Published in final edited form as:

J Alzheimers Dis. 2015 ; 44(4): 1193–1201. doi:10.3233/JAD-142319.

Sphingomyelin SM(d18:1/18:0) is Significantly Enhanced in Cerebrospinal Fluid Samples Dichotomized by Pathological Amyloid- β_{42} , Tau, and Phospho-Tau-181 Levels

Therese Koal^a, Kristaps Klavins^a, Daniele Seppi^a, Georg Kemmler^b, and Christian Humpel^{b,*}

^aBIOCRATES Life Sciences AG, Innsbruck, Austria

^bDepartment of Psychiatry and Psychotherapy, University Clinic of General and Social Psychiatry, Medical University of Innsbruck, Innsbruck, Austria

Abstract

Alzheimer's disease (AD) is a severe and chronic neurodegenerative disorder of the brain. The laboratory diagnosis is limited to the analysis of three biomarkers in cerebrospinal fluid (CSF): amyloid- β_{42} ($A\beta_{42}$), total tau, and phospho-tau-181 (P-tau-181). However, there is a need to find more biomarkers in CSF that can improve the sensitivity and specificity. The aim of the present study was to analyze endogenous small metabolites (metabolome) in the CSF, which may provide potentially new insights into biochemical processes involved in AD. One hundred CSF samples were dichotomized by normal ($n = 50$) and pathological decreased $A\beta_{42}$ and increased tau and P-tau-181 levels ($n = 50$; correlating to an AD-like pathology). These CSF samples were analyzed using the Absolute IDQ° p180 Kit (BIOCRATES Life Sciences), which included 40 acylcarnitines, 21 amino acids, 19 biogenic amines, 15 sphingolipids, and 90 glycerophospholipids. Our data show that two sphingomyelins (SM (d18:1/18:0) and SM (d18:1/18:1)), 5 glycerophospholipids (PC aa C32:0, PC aa C34:1, PC aa C36:1, PC aa C38:4 and PC aa C38:6), and 1 acylcarnitine (C3-DC-M/C5-OH) were significantly altered in the CSF with pathological "AD-like pathology". Sphingomyelin SM (d18:1/18:0) proved to be a specific (76%) and sensitive (66%) biomarker with a defined cut-off of 546 nM. Correct diagnoses for 21 out of 32 unknown samples could be achieved using this SM (d18:1/18:0) cut-off value. In conclusion, the sphingolipid SM (d18:1/18:0) is significantly increased in CSF of patients displaying pathological levels of $A\beta_{42}$, tau, and P-tau-181.

Keywords

Alzheimer's disease; cerebrospinal fluid; diagnosis; liquor; metabolomics; sphingolipids; SM(d18:1/18:0)

*Correspondence to: Dr. Christian Humpel, Department of Psychiatry and Psychotherapy, Anichstrasse 35, A-6020 Innsbruck, Austria. Tel.: +43 512 504 23712; Fax: +43 512 504 23713; christian.humpel@i-med.ac.at.

Authors' disclosures available online (<http://www.j-alz.com/disclosures/view.php?id=2606>).

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease of the brain that gradually leads to severe cognitive impairment. The neuropathological hallmarks of AD include amyloid- β ($A\beta$) plaques, neurofibrillary tau tangles, inflammation and glial responses, synapse loss, and cholinergic neurodegeneration. The diagnosis of possible or probable AD requires according to the current NINCDS-ADRDA criteria both the presence of cognitive impairment established by clinical and neuropsychological examination and the absence of other psychiatric or neurological diseases. Currently, the diagnosis of AD within the clinical routine is based on a time consuming combination of psychological testing, imaging, and the analysis of three well-established biomarkers in the cerebrospinal fluid (CSF) [1]. In the CSF of AD patients, $A\beta$ with 42 amino acids ($A\beta_{42}$) is reduced, whereas the tau protein (total tau) is increased. A more specific analysis can be obtained by measuring phosphorylated tau-181 (P-tau-181) [1-3]. However, there is an important need to discover new and more selective and specific biomarkers in CSF.

