

# Variation in the human lipidome associated with coffee consumption as revealed by quantitative targeted metabolomics

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**Abbreviations:** KORA, Cooperative Health Research in the Region of Augsburg; MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease; SM(OH), N-hydroxy-acyloylsphingosyl-phosphocholine; SM(OH,COOH), N-hydroxyldicarboacyloylsphingosyl-phosphocholine

**Keywords:** acylcarnitine, coffee, Metabolomics, MS/MS, sphingomyelin

## **ABSTRACT**

The effect of coffee consumption on human health is still discussed controversially. Here we report results from a metabolomics study of coffee consumption, where we measured 363 metabolites in blood serum of 284 male participants of the KORA (Cooperative Health Research in the Region of Augsburg) study population, aged between 55 and 79 years. A statistical analysis of the association of metabolite concentrations and the number of cups of coffee consumed per day showed that coffee intake is positively associated with two classes of sphingomyelins, one containing a hydroxy-group (SM(OH)) and the other having an additional carboxy-group (SM(OH,COOH)). In contrast long- and medium-chain acylcarnitines were found to decrease with increasing coffee consumption. It is noteworthy that the concentration of total cholesterol also rises with an increased coffee intake in this study group. The here observed association between these hydroxylated and carboxylated sphingolipid species and coffee intake may be induced by changes in the cholesterol levels. Alternatively, these molecules may act as scavengers of oxidative species which decrease with higher coffee intake. In summary, we demonstrate strong positive associations between coffee consumption and two classes of sphingomyelins and a negative association between coffee consumption and long- and medium-chain acylcarnitines.

## INTRODUCTION

Coffee is one of the most widely consumed beverages in the world [1]. Roasted, it consists of more than 800 components, including lipids, diterpenes such as cafestol and kahweol, carbohydrates, volatile and nonvolatile acids (e.g. acetic acid, chlorogenic acids), alkaloids such as caffeine, minerals and vitamins (e.g. niacin) [2]. The effects of coffee consumption on human health – beneficial as well as detrimental – are still discussed in the literature: Detrimental effects of coffee intake to health are thought to occur with respect to coronary heart disease: Most case-control studies suggest that the consumption of more than five cups of coffee per day leads to an increased risk for coronary heart disease or myocardial infarction [3-7]. However, the majority of the cohort studies could not confirm this observation [4, 8-12]. Also, the assumption that coffee induces hypertension is only supported by case-control studies [13-16], but not by cohort studies [8, 17-20]. The suspicion that coffee consumption may induce cancer could not be confirmed in several studies [21-23]. In contrast, a positive effect of coffee on cancer could be detected depending on the particular tissue: One meta-analysis and one review reporting the results of several case-control studies as well as of cohort studies show that, in the case of colorectal cancer, an inverse association between this cancer and coffee intake is observed and that, in contrast, cohort studies could not confirm such an association [24, 25]. However, for the hepatocellular carcinoma, the results of case-control as well as of cohort studies yielded a reduced risk of this cancer for heavy coffee drinkers [26-29].

Another potentially beneficial effect of coffee lies in the prevention of diabetes mellitus type 2. Higdon and Frei [8] reported that in six out of nine cohort studies a significant inverse association between coffee consumption and the risk for diabetes type 2 was found. For

example, a study with 1,000 Finnish twins found that the intake of more than seven cups of coffee per day reduces the risk of diabetes by 35% compared to a consumption of at most two cups per day [30]. An even larger effect, namely a decrease of 50%, was observed in a study with 17,000 Dutch individuals, again when more than seven cups of coffee per day were ingested [31]. Although an improvement of the glucose tolerance or of the insulin sensitivity by coffee consumption could not be determined in short-term trials, several cohort studies indicate that coffee consumption supports the maintenance of a normal glucose tolerance [32-35]. Coffee also seems to have a positive effect on the prevention of Parkinson's Disease [36-39]. Moreover, coffee consumption appears to lower the suicide risk [40-42] as well as the risk for dementia [43]. These studies demonstrate that coffee has many diverse effects on human health. However, it should be taken into account that some of these effects may also be explained by common, not yet identified covariates that may be traced back to general life style factors. Thus, more research in this field is needed.

