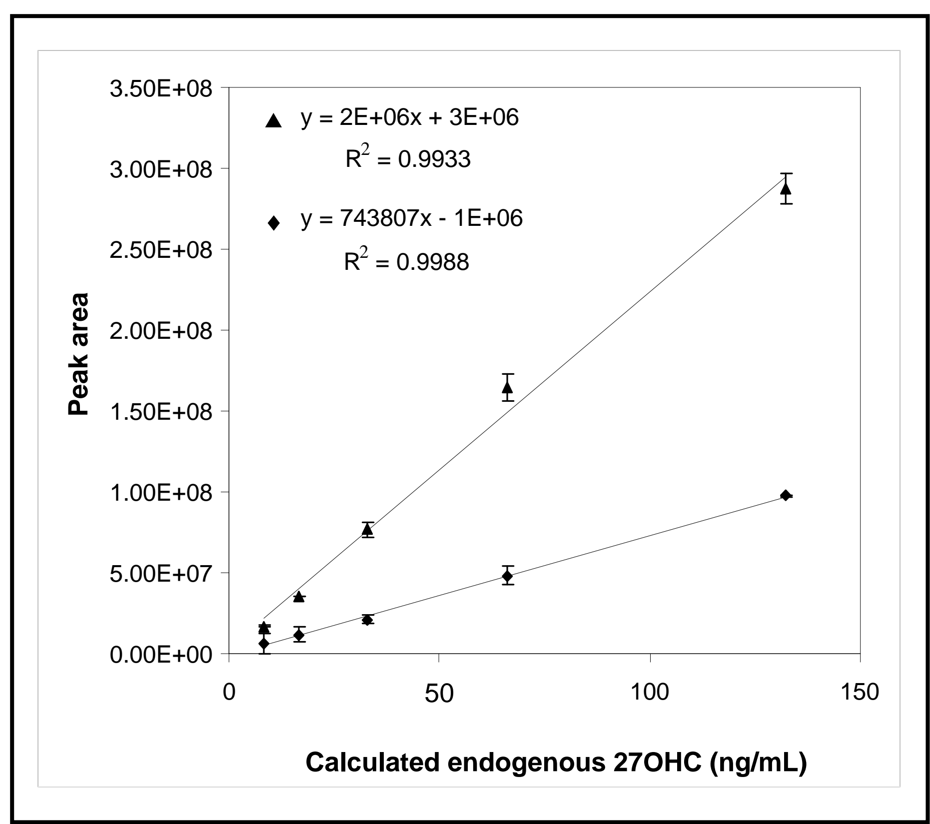

Figure 7. Dilution of 100 μL hydrolyzed plasma (▲) and 50 μL hydrolyzed plasma (♦)

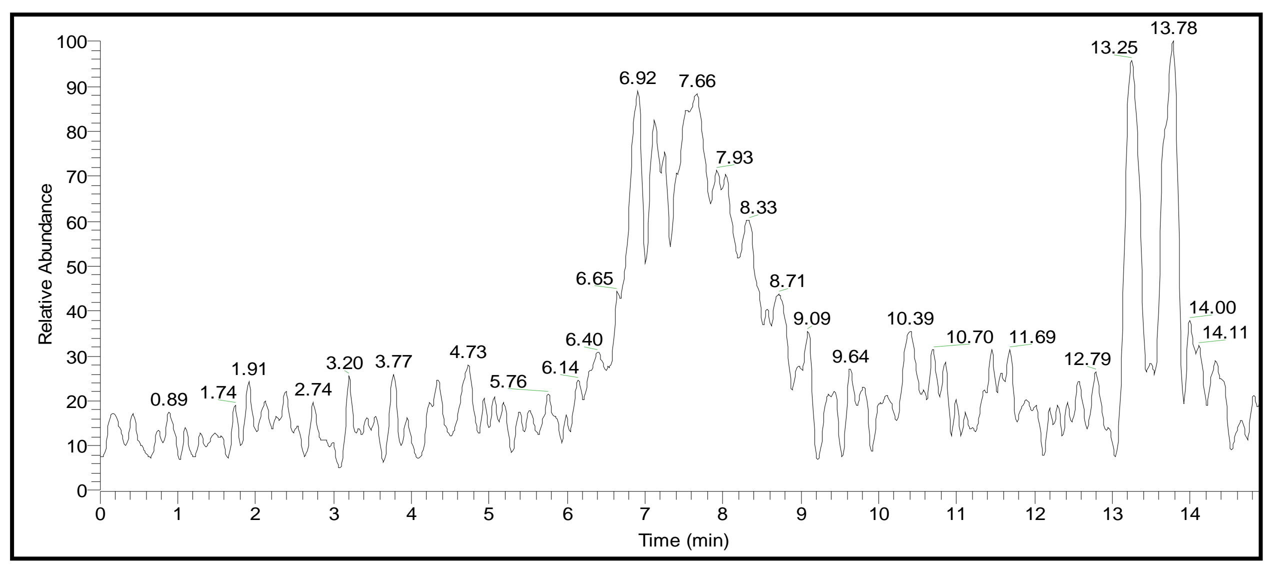

Figure 8. No ion suppression was observed during the 27OHC elution time (t_r ~10-11 min)

3.3.2. Method Validation

The calibration curve was constructed using standard solution in methanol during the whole validation process. The concentration range of the calibration curve was 10 to 250 ng/ml. The calibration curve was plotted as the peak area ratio of 27OHC to the internal standard *vs.* concentration. The calibration curve was linear and the least-square regression data (n=6) gave a regression line of $y = 0.0103x - 0.0443$ and a correlation coefficient of 0.9997.

In order to minimize the variability introduced during the analysis, the precision and accuracy of the method were confirmed in spiked, non-hydrolyzed and diluted plasma samples. Non-hydrolyzed plasma should only contain about 10-15% of the total endogenous 27OHC since the rest is esterified with fatty acids(19, 21). The precision and accuracy of the method was checked in 50 µL spiked diluted plasma and 15 µL spiked diluted plasma. Because the aim of the method is to analyze the total endogenous 27OHC in undiluted plasma samples, the between-days precision was also confirmed in hydrolyzed undiluted plasma samples, as this will be the procedure used for real samples. The LOQ was determined as the concentration when the imprecision remained below 15%. Thus, the LOQ was 10 ng/ml and 40 ng/ml for 50 µL and 15 µL samples, respectively.

The precision was defined as the coefficient of variation of the assayed concentrations. The accuracy was defined as the bias of the assayed concentration to the weight-in

concentration. The results are presented in Table 1. The chromatogram of 27OHC in a plasma sample is shown in Figure 9.

For the recovery test, defined amounts of 27OHC and the internal standards were added to plasma (samples) and to clean vials (standards) and analyzed as described in the “Experimental”. The recovery was determined by comparing the average peak areas of the samples to the peak areas of the standards. The recoveries for 27OHC were 91% (40 ng/ml) and 98% (100 ng/ml). The recoveries for the internal standard, 27OHC-d_6, were 100% (40 ng/ml) and 106% (100 ng/ml).

In comparison to the published LC-APCI-MS and GC-MS methods, the developed LC-MS method with DA-APPI is more sensitive, allowing the quantification of total 27OHC in plasma from a very low amount of sample (15 μL). Further advantage is that no prior derivatization is needed, unlike the LC-ESI-MS or the long-established GC-MS method. The sensitivity achieved broadens the usefulness of the LC-MS method in experimental and clinical applications.

Table 1: Precision and accuracy of 27OHC determination in plasma

Diluted unhydrolyzed plasma	**Weight-in concentration (ng/ml)**	**Replicates**	**Mean (ng/ml)**	**SD**	**Precision (%)**	**Accuracy (%)**
			Within-day			
50 μL	10	6	11.3	0.9	7.5	113.4
	40	6	36.4	1.6	4.5	90.9
15 μL	40	6	37.4	3.3	8.7	93.5
	125	6	136.2	17.7	13.0	108.9
	200	6	222.2	19.67	8.9	111.1
			Between-days			
50 μL	10	6	11.1	1.0	9.4	111.0
	40	6	39.7	3.9	9.9	99.3
15 μL	40	6	42.5	1.8	4.2	106.2
	125	6	125.4	2.8	2.2	100.3
	200	6	205.2	15.7	7.7	102.6
15 μL hydrolyzed plasma	-	6	254.7	27.9	10.9	-

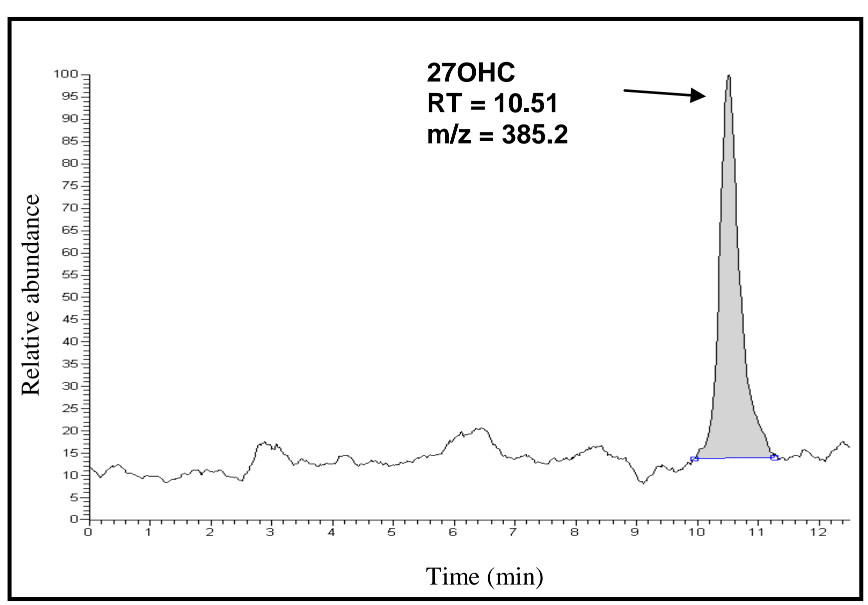

Figure 8. Chromatogram of an extracted plasma spiked with 40 ng/ml 27OHC and analyzed in SIM mode for m/z 385.2. The LC-MS condition was as explained in the "Experimental" section. RT: retention time (min).

3.4. Conclusions

We have demonstrated that DA-APPI (Dopant Assisted - Atmospheric Pressure Photoionization) improves the sensitivity of 27OHC analysis, allowing quantification from very low sample amounts (50 µL and 15 µL plasma). Critical parameters were the dopant used and the flow rate of the dopant, as well as the solvent used as the LC eluent. The method has been validated and is ready to be used in various studies where the amount of sample is very limited, such as biomarker evaluation studies in animal models or lipoprotein subfraction.

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4. PLASMA LEVELS OF 27-HYDROXYCHOLESTEROL IN HUMANS AND MICE WITH MONOGENIC DISTURBANCES OF HIGH DENSITY LIPOPROTEIN METABOLISM

R. Karuna[a], A.G. Holleboom[b], M.M. Motazacker[b], J.A. Kuivenhoven[b], R. Frikke-Schmidt[c], A. Tybjaerg-Hansen[c], S. Georgopoulos[d], K.M. Rentsch[a] and A. von Eckardstein[a]

a. Institute of Clinical Chemistry, University Hospital Zurich, and Competence Center of Systems Physiology and Metabolic Diseases, Zurich
b. Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam
c. Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital, Copenhagen
d. Department of Cell Biology, Biomedical Research Institute, Academy of Athens, Athens

[Atherosclerosis, submitted]

Abstract

Secretion of 27-hydroxycholesterol (27OHC) from macrophages has been proposed as an alternative mechanism to the high-density lipoprotein (HDL)-mediated reverse cholesterol transport. We therefore investigated 27OHC concentrations in plasma and lipoproteins of humans and mice with monogenic disorders of HDL metabolism. In humans, mutations in the genes encoding for apolipoprotein (apo) A-I, ATP binding cassette transporter A1 (ABCA1) and lecithin:cholesterol acyl transferase (LCAT) were associated with reduced concentrations of both HDL-cholesterol and HDL-27OHC; whereas mutations in cholesteryl ester transfer protein (CETP), scavenger receptor type B class 1 (SR-BI) and hepatic lipase (HL) were associated with elevated concentrations of HDL-27OHC and HDL-cholesterol. Likewise, plasma 27OHC were decreased in apoA-I and LCAT knockout mice and increased in SR-BI knock-out mice. Normalization for HDL-cholesterol revealed that mutations in LCAT caused less pronounced decreases of HDL-27OHC as compared to HDL-cholesterol. Moreover, LCAT activity appeared more strongly correlated with 27OHC-ester than to cholesteryl ester in HDL. The esterification of 27OHC appeared to be more sensitive to reduced LCAT activity than cholesterol esterification. Furthermore, 27OHC levels in apoB-containing lipoproteins were increased in LCAT or ABCA1, decreased in HL, and unaltered in mutation carriers of other HDL genes. In conclusion, mutations in several HDL genes led to distinct alterations in the quantity, esterification or lipoprotein distribution of 27OHC.

4.1. Introduction

27-Hydoxycholesterol (27OHC) is a metabolite of cholesterol formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27), an enzyme particularly expressed in the vascular endothelium, macrophages and the liver (1-4). Introduction of a hydroxyl group allows the otherwise hydrophobic cholesterol molecule to pass amphiphilic membranes more easily (5-6). Because of these physicochemical properties, 27OHC has been postulated to be secreted from cells independently of transporters and extracellular lipoprotein acceptors and thereby to facilitate an alternative route for apoA-I/HDL-mediated reverse transport of cholesterol from macrophages to the liver (7). In the liver, 27OHC is an important intermediary product of the so-called alternative bile acid synthesis pathway which contributes ~10% to *de novo* bile acid biosynthesis (4). Finally, 27OHC is an important activating ligand of liver-X-receptors (LXR), which are nuclear hormone receptors regulating the transcription of several genes involved in lipid and lipoprotein metabolism (8-9).

27OHC is the most abundant oxysterol (oxidized cholesterol) in the circulation (10) while plasma levels of 27OHC have been found to correlate with the cholesterol content in atherosclerotic lesions and the severity of coronary artery disease (11-13). Patients with genetic CYP27 deficiency suffer from cerebrotendinous xanthomatosis and develop premature atherosclerosis despite having normal levels of plasma cholesterol (14). Taken together, 27OHC has been proposed to be an anti-atherogenic molecule (1-2, 7, 15-17).

Since the classical reverse cholesterol transport is mediated by high-density lipoproteins (HDL), and because HDLs are important carriers of 27OHC in plasma (18), we investigated whether monogenic disorders of HDL metabolism affect plasma and lipoprotein concentrations of 27OHC. We used an LC-MS method which allows to quantify 27OHC in very small plasma volumes (15-50 μL) (19). Specifically, we analyzed plasmas of patients with functionally relevant mutations or mice with knock-outs of the following genes: apolipoprotein A-I (apoA-I) which is the main protein component of HDL; ATP-binding cassette transporter A1 (ABCA1) which mediates cholesterol and phospholipid efflux from cells to apoA-I; lecithin:cholesterol acyl transferase (LCAT) which esterifies cholesterol and thereby converts discoidal HDL precursors into mature spherical HDL particles; cholesteryl ester transfer protein (CETP, not expressed in mice) which exchanges cholesteryl ester of HDL against triglycerides of apoB-containing lipoproteins; scavenger receptor type B class 1 (SR-BI) which mediates selective uptake of cholesterol esters into the liver and steroidogenic

organs; and hepatic lipase (HL, only in humans) which hydrolyses triglyceride and phospholipids in HDL (20).

4.2. Methods

4.2.1. Origin of Plasma Samples from Mice and Humans

All animal procedures were approved by the Bioethical committee of the Biomedical Research Institute of the Academy of Athens. All animal experimentations were in agreement with the ethical recommendation of the European Communities Council Directive (86/609/EEC). Mutants as well as C57B/6 control mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on standard chow diets containing 5% fat (Harlan Teklad, Madison, WI, USA). Mice were fasted for 6 hours before the collection of blood samples. Samples from mutants and wild-type control mice were collected in EDTA-containing eppendorf tubes, kept in room temperature for 20 min and centrifuged to collect the plasma. All samples were stored in -80°C and transported in dry ice.

Forty-one Dutch patients with functionally relevant mutations in the genes encoding for either LCAT, ABCA1, APOA1, SR-BI, CETP or HL as well as 41 unaffected family members were investigated. Most of the families and the underlying defects were described previously (21-25). In addition, 4 Danish patients with mutations in apoA-I or CETP as well as 5 age- and sex-matched controls were included. The Medical Ethics Committee of the Academic Medical Center (AMC) in Amsterdam, The Netherlands, as well as the Danish Ethics Committee for Copenhagen and Frederiksberg, Denmark, approved all genetic and phenotypic studies described and all participants signed an informed consent to join the study. The characteristics of the study participants are shown in table 1. Fasting blood samples were collected after at least a 10-hours fast in the morning and EDTA-plasma was prepared through centrifugation of the blood at 3000 rpm for 10 min at 25°C by the study nurses of the AMC, Amsterdam, The Netherlands, and of the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark. Aliquots were immediately frozen at -80°C until later use.

Table 1. Characteristic of the study participants. Values represent mean ± SD.

Mutated gene	Number of defective alleles	Mutation[#]	Age (year)	Cholesterol (mM)	HDL-cholesterol (mM)	Non-HDL cholesterol (mM)	Triglyceride (mM)	Number of smokers
		DUTCH						
APOA-I	0		27 ± 14	4.59 ± 0.68	1.16 ± 0.06	3.43 ± 0.63	1.09 ± 0.29	0
	1	p.L202P (c.605T>C)	26 ± 17	3.61 ± 0.31	0.51 ± 0.35	3.10 ± 0.06	1.09 ± 0.51	0
ABCA1	0		44 ± 20	4.39 ± 0.89	1.47 ± 0.39	2.92 ± 0.62	0.95 ± 0.22	1
	1	p.L1056P (c.3167T>C) or p.C1477R (c.4429T>C)	57 ± 11	4.47 ± 1.08	0.94 ± 0.17	3.53 ± 0.96	1.01 ± 0.05	0
	2	p.L1056P (c.3167T>C, homozygote) or p.Q1038X (c.3112C>T) + p.N1800H (c.5398A>C) or p.C1477R (c.4429T>C) + IVS25+1G>C	53 ± 10	2.89 ± 2.39	0.10 / ND*	2.79 ± 2.39	2.29 ± 1.80	0
LCAT	0		49 ± 9	4.96 ± 0.86	1.33 ± 0.38	3.62 ± 0.97	1.29 ± 0.66	0
	1	p.T147I (c.440C>T), p.R322C (c.964C>T), p.N155D (c.463A>G), p.P34Q (c.101C>A), p.Y107X (c.321C>A), p.I202T (c.605T>C) or p.V333M (c.997G>A)	43 ± 13	4.27 ± 1.21	0.81 ± 0.28	3.45 ± 1.08	1.30 ± 0.55	1
	2	p.T147I (c.440C>T) + V333M	69 ± 4	3.26 ± 0.19	0.14 ± 0.01	3.11 ± 0.20	2.11 ± 0.49	0
SR-BI	0		54 ± 19	4.77 ± 0.89	1.17 ± 0.33	3.60 ± 0.79	1.21 ± 0.64	0
	1	p.P297S (c.889C>T)	45 ± 22	4.46 ± 1.21	1.73 ± 0.56	2.73 ± 0.81	0.97 ± 0.28	1
CETP	0		36 ± 16	4.14 ± 0.51	1.30 ± 0.21	2.85 ± 0.48	0.87 ± 0.40	1
	1	IVS7+1 (G>T)	39 ± 18	4.20 ± 0.51	1.56 ± 0.29	2.64 ± 0.77	0.76 ± 0.32	1

Table 1. (continued)

Mutated gene	Number of defective alleles	Mutation	Age (year)	Cholesterol (mM)	HDL cholesterol (mM)	Non-HDL cholesterol (mM)	Triglyceride (mM)	Number of smokers
HL (LIPC)	0		45 ± 19	5.23 ± 0.99	1.61 ± 0.54	3.62 ± 0.90	1.45 ± 1.05	3
	1	p.S289F (c.866C>T)	45 ± 15	4.92 ± 1.21	2.00 ± 0.68	2.92 ± 0.86	1.14 ± 0.43	1
				DANISH				
Controls	0		50 ± 9	5.84 ± 1.24	1.54 ± 0.24	4.30 ± 1.23	1.34 ± 0.62	1
APOA-I	1	p.L168R (c.503T>G)	63 ± 4	4.70 ± 0.28	0.85 ± 0.07	3.85 ± 0.35	1.27 ± 0.70	0
CETP	1	p.S349Y (c.1046C>A)	59 ± 4	6.85 ± 2.05	3.05 ± 1.77	3.80 ± 0.28	0.86 ± 0.23	1

* Either the detection limit of the assay or not detectable (ND)

 # Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by -24 amino acids, ABCA1 by -60 amino acids, LCAT by -24 amino acids, SR-BI by 0 amino acids, CETP by - 17 amino acids, HL by - 22 amino acids.