The brain has an extraordinarily high rate of metabolism [4] and it consumes 20% of the oxygen, although it accounts for only 2% of the body's weight. The brain's metabolic rate is so high because neurons need energy (ATP) to maintain the ion gradients between the cell membranes and for neurotransmission. Since the neuronal ATP is made available by the oxidative metabolism, the neurons are strongly dependent on mitochondrial function and the production of oxygen [5]. As early as 1987, it was suggested that AD is associated with metabolic disturbances [6, 7]. In parallel, mitochondrial dysfunction, stress, and an altered energy metabolism may cause changes in the nucleotides, citrate cycle, energy transfer, carbohydrates, neurotransmitters, and amino acid metabolic pathways in AD [5, 8, 9]. Further, a defect in glucose metabolism can contribute to the development of AD [10]. These metabolic changes may be very useful to identify novel biomarkers. In fact, a recent paper showed that plasma phospholipids and acylcarnitines, as marker of cell membrane integrity, may be very sensitive to predict phenocconversion to either amnesic mild cognitive impairment or AD within a 2–3 year timeframe with over 90% accuracy [11].

Modern analytical techniques can provide fast and efficient screening of thousands of genes by gene arrays or proteins by proteomics. In this respect, the analysis of the CSF metabolome can provide potentially new insights into biochemical processes into many brain disorders, including AD. The CSF metabolome database consists of more than 300 detectable endogenous metabolites with extensive information [12-14]. The composition of CSF is dependent upon the metabolic production rates in the brain, the metabolite concentrations in the blood, and transport processes at the blood-CSF barrier. The CSF metabolome analysis provides biochemical insights into central nervous system disorders and could result in the identification of biomarkers for disease, disease progression, or response to therapy [13, 15, 16]. The aim of the present study was to analyze the metabolome in CSF samples dichotomized by normal and pathological decreased $A\beta_{42}$ and enhanced tau and P-tau-181 levels (correlating to an AD-like pathology; we refer in the following to this term). We will quantitate 40 acylcarnitines metabolites, 21 amino acids, 19 biogenic amines, 15 sphingolipids, and 90 glycerophospholipids using the AbsoluteIDQ[®] p180 Kit (BIOCRATES Life Sciences AG).

MATERIALS AND METHODS

Grouping of CSF samples

It is well known that AD patients display reduced A β ₄₂ levels (<500 pg/mL), enhanced total tau (>550 pg/mL), and increased P-tau-181 levels (>60 pg/mL) [3]. Only the analysis of all three markers yields sensitivity and specificity of >90%. CSF samples were dichotomized by normal ($n = 50$) and pathological decreased A β ₄₂ and increased tau and P-tau-181 levels ($n = 50$; correlating to an AD-like pathology) (see Table 1). Only samples from patients >70 years were included. Samples were selected initially frozen in the years 2012, 2011, and 2010. In addition, 32 unknown CSF samples were included to verify the new diagnostic marker (blinded analysis). Since all samples have been processed for routine analysis for AD diagnosis in Austria as left-over samples, no clinical data are available for these individuals. Thus this study is a retrospective study.

CSF samples for analysis of A β ₄₂, tau, and P-tau-181

CSF was obtained by lumbar puncture in polypropylene tubes and frozen at -80°C maximal two days after collection. CSF was routinely analyzed for A β ₄₂, total tau, and P-tau-181 using the internationally established ELISA reference technique [3] from Innogenetics (NV, Gent, Belgium; now FujiRebio, Japan). The analysis was performed as described in detail in the manual.