In this study, we took a targeted quantitative metabolomics approach to detect coffee-induced changes on human metabolism. Targeted metabolomics aims at the quantitative measurement of ideally all key metabolites in a body fluid, thereby representing a snapshot of the full metabolic state of the organism. Changes in the metabolic profiles can be caused for example by specific food intake, disease or medication. Such changes can be detected in a very detailed manner using metabolomics techniques, so that the affected pathways may eventually be identified. This aim is now coming into reach with recent advances in the field of high resolution electrospray ionization tandem mass spectrometry. Here, we use this technology to determine the metabolic profiles of 284 participants from the KORA (Cooperative Health Research in the Region of Augsburg) study population [44]. Information on many different factors concerning life style (e.g. smoking and alcohol consumption), disease state or

medication use are available for each participant. These factors and their association with metabolic changes are presently analyzed in a number of separate studies which are based on this metabolomics dataset. Some of which have already been published for the association with nicotine consumption [45] and genetic variation [46]. The results from these studies also provide some kind of independent validation of this metabolomics dataset. In the present study we specifically examined the consequences of coffee consumption on the human metabolism.

## **MATERIAL & METHODS**

### *Experimental Setup*

KORA is a research platform for population-based surveys and subsequent follow-up studies in the fields of health economics, epidemiology, genetics and health care research [44]. It provides a multitude of different parameters, such as life style factors (nutrition, physical activity, smoking, alcohol consumption, etc.), sociodemographic variables, and medical history. The dataset presented here comes from the F3 study conducted in 2004-2005 as a follow-up of the third MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) survey (S3; 1994/1995). For this F3 study, 3006 of the 4856 participants of the S3 were reexamined for a second time, thus, 10 years apart from the baseline examination. From this group, 284 male individuals, aged between 55 and 79 years at the examination for F3, were chosen randomly for our analysis and were again recruited one to two years later for additional blood sampling. Out of this group, 239 of these individuals provided details concerning their coffee consumption (only participants younger than 75 were asked for this information). 82 participants (~34.3%) drank one to two cups of coffee per day, 100

individuals (~41.9%) had three to seven cups/day, twelve (~5.0%) regularly drank eight and more cups/day, and 45 (~18.8%) totally abstained from coffee consumption. As a control, information on the consumption of green tea and black tea was also analyzed. The study design was approved by an ethical committee and the informed consent of all participating subjects was obtained.

### ***Blood samples***

Blood samples for metabolic analysis were collected during 2006. To avoid variation due to circadian rhythm, blood was drawn in the morning between 8 and 10 am after a period of overnight fasting. Material was immediately horizontally shaken (10 min), followed by 40 min resting at 4°C to obtain complete coagulation. The material was then centrifuged (2000g; 4°C). Serum was aliquoted and stored for 2-4 hours at 4°C, after which it was deep frozen to -80°C until mass spectrometry analysis.

### ***Metabolite profiling***

Targeted metabolite profiling by electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was performed at *Biocrates life sciences AG*, Austria. The technique is described in detail by patent US 2007/0004044 (accessible online at <http://www.freepatentsonline.com/20070004044.html>). A summary of the method can be found in ([47]; [48]). A comprehensive overview of the field and the related technologies is given in the review paper by Wenk [49]. Briefly, the assay preparation was done by an automated robotics system (Hamilton Robotics GmbH) on a special double-filter 96 well plate containing isotope labeled internal standards. Assays used 10µl plasma and include Phenylisothiocyanate (PITC)-derivatisation of amino acids, extraction with organic solvent and several pipetting, drying, and centrifugation steps. Flow injection analysis coupled with

multiple reaction monitoring scans (FIA MS/MS) on a 4000 QTrap instrument (Applied Biosystems) was used for quantification of amino acids, acylcarnitines, sphingomyelins, phosphatidylcholines, and hexoses. LC-MS/MS methods using multiple reaction monitoring, neutral loss and precursor ion scans were applied for biogenic amines, eicosanoids and hydroxylated fatty acid derivatives as well as for intermediates of the energy metabolism. The quantification of the metabolites is achieved by reference to appropriate internal standards. The method is proven to be in conformance with 21CFR (Code of Federal Regulations) Part 11, which implies proof of reproducibility within a given error range. This measurement platform has been used in the past in different academic and industrial applications [45, 46, 50-52].