4.2.2. Quantification of 27OHC

The concentrations of 27OHC in total and apoB-depleted plasma were analyzed using dopant assisted-atmospheric pressure photoionization (DA-APPI) liquid chromatography-mass spectrometry (LC-MS) method as described previously (26). For the quantification of unesterified 27OHC, the hydrolysis step was omitted and the sample volume was increased to 100 µL due to the very low endogenous level of free 27OHC (only ~10-20% of total 27OHC) (18).

For the determination of 27OHC in HDL, apoB-containing lipoproteins were removed from 100 µL human EDTA plasma by precipitation with dextran-sulfate-Mg^{2+} (27). 27OHC concentrations assayed in apoB-depleted plasma were multiplied by 1.1 to correct for the dilution and to obtain the concentration of 27OHC in HDL. The difference between the concentrations of total 27OHC and HDL-27OHC is described as nonHDL-27OHC concentration. We have shown previously that this method recovers about 95% of 27OHC in the apoB-depleted fraction, as compared to density-gradient ultracentrifugation, and that nearly 90% of plasma 27OHC is contained in the lipoprotein fractions (18).

4.2.3. Quantification of Other Lipids, Apolipoproteins and LCAT

Total plasma cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol levels were measured with commercial kits (Wako, Neuss, Germany and Randox, Crumlin, UK) on a Cobas Mira autoanalyzer. Plasma apoB, apoA-I, and apoA-IIs were measured with commercial assays (Randox).

The unesterified cholesterol concentrations in the supernatant after precipitation of apoB-containing lipoproteins with polyethylenglycol (PEG) were assayed using a procedure that has been developed in house. Briefly, a mixture of reagent (200 µL) containing 7.3 mg/mL homovanilic acid, 0.1 M 3-[N-Morpholine]propanesulfonic acid, 1.25 mM taurocholate, 1.35 U/mL cholesterol oxidase and 10 U/mL peroxidase was added to 30 µL sample and assayed using the FLUOstar Galaxy fluorometer (BMG Labtech, Offenburg, Germany). The assay was based on the reaction of cholesterol with oxygen to give hydrogen peroxide, which subsequently reacted with homovanilic acid to give a fluorescence emission at 450 nm wavelength. Cholesteryl ester concentrations were calculated as the differences of total and unesterified cholesterol concentrations.

LCAT activity and cholesterol esterification rates (CER) were determined using proteoliposomes containing radioactive cholesterol as the exogenous substrate assay and after equilibration of plasma with ^{3}H-cholesterol as the endogenous substrate assay, respectively (28).

4.2.4. Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmont, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). Normal distribution was tested using Kolmogorov-Smirnov test. Since the data distribution was normal, unpaired student-t test was used to test for statistical significances of differences between two groups (assuming equal variance). Significance of a correlation was tested from the Pearson's correlation coefficient (r).

4.3. Results

4.3.1. Effects of Gender, Age and Statin Treatment on 27OHC Levels in Humans

Because of a strong effect of gender on plasma 27OHC levels (18), we stratified our data by sex. Because 27OHC concentrations in plasma are little affected by age (18), we did not further adjust the data for age. About 35% of all probands were treated with statins. However, when comparing family controls with and without statin treatment, this did not appear to affect plasma- and HDL- 27OHC concentrations: 0.47 ± 0.07 μM *vs.* 0.50 ± 0.15 μM, and 0.20 ± 0.05 μM *vs.* 0.23 ± 0.15 μM, respectively. We therefore did not stratify the data for statin treatment.

4.3.2. Effects of Inborn Errors of HDL Metabolism on 27OHC Levels

In table 2, we compared the concentrations of 27OHC in total plasma, HDL and non-HDL. As shown previously (18), we identified significant positive correlations between 27OHC and cholesterol levels in total plasma, HDL and non-HDL fractions (see figure 1). These plots as well as the ratios of 27OHC to cholesterol (figure 1 and table 3) allowed us to identify those conditions in which 27OHC levels were altered beyond cholesterol levels.

Homozygous defects in LCAT and ABCA1 were associated with significantly increased 27OHC/cholesterol ratios in plasma (table 3). All other differences were either not

statistically significant or not consistent: male but not female SR-BI mutation carriers showed lower 27OHC plasma levels as compared to all male controls, but not as compared to unaffected male relatives from the same family (table 2). The significantly decreased levels of 27OHC plasma levels in male and female carriers of the apoA-I(p.L202P) mutant were not retrieved in individuals carrying the apoA-I(p.L168R) mutation (table 2).

Defects in the genes of LCAT and ABCA1 were associated with gene-dosage-dependent decreases of HDL-27OHC levels. Heterozygous carriers of apoA-I mutations also showed significantly decreased 27OHC levels in HDL. Conversely, defects that increase HDL-cholesterol levels also tended to increase HDL-27OHC levels (table 2 and figure 1B). However, this effect was statistically significant for only male CETP and HL mutation carriers. After normalization for HDL-cholesterol levels, only defects in LCAT showed consistent and statistically significant associations with increased HDL-27OHC/HDL-cholesterol ratios (table 3).

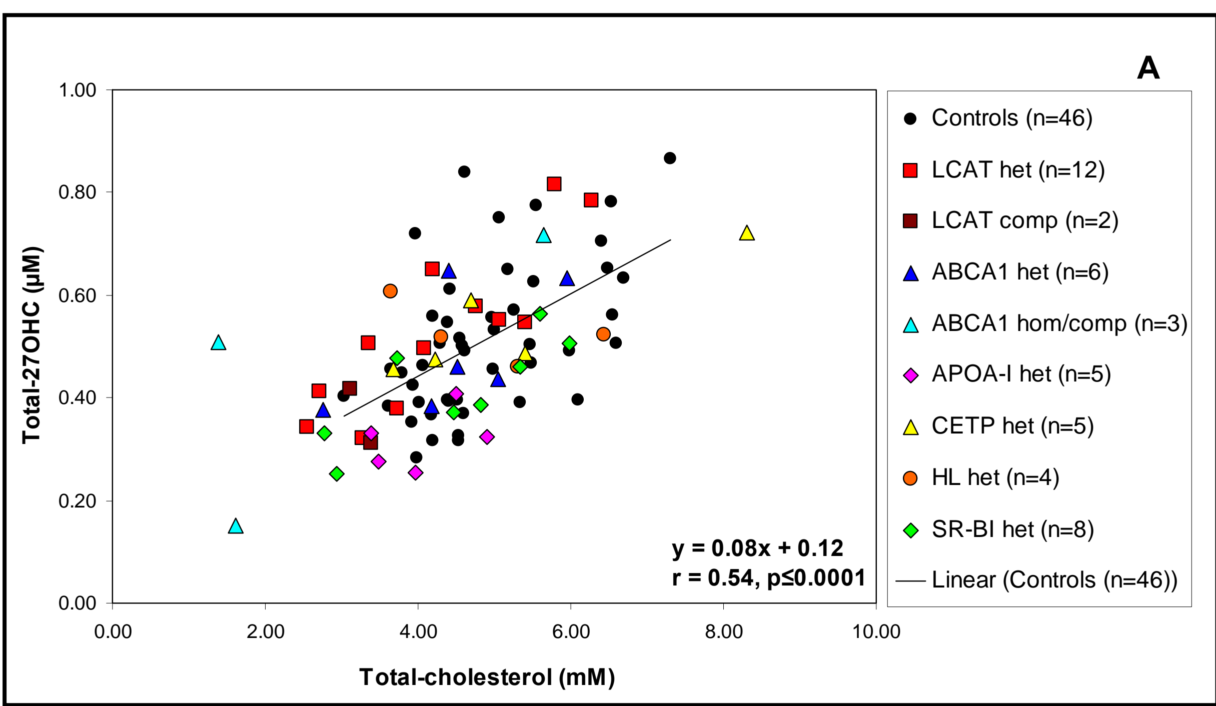

Figure 1. Plots of 27OHC *vs.* cholesterol concentrations in the plasma (A), HDL (B), and nonHDL (C) of individuals with mutations in HDL genes and their unaffected relatives. The Pearson correlation and regression have been calculated on data from all unaffected family members („Controls" of table 1 and 2). „Het" = heterozygous, „hom/comp" = homozygous / compound heterozygous. Please note the biases towards elevated 27OHC concentrations in the plasma, HDL and nonHDL of LCAT mutation carriers as well as the biases towards higher HDL-27OHC and lower nonHDL-27OHC concentrations in the carriers of HL mutations.

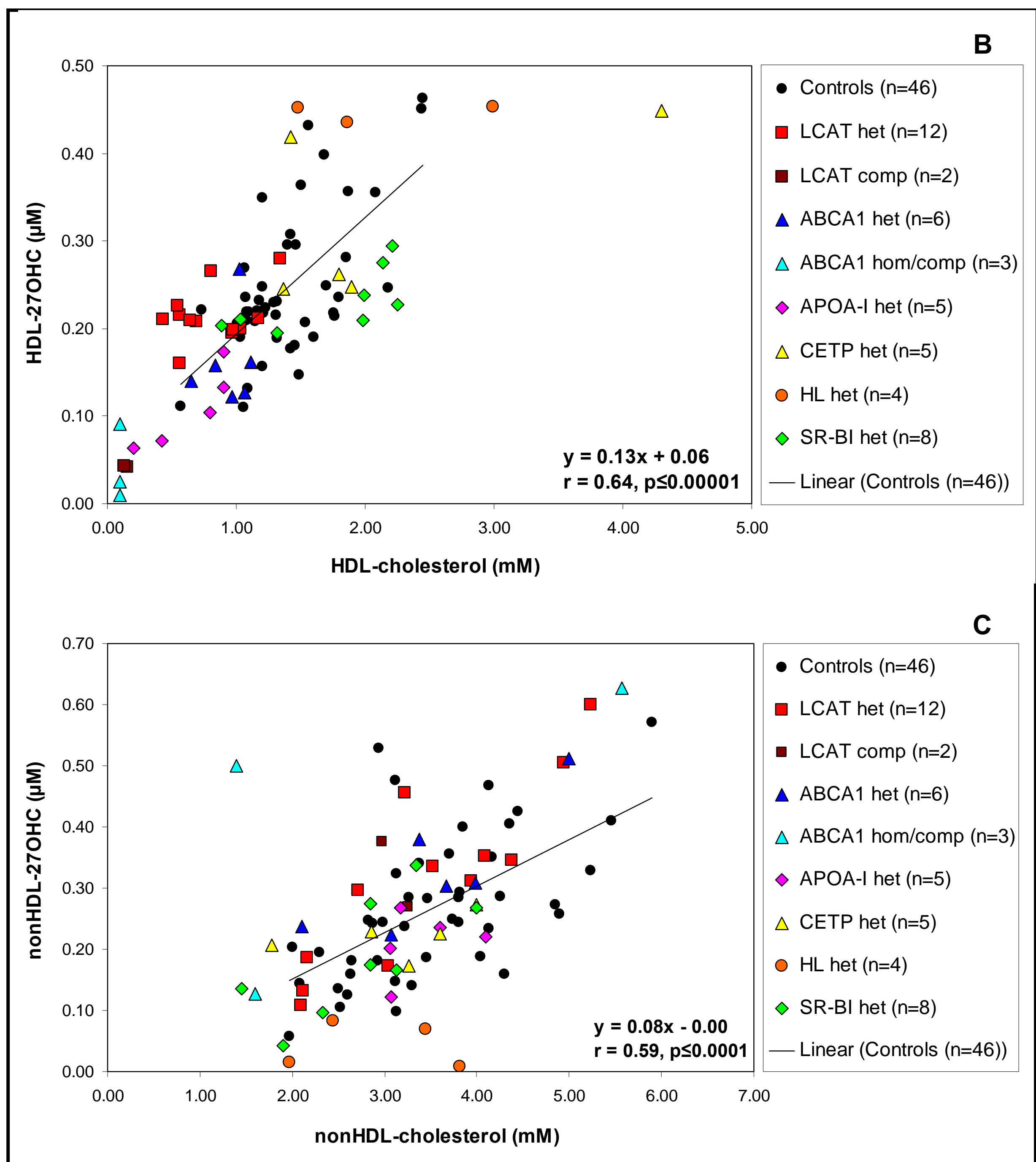

Figure 1. (continued)

Finally, 27OHC levels and the ratio of 27OHC/cholesterol were gene-dose-dependently increased in the nonHDL fractions of ABCA1 mutation carriers and female LCAT mutation carriers (table 2 and 3, figure 1C). By contrast, male heterozygotes for defects in apoA-I, SR-BI, CETP or HL had lower nonHDL-27OHC levels as compared to the unaffected male controls (table 2). In HL mutation carriers, nonHDL-27OHC levels and nonHDL-27OHC/nonHDL-cholesterol ratio were lower than in unaffected controls from either the same families or the entire population (table 3 and figure 1C).

Table 2. 27OHC levels in plasma and lipoproteins in families with monogenic disturbances of HDL metabolism. „Controls" = sum of all unaffected family members („0"), „0" = unaffected family members, „1" = heterozygous mutation carriers, „2" = homozygous or compound heterozygous mutation carriers. *, **, * and #, ##, ### indicate statistically significant differences (p≤0.05, p≤0.01, p≤0.001, respectively) as compared to „0" and „Controls", respectively.**

Mutated gene	Number of defective alleles	Number of participants		27OHC in total plasma (µM)		27OHC in HDL (µM)		27OHC in nonHDL (µM)	
		Female	Male	Female	Male	Female	Male	Female	Male
Controls	0	21	24	0.43 ± 0.10	0.60 ± 0.14	0.24 ± 0.09	0.26 ± 0.08	0.19 ± 0.06	0.34 ± 0.11
					Defects causing decreased levels of HDL cholesterol				
APOA-I	0	6	2	0.42 ± 0.08	0.74 ± 0.18	0.21 ± 0.04	0.26 ± 0.04	0.21 ± 0.06	0.48 ± 0.14
p.L202P (c.605T>C)	1	2	1	0.26 ± 0.01 *#	0.33 #	0.10 ± 0.04 **#	0.06 #	0.16 ± 0.06	0.27
p.L168R (c.503T>G)	1	2	0	0.37 ± 0.06		0.14 ± 0.05 *		0.23 ± 0.01	
ABCA1	0	4	4	0.38 ± 0.09	0.50 ± 0.11	0.20 ± 0.04	0.23 ± 0.05	0.18 ± 0.05	0.27 ± 0.06
	1	3	3	0.40 ± 0.03	0.58 ± 0.10	0.14 ± 0.02	0.18 ± 0.08	0.26 ± 0.05	0.40 ± 0.10
	2	2	1	0.43 ± 0.40	0.51	0.06 ± 0.05 **##	0.01 *##	0.38 ± 0.35 ##	0.50 *
LCAT	0	3	5	0.42 ± 0.04	0.64 ± 0.13	0.29 ± 0.06	0.27 ± 0.06	0.13 ± 0.03	0.36 ± 0.09
	1	4	8	0.47 ± 0.15	0.56 ± 0.17	0.20 ± 0.01 *	0.22 ± 0.04	0.27 ± 0.16	0.34 ± 0.15
	2	2	0	0.37 ± 0.07		0.04 ± 0.00 **##		0.32 ± 0.08 *##	
					Defects causing increased levels of HDL cholesterol				
SR-BI	0	4	4	0.42 ± 0.11	0.55 ± 0.15	0.17 ± 0.06	0.19 ± 0.05	0.25 ± 0.06	0.37 ± 0.13
	1	5	3	0.42 ± 0.09	0.41 ± 0.14 #	0.24 ± 0.04	0.22 ± 0.02	0.18 ± 0.09	0.19 ± 0.13 #

Table 2. (continued)

Mutated gene	Number of defective alleles	Number of participants		27OHC in total plasma (µM)		27OHC in HDL (µM)		27OHC in nonHDL (µM)	
		Female	Male	Female	Male	Female	Male	Female	Male
CETP	0	5	3	0.43 ± 0.09	0.76 ± 0.17	0.22 ± 0.03	0.30 ± 0.06	0.20 ± 0.07	0.46 ± 0.12
(IVS7+1 (G>T))	1	1	2	0.45	0.53 ± 0.08	0.25	0.33 ± 0.12 #	0.21	0.20 ± 0.04
p.S349Y(c.1046C>A)	1	1	1	0.49	0.72	0.26	0.45 #	0.22	0.27
HL (LIPC)	0	3	7	0.51 ± 0.17	0.58 ± 0.12	0.36 ± 0.16	0.29 ± 0.10	0.15 ± 0.04	0.29 ± 0.11
	1	1	3	0.52	0.53 ± 0.07	0.45 #	0.49 ± 0.08 **###	0.07	0.04 ± 0.04 **###

Table 3. 27OHC/cholesterol ratios in plasma, HDL and non HDL of families with monogenic disturbances of HDL metabolism. „Controls" = sum of all unaffected family members („0"), „0" = unaffected family members, „1" = heterozygous mutation carriers, „2" = homozygous or compound heterozygous mutation carriers. *, **, * and #, ##, ### indicate statistically significant differences (p≤0.05, p≤0.01, p≤0.001, respectively) as compared to „0" and „Controls", respectively.**

Mutated gene	Number of defective alleles	Ratio total-27OHC (µM) / total-cholesterol (mM)		Ratio HDL-27OHC (µM) / HDL-cholesterol (mM)		Ratio nonHDL-27OHC (µM) / nonHDL-cholesterol (mM)	
		Female	Male	Female	Male	Female	Male
Controls	0	0.09 ± 0.01	0.12 ± 0.02	0.15 ± 0.03	0.21 ± 0.04	0.06 ± 0.01	0.09 ± 0.03
HDL Maturation							
APOA-I	0	0.08 ± 0.02	0.13 ± 0.01	0.15 ± 0.04	0.20 ± 0.01	0.06 ± 0.01	0.11 ± 0.01
p.S349Y (c.1046C>A)	1	0.07 ± 0.01	0.10	0.16 ± 0.02	0.31 #	0.05 ± 0.02	0.08
p.L168R (c.503T>G)	1	0.08 ± 0.02		0.16 ± 0.04		0.06 ± 0.01	