CSF sample metabolome analysis

The endogenous metabolites were analyzed with a targeted quantitative and quality controlled metabolomics approach using the AbsoluteIDQ[®] p180 Kit (BIOCRATESLife Science AG, Innsbruck, Austria). This validated assay allows the comprehensive identification and the quantification of a large number of endogenous metabolites, including amino acids, acylcarnitines, biogenic amines, phospho- and sphingolipids (phosphatidylcholines, lyso-phosphatidylcholines, sphingomyelins). The CSF metabolome have been already characterized in detail using this kit assay [14, 17]. Briefly, the frozen CSF samples (-80°C) were thawed on ice, then vortexed and centrifuged at $10,000 \times g$. Due to the lower concentration of most metabolites in CSF compared with plasma, 30 μL CSF sample volume was required instead the standard volume of 10 μL used for plasma. Volume higher than 30 μL of CSF samples is not recommended due to the significant increase of matrix effects in the mass-spectrometry based analysis. The filter paper in the upper 96 well-plate of the kit has a limited absorption capacity, therefore the 30 μL of samples was added in two steps - 15 μL each. The filter spot was dried for 30 min under the nitrogen flow after each step. Afterwards the CSF samples were processed as described in the AbsoluteIDQ[®] p180 Kit user manual. In brief, 50 μL of a 5% v/v solution of phenyl-isothiocyanate was added for derivatization (of amino acids and biogenic amines). After incubation, the filter spots were dried again under the nitrogen flow. Extraction of the metabolites was then achieved by adding 300 μL of 5 mM ammonium acetate solution in methanol. The plate was centrifuged and the extract was collected into the pre-installed lower 96 deep capture well-plate. The extract for HPLC-MS/MS analysis was prepared by pipetting 150 μL of the original extract into a second 96-deep well plate and diluting it with 150 μL of HPLC grade water. 400 μL of MS running solvent was added to the remaining extract in the primary 96-

deep well plate for the flow injection analysis (FIA-MS/MS). Both prepared sample extract plates were subsequently analyzed with FIA-MS/MS and HPLC-MS/MS using a 4000 QTRAP® System (AB Sciex) triple quadrupole mass spectrometer operating in MRM mode combined with Agilent 1200 binary HPLC pump. FIA-MS/MS analysis (both in positive and negative ionization mode) was carried out for the identification and quantitation of hexose, acylcarnitines, phospho- and sphingolipids. HPLC-MS/MS was used for the quantitative analysis of amino acids and biogenic amines. MetIDQ® software (part of the AbsoluteIDQ® p180 Kit, BIOCRATES Life Sciences AG, Innsbruck, Austria) was used to supervise the whole kit work flow. This comprises among others the quantification of the metabolite concentrations in the FIA-MS/MS analysis, the data compilation of FIA-MS/MS and HPLC-MS/MS, the analytical data evaluation, and the basic statistical analysis.

Statistical analysis

Analysis of individual markers—As a large number of potential markers was tested, Bonferroni corrected *p*-values were calculated in addition to the raw *p*-values. The ability of the individual laboratory parameters to discriminate between diagnostic groups was tested by Student's *t*-test. Parameters tested were SM (d18:1/18:0), PC aa C32:0, PC aa C36:1, PC aa C34:1, PC aa C38:4, PC aa C38:6, SM (d18:1/18:1), and C3-DC-M/C5-OH. Laboratory parameters with a skewed distribution (skewness >1 or <-1) were subjected to an appropriate “normalizing” transformation, e.g., log transformation, prior to the analysis.

Prediction of group membership by logistic regression analysis—Multiple logistic regression with forward stepwise variable selection was used in order to identify those laboratory parameters which best predict group separation. A second analysis with backward stepwise selection was done for “normal CSF”. As both analyses yielded different results, the findings of both analyses are shown. The predicted probabilities obtained from logistic regression were entered into a receiver operating characteristic (ROC) analysis to obtain estimates of the sensitivity and specificity and of the area under the ROC curve (AUC). Optimal cut-off levels were determined such that the sum of sensitivity and specificity was maximized. Corrected estimates of sensitivity and specificity were obtained using leave-one-out cross-validation [18].

RESULTS

CSF metabolome analysis

In a first screening CSF samples from the years 2010–2012 were analyzed. Three sphingolipids, 13 glycerophospholipids, 3 acylcarnitines, 2 amino acids, and one biogenic amine were identified to be altered (Table 2). However, when the data were pooled only 2 sphingolipids, 7 glycerophospholipids, and one acylcarnitine was still significant between controls and AD patients (Tables 2 and 3; Fig. 1). Out of those 10 metabolites, only 3 were highly significantly different ($p < 0.0001$): SM (d18:1/18:0), PC aa C32:0 and PC aa C34:1 (Table 3). Analysis of the log-transformed variable $\ln$ SM(d18:1/18:0), by One Way ANOVA showed that there were no significant differences in SM(d18:1/18:0) CSF levels between years ($F = 1.50$, $df = 3$, $p = 0.208$).