### ***Metabolite spectrum***

Concentrations of all analyzed metabolites are reported in  $\mu\text{M}$ . In total, 363 different metabolites were screened and detected in plasma: 18 amino acids, nine reducing mono-, di- and oligosaccharides (abbreviated as H $n$  for  $n$ -hexose, dH for desoxyhexose, UA for uronic acid, HNAc for N-acetylglucosamine), 21 acylcarnitines (C $x$ : $y$ , where  $x$  denotes the number of carbons in the side chain and  $y$  the number of double bonds), seven hydroxylacylcarnitines (C(OH) $x$ : $y$ ) and dicarboxylacylcarnitines (C $x$ : $y$ -DC), free carnitine (C0), seven biogenic amines, seven prostaglandins and 293 lipids. These lipids are subdivided into 14 different ceramides (Cer) and glucosylceramides (GlcCer), 71 different sphingomyelins (SM $x$ : $y$ ) and sphingomyelin-derivatives, such as N-hydroxyldicarboacyloylsphingosyl-phosphocholine (SM(OH,COOH) $x$ : $y$ ) and N-hydroxylacyloylsphingosyl-phosphocholine (SM (OH) $x$ : $y$ ), five glycerophosphatidic acids (PA), 124 glycerophosphatidylcholines (PC), 42 glycerophosphatidylethanolamines (PE), four phosphatidylglycerols (PG), 30 glycerophosphatidylinositols (PI) and glycerophosphatidylinositol-bisphosphate (PIP2), and three

glycero-phosphatidylserines (PS). Glycero-phospholipids are further differentiated with respect to the presence of ester (*a*) and ether (*e*) bonds in the glycerol moiety, where two letters (*aa*, *ae*, or *ee*) denote that the first as well as the second position of the glycerol unit are bound to a fatty acid residue, while a single letter (*a* or *e*) indicates a bond with only one fatty acid residue. E.g. PC *ae* 33:1 denotes a plasmalogen phosphatidylcholine with 33 carbons in the two fatty acid side chains and a single double bond in one of them. In some cases, the mapping of metabolite names to individual masses can be ambiguous. For example, stereochemical differences are not always discernable, neither are isobaric fragments.

### ***Statistical analysis***

The statistical analysis system R (<http://www.r-project.org/>) and SPSS for Windows (Version 16.0, Chicago: SPSS Inc.) were used for the statistical analysis. For the identification of metabolites influenced by coffee consumption, a Kendall's test was applied to each variable (metabolite concentration). Kendall's test is a non-parametric test that calculates the correlation of two variables based on the rank of their data. This test was chosen as it does neither require a linear correlation of the variables nor a normal distribution of the data. To control for the effect of testing multiple hypotheses, the positive false discovery rate (q-value) was computed, which is a measure for the fraction of false positives appearing even if the test itself was significant [53]. The rank correlation coefficient Kendall's tau was determined for every metabolite to indicate the direction of the correlation.



## RESULTS

We analyzed the metabolic dataset to identify those metabolites that are most strongly associated with coffee consumption. The results of the Kendall tests are given in *Table 1*. Most remarkable is the association of two sphingomyelin classes (SM(OH,COOH)<sub>x:y</sub> and SM(OH)<sub>x:y</sub>) with coffee intake, with p-values ranging between  $7.5 \times 10^{-4}$  and  $2.4 \times 10^{-5}$ . The Kendall q-value confirms a very low false positive rate (smaller than 2.5%). The positive values of the Kendall's tau for all sphingomyelins listed in Table 1 suggest that the sphingomyelins species SM(OH,COOH)<sub>x:y</sub> and SM(OH)<sub>x:y</sub> positively correlate with coffee intake. In order to further test this hypothesis, we calculated the sum of the concentrations of all SM(OH,COOH)<sub>x:y</sub> as well as the sum of the concentrations of all SM(OH)<sub>x:y</sub> and applied the Kendall's test to each. The sums of these sphingomyelin classes also correlate positively with coffee consumption, as shown in Figures 1 and 2.

Besides the sphingomyelins, also long- and medium-chain acylcarnitines showed low p-values concerning the association to the coffee intake (*Tab.1; Supplemental Tab.1*). However, this association concerns mainly the two groups of coffee drinkers consuming 1-2 cups/day and 3-7 cups/day respectively. For the individual long- and medium-chain acylcarnitines as well as for the sum of all acylcarnitines with a chain length of at least 10, the Kendall's tau yielded negative values. Thus, the coffee consumption and the plasma concentration of long- and medium-chain acylcarnitines are inversely associated.