Table 3. (continued)

Mutated gene	Number of defective alleles	Ratio total-27OHC (µM) / total-cholesterol (mM)		Ratio HDL-27OHC (µM) / HDL-cholesterol (mM)		Ratio nonHDL-27OHC (µM) / nonHDL-cholesterol (mM)	
		Female	Male	Female	Male	Female	Male
ABCA1	0	0.08 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.19 ± 0.02	0.06 ± 0.01	0.09 ± 0.01
	1	0.11 ± 0.03	0.12 ± 0.02	0.16 ± 0.05	0.19 ± 0.07	0.09 ± 0.02	0.10 ± 0.02
	2	0.11 ± 0.02 #	0.36 ***###	0.58 ± 0.46 ###	0.09 **##	0.10 ± 0.02 *###	0.39 ***###
LCAT	0	0.09 ± 0.01	0.12 ± 0.02	0.17 ± 0.02	0.25 ± 0.05	0.05 ± 0.02	0.09 ± 0.02
	1	0.11 ± 0.03 #	0.13 ± 0.02	0.22 ± 0.06 ###	0.33 ± 0.10 ###	0.08 ± 0.04 #	0.09 ± 0.02
	2	0.11 ± 0.03 #		0.30 ± 0.03 **###		0.10 ± 0.03 ###	
HDL Catabolism							
SR-BI	0	0.08 ± 0.01	0.12 ± 0.04	0.13 ± 0.03	0.19 ± 0.01	0.07 ± 0.01	0.11 ± 0.05
	1	0.09 ± 0.02	0.10 ± 0.02	0.12 ± 0.02	0.18 ± 0.06	0.07 ± 0.03	0.06 ± 0.04
CETP	0	0.09 ± 0.02	0.14 ± 0.03	0.15 ± 0.03	0.22 ± 0.02	0.06 ± 0.02	0.12 ± 0.03
(IVS7+1 (G>T))	1	0.12	0.12 ± 0.01	0.13	0.24 ± 0.08	0.12 ###	0.07 ± 0.02
p.S349Y (c.1046C>A)	1	0.09	0.09	0.15	0.10 *##	0.06	0.08
HL (LIPC)	0	0.09 ± 0.02	0.11 ± 0.01	0.17 ± 0.04	0.21 ± 0.04	0.05 ± 0.00	0.07 ± 0.02
	1	0.08	0.12 ± 0.04	0.15	0.30 ± 0.06 **###	0.02 *##	0.01 ± 0.02 **###

4.3.3. Esterification of 27OHC in Patients with HDL Deficiency

LCAT activity was decreased to about 75% of normal in female and male heterozygotes for LCAT defects (table 4). This decreased LCAT activity affected neither the esterification rate nor the cholesteryl ester/total cholesterol ratios in total plasma and HDL. By contrast, the percentage of 27OHC-ester was significantly decreased while the ratio of free-27OHC/free-cholesterol was significantly increased in both plasma and HDL of heterozygous LCAT mutation carriers as compared to unaffected family members. A regression analysis revealed a stronger correlation of LCAT activity with 27OHC-ester concentration than with cholesteryl ester concentration in HDL (figure 2). Interestingly, the percentage of esterified 27OHC was also significantly decreased in plasma and HDL of heterozygous apoA-I(p.L202P) carriers, but not of heterozygotes for the apoA-I(p.L168R) mutation. No significant changes were observed in the degree of 27OHC esterification in carriers of ABCA1 defects.

Table 4. Esterification of 27OHC and cholesterol in plasma and lipoproteins of LCAT mutation carriers. * and * indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.001$, respectively) between „1" and „0".**

	Unaffected family members („0")	Heterozygous mutants („1")
Number (male/female)	5/3	8/4
LCAT activity (nmol cholesteryl ester/hour*mL)	8.37 ± 1.21	6.58 ± 1.01 ***
% 27OHC ester in plasma	92.3 ± 6.7	89.8 ± 4.3
% cholesteryl ester in plasma	87.7 ± 2.4	88.4 ± 1.4
Ratio 27OHC ester (µM) / cholesteryl ester (mM) in plasma	0.16 ± 0.04	0.16 ± 0.03
Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in plasma	0.09 ± 0.06	0.15 ± 0.08
% 27OHC ester in HDL	88.4 ± 4.4	79.8 ± 10.6 *
% cholesterol esters in HDL	80.9 ± 3.0	82.5 ± 3.6
Ratio 27OHC ester (µM) / cholesteryl ester (mM) in HDL	0.22 ± 0.06	0.21 ± 0.07
Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in HDL	0.12 ± 0.04	0.28 ± 0.20 *
% 27OHC ester in nonHDL	93.3 ± 23.9	95.8 ± 9.6
% cholesterol esters in nonHDL	92.5 ± 1.4	91.4 ± 2.0
Ratio 27OHC ester (µM) / cholesteryl ester (mM) in nonHDL	0.13 ± 0.06	0.14 ± 0.05
Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in nonHDL	0.14 ± 0.19	0.10 ± 0.10

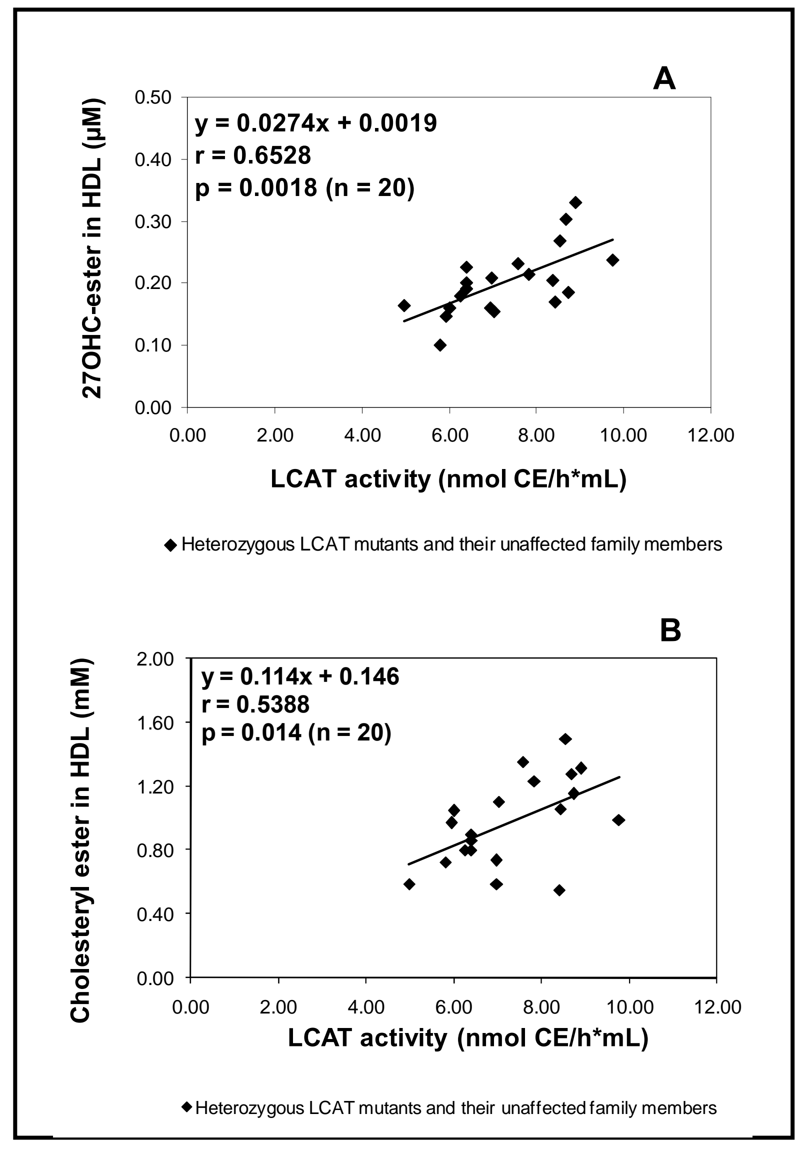

Figure 2. Correlations of LCAT activities with the concentrations of 27OHC-ester (A) and cholesteryl ester (B) in HDL in families with LCAT defects.

4.3.4. 27OHC in Mice with Various Knock-outs of HDL Genes

In normal murine plasma both cholesterol and 27OHC is almost exclusively carried by HDL (table 5). We therefore analyzed the different genetic mouse strains for total 27OHC plasma levels only. Compared to wild-type mice, apoA-I or LCAT knock-out mice were characterized by more than 50% lowered plasma 27OHC concentrations, whereas SR-BI knock-out mice had about 5-fold higher 27OHC levels. After normalization for total cholesterol, the differences remained statistically significant for LCAT and SR-BI knock-out mice only. In mouse plasmas, by contrast to HDL in human plasma, the ratio 27-OHC/cholesterol was lowered rather than increased. In addition, ABCA1 deficient mice

showed an increased 27OHC/cholesterol ratio in the presence of normal 27OHC levels (table 6).

Table 5. Comparison between the plasma and HDL concentrations of 27OHC and cholesterol in mice with knock-outs of apoA-I and SR-BI. „+/+“ = wild-type controls, „+/-“ = heterozygous knockouts, „-/-“ = homozygous knockouts.

Mutants	Number of mice (n)	27OHC concentration (µM)		Cholesterol concentration (mM)	
		plasma	HDL	plasma	HDL
+/+	3	0.13 ± 0.04	0.10 ± 0.02	1.77 ± 0.35	1.67 ± 0.12
APOA-I -/-	2	0.08 ± 0.00	0.07 ± 0.01	1.25 ± 0.35	1.15 ± 0.21
SR-BI +/-	3	0.20 ± 0.06	0.20 ± 0.04	2.87 ± 0.06	2.17 ± 0.12
SR-BI -/-	3	1.11 ± 0.12	1.08 ± 0.32	3.50 ± 0.26	3.13 ± 0.25

Table 6. 27OHC plasma levels in mice with knock-outs of LCAT, apoA-I, ABCA1 or SR-BI. „+/+“ = wild-type controls, „+/-“ = heterozygous knockouts, „-/-“ = homozygous knockouts. *, **, * indicate statistically significant differences ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively) as compared to „+/+“.**

Mutants	Number of mice (n)	27OHC in total plasma (µM)	Ratio total-27OHC (µM) / total-cholesterol (mM)
+/+	28	0.15 ± 0.08	0.08 ± 0.04
APOA-I -/-	5	0.06 ± 0.04 *	0.05 ± 0.05
ABCA1 +/-	3	0.08 ± 0.01	0.06 ± 0.02
ABCA1 -/-	6	0.11 ± 0.06	0.19 ± 0.08 ***
LCAT -/-	8	0.06 ± 0.02 **	0.03 ± 0.01 ***
SR-BI +/-	9	0.18 ± 0.04	0.06 ± 0.02
SR-BI -/-	10	0.96 ± 0.32 ***	0.29 ± 0.10 ***

4.4. Discussion

We have previously shown for normolipidemic individuals that about 50% and 30% of circulating 27OHC in healthy individuals is carried by HDL and LDL, respectively (18). We also observed statistically significant correlations between concentrations of cholesterol and 27OHC, which were stronger in total plasma (r = 0.54) and nonHDL (r = 0.72) than in HDL (r = 0.34) (18). In the present study these correlations were even stronger (figure 1). Following the strong correlation between HDL-cholesterol and HDL-27OHC (r = 0.64 in normolipidemic individuals of Dutch descent without any mutation in the HDL genes), functionally relevant mutations in apoA-I, ABCA1 and LCAT that decrease HDL-cholesterol were associated with reduced concentrations of 27OHC in HDL, whereas mutations in the genes of CETP, SR-BI and HL that increase HDL-cholesterol levels were associated with elevated HDL-27OHC concentrations. For defects in ABCA1 or LCAT, for which we could investigate the effects of both monoallelic and biallelic mutations, HDL-27OHC levels and HDL-cholesterol levels showed similar gene-dose-dependent alterations. Likewise, HDL deficient mice with knock-outs of apoA-I, ABCA1 or LCAT presented with decreased 27OHC plasma levels. Conversely, 27OHC plasma levels were strongly elevated in SR-BI knock-out mice, which are hypercholesterolemic.

Taken together, in humans and mice with inborn errors of HDL metabolism, HDL-27OHC levels appeared simply to change passively in parallel with HDL-cholesterol levels. However, normalization of HDL-27OHC levels for HDL-cholesterol levels revealed that loss of LCAT function is an interesting exception. In both genders, mutations in LCAT caused (dose-dependently) relatively less pronounced decreases in HDL-27OHC than HDL-cholesterol levels in comparison with unaffected family members and the entire mutation-free control population as well as other genetic low HDL conditions. This happened although reduced LCAT activity has a stronger impact on 27OHC-ester formation than on cholesteryl ester formation. Thus, as it has been shown previously (18) as well as in this study, the proportion of 27OHC-esters is higher than the proportion of cholesteryl esters in both plasma and HDL (table 4). Moreover, heterozygous LCAT mutation carriers showed reduced 27OHC-ester/total-27OHC ratios, but normal cholesteryl ester/total cholesterol ratios, in HDL. Finally, LCAT activity showed a stronger correlation with 27OHC-ester concentrations than with cholesteryl ester concentrations in HDL (figure 2). This observational data do not allow any conclusion on the biochemical basis for these differences. One reason may be differences in the reaction kinetics (affinity and/or maximal reaction velocity) of LCAT towards 27OHC and cholesterol, so that reduced LCAT activity in mutation carriers affects

27OHC esterification more strongly than cholesterol esterification. Alternatively, esterification of 27OHC may depend more strictly on LCAT than cholesterol, for example because it is not esterified by acyl-CoA:cholesterol acyl transferase (ACAT) (29-30). However, since 27OHC-ester/total-27OHC ratio was decreased in HDL but not in apoB-containing lipoproteins of LCAT mutation carriers, and since ACAT is especially relevant for the secretion of cholesteryl esters in apoB containing lipoproteins (30), the former explanation is more likely than the latter one. In any case, it is important to emphasize that the elevated 27OHC/cholesterol ratio in LCAT mutation carriers is the result of a relatively high concentration of unesterified 27OHC, rather than of esterified 27OHC. It thus appears that reduced LCAT activity increases the capacity of HDL particles to accommodate unesterified 27OHC.

Another interesting finding was the counter-reactive increase of 27OHC levels in the non-HDL fraction. It was most prominent in HDL deficient individuals in that compound heterozygosity for mutations in LCAT or ABCA1 showed increased concentrations of 27OHC, both in absolute concentrations and relative to cholesterol, in non-HDL fraction, that is apoB-containing lipoproteins. Vice versa, heterozygous carriers of HL mutations presented with strongly decreased 27OHC concentrations in the nonHDL fraction, both in absolute concentrations and relative to cholesterol. It hence appears that the changes in HDL particle numbers or composition due to mutations in LCAT, ABCA1 and HL result in a redistribution of 27OHC among lipoproteins. In this regard, it is important to emphasize that the plasma concentration of 27OHC (0.5 μmol/L in average), unlike the concentration of cholesterol (5 mmol/L in average), is considerably lower than the particle concentration of both HDL (about 20 μmol/L in average) and LDL (about 2 μmol/L in average). Therefore, and also because of its higher water-solubility (6), 27OHC may passively equilibrate among the various lipoprotein fractions.

It has been suggested that HDL-independent 27OHC secretion represents an alternative pathway to HDL-mediated cholesterol efflux by which macrophages protect themselves from cholesterol overload (1-2, 7, 15-17). This observational study does not provide conclusive arguments either in support or in contradiction to this concept. On the one hand, the strong correlation between HDL-cholesterol and HDL-27OHC in normolipidemic population as well as the parallel changes of HDL-cholesterol and HDL-27OHC observed in this study rather contradicts the concept of an HDL-independent reverse cholesterol transport pathway. On the other hand, neither in humans nor in mice ABCA1 deficiency had any effect on 27OHC levels. Rather by contrast, 27OHC/cholesterol ratios were increased in the plasma

of both ABCA1 knock-out mice and humans with two defective ABCA1 alleles, which may be taken as indirect indication that 27OHC is secreted independently from ABCA1.

In conclusion, in most cases, defects in HDL metabolism affect 27OHC concentrations in HDL to a similar degree as cholesterol in this lipoprotein. The most important exception were mutations in LCAT, possibly because reduced LCAT activity impairs 27OHC esterification in the plasma compartment more pronouncedly than cholesterol esterification. In addition, several defects in HDL metabolism led to counter-reactive changes of 27OHC levels in apoB-containing lipoproteins, which were not observed for cholesterol. Finally, our data in humans and mice with inborn errors of HDL metabolism neither support nor contradict the concept that 27OHC represents an HDL-independent mode of reverse cholesterol transport.

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5. PLASMA LEVELS OF SPHINGOSINE-1-PHOSPHATE IN PATIENTS WITH MONOGENIC DISTURBANCES OF HIGH DENSITY LIPOPROTEIN METABOLISM

R. Karuna[a,b], A.G. Holleboom[c], M.M. Motazacker[c], J.A. Kuivenhoven[c], L. Rohrer[a], K.M. Rentsch[a,b] and A. von Eckardstein[a,b]

a. Institute of Clinical Chemistry, University Hospital Zurich, Zurich
b. Competence Center of Systems Physiology and Metabolic Diseases, Zurich
c. Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam

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Abstract

Approximately 60% of plasma sphingosine-1-phosphate (S1P) is carried by high density lipoproteins (HDL) and contributes to several beneficial cardiovascular effects of HDL. To explore whether HDL-cholesterol levels determine S1P levels in plasma, we analyzed S1P levels in total and apoB-depleted plasmas of 48 patients with monogenic disorders of HDL metabolism as well as in their 51 unaffected relatives. Compared to unaffected family members, S1P levels in total and apoB-depleted plasma were significantly decreased in hypoalphalipoproteinemic individuals with mutations in apoA-I (heterozygotes: -23% and -29%, respectively), ABCA1 (heterozygotes: -37% and -45%, respectively; compound heterozygotes: -64% and -79%, respectively), or LCAT (heterozygotes: -32% and -22%, respectively; homozygotes: -53% and -62%, respectively). No difference was found when comparing the heterozygotes for the mutations in CETP, SR-BI or HL (which have high HDL-cholesterol levels) with their unaffected relatives. In addition, the regression analyses of data from all individuals without mutations revealed statistically significant correlations between S1P levels and HDL-cholesterol levels if apoA-I levels were in the lowest tertile (<1.48 g/L), which became weaker and statistically insignificant as apoA-I levels increased. Finally, the infusion of reconstituted HDL (rHDL) into the heterozygous mutation carriers of apoA-I or LCAT increased plasma S1P levels in vivo. We concluded that the plasma levels of HDL-cholesterol and apoA-I determined plasma S1P concentrations, but only at the lower end of the distribution curve.

5.1. Introduction

Sphingosine-1-phosphate (S1P) is produced by the degradation of ceramides into sphingosine which is subsequently phosphorylated by sphingosine kinase (1). As the ligand of at least five different G-protein coupled receptors, S1P exerts many biological activities (2). Recently, erythrocytes have been established as the main source (~90%) of plasma S1P (3). Platelets and endothelial cells are considered as important contributors for the remaining ~10% of S1P found in plasma (4-6).

Published plasma S1P concentrations varied from ~200 nM up to ~2000 nM (7-9). The S1P levels in blood and body fluids are 20 to 100 fold higher than the K_d values of its receptors (8, 10, 11). Circulating S1P is transported by high-density lipoproteins (HDL, ~50-70%), albumin (~30%), low and very-low density lipoproteins (LDL and VLDL, respectively; together <10%). Because of approximately 10 to 20 fold lower particle numbers, however, each LDL particle carries more S1P than each HDL particle (>50 mmol/mol and <10 mmol/mol, respectively) (8, 11-14).