Classification of CSF samples on the basis of SM (d18:1/18:0)

Logistic regression with forward variable selection identified SM (d18:1/18:0) as the only significant predictor for discriminating AD-like CSF from normal CSF (Wald = 15.46, $p < 0.0001$). No further significant predictor was detected by the stepwise regression procedure, though PC aa 36:1 almost reached the 0.05 significance level (Wald = 3.74, $p = 0.053$). A summary of the most important measures of diagnostic accuracy based on the biomarker SM (d18:1/18:0) is given in Table 4. The ROC curve is shown in Fig. 1. The AUC of the ROC curve indicated satisfactory discrimination between AD-like CSF and normal CSF (AUC = 0.759). At the optimal cut-off level for SM (d18:1/18:0) (546 nM), fairly good specificity (0.76), but only moderate sensitivity (0.66) was observed. Overall accuracy, i.e., the proportion of correctly classified cases, amounted to 71%. Bias-correction by cross-validation led to a very minor reduction in specificity and accuracy, while sensitivity remained unchanged. There were no significant differences in SM(d18:1/18:0) CSF levels between male and female subjects ($t = 0.63$, $p = 0.531$). Moreover, separate ROC analyses for male and female subjects yielded very similar results for both sexes (males: AUC = 0.751, sensitivity = 0.667, specificity = 0.727; females: AUC = 0.755, sensitivity = 0.667, specificity = 0.731).

Combination of lipid biomarkers to enhance accuracy

Logistic regression with backward elimination of variables identified SM (d18:1/18:0) (Wald = 11.65, $p < 0.001$), PC aa 36:1 (Wald = 6.74, $p = 0.009$), and PC aa 32:0 (Wald = 4.28, $p = 0.039$), as predictors for discriminating AD-like CSF and normal CSF. The corresponding uncorrected values of AUC (0.794), specificity (0.74), sensitivity (0.74), and overall accuracy (0.74) were somewhat higher than those for SM (d18:1/18:0) alone. However, bias-correction by cross-classification led to a considerable reduction in sensitivity (0.68) and a small decrease in specificity (0.72), resulting in an overall accuracy of 70%. Hence, addition of the two parameters did not lead to a gain in diagnostic accuracy. We also checked if a combination of one of the established biomarkers, A β_{42} or tau, with the new marker SM (d18:1/18:0), would lead to an improved prediction. Due to the non-normal distribution of SM(d18:1/18:0) and A β_{42} , Spearman rank correlation coefficients (r_s) were determined. SM(d18:1/18:0) was significantly positively correlated with Tau ($r_s = 0.462$, $p < 0.001$) and significantly negatively with A β_{42} ($r_s = -0.309$, $p < 0.001$), as expected.

Verification of diagnosis with SM (d18:1/18:0) (blinded study)

In order to verify the diagnosis if SM (d18:1/18:0) can distinguish AD from controls in unknown samples, a cut-off value of 546 nM was defined (Fig. 1). When 32 unknown CSF samples were analyzed, our data shows that only 21 were correctly diagnosed (Table 5). This again verifies that SM (d18:1/18:0) has an accuracy of ca. 70%. Optimal cut-off levels for SM(d18:1/18:0) did not differ significantly between sexes, as was shown by an additional logistic regression analysis with diagnosis (normal and AD-like) as the dependent variable and both SM(d18:1/18:0) and sex as independent variables. In this analysis the variable sex did not attain statistical significance (Wald = 0.095, $p = 0.751$).

DISCUSSION

In the present study, we analyzed 100 CSF samples dichotomized by normal ($n = 50$) and pathological decreased $A\beta_{42}$ and increased tau and P-tau-181 levels ($n = 50$; correlating to an AD-like pathology). Our data provide evidence that 8 metabolites are significantly altered, in particular SM (d18:1/18:0), which was significantly increased in CSF samples with AD-like pathology.