From the remaining metabolites none was associated with coffee consumption with a positive False Discovery Rate (pFDR; q-value) lower than 5%. As a positive control, showing that null findings within this rest of the metabolites cannot be attributed to a potentially poor data quality, we note the identification of a number of very significant associations in other studies concerning different metabolites from this same metabolomics dataset, such as different

phosphatidyl-choline, -ethanolamine and -inositol species, as well as amino acids and short and medium chain length acyl carnitines, with p-values down to  $10^{-21}$  as documented by [46] and [45]. As a negative control, the effect of green tea as well as that of black tea on the presented set of metabolites was analyzed. For neither kind of tea a significant correlation with any metabolite could be found, especially all q-values were larger than 0.1. This fact emphasizes the significance of the observed associations between the coffee consumption and the two reported sphingomyelin species.

## **DISCUSSION**

In this paper, we analyzed the influence of coffee consumption on the metabolism of 239 male participants of the KORA project. The measurement of 363 metabolites by tandem mass spectrometry provides a deeper insight into changes in the metabolic state caused by coffee intake. Humans are exposed to many different environmental influences, such as various nutrition, physical activity or medication. Therefore, it is remarkable that three groups of metabolites clearly associated with coffee consumption could be identified in this study. Among these metabolites are the long- and medium-chain acylcarnitines, whose concentrations decrease with rising coffee consumption. One explanation might be the effect of niacin, which is a component of coffee [54]. Niacin lowers the concentration of triglycerides and free fatty acids in the plasma [55-59]. A reduced concentration of these triglycerides and free fatty acids causes a decrease of the concentration of acylcarnitines, which are synthesized during the transport of fatty acids into the mitochondrium for  $\beta$ -oxidation. Thus, an increase of fat storage could be a consequence.

The sphingomyelin classes  $SM(OH,COOH)_{x:y}$  and  $SM(OH)_{x:y}$  showed a positive association to coffee intake. The synthesis of sphingomyelin is located in the golgi apparatus and is based on the transfer of the phosphorylcholine from a phosphatidylcholine to a ceramide [60, 61]. A ceramide consists of a sphingosine and a fatty acid that is added to the C2 of the sphingosine by N-acetylation [62].  $SM(OH)_{x:y}$  is formed by adding an  $\alpha$ -hydroxylated fatty acid [63]. For  $SM(OH,COOH)_{x:y}$  this fatty acid additionally carries a carboxyl group, presumably at the  $\omega$ -position (Figure 3). In contrast to  $SM(OH)_{x:y}$ , the sphingomyelin class  $SM(OH,COOH)_{x:y}$  is not described in much detail in the literature. In principle, there are two mechanistic explanations for the biogenic formation of this molecule: Either an unsaturated fatty acid chain in a sphingomyelin is oxidized, or an existing  $\alpha$ -hydroxy-1, $\omega$ -dicarboxyl fatty acid is added to the C2 position of the sphingosine.  $\alpha$ -hydroxy-1, $\omega$ -dicarboxyl fatty acids have been found in the cell wall of *Legionella* in a study by Sonesson *et al.* [64]. An alternative explanation for the formation of  $SM(OH,COOH)_{x:y}$  might be an oxidative attack on a double bond of the fatty acid moiety. During this process the carboxyl group might be built at the  $\omega$ -position. Hence,  $SM(OH,COOH)_{x:y}$  could function as a scavenger and might therefore reduce the risk of oxidative damage leading for instance to cancer. Sargis and Subbaiah [65] reported that sphingomyelins are able to protect cholesterol from oxidation, but the exact mechanism is still unknown and requires further investigation.