The S1P carried by HDL has been thought responsible for the many cardioprotective effects of this lipoprotein class (8, 14-16). For example, HDL-S1P inhibits the migration of vascular smooth muscle cells (VSMCs), promotes vasodilation by stimulating endothelial nitric oxide synthase (eNOS) and thereby nitric oxide production, and promotes endothelial cell growth and survival by inducing cell proliferation, migration, and tube formation as well as by inhibiting apoptosis and reactive oxidative species (ROS) production. In addition, HDL-S1P interferes with the recruitment of monocytes and lymphocytes into the intima of the arterial wall by inhibiting the endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in response to TNF-α. By contrast, intracellular S1P appears to exert adverse effects such as stimulating the expression of VCAM-1 and ICAM-1, mediating the pro-inflammatory effects of cytokines, and inducing vasoconstriction (8, 14, 16, 17).

To date, little is known on the determinants of S1P levels in plasma. Since the main portion of S1P has been reported to be carried by HDL, the effects of plasma HDL levels on plasma S1P concentration was studied. Specifically, the S1P levels in total and apoB-depleted plasmas of patients with functional mutations in the following genes were investigated: apolipoprotein A-I (apoA-I), which is the main protein component of HDL; ATP-binding cassette transporter A1 (ABCA1), which mediates cholesterol and phospholipid efflux from

cells to apoA-I; lecithin:cholesterol acyl transferase (LCAT), which esterifies cholesterol and thereby converts nascent HDL into mature HDL; cholesteryl ester transfer protein (CETP), which exchanges cholesteryl ester from HDL with triglycerides from apoB-containing lipoproteins; scavenger receptor type B class 1 (SR-BI), which mediates selective uptake of cholesteryl esters into the liver and steroidogenic organs; hepatic lipase (HL), which hydrolyses triglyceride and phospholipids in HDL; and endothelial lipase (EL), which preferentially hydrolyses HDL-phospholipids (18, 19). HDL-cholesterol levels are low in patients with mutations in either apoA-I, ABCA1 or LCAT and elevated in patients with mutations in either CETP, SR-BI, HL or EL. Since we found S1P levels to be modulated by low HDL levels, but not by high HDL levels, we also tested the effects of infusing reconstituted HDL (rHDL) on S1P levels in patients with apoA-I and LCAT mutations.

5.2. Methods

5.2.1. Subjects and Sample Collection

Forty-eight Dutch patients with functional mutations in the genes encoding for either LCAT, ABCA1, apoA-I, SR-BI, CETP, HL or EL as well as 51 unaffected family members were investigated. Most of the respective defects have been described previously (20-29). Mutations that have not yet been reported for ABCA1 are c.3167T>C (p.L1056P), c.3112C>T (p.Q1038X), and c.5398A>C (p.N1800H), the homozygosity for p.L1056P and the compound heterozygosity for p.Q1038X and p.N1800H (paper submitted). The as yet undescribed p.R322C mutation caused 38% loss on the LCAT activity when analyzed using a proteoliposome substrate assay. A frameshift mutation in LIPG was anticipated to be functional since the deletion resulted in a premature truncation of the enzyme (p.L130fsX165).

The Medial Ethics Committee of the Academic Medical Center in Amsterdam, The Netherlands, approved all the genetic and phenotypic studies described and all participants signed informed consents to join the study. The characteristics of the study participants are shown in table 1. Fasting blood samples were collected after at least a 10-hour fast in the morning and EDTA-plasma was prepared through centrifugation of the blood at 3000 rpm for 10 min at 25°C. Aliquots were immediately frozen at -80 degrees until later use.

Table 1. Characteristics of the study participants. Values are presented as mean ± SD. The apolipoprotein levels in mg/dL were converted into μM based on apoA-I, apoB-100 and dimeric apoA-II molecular weights (28’300, 512’000 and 17’400 Da, respectively).

Mutated gene	Number of defective alleles	Mutation	Age (year)	Cholesterol (mM)	HDL-cholesterol (mM)	Triglyceride (mM)	ApoA-I (μM)	ApoB (μM)	ApoA-II (μM)
ApoA-I	0		27 ± 14	4.59 ± 0.68	1.16 ± 0.06	1.09 ± 0.29	57.57 ± 4.68	1.89 ± 0.30	16.96 ± 0.64
	1	p.L202P (c.605T>C)	26 ± 17	3.61 ± 0.31	0.51 ± 0.35	1.09 ± 0.51	30.64 ± 13.52	1.67 ± 0.13	10.77 ± 3.71
ABCA1	0		44 ± 20	4.39 ± 0.89	1.47 ± 0.39	0.95 ± 0.22	56.44 ± 10.18	1.64 ± 0.24	17.35 ± 2.02
	1	p.L1056P (c.3167T>C) or p.C1477R (c.4429T>C)	57 ± 11	4.47 ± 1.08	0.94 ± 0.17	1.01 ± 0.05	43.68 ± 4.17	1.92 ± 0.43	15.89 ± 1.15
	2	p.L1056P (c.3167T>C, homozygote) or p.Q1038X (c.3112C>T) + p.N1800H (c.5398A>C) or p.C1477R (c.4429T>C) + IVS25+1G>C	53 ± 10	2.89 ± 2.39	0.10 / ND*	2.29 ± 1.80	1.53 ± 0.88	2.27 ± 1.29	0.43 ± 0.75
LCAT	0		49 ± 9	4.96 ± 0.86	1.33 ± 0.38	1.29 ± 0.66	60.24 ± 7.63	1.84 ± 0.47	17.75 ± 1.18
	1	p.T147I (c.440C>T), p.R322C (c.964C>T), p.N155D (c.463A>G), p.P34Q (c.101C>A), p.Y107X (c.321C>A), p.I202T (c.605T>C) or p.V333M (c.997G>A)	43 ± 13	4.27 ± 1.21	0.81 ± 0.28	1.30 ± 0.55	43.26 ± 9.71	1.90 ± 0.56	15.12 ± 2.78
	2	p.T147I (c.440C>T) + V333M	69 ± 4	3.26 ± 0.19	0.14 ± 0.01	2.11 ± 0.49	18.50 ± 1.22	1.74 ± 0.38	2.15 ± 0.13
SR-BI	0		54 ± 19	4.77 ± 0.89	1.17 ± 0.33	1.21 ± 0.64	51.43 ± 7.62	1.74 ± 0.36	15.78 ± 1.50
	1	p.P297S (c.889C>T)	45 ± 22	4.46 ± 1.21	1.73 ± 0.56	0.97 ± 0.28	65.04 ± 11.67	1.34 ± 0.34	18.96 ± 3.06

Table 1. (continued)

Mutated gene	Number of defective alleles	Mutation	Age (year)	Cholesterol (mM)	HDL-cholesterol (mM)	Triglyceride (mM)	ApoA-I (μM)	ApoB (μM)	ApoA-II (μM)
CETP	0		36 ± 16	4.14 ± 0.51	1.30 ± 0.21	0.87 ± 0.40	56.69 ± 5.25	1.52 ± 0.26	17.29 ± 0.89
	1	IVS7+1 (G>T)	39 ± 18	4.20 ± 0.51	1.56 ± 0.29	0.76 ± 0.32	59.56 ± 4.47	1.45 ± 0.28	15.67 ± 2.47
HL (LIPC)	0		45 ± 19	5.23 ± 0.99	1.61 ± 0.54	1.45 ± 1.05	56.67 ± 10.89	2.51 ± 0.48	
	1	p.S289F (c.866C>T)	45 ± 15	4.92 ± 1.21	2.00 ± 0.68	1.14 ± 0.43	71.03 ± 20.49	2.06 ± 0.45	
EL (LIPG)	0		41 ± 21	5.43 ± 1.24	1.82 ± 0.50	0.74 ± 0.27	61.66 ± 14.03	1.64 ± 0.46	
	1	p.L130fsX165 (premature stop) or p.R476W	45 ± 21	5.41 ± 1.23	1.83 ± 0.51	1.37 ± 0.82	63.01 ± 16.67	1.86 ± 0.47	

* Either the detection limit of the assay or not detectable (ND)

Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by -24 amino acids, ABCA1 by -60 amino acids, LCAT by -24 amino acids, SR-BI by 0 amino acids, CETP by - 17 amino acids, HL by -22 amino acids, EL by 0 amino acids.

5.2.2. Infusion of rHDL

One 29 years old female patient with fish-eye disease (a compound heterozygous for p.T147I and p.V333M), one 42 years old female patient with Tangier disease (a homozygous for p.L1056P), two heterozygotes for mutations in apoA-I (p.L202P; male, 61 years old) or LCAT (male, 56 years old), as well as one 57 years old male control received an infusion of rHDL (ZLB Behring, Bern, Switzerland) at a dose of 40 mg/kg body weight over a period of 3 hours (30). Blood was drawn at baseline (Pre) and 7 days (Day = 7) thereafter and S1P levels were analyzed.

5.2.3. Lipoprotein Fractionation

Lipoproteins were isolated from pooled plasmas of two healthy blood donors (Zürcher Blutspendedienst, Zurich, Switzerland) by stepwise ultracentrifugation at 59'000 rpm and 15°C using a Beckman Coulter Optima L90K ultracentrifuge (Beckman Coulter, Brea, CA, USA) (31).

For some measurements, apoB-containing lipoproteins were removed from 100 µL EDTA plasma by precipitation with dextran-sulfate-Mg^{2+} (0.9 g/L dextran sulfate and 45 mmol/L Mg^{2+} as the final concentration after being added to the sample) (32). The S1P concentration in the LDL/VLDL fraction was calculated by subtracting the S1P levels measured in apoB-depleted-plasma from the S1P levels measured in total plasma.

5.2.4. Sample Preparation

Samples were prepared by protein precipitation without any further lipid extraction. An aliquot of 25 µL EDTA plasma or apoB-depleted plasma as well as 25 µL, 1.09 nM, internal standard (IS) solution in methanol (C17-S1P; Avanti Polar Lipids, Alabaster, AL, USA) were added to 200 µL methanol. The mixture was vortexed immediately each time after the addition of methanolic solution to plasma to minimize anisotropic precipitation. The mixture was then centrifuged at 10'000x g for 10 min at 4°C (Centrifuge 5415R, Vaudaux-Eppendorf, Schönenbuch, Switzerland). The precipitated proteins were discarded and 5 µL of the supernatant was directly injected into the LC-MS system.

5.2.5. Blanks, Calibration and Quality Control (QC)

To prepare S1P-free plasma for blanks, calibrators, and quality control (QC) matrix, activated charcoal was added to EDTA plasma from a healthy volunteer (100 mg charcoal per mL plasma) and mixed for at least 12 hr. The mixture was then centrifuged (10'000x g, 15 min at room temperature) a few times until the supernatant was clear. Charcoal and the bound S1P were removed from the supernatant by sequential passaging through filters with 0.45 μm and 0.22 μm pore diameters.

S1P (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in DMSO:concentrated-HCl (100:2 by volume) to make 0.28 μM stock solutions. Standards were prepared by further dilution of the stock solution in methanol (0.06, 0.14, 0.28, 0.57, 1.42, 2.27 nM).

The standards (25 μL), IS (25 μL) and methanol (175 μL) were added to the charcoal-treated plasma (25 μL) and processed as described in "Sample Preparation" above. A calibration curve was constructed by plotting the peak area ratios of S1P/IS and the concentrations of the S1P standards. The S1P concentration in samples was derived from the S1P/IS peak area ratios relative to the calibration curve. The calibration curve was obtained for each series of samples which was analyzed within one day.

Two quality controls were prepared using S1P standards in methanol (0.45 and 1.14 nM) and assayed at the beginning and at the end of each sample series. Blank samples (blanks) were prepared by adding the IS (25 μL) to the charcoal-treated plasma (25 μL) and methanol (200 μL), then processed as in "Sample Preparation". Blanks were assayed before each run of the calibrators and samples for the purpose of baseline correction and to correct for any carryover in the system.

5.2.6. Liquid Chromatography – Mass Spectrometry (LC-MS)

The LC-MS system consisted of an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) with cooled sample trays (4°C), a Rheos 2000 HPLC pump (Flux Instruments, Basel, Switzerland) and a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic conditions for the reversed-phase separation of S1P have been modified from Schmidt H *et al* (33). We used a Nucleosil C18 HD column (125x2 mm, 100 Å, 5 μm) which was connected to a Nucleosil C18 HD

guard column (8x4 mm, 100 Å, 5 μm) and heated at 30°C with a HotDog 5090 column oven (Prolab Instruments, Reinach, Switzerland). The mobile phase A was a mixture of methanol/tetrahydrofuran/0.1% formic acid (2/2/3) and the mobile phase B was a mixture of methanol/tetrahydrofuran (1/1). Gradient elution was performed always at 200 μL/min flow rate and started with 100% mobile phase A for the first 0.6 min. The mobile phase B was subsequently increased to 100% within 4.4 min, then kept constant for 4 min. The column was then re-equilibrated with 100% mobile phase A for 15.5 min. The S1P and IS eluted at t_r ~7.7 min.

The carryover of S1P from the injection system was minimized by washing the syringe and the injector valve 12 times with a mixture of methanol/acetone/isopropanol (1/1/1) containing 0.1% formic acid and 3 times with a mixture of acetone/methanol/water (2/2/1) after each injection. In addition, the column was flushed with methanol at least twice after each sample run. The column was also flushed with a mixture of methanol/aceton/isopropanol (1/1/1) after an injection of highly concentrated samples or calibrators to further minimize the carryover of S1P from the column.

The ionization of S1P was performed using electrospray ionization (ESI). The detection was conducted in the positive mode, monitoring $[M+H]^+$ ions using selective reaction monitoring (SRM) for the transition of m/z 380.2 → 264.2 (16 V, 1 mTorr) for the target analyte (S1P) and m/z 366.2 → 82.06 (34 V, 1 mTorr) for the IS. The MS parameters were as follow: 5000 V spray voltage, 10 V skimmer offset, and 150°C ion-transfer capillary temperature. Further parameters were optimized by tuning the system with S1P standards. The data analysis was performed on XCalibur 2.0.7 SP1 software.

5.2.7. Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmont, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). Normal distribution was tested using Kolmogorov-Smirnov test. Since the distribution was normal, unpaired student-t test was used to test statistical significances for the differences between two groups (assuming equal variance). Significance of a correlation was tested from the Pearson's correlation coefficient (r).

5.3. Results

5.3.1. Analytical Method Validation

Before analyzing samples, the newly established LC-MS method was validated. Recoveries of 105.7% and 103.7% were obtained for charcoal-treated plasma spiked with 0.14 µM and 0.57 µM S1P, respectively, suggesting the binding of endogenous S1P to proteins was completely disrupted during protein precipitation. At 0.06 µM, 0.14 µM and 0.57 µM concentrations, the intra-day imprecision amounted to 10.9%, 1.8% and 6.3%, respectively, and the inter-day imprecision to 10.2%, 8.2% and 3.9%, respectively. At the same concentrations, the intra-day accuracy amounted to 111.3%, 98.5% and 100.9%, respectively, and the inter-day accuracy to 103.2%, 92.35% and 95.2%, respectively.

5.3.2. Lipoprotein Distribution of S1P in Normolipidemic Blood Donors

In line with the published literature (34, 35), nearly 60% of circulating S1P in plasma was recovered in HDL. Nearly 30% of plasma S1P was recovered in the lipoprotein-depleted fraction (LPDS). S1P in LDL and triglyceride-rich lipoproteins (chylomicrons and VLDL) accounted for only 8.1% and 3.3%, respectively. After precipitation of apoB-containing lipoproteins with dextran-sulfate-Mg^{2+}, 85.3% S1P instead of 88.5% S1P expected from the ultracentrifugation data was recovered in the supernatant (containing S1P in HDL and LPDS).

5.3.3. Plasma S1P Levels in Normolipidemic Individuals

In the entire sub-cohorts of relatives without any mutation in the HDL genes, the plasma levels of S1P amounted to 0.89 ± 0.34 µM, of which 73.1 ± 9.9% could not be precipitated together with the apoB-containing lipoproteins. When comparing men (n = 26) and women (n = 25), there were no significant difference in the S1P concentrations in either plasma (0.83 ± 0.34 µM *vs.* 0.86 ± 0.35 µM), the supernatant after precipitation of apoB-containing lipoproteins (0.60 ± 0.31 µM *vs.* 0.60 ± 0.29 µM) or the LDL/VLDL-fraction (0.26 ± 0.12 µM *vs.* 0.26 ± 0.12 µM). Regression analyses showed the absence of correlations between age and S1P levels in total (r = 0.0768) or apoB-depleted plasma (r = 0.1113). These

results allowed us to compare S1P levels of patients with disorders in HDL metabolism and normolipidemic controls without any stratification for age or gender.

5.3.4. Plasma S1P Levels in Patients with Inborn Errors of HDL Metabolism

As shown in table 2, significantly decreased S1P plasma levels were observed in patients with low HDL-cholesterol levels due to mutations in LCAT, ABCA1 or apoA-I. The absence of HDL in the compound heterozygotes for mutations in LCAT or ABCA1 showed the most prominent decreases in S1P plasma levels (-50% and -65%, respectively). The 20 to 30% decreased S1P plasma concentrations of the heterozygotes for apoA-I or ABCA1 mutations as well as the 65% decreased S1P levels of the compound heterozygotes for ABCA1 mutations were exclusively explained by the decreases in S1P levels in the apoB-depleted fractions. After normalization for HDL-cholesterol or apoA-I levels, S1P levels did not differ between the controls and the heterozygous carriers of mutations in LCAT or ABCA1 (table 2). However, the heterozygous carriers of apoA-I mutation as well as the compound heterozygotes for LCAT or ABCA1 mutations had significantly elevated ratios of S1P to HDL-cholesterol and apoA-I in the apoB-free plasma (table 2).

Interestingly, both the heterozygotes and compound heterozygotes for LCAT mutations also showed decreased S1P levels in both apoB-containing and apoB-depleted fractions of plasma. Also, after normalization for the plasma levels of apoB or nonHDL-cholesterol, carriers of LCAT mutations, but not carriers of other mutations, differed from their unaffected relatives by decreased S1P concentrations in apoB-containing lipoproteins (table 2).