CSF metabolome

The human CSF metabolome contains ca. 500 fully identified and quantified metabolites. Several of them have been linked to neurological/psychiatric diseases, such as schizophrenia [19] or AD [20]. Because of the hydrophilic nature of the CSF, high diversity in metabolite physical and chemical properties as well as limited sample amount mass spectrometry is the most powerful metabolomic platform for measuring CSF metabolites. It provides high selectivity, sensitivity, robustness, and high throughput capabilities with limited sample amount. In recent years, mass spectrometry has become method of choice in field of metabolomics. As a matter of fact, the recent improvements in mass spectrometry technique allowed significant development of metabolomics including comprehensive characterization of the CSF metabolome [14]. With increased number of studies and amount of data produced in recent years the topic of measurement and data standardization has emerged in the field of metabolomics. Up to now, it has been overlooked in majority of the performed studies; however, standardization is essential in order to ensure comprehensive and correct lab-to-lab data comparison, evaluation, and interpretation. In this perspective, the AbsoluteIDQ® p180 Kit is the optimal platform for metabolome analysis as it offers validated and standardized solution for identification and quantification of large number of endogenous metabolites including amino acids, biogenic amides, acylcarnitines, glycerophospholipids, sphingolipids, and sugars.

Metabolites analyzed and nomenclature

In total, 183 different metabolites have been analyzed: 40 acylcarnitines, 21 amino acids 19 biogenic amines, the sum of hexoses, 76 phosphatidylcholines, 14 lyso-phosphatidylcholines, and 15 sphingomyelins. The targeted mass spectrometry-based analysis in the kit format allow the highly sensitive, simultaneous, and quantitative analysis of these endogenous metabolites in 30 μ L CSF sample volume. Glycerophospholipids are differentiated related to the presence of ester (a) and ether (e) bonds in the glycerol moiety, where two letters (aa = diacyl, ae = acyl-alkyl, ee = dialkyl) denote that two glycerol positions are bound to a fatty acid residue, while a single letter (a = acyl or e = alkyl) indicates the presence of a single fatty acid residue. Lipid side chain composition is abbreviated as Cx:y, where x denotes the number of carbons in the side chain and y the number of double bonds, e.g., “PC ae C38:1” denotes a plasmalogen/plasmenogen phosphatidylcholine with 38 carbons in the two fatty acid side chains and a single double bond in one of them. Our data shows that the sphingolipid SM (d18:1/18:0) is highly significantly enhanced in CSF with an AD-like pathology. SM (d18:1/18:0) is a sphingomyelin with systematic name N-(octadecanoyl)-sphing-4-enine-1-phosphocholine. Sphingomyelin consist of a ceramide unit with a phosphocholine group linked at position 1.

Lipid side chain composition is abbreviated in similar manner as for rest of the lipids: d18:1 indicates the number of carbon atom and double bond number in side chain of sphingoid base, and 18:0 indicates number of carbon atoms and double bonds in the amide linked side chain. In addition, we show that two metabolites (PCaa C38:6 and less pronounced PC aa C36:6) are altered in CSF with an AD-like pathology, these are the same, which have been recently reported to be altered also in plasma of AD patients [11].

Sphingomyelin and AD

Neuronal membranes contain sphingolipids (ceramides, sphingomyelins, and glycosphingolipids including cerebroside and gangliosides), which play a major role in several signal transduction processes in the brain. There is now clear evidence that the sphingolipid catabolism is directly linked to neurodegenerative disorders in the brain [21, 22]. Recent findings have identified pathways of the sphingolipid and sphingomyelin metabolism that contribute to the AD pathology [19, 20, 23-28]. Further, there is a clear indication that A β ₄₂ is linked to the sphingomyelin catabolisms in AD [25, 29]. In addition, the central metabolites of the sphingolipid pathways, the ceramides, have also been linked to tau phosphorylation [30-32]. Recently, Fiedorowicz and colleagues [22] reported that sphingolipid profiles were altered in prefrontal cortex of rats with acute hyperglycemia. Indeed they showed that several ceramide metabolites (C16:0, C18:0, C20:0, C22:0, C18:1, C24:) and the sphingomyelins SM-C16:0 and SM-C18:1 were altered. Han et al. [20] analyzed plasma from AD patients and found that sphingomyelins were altered, particularly those with long aliphatic chains (such as with 22 and 24C atoms). In addition, Mapstone et al. [11] discovered a set of 10 lipids (such as PC aa C36:6, PC aa C38:0 and :6, PC aa C40:1, :2 and :6 or PC ae C40:6) in plasma which predicted conversion to AD. Thus our data fully fit that lipid metabolites and sphingomyelins should indeed be affected in AD.