Sphingomyelins are mainly located in biological membranes, including the myelin sheath of nerve cell axons. These membranes are mainly composed of different lipids, proteins and further components such as cholesterol, which interacts with phospholipids to stabilize them and thus supports their ability to build membranes. Among the phospholipids sphingomyelins show the strongest interaction with cholesterol [66, 67]. Moreover the analysis of Gronberg *et*

*al.* [67] showed that cholesterol increases the packing of SM(OH)<sub>x:y</sub> in the membrane. Both, SM(OH,COOH)<sub>x:y</sub> and SM(OH)<sub>x:y</sub>, were positively associated with coffee consumption in our study. This could be linked to an increased cholesterol level in plasma. It is known, that cholesterol stabilizes and increases the packing of sphingomyelins and, hence, supports the formation of membranes. In course of the KORA project, blood parameters, including cholesterol, have been determined for each participant. Our analysis showed that total cholesterol increases with coffee consumption in this study group (Kendall p-value:  $1.1 \cdot 10^{-4}$ ; Kendall's tau: 0.165, Figure 4). Using data from the entire F3-population indicates that this positive association is still significant after adjustment for age and sex (data not shown). Based on this observation, and the described function of cholesterol, one may, thus, speculate that the increased cholesterol levels and the increased SM(OH,COOH)<sub>x:y</sub> and SM(OH)<sub>x:y</sub> are functionally related.

Whether the intake of coffee really increases the concentration of cholesterol is still discussed in literature. In the 1980s, evidence was provided that coffee consumption causes a higher concentration of cholesterol in blood [68]. But the subsequent studies found that this effect is based mainly on the diterpenes cafestol and kahweol, which are only present in unfiltered coffee [69-71]. Thus, this would indicate that there is no association between coffee consumption and cholesterol. This aspect is important, since the people in southern Germany (location of the KORA project) mainly drink filtered coffee. Exactly the opposite result, a positive association of filtered coffee intake with the concentration of cholesterol in blood, was reported by the studies of [72], [73], [74] and [75]. So we can conclude that there are indicators for this positive correlation between filtered coffee and cholesterol, but still more evidence is needed.

An additional aspect worth considering is the association of sphingomyelin with atherosclerosis. Since decades it is known, that sphingomyelin accumulates in atheromas [76]. Recent studies have confirmed the association of atherosclerosis with an increased sphingomyelin level in animals as well as in humans [77-80]. In this context one might speculate that coffee consumption heightens the risk of atherosclerosis by increasing the levels of the two resulting classes of sphingomyelins.

Concerning these sphingomyelins, an interesting association was identified in a genome-wide association study. In this study, the above discussed (coffee-associated) sphingomyelin species are found to be strongly associated with a genetic variant (SNP) in the PLEK gene. The PLEK gene codes for pleckstrin, a protein that has been proposed to facilitate protein/lipid interactions and to affect membrane structure. It is supposed to enable the localization to the cell membrane by acting as a signal adapter molecule. Ma *et al.* [81] report, that pleckstrin associates with human platelet membranes and supports the formation of membrane projections from transfected (Cos-1) cells. Pleckstrin is recruited into natural killer cell membranes, when these cells are activated [82], and it associates with the membranes of stimulated macrophages [83]. This observation raises the question, whether coffee consumption and variation in the PLEK gene may have an interacting effect on the level of the described sphingomyelins, and possibly related medical outcomes.

Our results demonstrate that the quantitative measurement of more than 360 metabolites by tandem mass spectrometry may detect unexpected correlations in human metabolism. Especially the influence of coffee on the two classes of sphingomyelins is a remarkable result of our analysis. This could be a starting point for further research on the function of these sphingomyelins.

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## **AUTHOR DISCLOSURES**

K.M.W. is an employee of Biocrates Life Sciences AG. This private company offers products and services in the field of targeted quantitative metabolomics research. The other authors have nothing to declare.