Table 2. S1P in patients with monogenic disturbances of HDL metabolism. „Controls" = all unaffected relatives, „0" = unaffected relatives from specific mutation, „1" = heterozygous mutant carriers, „2" = homozygous or compound heterozygous mutant carriers. *, **, * and #, ##, ### indicate statistically significant differences (p≤0.05, p≤0.01, p≤0.001, respectively) as compared to „0" and „Controls", respectively.**

Mutated gene	Number of defective alleles	Number of participants		S1P in total plasma (µM)	S1P in apoB-depleted fraction			S1P in LDL/VLDL fraction		
		Female	Male		(µM)	per mM HDL-cholesterol	per µM apoA-I	(µM)	per mM nonHDL-cholesterol	per µM apoB
Controls	0	25	26	0.89 ± 0.34	0.65 ± 0.29	0.48 ± 0.23	0.012 ± 0.006	0.26 ± 0.12	0.08 ± 0.04	0.15 ± 0.08
					Defects affecting HDL maturation					
ApoA-I	0	1	2	1.13 ± 0.11	0.84 ± 0.02	0.73 ± 0.04	0.015 ± 0.002	0.29 ± 0.12	0.08 ± 0.03	0.15 ± 0.04
	1	1	2	0.87 ± 0.13	0.60 ± 0.03 ***	1.66 ± 1.15 ###	0.022 ± 0.010 ##	0.27 ± 0.14	0.09 ± 0.04	0.16 ± 0.07
ABCA1	0	4	4	1.38 ± 0.41	1.04 ± 0.44	0.75 ± 0.30	0.019 ± 0.009	0.34 ± 0.21	0.11 ± 0.06	0.20 ± 0.10
	1	3	3	0.87 ± 0.29 *	0.57 ± 0.15 *	0.65 ± 0.25	0.013 ± 0.004	0.29 ± 0.21	0.08 ± 0.04	0.15 ± 0.07
	2	1	2	0.50 ± 0.14 **	0.22 ± 0.05 **	2.17 ± 0.49 ***###	0.168 ± 0.069 ***###	0.29 ± 0.13	0.12 ± 0.04	0.13 ± 0.01
LCAT	0	5	3	0.69 ± 0.17	0.42 ± 0.12	0.34 ± 0.14	0.007 ± 0.002	0.27 ± 0.08	0.08 ± 0.02	0.15 ± 0.04
	1	8	4	0.47 ± 0.12 **###	0.33 ± 0.06 *###	0.44 ± 0.14	0.008 ± 0.002	0.15 ± 0.06 ***##	0.05 ± 0.02 **##	0.08 ± 0.05 **##
	2	0	2	0.33 ± 0.002 *##	0.16 ± 0.00 **#	1.15 ± 0.12 ***###	0.009 ± 0.001	0.17 ± 0.00	0.05 ± 0.00	0.10 ± 0.02
					Defects affecting HDL catabolism					
SR-BI	0	4	5	0.82 ± 0.06	0.59 ± 0.05	0.55 ± 0.21	0.012 ± 0.002	0.23 ± 0.06	0.07 ± 0.02	0.14 ± 0.04
	1	3	4	0.90 ± 0.16	0.69 ± 0.16	0.44 ± 0.16	0.011 ± 0.003	0.22 ± 0.09	0.08 ± 0.03	0.16 ± 0.06

Table 2. (continued)

Mutated gene	Number of defective alleles	Number of participants		S1P in total plasma (μM)	S1P in apoB-depleted fraction			S1P in LDL/VLDL fraction		
		Female	Male		(μM)	per mM HDL-cholesterol	per μM apoA-I	(μM)	per mM nonHDL-cholesterol	per μM apoB
CETP	0	2	1	1.02 ± 0.13	0.59 ± 0.08	0.47 ± 0.13	0.011 ± 0.002	0.42 ± 0.09	0.15 ± 0.03	0.28 ± 0.05
	1	2	1	1.03 ± 0.15	0.60 ± 0.03	0.40 ± 0.08	0.010 ± 0.001	0.43 ± 0.18	0.19 ± 0.14 ###	0.32 ± 0.20 ###
HL	0	7	3	0.67 ± 0.18	0.51 ± 0.17	0.33 ± 0.10	0.009 ± 0.002	0.16 ± 0.05	0.05 ± 0.02	0.07 ± 0.03
	1	3	1	0.68 ± 0.13	0.48 ± 0.14	0.27 ± 0.13	0.007 ± 0.003	0.19 ± 0.04	0.07 ± 0.03	0.10 ± 0.03
EL	0	3	7	0.94 ± 0.24	0.69 ± 0.21	0.40 ± 0.16	0.013 ± 0.006	0.26 ± 0.06	0.08 ± 0.03	0.17 ± 0.05
	1	6	4	0.85 ± 0.26	0.62 ± 0.21	0.40 ± 0.23	0.011 ± 0.006	0.22 ± 0.07	0.07 ± 0.03	0.12 ± 0.03

Patients with mutations in SRB1, CETP, HL or EL, who have increased HDL-cholesterol levels, remarkably did not show any statistically significant difference in S1P levels in either total or apoB-depleted plasmas. Normalization for HDL-cholesterol or apoA-I levels revealed no significant difference as well (table 2).

5.3.5. Correlations of S1P Levels with Other Lipid Parameters in Family Members without any Mutation in the HDL Genes

Univariate regression analyses of data from 51 mutation-free family controls revealed inverse correlations between apoB-depleted-S1P levels with the concentrations of total cholesterol, nonHDL-cholesterol and apoB as the only ones with statistical significance. Interestingly, S1P levels in total plasma or apoB-free plasma did not correlate significantly with the plasma concentrations of HDL-cholesterol or apoA-I. Also of note, we did not find any significant correlation of S1P levels in apoB-containing lipoproteins with nonHDL-cholesterol or apoB levels (table 3).

Table 3. Regression analyses of all cohorts of non-mutation carriers. Value represents correlation coefficient (r). * and ** indicate statistically significant Pearson's correlation coefficient ($p \leq 0.05$ and $p \leq 0.01$, respectively).

	Total-S1P	ApoB-depleted-S1P	LDL/VLDL-S1P
Total cholesterol	-0.1949	-0.2811 *	0.0934
HDL-cholesterol	0.1414	0.0316	-0.0689
NonHDL-cholesterol	-0.2366	-0.3225 **	0.1353
ApoA-I	0.0447	-0.1342	0.0327
ApoA-I + ApoA-II	0.0775	-0.0447	0.2324
ApoB	-0.2683	-0.2966 *	0.0837

Since S1P levels were reduced in individuals with low HDL-cholesterol and not increased in individuals with high HDL-cholesterol, we tested the hypothesis that HDL limits S1P levels only up to a certain threshold concentration and repeated the regression analyses in the sub-cohorts of non-mutation carriers who were stratified by apoA-I tertiles (figure 1). The

subgroup of individuals with apoA-I levels in the lowest tertile (that is: <52.5 μM or <1.48 g/L, figure 1A) showed statistically significant correlations of S1P levels with HDL-cholesterol and apoA-I, respectively. These correlations became weaker and statistically insignificant in individuals with apoA-I levels in the second (52.5-61.3 μM or 1.48-1.74 g/L, figure 1B) or third (>62.3 μM or >1.74 g/L, figure 1C) tertiles. Accordingly, we identified a statistically significant correlation between LDL/VLDL-S1P and apoA-I only in the lowest apoA-I tertile (figure 1D).

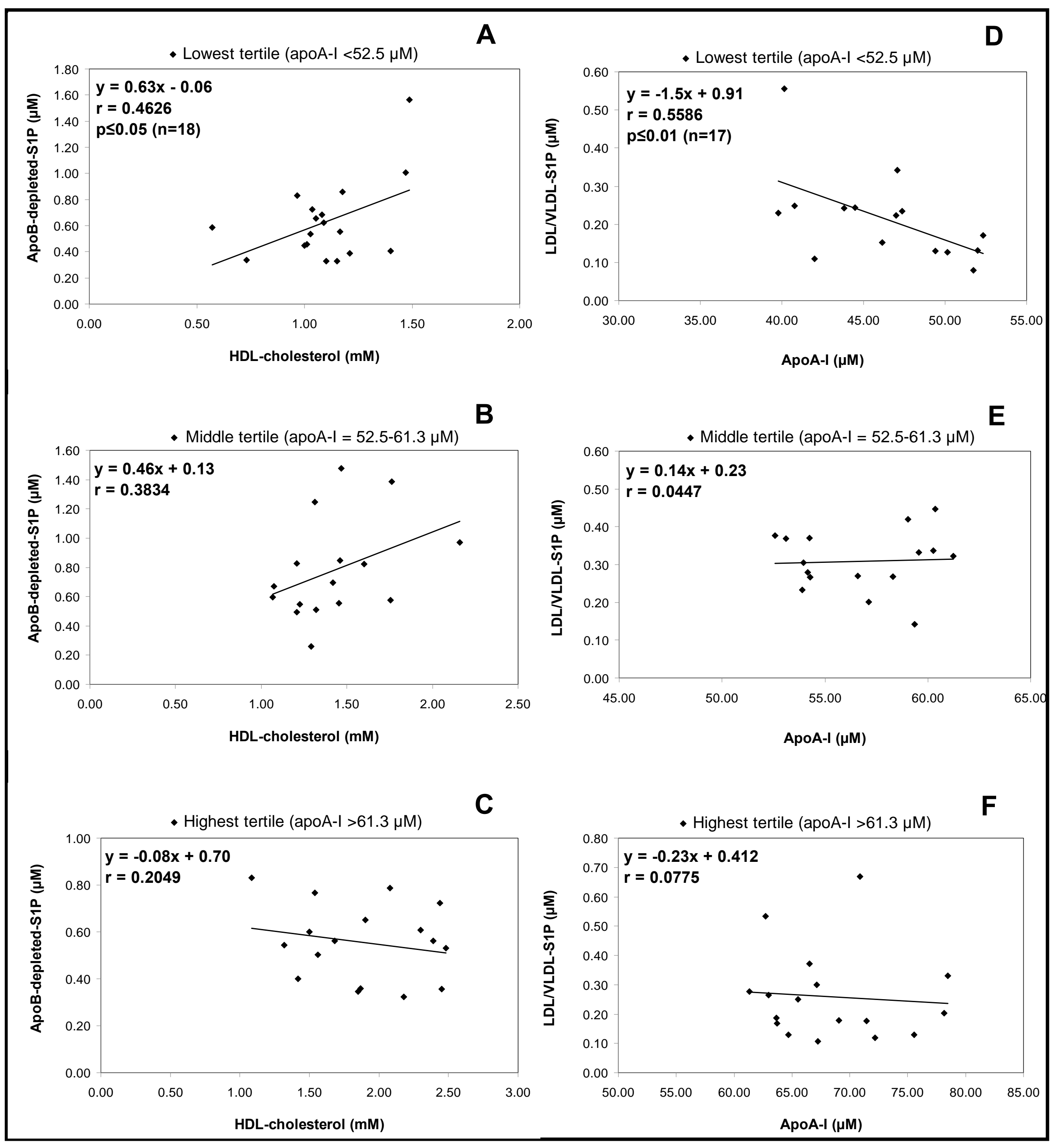

Figure 1. Regression analyses of sub-cohorts of non-mutation carriers who were stratified by tertiles of apoA-I.

5.3.6. Effect of rHDL Infusion on S1P Levels

The findings of both the low S1P levels in the hypolaphalipoproteinemic patients with inborn errors of HDL metabolism and the significant correlation of S1P levels with HDL-cholesterol levels in the mutation-free controls with apoA-I levels <1.48 g/L suggested that a low HDL level limits the abundance of S1P in plasma. We therefore investigated the effect of rHDL infusion on S1P plasma levels in patients with familial low HDL-cholesterol levels as compared to control. In all patients, except for the compound heterozygote for LCAT mutations, S1P levels in total and apoB-depleted plasma (figure 2) increased in response to rHDL infusion and stayed higher than baseline for at least one week.

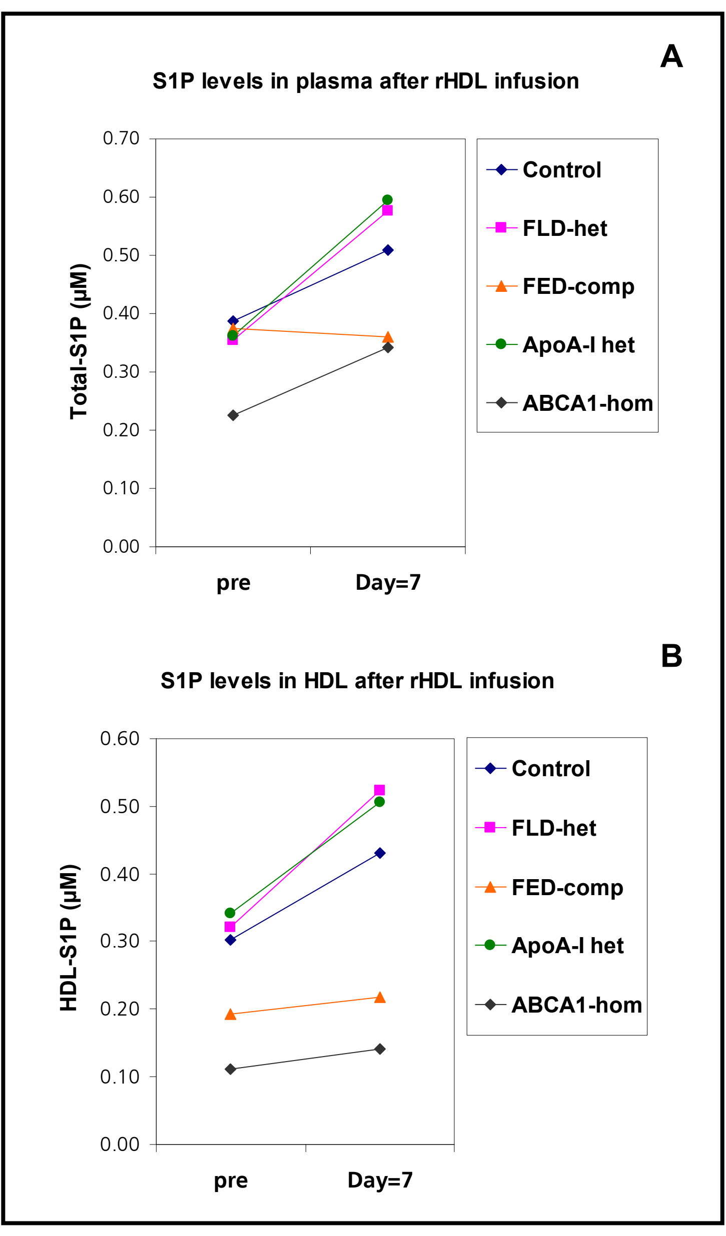

Figure 2. Changes in S1P levels after infusion of rHDL in patients with familial low HDL-cholesterol.

6.4. Discussion

Plasma concentration of S1P varied widely among normolipidemic individuals, for example from 0.49 μM to 1.21 μM in the Dutch population of 47 family members without any mutations in the HDL genes. As yet, the determinants of S1P plasma concentrations have not been well explored. One obvious candidate is the plasma concentration of HDL, which induces S1P efflux from erythrocytes (34, figure 3) and carries about 60% of S1P in normal plasma. In fact, Zhang *et al* have previously reported very strong correlations between S1P levels with HDL-cholesterol ($r = 0.82$) or apoA-I levels ($r = 0.91$) (9). In contrast to these previous observations in a relatively small group of Japanese individuals ($n = 16$), we observed a more complex relationship between HDL and S1P levels.

By investigating S1P levels in about 50 Dutch individuals with defined monogenic errors of HDL metabolism and approximately 50 unaffected family members, we found that the previous observations were only true for those individuals with relatively low plasma concentrations of HDL-cholesterol or apoA-I. Mutations in the genes of apoA-I, ABCA1 or LCAT decreased the plasma concentrations of HDL-cholesterol, apoA-I and S1P in gene-dose-dependent fashions. The lowest S1P levels were found in patients with HDL deficiency due to two mutated ABCA1 or LCAT alleles. Specifically, they presented with 30 and 50% of normal S1P levels in total plasma or 20 and 40% of normal S1P levels in apoB-depleted plasma, respectively. By contrast, mutations in CETP, SR-BI, EL or HL, which increase HDL-cholesterol and apoA-I levels, did not affect S1P levels in either total or apoB-depleted plasma.

Furthermore, in the subgroup of individuals without any mutation in one of the HDL genes, S1P levels in total or apoB-depleted plasma correlated significantly with HDL-cholesterol levels only in individuals who presented with apoA-I levels in the lowest tertile, that is below 1.48 g/L. The correlations of S1P levels with HDL-cholesterol levels weakened and diminished with the increasing apoA-I or HDL-cholesterol tertiles.

We made further observation that low HDL levels are the limiting factor for S1P plasma levels by an additional experiment. In vivo, infusion of rHDL increased S1P levels more profoundly in the heterozygotes for apoA-I or LCAT mutations than in a normolipidemic individual.

Our finding that the low plasma concentrations of HDL might limit plasma S1P levels has been in agreement with the fact that the concentration of S1P is nearly 100-fold and

10’000-fold lower than those of apoA-I and HDL, respectively. Assuming that every HDL particle contains 2 to 4 apoA-I molecules, only 2 to 5% of HDL particles in normal plasma will transport one S1P molecule in average. Thus, even the heterozygosity for HDL-deficiency syndromes, which are characterized by approximately half-normal levels of HDL-cholesterol or apoA-I, should not limit the abundance of S1P in plasma. Our contrary finding suggested that S1P has been transported by specific HDL subclasses which were critically lowered in patients with low HDL-cholesterol. In fact, Kontush *et al* reported that in normolipidemic subjects, S1P is preferentially enriched in small dense HDL_3 particles, rather than in large HDL_2 particles (36). Interestingly, mutations in CETP, EL, HL and SR-BI increase the concentrations of HDL_2 particles more pronouncedly than of HDL_3 particles (37), which may explain the missing effect of mutations in these genes on S1P levels. It will be hence interesting to investigate the molecular determinants of S1P uptake and/or transport by specific HDL subclasses.

About 10% of S1P in normolipidemic plasma is transported by LDL and VLDL. Similar to HDL, the concentration of S1P in LDL and VLDL (about 0.2 μM) is about 10-fold lower than the concentration of apoB-containing particles (about 2 μM). Zhang and colleagues have previously reported a strong correlation between the S1P levels in LDL with the plasma levels of LDL cholesterol or apoB (9). In our study, however, we did not observe any statistically significant correlation between S1P levels in apoB-containing lipoproteins with the plasma concentrations of apoB or nonHDL-cholesterol. Rather by contrast, in individuals with apoA-I levels in the lowest tertile, we observed an inverse correlation of S1P levels with apoB or total cholesterol concentrations as well as of S1P levels in the LDL/VLDL fraction with apoA-I concentrations. Similar to the positive correlations between HDL-cholesterol and S1P in total or apoB-depleted plasma, these inverse correlations became weaker and finally disappeared with the increasing tertiles of apoA-I levels. These inverse relationships indicated an interaction of HDL particles and apoB-containing lipoproteins, by which in individuals with the low levels of HDL-cholesterol or apoA-I, S1P was shifted to apoB-containing lipoproteins and partially lost from the plasma compartment. Despite this interaction in normolipidemic relatives of mutation carriers, only defects in the LCAT gene influenced S1P levels in apoB-containing lipoproteins such that the S1P levels in VLDL and LDL were lower in homozygous or heterozygous mutant carriers than in unaffected family members.

In conclusion, we confirmed in this study that HDL carried about 60% of S1P in normal plasma. Although only ≤5% of HDL particles carry one S1P molecule, both mildly and severely lowered HDL concentrations due to multifactorial reasons or mutations in the genes of apoA-I, ABCA1 or LCAT limited the quantity of S1P in plasma. By contrast, high concentrations of HDL-cholesterol and apoA-I, neither because of mutations in the genes of CETP, SR-BI, EL or HL nor because of multifactorial reasons, influenced S1P levels in plasma. Since S1P exerts several anti-atherogenic functions of HDL, these findings have agreed with the concept of a threshold concentration of HDL or apoA-I which is needed for atheroprotection (38).