Is SM (d18:1/18:0) a useful CSF biomarker in AD?

Recently Mielke et al. [33] found a cross-sectional positive association between CSF ceramide C18:0 and total sphingomyelins and CSF A β ₄₂ and tau levels in a cohort of cognitively normal individual aged 36–69 years with a confirmed parental history of AD. As stated in their limits, their samples consisted of individuals with “normal” CSF levels of A β ₄₂ and tau and it is not known whether these correlations will be as robust in patients with MCI and AD. Thus, our data show now in agreement with their study changes in SM (d18:1/18:0), and we show for the first time that SM (d18:1/18:0) is indeed significantly enhanced in CSF with an AD-like pathology. Further we can provide an indication that SM (d18:1/18:0) could become a novel robust biomarker for AD to dichotomize AD-like pathology in unknown samples with 70% accuracy. However, the changes are relatively small and it cannot be concluded that SM (d18:1/18:0) is a superior biomarker which can replace the established markers A β ₄₂, tau, and P-tau-181.

Limits of the study

There are several limits of this study. First, the number of samples was relatively small, although the changes were highly significant. Second, cross-sectional longitudinal studies need to be performed. Third, and possibly this is the major limit of this study, the patients were not clinically diagnosed. We took advantage of the highly specific diagnostic criteria

of the well-established CSF parameters A β ₄₂, tau, and P-tau-181. Since the samples were collected during routine analysis, no further clinical data are available. On the other hand, this is exactly what an easy diagnostic tool should provide: a diagnosis based only on fluids without any additional clinical criteria. Fourth, we could not include patients with other forms of dementia, such as MCI or vascular dementia, but our data indicate that SM (d18:1/18:0) will not provide any statistical significance to distinguish non-AD (demented) samples from controls.

In conclusion, we show that the sphingolipid SM (d18:1/18:0) is significantly increased in CSF of patients displaying pathological levels of A β ₄₂, tau, and P-tau-181.

ACKNOWLEDGMENTS

This study has been supported by the Austrian Nationalbank Jubiläumsfonds (Nr. 15887) and by a Bridge Early Phase grant (FFG).