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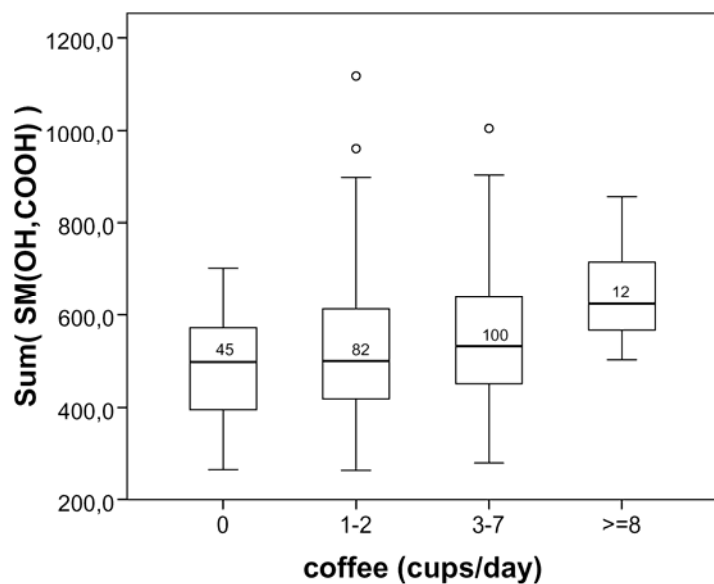
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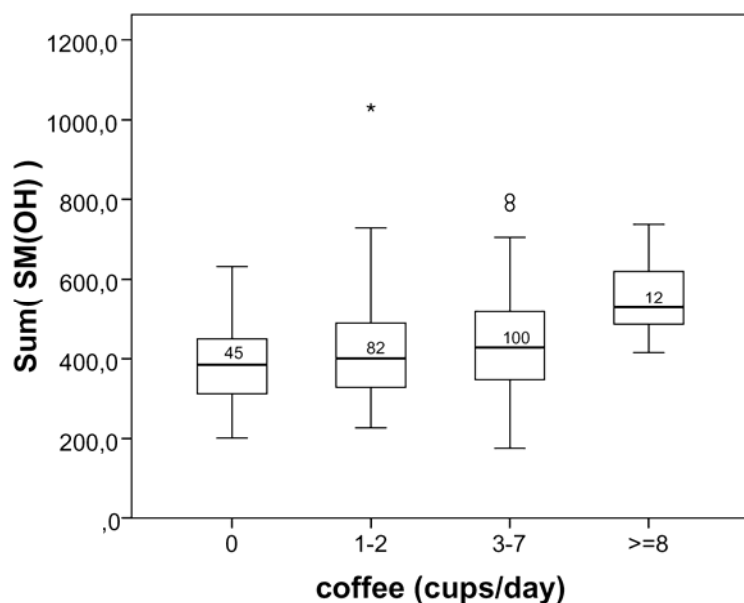
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## FIGURES AND TABLES

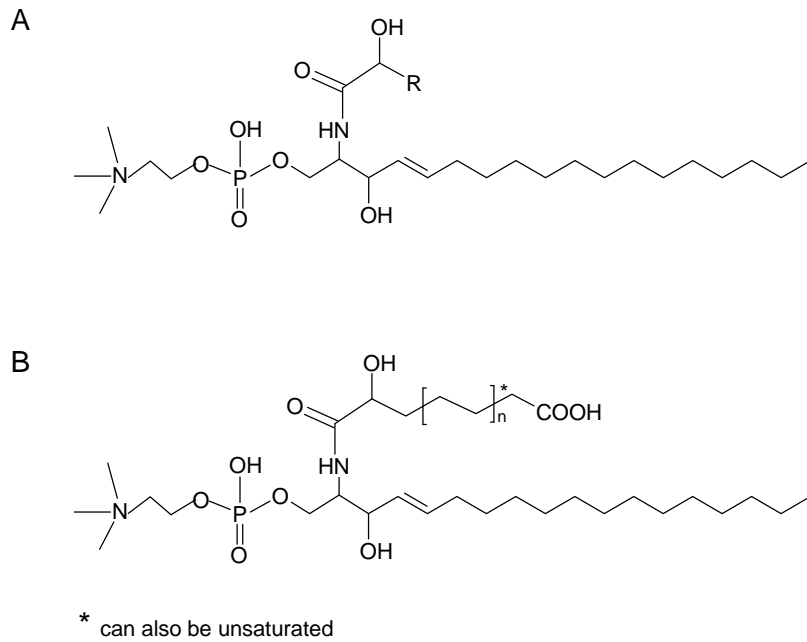


**FIGURE 1:** Boxplots of the plasma concentrations [ $\mu\text{M}$ ] of the sum of all sphingomyelin species SM(OH,COOH)<sub>x:y</sub> as a function of coffee consumption. The Kendall p-value for the association is  $4.9 \times 10^{-5}$ . Boxes extend from 1<sup>st</sup> quartile (Q<sub>1</sub>) to 3<sup>rd</sup> quartile (Q<sub>3</sub>); median is indicated as a horizontal line; whiskers are drawn to the observation that is closest to, but not more than a distance of  $1.5(Q_3 - Q_1)$  from the end of the box. Observations that are more distant than this are shown individually on the plot. The number of individuals in each group is given in the boxes.