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6. METHOD DEVELOPMENT FOR SPHINGOLIPID AND PHOSPHOLIPID PROFILING USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MULTIPLE PARENT-ION SCANNING

R. Karuna, A. von Eckardstein and K.M. Rentsch

Institute of Clinical Chemistry, University Hospital Zurich, and Competence Center for Systems Physiology and Metabolic Diseases, Zurich, Switzerland

Abstract

Sphingolipids and phospholipids are ubiquitous lipid groups encompassing more than 1000 different lipid species. Their structural heterogeneity makes the profiling of sphingo- and phospholipids a challenge. We developed an LC-MS/MS method for the analysis of sphingomyelins (SM), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidic acids (PA), phosphatidylinositols (PI), phosphatidylserines (PS) and phosphatidylcholines (PC). Different lipid classes were separated based on their head-group rentention on a diol silica-based HPLC column. Parent-ion scanning was applied both for screening each lipid species and for the selectivity of the class determination. The feasibility of the method has been shown by analyzing plasma of patients with stable coronary heart diseases, allowing the analysis of both the total endogenous amount of lipids within the same class as well as the concentration of each lipid species within a given class.

6.1. Introduction

Sphingolipids and phospholipids are ubiquitous lipid groups encompassing more than 1000 different lipid species. They function as essential building blocks of cell membranes and lipoproteins. They also act as first and second messengers in mediating cell-cell recognition and interaction (1). Despite their great functional diversities, sphingo- and phospholipids have been rarely investigated for their pathogenetic contribution to diseases or exploited for diagnostic purposes.

Sphingo- and phospholipids are characterized by a sphingoid backbone and a glycerophosphate group, respectively (figure 1). Variation in their head groups determines their lipid classes. Each lipid class comprises at least 20 different lipid species, which differ in the number of carbons and double bonds of the fatty-acid tails (2-3).

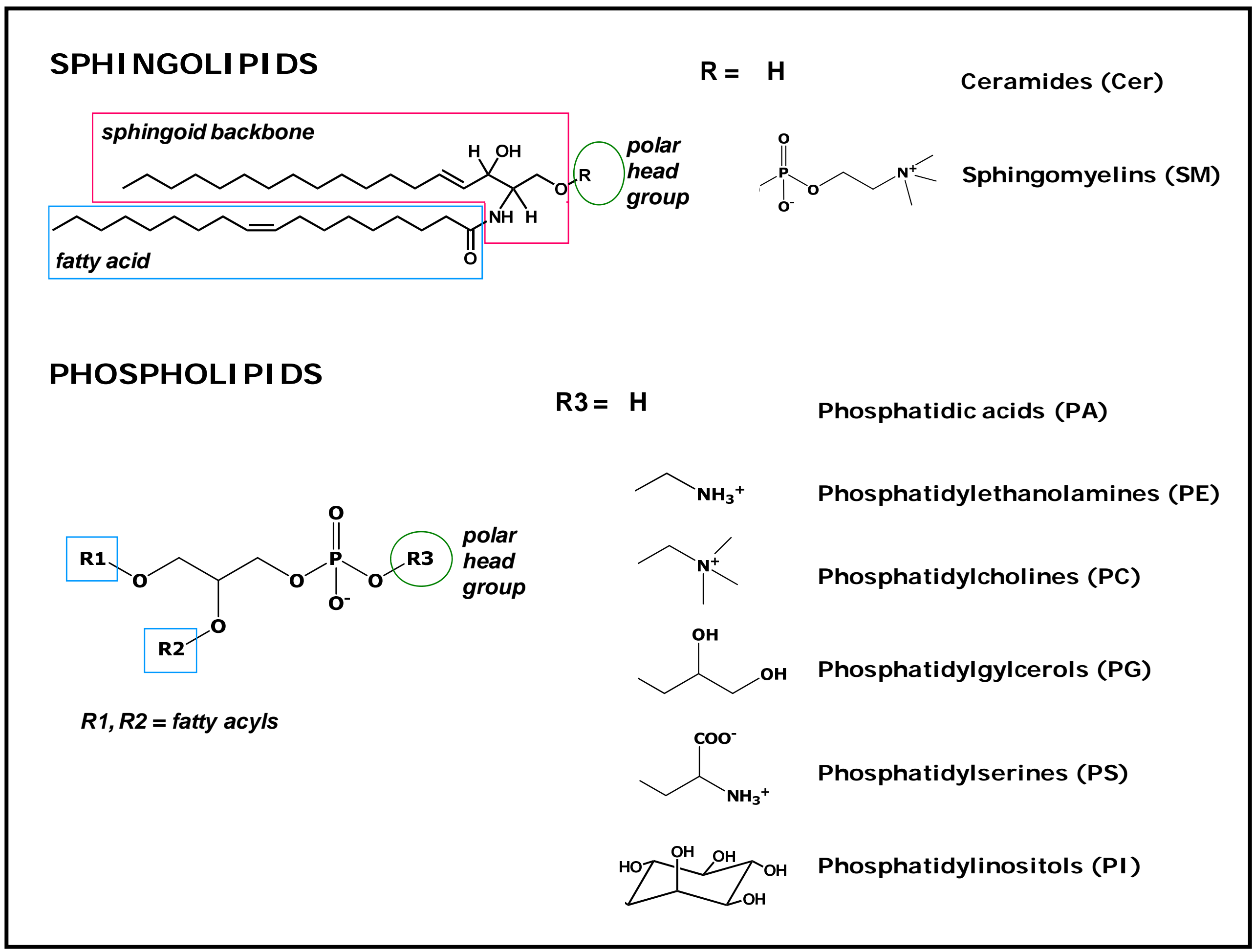

Figure 1. Sphingolipid and phospholipid structures

Due to their structural heterogeneity, profiling of sphingo- and phospholipids is a challenge. Amongst the many efforts to achieve this, one interesting approach is the so-called “shotgun lipidomics”. Principally an intra-source separation and selective ionization of phospholipids and their lithium adducts (4-5), shotgun lipidomics offers sensitivity and the efficiency of single parent-ion scan on a triple quadrupole mass spectrometer. The method utilizes a syringe infusion for less than 30 min run-time and omits any chromatographic separation step. Ion suppression of the low abundance lipids, however, is one of the weaknesses. Another major drawback is contamination of the instrument with lithium ions (5).

Separation with normal or reverse phase HPLC has been commonly applied to reduce the complexity of the lipids prior to mass spectrometric analysis and to minimize the risk of ion suppression. Various LC-MS/MS-based methods have been developed; for example, parent-ion or neutral-loss scans under data-dependent acquisition control on a hybrid Q-TOF (6-10), product-ion or parent-ion scan-dependent multiple reaction monitoring (MRM) on a Q-Trap (11-12), or linear trap/FT-ICR(13) and MS(n) on an Orbitrap mass analyzer (14-16). The LIPID MAPS Consortium (www.lipidmaps.org), for example, has been developing standard protocols based on product-ion scans and MRM for cataloguing lipid species in mammalian cells (12, 17).

For the purpose of non-targeted analysis of sphingo- and phospholipids in a relatively large amount of samples, we developed an LC-MS/MS method combining HPLC separation with multiple parent-ion scans on a triple quadrupole mass spectrometer. The different lipid classes (sphingomyelins (SM), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidic acids (PA), phosphatidylinositols (PI), phosphatidylserines (PS) and phosphatidylcholines (PC)) were separated based on their polar head group retention on normal phase HPLC. Parent-ion scanning was applied both for screening each lipid species and for the selectivity of the class determination. The feasibility of the method has been shown by applying the method to analyze plasmas of patients with stable coronary heart diseases.

6.2. Materials and Methods

6.2.1. Chemicals and Reagents

Sphingo- and phospholipid standards (PG(17:0/17:0), PG(17:1/0:0), PE(17:0/17:0), PE(17:1/0:0), PA(17:0/17:0), PA(17:0/0:0), PC(17:0/17:0), PC(17:0/0:0), PS(17:0/17:0), PI(12:0/13:0), PI14:0/17:0), L-α-PI (bovine liver), C17- sphingosine-1-phosphate (C17-S1P), SM(d18:1/6:0), SM(d18:1/6:0), SM(d18:1/12:0), Cer(d18:1/17:0)) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). All solvents were of HPLC grade except for methyl-tert butyl ether, which was of pro analysis grade. Methanol, hexane and isopropanol were obtained from SDS (Cedex, France). Chloroform, n-butanol and methyl-tert butyl ether were obtained from Sigma Aldrich (Saint Louis, MO, USA). Citric acid and disodium phosphate were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

6.2.2. Lipid Nomenclature

Lipid names were abbreviated according to the newly updated nomenclature used by the LIPID MAPS Consortium (18), where "headgroup(sn1/sn2)' format is used to describe a species with one or two radyl side-chains without specifying their stereochemistry (by default, attachment of head groups at sn3 position is assumed). Numbers between parentheses describe the side-chain structures, with the number before the colon refers to the number of carbons, while the number after the colon refers to the number of double bonds. For examples: PC(16:0/16:0) refers to 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine, PC(16:0/0:0) refers to 1-hexadecanoyl-sn-glycero-3-phosphocholine. Monoacyl species are also termed "lyso-".

For sphingolipids, the sn1 position refers to the typical sphingolipid backbone. For example, SM(d18:1/6:0) refers to sphingomyelins with sphingosine or 2-amino-4-octadecene-1,3-diol as the backbone ('d' denotes the 'double' hydroxyl group attachment) and 6 carbon chain as the fatty-acid tail.

In case of undifferentiated fatty-acid tails as in 'Feasibility studies in patients and healthy volunteers', the sum of the carbon and double bond numbers of the two acyl side-chains was given. For example: PC(32:0) refers to any structural combination of two fatty-acid tails (either PC(16:0/16:0) or PC(18:0/14:0) or PC(14:0/18:0), and so on), that together have 32 carbon atoms and 0 double bonds.

6.2.3. Plasma Samples

Blood samples from two female and one male patients (67 ± 9 years old) with stable coronary heart diseases as well as 3 healthy female volunteers (29 ± 2 years old) were collected and EDTA-plasma was obtained by centrifugation at 1350x g for 10 min at 25°C (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany). Samples were stored at -80°C until use.

6.2.4. Sample Pre-treatment

6.2.4.1. Liquid-liquid Extraction for Sample Pre-treatment Optimization

Different extraction methods using different solvents were compared. Liquid-liquid extraction of 100 µL sample was conducted using either methanol-chloroform as in the Bligh and Dyer extraction method (19), methyl-tert butyl ether (MTBE) (20), n-butanol (21) or a mixture of MTBE and n-butanol as in "Optimized liquid-liquid extraction for sample analysis". The internal standard (IS) mixture solution contained 10 µg/mL each of PC(17:0/17:0), PC(17:0/0:0), C17-S1P, SM(d18:1/6:0), and Cer(d18:1/17:0).

For the Bligh and Dyer procedure, 250 µL methanol, 125 µL chloroform and 10 µL IS mixture solution were added to 100 µL sample. The mixture was sonicated for 10 s. Additional 100 µL water and 125 µL chloroform were added. The mixture was shaken for 15 min and centrifuged at 1272x g for 5 min at 25°C with Sigma 4-15 (Sigma Laborzentrifugen, Osterode am Harz, Germany). The lower phase was collected and additional 250 µL chloroform was added. The mixture was again shaken for 15 min and centrifuged at 1272x g for 5 min at 25°C. All lower phases were combined.

For the extraction procedure using MTBE solvent, 750 µL methanol, 2.5 mL MTBE and 10 µL IS mixture solution were added to 100 µL sample. The mixture was shaken for 1 hr. Additional 625 µL water was added and the mixture was shaken again for 10 min. The mixture was centrifuged at 1272x g for 5 min at 25°C using Sigma 4-15 centrifuge. The upper phase was collected and the lower phase was re-extracted with 1 mL mixture of MTBE/methanol/water (10:3:2.5, v/v/v). All upper phases were combined.

For the extraction procedure using n-butanol solvent, 300 µL buffer (containing 30 mM citric acid and 40 mM disodium phosphate, pH 4), 2 mL n-butanol, 1 mL water-saturated n-butanol and 10 µL IS mixture solution were added to 100 µL sample. The mixture was

shaken for 15 min and centrifuged at 1272x g for 5 min at 25°C using Sigma 4-15 centrifuge. The upper phase was collected.

The collected lower or upper phases from each of the extraction procedures were dried by evaporation at 45°C (Rotavapor, Büchi, Flawil, Switzerland). The residue was reconstituted in 100 µL mobile phase mixture (70% hexane and 30% mixture of isopropanol:methanol:1% ammonium hydroxide (75:22.5:2.5)) and 10 µL was injected into the LC-MS system.

6.2.4.1. Optimized Liquid-liquid Extraction for Sample Analysis

Extraction with a solvent mixture of MTBE and water-saturated n-butanol (80:20 by volume) was conducted as follows: 400 µL solvent mixtures and 10 µL IS mixture solution were added to 100 µL sample. The mixtures were always shaken for 15 min and centrifuged at 1272x g for 5 min at 25°C using Sigma 4-15 centrifuge. The procedure was repeated 3 times with water-saturated n-butanol used for the third re-extraction. All upper phases were combined and dried by evaporation at 45°C (Rotavapor, Büchi, Flawil, Switzerland). The residue was reconstituted in 100 µL mobile phase mixture (70% hexane and 30% mixture of isopropanol:methanol:1% ammonium hydroxide (75:22.5:2.5)) and 10 µL was injected into the LC-MS system.

The IS mixture solution contained 10 µg/mL each of PG(17:0/17:0), PG(17:1/0:0), PE(17:0/17:0), PE(17:1/0:0), PA(17:0/17:0), PA(17:0/0:0), PC(17:0/17:0), PC(17:0/0:0), PS(17:0/17:0), PI(12:0/13:0) or PI14:0/17:0), and SM(d18:1/6:0) or SM(d18:1/12:0).

6.2.5. LC-MS/MS

The LC-MS system consisted of a Rheos 2000 pump (Flux Instruments, Basel, Switzerland), an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a TSQ Quantum Ultra triple quadrupole mass analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Normal phase HPLC separation was conducted on a diol silica-based column (QS Uptisphere 6 OH, 150 x 2.1 mm, 5 µm). Mobile phase composition was 70% hexane and 30%

mixture of isopropanol:methanol:1% ammonium hydroxide (75:22.5:2.5). Elution was performed isocratically at a flow of 200 µL/min for 60 min.

The MS parameters were set as follows: 4000 V spray voltage, 36 V skimmer voltage, 250°C capillary temperature, and 30 and 5 (arbitrary) sheath and auxiliary gas, respectively. Tube lens voltages were tuned using a standard mixture of PG(16:0/0:0), PG(16:0/16:0), PA(16:0/16:0) and L-α-PI (bovine liver), covering a wide molecular mass range. Both Q1 and Q3 resolution were set at 0.7 peak-width (at full-width half-maximum). Multiple parent-ion scan events with polarity switching were performed using 0.5 mTorr collision gas (figure 2). Acquired data was analyzed using the Xcalibur software (version 2.0.7 SP1).

6.2.6. Data Extraction

From each scan event, a mass-peak list containing parent-ion masses (m/z) and their intensities were generated using Xcalibur. The noise cut-off was determined by visually examining the mass spectra. The mass-peak list was entered to an online lipid database (www.lipidmaps.org) using a mass tolerance of ±0.5 Da. The database search output gave both the lipid classes and species, listing all possible combinations of fatty-acid tails. However, individual fatty-acid tail structure could have been obtained only by product-ion scans in the negative mode; hence only the sum of carbon and double bond numbers in the side-chains could be determined. All data were processed manually using Microsoft Excel (Microsoft, Redmont, WA, USA).

Parent-ion scanning of m/z 153 was selective for glycerophospholipids, hence the database search output might give more than one possible phospholipid class. Other parameters (nitrogen rule, retention time, and ionization efficiency) were then considered for the lipid identification. Subsequently, a shortened list of tentative sphingo- and phospholipid species was generated, listing the determined lipid classes, each species with summed number of carbons and double bonds of the side chains, the observed masses (m/z), the absolute intensities, and intensities relative to the corresponding IS (each class has a non-endogenous (mono- and) diacyl lipid species as IS, see “Sample pre-treatment”). Comparison between samples was conducted based on the ratio intensity to the IS of the same tentative lipid species with the same parent mass (m/z).

6.3. Results and Discussions

6.3.1. Sample Pre-treatment Optimization

Sphingo- and phospholipids differ in chemical and physical characteristics and interact with other endogenous compounds such as proteins (for example: lipoproteins). Hence, the quantitative extraction of all sphingo- and phospholipids was a challenge. Various solvents or solvent combinations have been suggested for extracting lipids from specific matrices (22-25). However, many analysts generally used the "standard" Folch procedure (26) and its Bligh-and-Dyer (BD) variation (19) which utilize chloroform-methanol and the matrix's water in a ternary system.

Table 1. Comparison of different solvent and procedures for liquid-liquid extraction of several lipids from plasma.

Lipid class	Standards	Extraction recovery from plasma (%)			
		BD	MTBE	n-Butanol	MTBE/n-Butanol
Cer	Cer(d18:1/17:0)	82.0	17.3	5.5	73.5
SM	SM(d18:1/6:0)	84.3	18.5	95.5	95.2
PC	PC(17:0/17:0)	95.8	82.2	100.9	84.0
LPC	PC(17:0/0:0)	86.6	50.9	101.3	96.1
S1P	C17-S1P	65.5	27.3	44.5	114.5

Since chloroform is very toxic, we investigated other solvent combinations for liquid-liquid extraction of lipids from plasma. We initially tested standards from 5 different lipid classes covering a wide range of different polarities (Cer, SM, PC, LPC and S1P: see figure 1). When available, internal standards have been chosen from non-endogenous lipids which have odd-numbered fatty-acid chains. As shown in table 1, re-extraction of methyl-tert butyl ether (MTBE) extract with water-saturated n-butanol increased the extraction recovery of the more polar lipids. Improvement of the extraction recovery of highly polar lipids by re-extraction with water-saturated n-butanol after a Folch procedure has also been shown in another published method (27). Thus, MTBE/n-butanol was used as the extraction solvent for sample analyses as it gave acceptable recoveries (>80%) for all the lipid classes in the text-mixture, except for Cer. However, Cer was later excluded from this current ESI method to be profiled using a different APCI method since the ionization efficiency of Cer in APCI was 10

times higher than in ESI. Table 2 shows the extraction recoveries of the IS used for sphingo- and phospholipid profiling.

Table 2. Extraction recoveries of different sphingo- and phospholipid classes

Lipid class	Internal standards	Extraction recovery from plasma (%)
PG	PG(17:0/17:0)	94.0
	PG(17:1/0:0)	82.5
PE	PE(17:0/17:0)	103.6
	PE(17:0/0:0)	95.1
PA	PA(17:0/17:0)	82.3
	PA(17:0/0:0)	90.5
PS	PS(17:0/17:0)	102.2
PI	PI(17:0/14:1)	89.4
PC	PC(17:0/17:0)	95.4
	PC(17:0/0:0)	104.8
SM	SM(d18:1/6:0)	82.9

6.3.2. Separation with Normal Phase HPLC

Sphingo- and phospholipids consist of two different moieties: the apolar fatty-acid tails and the head group which determines their lipid classes. The separation of sphingo- and phospholipid classes requires normal phase chromatography since the polar head groups should be retained on the silica column material. Resolution, however, was difficult to optimize due to the small differences in the molecular structures between classes (data not shown). Moreover, each lipid class can comprise more than 20 lipid species, with 16 to 26 carbon chains from each of the fatty-acid tails plus their variation in the number of double bonds and hydroxyl groups, giving the heterogeneity in the molecular weights carried within each class. This and the fact that basic moieties tend to be adsorbed to the silica column material caused peak tailing and broadening and further decrease in the resolution of some lipid classes. The use of a diol silica-based column gave a better resolution than a normal silica column under the chromatographic condition as described in “LC-MS/MS” (data not shown). Further optimization of the separation by changing chromatographic conditions (solvent, solvent composition, pH, gradient elution) or by using either a combination of 2

different columns (a normal silica column and a diol column) or a hydrophilic interaction chromatography (HILIC) column did not give satisfactory results (data not shown). Finally, the HPLC method was developed on the diol silica-based column under the condition as described in “LC-MS/MS” (figure 2).