REFERENCES

- [1]. Humpel C. Identifying and validating biomarkers for diagnosing Alzheimer's disease. *Trends Biotechnol.* 2011; 29:26–32. [PubMed: 20971518]
- [2]. Blennow K. CSF biomarkers for mild cognitive impairment. *J Intern Med.* 2005; 256:224–234. [PubMed: 15324365]
- [3]. Mattsson N, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, Bocchio-Chiavetto L, Blankenstein MA, Carrillo MC, Chalbot S, Coart E, Chiasserini D, Cutler N, Dahlfors G, Duller S, Fagan AM, Forlenza O, Frisoni GB, Galasko D, Galimberti D, Hampel H, Handberg A, Heneka MT, Herskovits AZ, Herukka SK, Holtzman DM, Humpel C, Hyman BT, Iqbal K, Jucker M, Kaeser SA, Kaiser E, Kapaki E, Kidd D, Klivenyi P, Knudsen CS, Kummer MP, Lui J, Lladó A, Lewczuk P, Li QX, Martins R, Masters C, McAuliffe J, Mercken M, Moghekar A, Molinuevo JL, Montine TJ, Nowatzke W, O'Brien R, Otto M, Paraskevas GP, Parnetti L, Petersen RC, Prvulovic D, de Reus HP, Rissman RA, Scarpini E, Stefani A, Soininen H, Schröder J, Shaw LM, Skinningsrud A, Skrogestad B, Spreer A, Talib L, Teunissen C, Trojanowski JQ, Tumani H, Umek RM, Van Broeck B, Vanderstichele H, Vecsei L, Verbeek MM, Windisch M, Zhang J, Zetterberg H, Blennow K. The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers. *Alzheimers Dement.* 2011; 7:386–395. [PubMed: 21784349]
- [4]. Perry G, Nunomura A, Raina AK, Aliev G, Siedlak SL, Harris PL, Casadesus G, Petersen RB, Bligh-Glover W, Balraj E, Petot GJ, Smith MA. A metabolic basis for Alzheimer disease. *Neurochem Res.* 2003; 28:1549–1552. [PubMed: 14570400]
- [5]. Gibson GE, Park LC, Zhang H, Sorbi S, Calingasan NY. Oxidative stress and a key metabolic enzyme in Alzheimer brains, cultured cells, and an animal model of chronic oxidative deficits. *Ann N Y Acad Sci.* 1999; 893:79–94. [PubMed: 10672231]
- [6]. Grady CL, Haxby JV, Horwitz B, Berg G, Rapoport SI. Neuropsychological and cerebral metabolic function in early vs late onset dementia of the Alzheimer type. *Neuropsychologia.* 1987; 25:807–816. [PubMed: 3501553]
- [7]. Blass JP. Brain metabolism and brain disease: Is metabolic deficiency the proximate cause of Alzheimer dementia? *J Neurosci Res.* 2001; 66:851–856. [PubMed: 11746411]
- [8]. Smith MA, Nunomura A, Zhu X, Takeda A, Perry G. Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. *Antioxid Redox Signal.* 2000; 2:413–420. [PubMed: 11229355]
- [9]. Moreira PI, Zhu X, Lee HG, Honda K, Smith MA, Perry G. The (un)balance between metabolic and oxidative abnormalities and cellular compensatory responses in Alzheimer disease. *Mech Ageing Dev.* 2006; 127:501–506. [PubMed: 16516950]