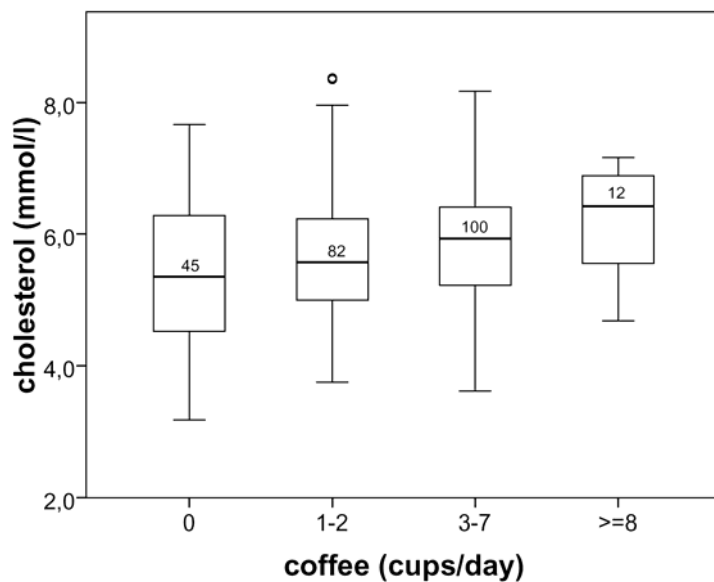


**FIGURE 2:** Boxplots for the sphingomyelin species SM(OH)<sub>x:y</sub> (legend see Fig. 1). The Kendall p-value for the association is  $1.0 \times 10^{-4}$ .





**FIGURE 3:** Assumed structure of the sphingomyelin species SM(OH) $x$ : $y$  (A) and SM(OH,COOH) $x$ : $y$  (B).



**FIGURE 4:** Boxplots of the plasma concentrations of total cholesterol levels (legend see Fig. 1). The Kendall p-value for the association is  $1.1 \times 10^{-4}$ .

**Table 1:** Results of the Kendall rank test, with p- and q-value as well as the Kendall’s tau; all tested metabolites yielding a Kendall p-value smaller than  $10^{-3}$  and a q-value smaller than  $3 \times 10^{-2}$  are listed. The sums of SM(OH,COOH) $x:y$ , of SM(OH) $x:y$  and of all acylcarnitines C $x:y$  with chain length greater or equal to ten are indicated in italics. In cases where alternative assignments of the metabolites are possible, these are indicated by a ‘\*’.

metabolites	mean	standard deviation	Kendall p-value	Kendall q-value	Kendall’s tau
	$\mu M$	$\mu M$			
SM (OH,COOH) 20:2	64.741	22.394	2.4E-05	3.1E-03	0.179
SM (OH,COOH) 16:2 *	84.938	26.854	2.6E-05	3.2E-03	0.179
SM (OH) 20:3	172.874	53.820	4.2E-05	4.2E-03	0.174
C16:1	0.036	0.014	4.3E-05	4.2E-03	-0.174
<i>Sum(SM(OH,COOH)<math>x:y</math>)</i>	528.757	149.989	4.9E-05	2.2E-03	0.172
C10:1	0.166	0.075	9.8E-05	6.8E-03	-0.165
<i>Sum(SM(OH)<math>x:y</math>)</i>	423.809	126.783	1.0E-04	3.0E-03	0.165
SM (OH,COOH) 18:2	92.451	30.334	1.1E-04	7.3E-03	0.164
C12:1	0.122	0.054	1.3E-04	7.9E-03	-0.163
C14:1	0.126	0.064	1.4E-04	8.3E-03	-0.162
SM (OH) 22:1	44.567	17.038	1.4E-04	8.5E-03	0.162
C14:2	0.052	0.024	1.7E-04	9.9E-03	-0.159
<i>Sum(C<math>x10:y</math>)</i>	1.463	0.510	1.9E-04	3.3E-03	-0.159
SM (OH,COOH) 24:0	20.928	8.206	2.2E-04	1.2E-02	0.157
SM (OH,COOH) 18:1	19.272	6.467	4.0E-04	1.7E-02	0.150
SM (OH) 28:0	59.325	22.334	5.0E-04	1.9E-02	0.148
C6	0.089	0.036	7.5E-04	2.5E-02	-0.143