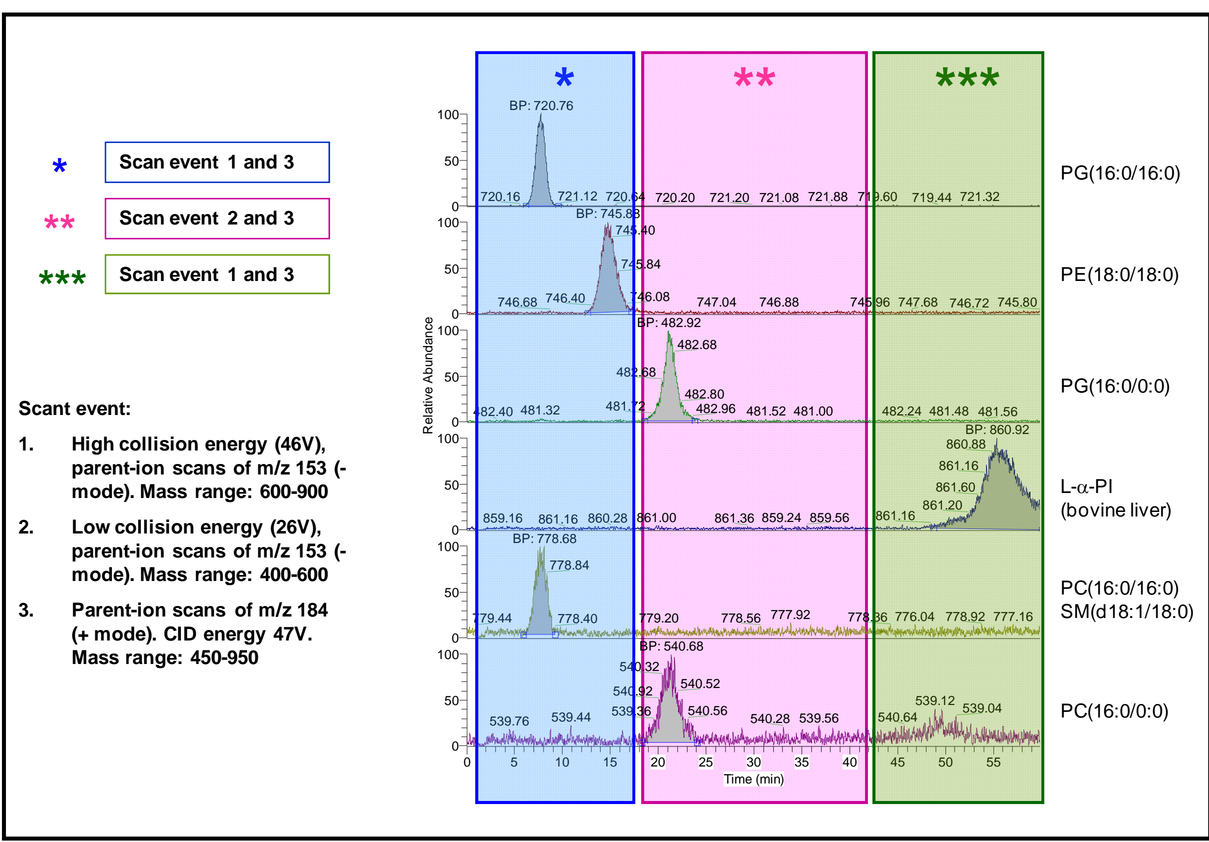

Figure 2. Chromatogram showing the separation of different sphingo- and phospholipids using LC-MS in full scan mode as well as multiple parent-ion scanning (scan events) in different time-segment during a single chromatographic run.

6.3.3. Parent-ion Scanning of Sphingo- and Phospholipids

The head groups of sphingo- and phospholipids break down during mass spectrometric fragmentation, giving unique product ions which are the characteristics of different lipid classes (table 3).

The monitoring of sphingo- and phospholipids has been done in parent-ion scanning mode on a triple quadrupole mass analyzer, in which full scans of precursor-ions have been obtained for the typical product-ions of certain lipid classes. During one chromatographic run, different parent-ion scans could be applied during the different time-segments depending on

which lipid classes were being eluted (figure 2). However, as baseline chromatographic resolution for each single lipid class could not be achieved (see figure 2), multiple parent-ion scans had then to be performed on the same time-segment, in which each full parent-ion scan had to be conducted sequentially. Moreover, due to the structural heterogeneity of lipid species, parent-ions need to be scanned at a wide mass range (the masses of lysolipids range between 400 to 600 amu, while the masses of di- or mixed acyl lipids range between 600 to at least 1000 amu). Hence, although a quadrupole gains a very efficient duty cycle during single ion monitoring of a product ion, efficiency was later severely reduced during multiple parent-ion scans. This led to a reduced number of scans and ion counts, resulting in decreased sensitivity and precision of the less abundant or the less efficiently ionized lipid species. Figure 3 shows two mass spectra obtained from parent-ions scans of m/z 184 (identifying phosphocholine-containing lipids: PC, LPC, SM; see table 3) from the same plasma sample. The spectrum in figure 3A was obtained together with two other multiple parent-ion scans (of m/z 153 and m/z 196, see table 3), while the spectrum in figure 3B was obtained with one other multiple parent-ion scans (of m/z 153). Figure 3B clearly shows a richer spectrum as compared to figure 3A; thus, the more parent-ion scans were conducted, the less information (in terms of the number of peaks) was obtained. Therefore, only two different parent-ion scans were applied for each time-segment during a single chromatographic run (figure 2): for glycerophosholipids or lysophospholipids (parent-ion scans of m/z 153 with high or low collision energy, respectively, in a negative mode) and for phosphocholine-containing lipids (parent-ion scans of m/z 184 in a positive mode).

Table 3. Characteristic product ions of different sphingo- and phospholipid classes

Lipid class	Detection	Product-ion (m/z)	Fragment structure (28)	Optimal collision energy at 0.5 mTorr* (V)
Glycerophospholipids:				
PG (or lyso-PG)	$[M]^-$			44 (or 34)
PE (or lyso-PE)	$[M-H]^-$			43 (or 30)
PA (or lyso-PA)	$[M]^-$	153		38 (or 21)
PS	$[M]^-$			44
PI	$[M]^-$			46
Other unique fragmentation:				
PE (or lyso-PE)	$[M-H]^-$	196		39 (or 23)
PI	$[M]^-$	241		45
Phosphocholine-containing lipids:				
PC (or lyso-PC)	$[M+H]^+$	184		35 (or 21)
SM	$[M+H]^+$			27

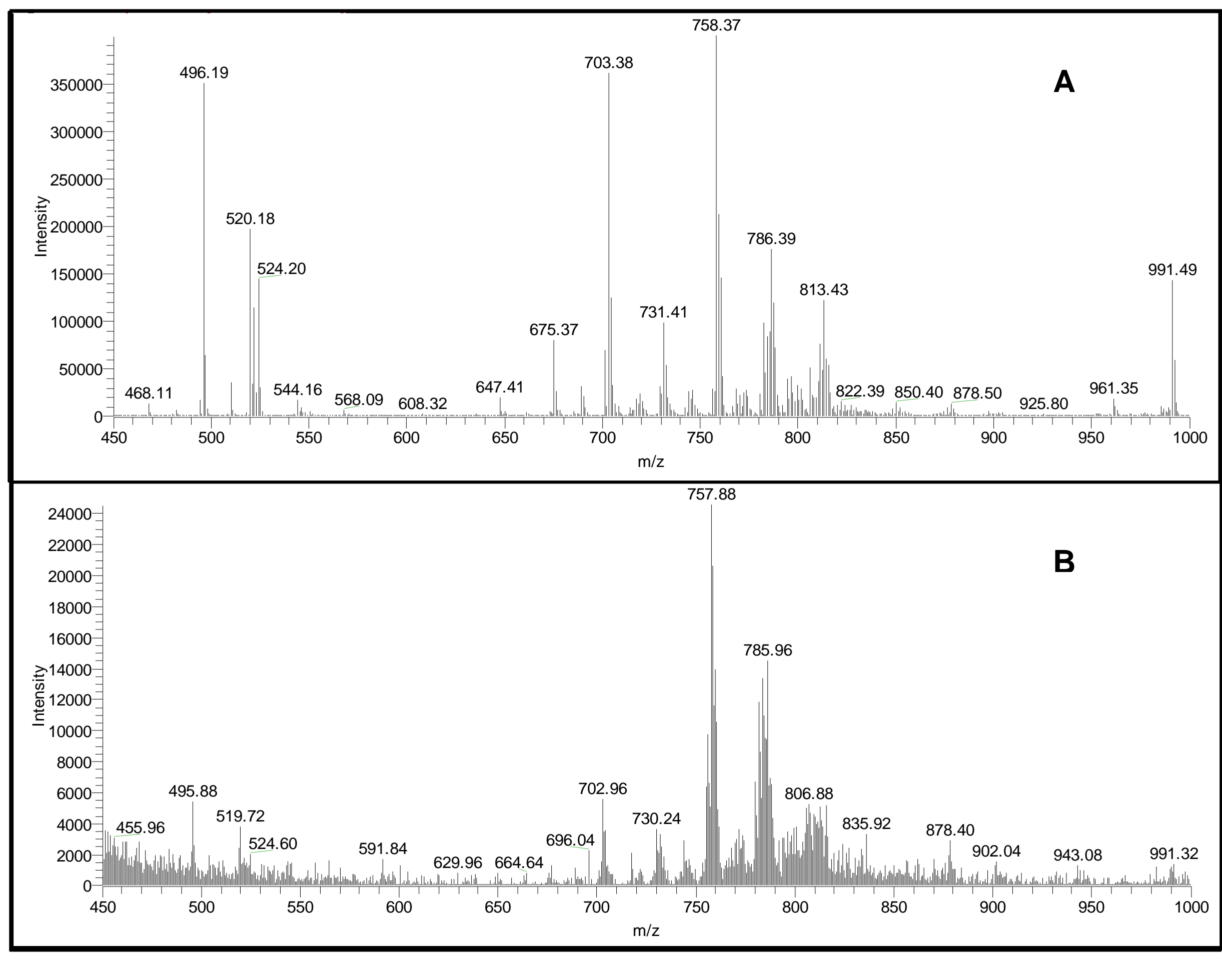

Figure 3. Mass spectra of phosphocholine-containing lipids (parent-ion spectra of m/z 184) from the same plasma samples but obtained using different number of scan events. A: 3 scant events (parent-ion scans of m/z 184, 153 and 196). B: 2 scant events (parent-ion scans of m/z 184 and 153).

Since full parent-ion scans were conducted on a wide mass range, the scan time affected the quality of the spectra acquired. Figure 4 shows spectra of PG(16:0/16:0) and PG(18:0/0:0) standards obtained with 0.2 s scan time (A, C) and 1 s scan time (B, D) for the same mass range. Thus a faster scan time increases the number of scans but decreases the mass accuracy and isotopic resolution which then can lead to a false identification of the lipid species.

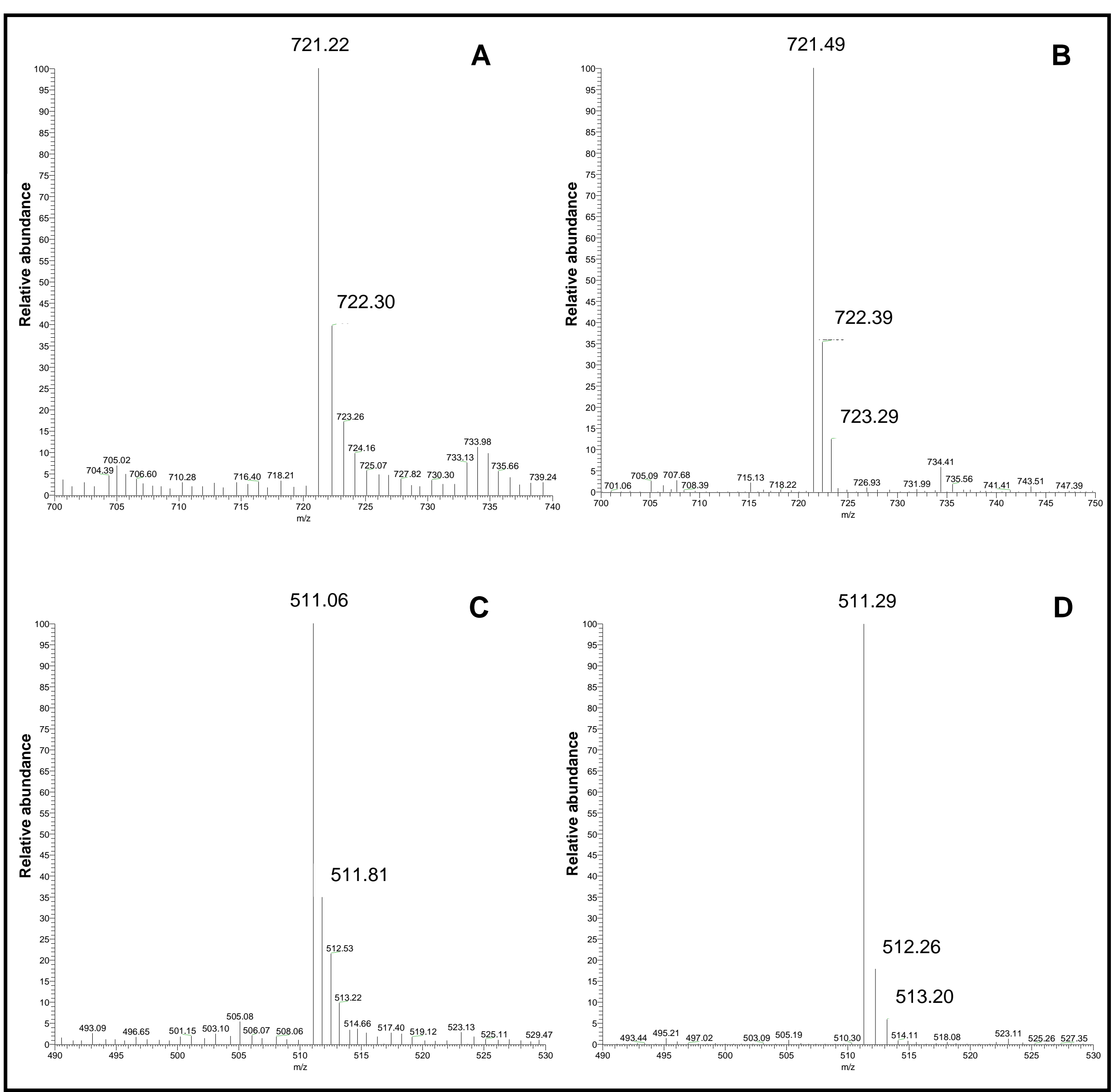

Figure 4. Parent-ion spectra of PG(16:0/16:0), m/z 721.2 (A, B) , and PG(18:0/0:0), m/z 511.2 (C, D), obtained with 0.2 s (A, C) and 1 s (B, D) scan time. The longer scan time improved the mass accuracy and isotopic resolution (C, D).

6.3.4. Sensitivity and Reproducibility of the Method

Accurate quantification of the different lipid species tends to be difficult due to still many limitations on the methods developed for lipid profiling (29-30). Hence, reports on sphingo- and phospholipid endogenous levels are based on quantification of the total amount of a lipid class instead of each lipid species. Moreover, different fatty-acid chain structures

within one lipid class already showed different ionization efficiencies (31). Hence, it is difficult to determine the sensitivity needed to be able to reliably quantify a certain endogenous lipid species. Table 4 gives an indication of the sensitivity and reproducibility obtained for different lipid classes, based on investigation of their IS. The endogenous lipid species, however, might behave differently due to the difference in fatty-acid structures and the presence of different co-eluting lipids from different matrices. As shown in table 4, relative signal intensities could be measured with better reproducibility than absolute intensities. The higher relative signal intensity improved the reproducibility of the analysis, as shown by the relative standard deviation (RSD) of the relative intensities of different lipids in each scan event.

Table 4 also shows big differences in the ionization efficiencies of different lipid classes. Some classes (especially PG and lyso-PA) consistently appeared as the base peaks in the particular scan event. Other lipid classes co-eluted with these lipids might undergo ion suppression; hence their true abundance in biological samples might be undetermined.

Table 4. Reproducibility of lipid standards, expressed as relative standard deviation (RSD) of the peak intensities and relative intensities of the parent ions. Relative intensity is the intensity of a parent ion as compared to the base peak (100%) of a particular scan event.

Scan event*	Internal standard	Exact mass	Concentration (nM)	Intensity (arb)		Relative intensity (%)	
				mean ± SD	RSD	mean ± SD	RSD
1 (1st)	PG(17:0/17:0)	749.533	3.34	2’480 ± 207	8.3%	100.0 ± 0.0	0.0%
	PE(17:0/17:0)	719.547	6.95	584 ± 60	10.2%	24.3 ± 1.5	6.3%
2	PA(17:0/0:0)	423.251	5.91	5’390 ± 1’041	19.2%	100.0 ± 0.0	0.0%
	PG(17:1/0:0)	495.272	4.39	1’392 ± 207	14.9%	26.0 ± 2.0	7.7%
	PE(17:1/0:0)	465.286	8.32	807 ± 60	7.5%	17.3 ± 1.2	6.7%
1 (2nd)	PI(12:0/13:0)	693.376	3.75	806 ± 98	12.2%	100.0 ± 0.0	0.0%
	PS(17:0/17:0)	762.528	6.56	367 ± 13	3.5%	49.0 ± 1.4	2.9%
	PA(17:0/17:0)	675.496	7.40	267 ± 26	9.8%	36.0 ± 3.5	9.6%
3	PC(17:0/17:0)	761.593	6.57	93’200 ± 10’022	10.8%	100.0 ± 0.0	0.0%
	SM(d18:1/12:0)	646.505	7.73	56’667 ± 3’400	6.0%	62.7 ± 2.3	3.7%
	PC(17:0/0:0)	509.348	9.82	5’030 ± 511	10.2%	8.7 ± 0.6	6.7%

* See figure 2

6.3.5. Feasibility studies in patients and healthy volunteers

The developed method has been applied for sphingo- and phospholipid analysis of 3 patients having stable coronary heart disease as well as in 3 healthy volunteers. About 137 lipid species from different lipid classes have been identified (figure 6).

Sphingo- and phospholipids are present ubiquitously and their endogenous levels are influenced by many factors including fasting state, daytime and dietary behavior. Some differences in the concentrations of each lipid species between patients and controls might be caused by interindividual variation, rather than by the disease. Nevertheless, certain diseases have been shown to be associated with distinct fatty-acid side-chain structures (32). Hence, comparison of both the total amount of each lipid class and the concentrations of each lipid species within a given class needs to be performed in order to search for disease-related differences. The developed method has allowed the measurement of not only the total endogenous level of different lipid classes, but also each concentration of the lipid species, thus allowing lipid pattern comparisons between patients and controls.

Multivariate analysis is clearly needed to reveal which lipid species or lipid classes that are consistently different between patients and controls. At present, the number of samples presented here is too small to be subjected to multivariate analysis. Moreover, the resolution of the data needs to be improved to maximize the differences between data clusters, for example by employing accurate mass measurement and incorporating retention time information. Furthermore, well-matched controls as well as stricter exclusion criteria for patients are needed to limit any difference being caused by non-disease related variation and for a better interpretation of the results.

Due to the fact that different fatty-acid chain structures within one lipid class has different ionization efficiencies (31) and that non-endogenous, odd fatty-acid chains, lipid internal standards have been used in this method, the analysis of sphingo- and phospholipids has been only semi-quantitative. Thus, the method provided a screening of the candidate lipids that potentially made the difference between patients and controls. These candidate lipids need to be further analysed, by identifying their distinct molecular structures (from their product-ion spectra in the negative mode) and measuring their absolute concentrations, so that they can be later evaluated in multiple clinical studies.

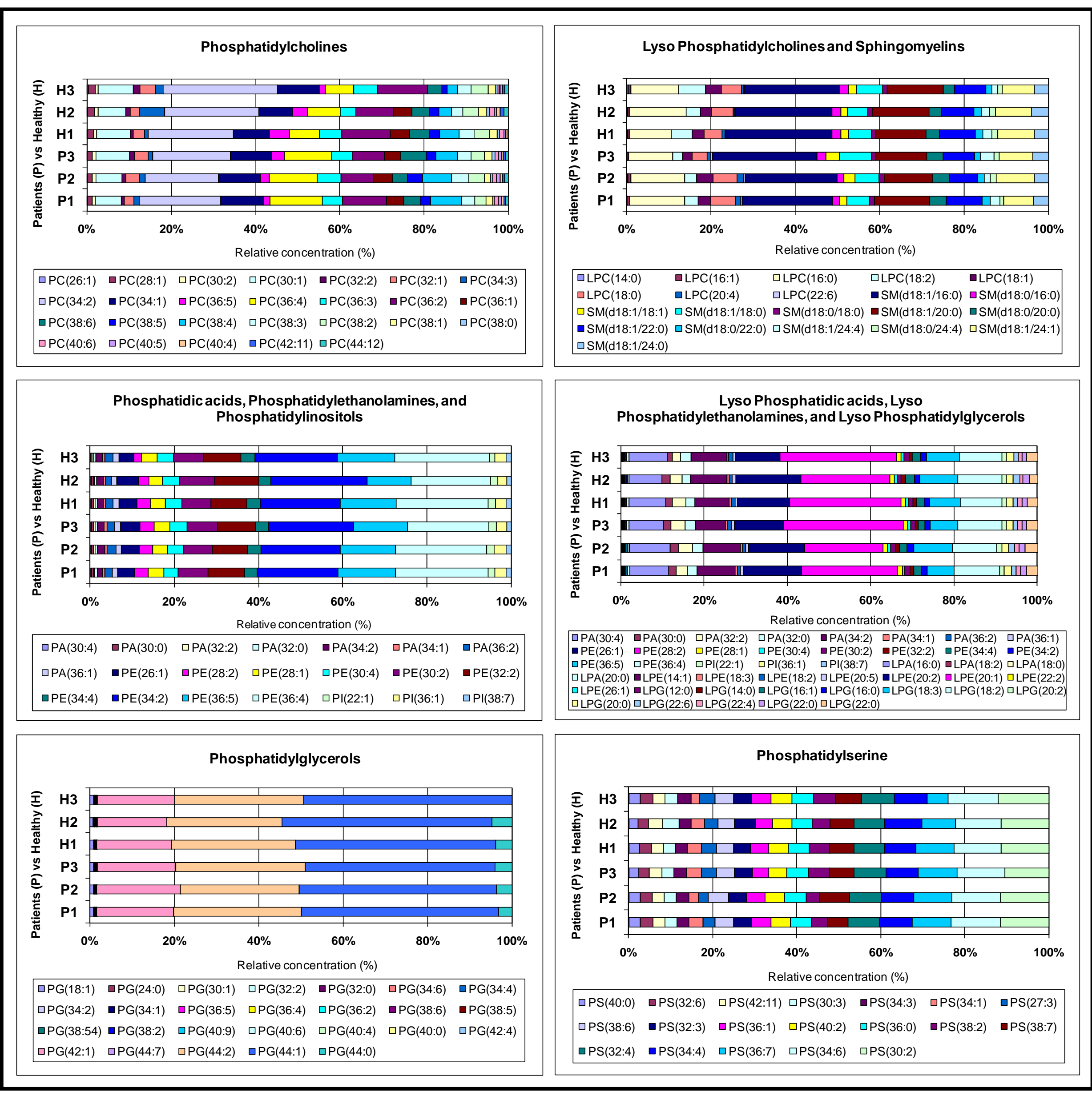

Figure 6. Different patterns of sphingo- and phospholipids in patients with stable coronary heart disease and healthy individuals.

6.4. Conclusions

An LC-MS/MS method combining normal phase HPLC separation and multiple parent-ion scanning on a triple quadrupole mass analyzer has been developed. The method allowed the analysis of both the total endogenous amount of lipids within the same class as well as the concentration of each lipid species within a given class. The method was applied in a feasibility study in patients having stable coronary heart diseases and lipid species from 7 different lipid classes (SM, PG, PE, PA, PI, PS and PC) have been semi-quantified. Improvements of the method are still needed before the method can be applied to large-scale clinical studies. For example: automation of the data processing (lipid identification and semi-quantification) as well as multivariate data analyses. The method provided a screening of candidate lipids potential for further evaluation by more targeted, quantitative analysis.

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7. GENERAL DISCUSSION AND OUTLOOK

7.1. General Discussion

The project has emerged from the need to find markers that can improve the reliability of cardiovascular disease (CVD) prevention. As described in the "Introduction", the current strategies for stratifying patients at risk for CVD have high false positive rates. For instance, the risk prediction from the Munster Heart Study (PROCAM) gives false-positive rates of 68 and 86% for high and intermediate risks, respectively (1). This means that, for example, out of 100 individuals who are diagnosed as having high risk for CVD, 68 persons receive aggressive medications to lower their LDL-cholesterol and blood pressure, while they actually will not develop CVD. Novel biomarkers are therefore needed to optimize the cost-benefit relationship of preventive measures. Moreover, they give a better understanding on the pathophysiology of the disease, leading to better therapeutic interventions in different stages of CVD progression.

Decades of research have revealed that HDL plays an important role in the development of atherosclerosis, the major contributor for CVD (2-4). The cardioprotective effect of HDL has been mainly attributed to its ability to limit cholesterol accumulation in peripheral cells via reverse cholesterol transport (4). Secretion and plasma transport of 27-hydroxycholesterol (27OHC), an oxidized metabolite of cholesterol, has been proposed as an alternative route in the situations of disturbed HDL-dependent reverse cholesterol transport (5-7). However, as has been shown previously, about 50% of plasma 27OHC in normolipidemic population is actually carried by HDL (8). As the beneficial effects of HDL on the cardiovascular system are not limited to its role in reverse cholesterol transport, but include anti-inflammation, anti-oxidative and cytoprotective as well, other candidate biomarkers have also emerged, for example sphingosine-1-phosphate (S1P). Little is known on the determinants of S1P levels in plasma. However, S1P has been suggested as the component responsible for many anti-inflammatory and cytoprotective effects of HDL (9-12).

Atherosclerosis is a multifactorial disease whose progression is determined by many risk factors such as heredity and environment. Thus, our strategy was analyzing the candidate biomarkers in patients having monogenic disorders affecting their HDL metabolism together with their unaffected family members as controls, as well as in genetic animal models with knock-outs of the same genes. For this purpose, a very sensitive LC-MS method with an

atmospheric pressure photoionization (APPI) interface has been developed and validated for quantifying 27OHC in plasma and the HDL fraction. The method was more sensitive than the published LC-APCI-MS or GC-MS methods; enabling us to analyze as little as 15 µL sample volume with lower a limit of quantification (LLOQ) of 40 ng/mL or 4.9 pmol on column.

At a first glance, 27OHC in humans and mice with monogenic disorders of HDL genes seemed to behave similarly as cholesterol, prompting us to question whether measuring 27OHC is not just a laborious way to obtain the same information as with cholesterol measurements. However, normalization of HDL-27OHC levels for HDL-cholesterol levels revealed at least one interesting exception, which was consistent in both genders and gene-dosage dependent, namely mutations in lecithin:cholesterol acyl transferase (LCAT), the enzyme responsible for cholesterol esterification and its packaging into HDL. Our observational study suggested that 27OHC is more sensitive to reduced LCAT activity than cholesterol.

The analysis of S1P in patients with monogenic disorders of HDL metabolism showed an interesting feature: the S1P concentration was reduced in patients having HDL deficiency, while it was not elevated in patients with high HDL-cholesterol levels. Further investigations by stratifying our data and additional experiments indicated the existence of a threshold HDL level which limited the concentration of S1P in plasma. Since some of the atheroprotective effects of HDL are exerted by S1P, this threshold concentration could be the HDL level needed for atheroprotection. This might answer to the current controversial data which shows that increasing HDL-cholesterol level (as targeted by novel HDL-modifying therapies) does not always prevent cardiovascular events (13-14).

As yet, only total plasma concentrations of S1P have been investigated for their association with CVD. At a first glance, there seems to be contrary findings as S1P plasma levels have been shown in one study to increase with the degree of arterial stenosis (15), while another much smaller study has shown that S1P plasma levels were lowered in patients with myocardial infarction (16). It may however be more important to investigate the association of coronary artery disease with HDL-S1P, rather than with total S1P plasma concentrations, also because HDL-associated S1P rather than total-S1P appears to exert cardioprotective effects (9, 11-12).

Because multiple lipid classes and species have been shown to be involved in the pathogenesis of atherosclerosis, profiling of lipids may reveal additional relevant risk factors.

Therefore, a sphingo- and phospholipid profiling project has been started in our institute, in which a method has been developed using a combination of on-line normal phase HPLC separation and multiple parent-ion scanning on a triple quadrupole mass spectrometer. Feasibility has been shown by applying the method for analyzing plasma samples of patients with stable coronary heart diseases. Automation of the data mining and lipid identification is still a problem that hinders the analysis of larger numbers of samples.

As has been described in the "Introduction", different techniques exist for the profiling of lipids, in which either parent-ion or neutral-loss scan experiments is one of the most used approaches to selectively determine lipid classes. Parent-ion or neutral-loss scans on a triple quadrupole mass spectrometer has been usually employed with direct infusion and intra-source separation (17-18). This approach has been applied for comparative profiling of specific sphingo- or phospholipid classes in biological samples (19-21). To minimize ion suppression effects as well as to help resolving some isobaric species, chromatographic separation is better employed prior to the mass spectrometric analysis. An improvement in the separation of different lipids has been shown for example by Houjou *et al*, who utilized an off-line two dimensional separation of normal and reverse phase HPLC (22).

A few publications have shown the application of lipid profiling to investigate the lipid changes in hypertensive and high HDL-cholesterol patients (23-25). One of the most recent studies was reported by the group of Oresic *et al*, who analyzed the lipid composition of HDL from well characterized high and low HDL-cholesterol subjects. The lipid analysis was performed using ultra-performance liquid chromatography (UPLC) separation combined with multiple parent-ion scans (MPIS) technique on the high resolution high accuracy Q-TOF instrument. For the first time, lipid profiling was combined with a dynamic modeling of lipoprotein structure to show the spatial re-distribution of lipids within the HDL particles (25).

Similar to the Indian fable of the "blind men and an elephant", differential approaches are useful to have a more complete picture of the pathophysiology of atherosclerosis. Technological developments in mass spectrometry and bioinformatics have made possible to analyze hundreds of analytes within a single run. However, several problems still wait to be solved; of particular importance is the accurate and reproducible quantification of all components detected. A lesson from the more advanced field of proteomics, for example as nicely reviewed by Nita-Lazar *et al* (26), tells us that accuracy, selectivity and sensitivity are pivotal for a robust and reliable quantification. The impact is that profiling serves as a pre-

screening method for a later more targeted, quantitative analysis. Any findings from a profiling approach should be treated not as a conclusion, but as a novel hypothesis which then should be confirmed by further more specific, well-controlled studies including *in vitro* and animal studies. Decades of merit of the time-consuming targeted analysis should not be forgotten. As has been shown in chapter 3 and 4, targeted analysis has led us to describe specific roles of the compounds of interest in HDL and cholesterol metabolism.

7.2. Outlook: Current Challenges and Next Works

7.2.1. Targeted Lipid Analysis

27OHC has been proposed as an alternative to the HDL-dependent reverse cholesterol transport pathway. However, our observational studies in patients with monogenic disorders of HDL metabolism neither support nor contradict this concept. Changes in plasma and HDL-27OHC in our dyslipidemic patients do not immediately reflect specific changes in the regulation of CYP27, the enzyme responsible for the conversion of cholesterol to 27OHC, in macrophages. This is because 27OHC is also generated in other cells, notably in the liver for an alternative bile acid biosynthetic pathway (27). Measurement of CYP27 enzyme activity in combination with 27OHC levels in plasma will certainly help to understand the regulation of 27OHC and its role in atherogenesis. Furthermore, due to its physiochemical property, 27OHC has been postulated to be excreted from cells independently of transporters and extracellular acceptors (28-29). This assumption needs to be confirmed by *in vitro* studies of the transport of 27OHC from macrophages, with or without the presence of (lipo)-protein acceptors and with specifically expressed transporters or receptors, in order to better interpret the results from the analysis of 27OHC concentrations in plasma.

A previous *in vitro* study by Bjorkhem *et al* has shown that 27OHC is actually not the end product of the CYP27 enzyme (6). It is further oxidized into its carboxylic acid form (3β-hydroxy-5-cholestenoic acid), which is even more hydrophilic and might be more easily transported into the blood circulation than its precursors (27OHC and cholesterol). Since cholesterol, 27OHC and 3β-hydroxy-5-cholestenoic acid altogether compete for the same enzyme and are all transported into the circulation, measuring 3β-hydroxy-5-cholestenoic acid concentrations in plasma may characterize dyslipidemic patients. Moreover, as 27OHC also contributes to the *de novo* bile acid biosynthesis as an alternative precursor, it is also

important to combine the measurement of 27OHC with the analysis of bile acids and their classical precursors (7α-hyxdroxy-4-cholesten-3-one).

Our data suggested a threshold HDL level which limits the plasma concentration of S1P. This may be due to the fact that S1P is transported by specific HDL subclasses or constituents, which are critically lowered in patients with HDL deficiency, but not pronouncedly increased in patients having elevated HDL-cholesterol. For example, the minor HDL protein constituents, apolipoprotein M (apoM), has been suggested to act as a lipocalin (a family of diverse proteins with typical crystal structures which transport small hydrophobic molecules) which binds to S1P on 1:1 molar basis (30). Since apoM has similarly low plasma concentrations like S1P, this may explain the limited abundance of S1P in HDL. Another publication has reported that the S1P content in HDL is elevated in parallel with increasing HDL density such that S1P is enriched in the HDL_3 subclass (31). Thus, it will be interesting to investigate the molecular determinants of S1P uptake and/or transport by specific HDL subclasses.

7.2.2. Lipid Profiling

At present, the actual application of our sphingo- and phospholipid profiling method is still laborious and time-consuming, particularly at the data mining step. The development of software enabling to automatically process the characterization and semi-quantification of the detected lipid species will tremendously help in analyzing a much larger amount of samples. To the best of our knowledge, a software package which can help us completely in all our data mining steps currently does not exist publicly and most research groups develop their own home-made software specific for their types of experiments.

As has been discussed in the "Introduction", several different types of experiments can be done in lipid profiling. Thorough investigations comparing these different analytical methods, particularly in terms of sensitivity and reproducibility, will be useful for the generation of reliable data required for multivariate data analysis. The choice of suitable sample cohorts as well as the developed analytical method(s) should be able to distinguish between interindividual variation and changes caused by the disease for a better biological interpretation. Finally, any outcome needs to be carefully verified and validated by well-

designed targeted studies involving in vitro and animal studies as well as epidemiological or clinical studies.

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CURRICULUM VITAE

Personal Data

1977	Born on January 11 in Surabaya, Indonesia
1983 - 1989	Elementary school in Surabaya, Indonesia
1989 - 1995	Junior and senior high school in Surabaya, Indonesia
1995 - 1999	Studies in Pharmacy, Airlangga University, Surabaya, Indonesia
1999	Graduation with BSc. in Pharmacy
1999 - 2000	Training in Pharmacy, Airlangga University, Surabaya, Indonesia
2000	Graduation with Apt. (apothecary)
2001	Research technician, Pharmaceutical - Biotechnology Laboratory, Faculty of Pharmacy, Airlangga University, Indonesia
2001 - 2004	Master studies in Bio-pharmaceutical Sciences, Division of Analytical Biosciences, Leiden University, The Netherlands Major project: Global screening of human plasma using liquid chromatography with electrochemical detection
2003 - 2004	Research technician, Beyond Genomics, Inc.
2004	Graduation with MSc. in Bio-pharmaceutical Sciences
2004 - 2005	Research assistant for mass spectrometry, Division of Pharmacology, Leiden University, The Netherlands
2006 - 2010	Doctoral candidate, Department of Chemistry and Applied Biosciences, ETH Zürich, Switzerland (Prof. Dr. Renato Zenobi)

Awards

2001 - 2002 Delta Scholarship, Netherlands Organization for International Cooperation in Higher Education (Nuffic)

2002 - 2003 Huygens Scholarship, Nuffic

2001 - 2003 Tuition-fee waiver, Leiden University, The Netherlands

2006 - 2007 Wilhelm Simon Fellowship, CEAC (Center of Excellence in Analytical Chemistry) and ETH Zurich

Publications

1. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2009. Dopant assisted-atmospheric pressure photoionization (DA-APPI) liquid chromatography-mass spectrometry for the quantification of 27-hydroxycholesterol in plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 877: 261-268.

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Selected Oral and Poster Presentations

1. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2006. Development of analytical methods for lipids as biomarkers for cardiovascular disease.

Center of excellence in analytical chemistry (CEAC) winter seminar, November 23, 2006, Zurich, Switzerland. (oral presentation as Wilhelm Simon Fellowship winner)

2. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2008. LCMS-APPI-based method for the analysis of 27-hydroxycholesterol as candidate biomarker for atherosclerosis.

   26th Informal meeting on mass spectrometry (IMMS), May 4-8, 2008, Fiera di Primiero, Italy. (oral presentation)

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   Swiss Medlab, international congress of laboratory medicine and clinical chemistry, September 16-19, 2008, Montreux, Switzerland. (poster)

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   Swiss group for mass spectrometry (SGMS) meeting, November 6-7, 2008, Beatenberg, Switzerland. (oral presentation)

6. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2009. LC-APPI-MS for the Analysis of 27-Hydroxycholesterol as Candidate Biomarker for Atherosclerosis

   23rd Annual meeting of the German atherosclerosis society and 4th joint meeting of the French, Swiss and German atherosclerosis societies, March 12-14, 2009, Blaubeuren, Germany. (poster)

7. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2009. Sphingo- and phospholipidomic approach in the search for biomarkers for atherosclerosis.

   27th Informal meeting on mass spectrometry (IMMS), May 4-7, 2009, Retz, Austria. (oral presentation)

8. Karuna, R., A. von Eckardstein, and K. M. Rentsch. 2009. Sphingo- and phospholipidomic approach in the search for biomarkers for cardiovascular diseases.

   18th International mass spectrometry conference (IMSC), September 2-4, 2009, Bremen, Germany. (poster)

9. Karuna, R., A.G. Holleboom, M.M. Motazacker, J.A. Kuivenhoven, L. Rohrer, K.M. Rentsch and A. von Eckardstein. Sphingosine-1-phosphate in patients with monogenic disturbances of HDL metabolism.

   9th Day of clinical research, April 8, 2010, University Hospital Zurich, Switzerland. (poster)