- [10]. Ahmad W. Overlapped metabolic and therapeutic links between Alzheimer and diabetes. *Mol Neurobiol.* 2013; 47:399–424. [PubMed: 23011810]
- [11]. Mapstone M, Cheema AK, Fiandaca MS, Zhong X, Mhyre TR, MacArthur LH, Hall WJ, Fisher SG, Peterson DR, Haley JM, Nazar MD, Rich SA, Berlau DJ, Peltz CB, Tan MT, Kawas CH, Federoff HJ. Plasma phospholipids identify antecedent memory impairment in older adults. *Nat Med.* 2014; 20:415–418. [PubMed: 24608097]
- [12]. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorn Dahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. HMDB 3.0—The Human Metabolome Database in 2013. *Nucleic Acids Res.* 2013; 41:D801–D807. Database issue. [PubMed: 23161693]
- [13]. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, Sawhney S, Bamforth F, Greiner R, Li L. The human cerebrospinal fluid metabolome. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2008; 871:164–173.
- [14]. Mandal R, Guo AC, Liu CK, Yallou P, Dong FS, Aziat E, Wishart F. Multi-platform characterization of the human cerebrospinal fluid metabolome: A comprehensive and quantitative update. *Genome Med.* 2012; 4:38. DS. [PubMed: 22546835]
- [15]. Foneth AN, Harrington RJ, Huhmer AF, Biringer RG, Riggins JN, Harrington MG. Identification of disease markers in human cerebrospinal fluid using lipidomic and proteomic methods. *Dis Marker.* 2006; 22:39–64.
- [16]. Kaddurah-Daouk R, Krishnan KRR. A global approach to the study of central nervous system diseases. *Neuropsychopharmacology.* 2009; 34:173–186. [PubMed: 18843269]
- [17]. Illig T, Gieger C, Zhai G, Römisch-Margl W, Wang-Sattler R, Prehn C, Altmäier E, Kastenmüller G, Kato BS, Mewes HW, Meitinger T, de Angelis MH, Kronenberg F, Soranzo N, Wichmann HE, Spector TD, Adamski J, Suhre KA. Genome-wide perspective of genetic variation in human metabolism. *Nat Genet.* 2010; 42:137–141. [PubMed: 20037589]
- [18]. Vittinghoff, E.; McCulloch, C.; Glidden, D.; Shiboski, S. Linear and non-linear regression methods in epidemiology and biostatistics. In: Rao, CR.; Miller, JR.; Rao, DC., editors. *Handbook of Statistics vol. 27: Epidemiology and Medical Statistics.* Elsevier; Amsterdam: 2008. p. 148-186.
- [19]. He X, Huang Y, Li B, Gong CX, Schuchman EH. Deregulation of sphingolipid metabolism in Alzheimer's disease. *Neurobiol Aging.* 2010; 31:398–408. [PubMed: 18547682]
- [20]. Han X, Rozen S, Hellegers C, Cheng H, Burke JR, Welsh-Bohmer KA, Doraiswamy PM, Kaddurah-Daouk R. Metabolomics in early Alzheimer's disease: Identification of altered plasma sphingolipidome using shotgun lipidomics. *PLoS One.* 2014; 6:e21643. [PubMed: 21779331]
- [21]. Farooqui AA, Horrocks LA, Farooqui T. Interactions between neural membrane glycerophospholipid and sphingolipid mediators: A recipe for neural cell survival or suicide. *J Neurosci Res.* 2007; 85:1834–1850. [PubMed: 17393491]
- [22]. Fiedorowicz A, Prokopiuk S, Zendzian-Piotrowska M, Chabowski A, Car H. Sphingolipid profiles are altered in prefrontal cortex of rats under acute hyperglycemia. *Neuroscience.* 2014; 256:282–291. [PubMed: 24161280]
- [23]. Haughey NJ, Bandaru VV, Bae M, Mattson MP. Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis. *Biochim Biophys Acta.* 2010; 1801:878–886. [PubMed: 20452460]
- [24]. Mielke MM, Lyketsos CG. Alterations of the sphingolipid pathway in Alzheimer's disease: New biomarkers and treatment targets? *Neuromolecular Med.* 2010; 12:331–340. [PubMed: 20571935]
- [25]. Katsel P, Li C, Haroutunian V. Gene expression alterations in the sphingolipid metabolism pathways during progression of dementia and Alzheimer's disease: A shift toward ceramide accumulation at the earliest recognizable stages of Alzheimer's disease? *Neurochem Res.* 2007; 32:845–856. [PubMed: 17342407]
- [26]. Mahfoud R, Garmy N, Maresca M, Yahi N, Puigserver A, Fantini J. Identification of a common sphingolipid-binding domain in Alzheimer, prion, and HIV-1 proteins. *J Biol Chem.* 2002; 277:11292–11296. [PubMed: 11792705]

- [27]. Bandaru VV, Troncoso J, Wheeler D, Pletnikova O, Wang J, Conant K, Haughey NJ. ApoE4 disrupts sterol and sphingolipid metabolism in Alzheimer's but not normal brain. *Neurobiol Aging*. 2009; 30:591–599. [PubMed: 17888544]
- [28]. Tamboli IY, Tien NT, Walter J. Sphingolipid storage impairs autophagic clearance of Alzheimer-associated proteins. *Autophagy*. 2011; 7:645–646. [PubMed: 21460623]
- [29]. Yuyama K, Sun H, Mitsutake S, Igarashi Y. Sphingolipid-modulated exosome secretion promotes clearance of amyloid- β by microglia. *J Biol Chem*. 2012; 287:10977–10989. [PubMed: 22303002]
- [30]. Chalfant CE, Kishikawa K, Mumby MC, Kamibayashi C, Bielawska A, Hannun YA. Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. *J Biol Chem*. 1999; 274:20313–20317. [PubMed: 10400653]
- [31]. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. *J Biol Chem*. 1993; 268:15523–15530. [PubMed: 8393446]
- [32]. Mukhopadhyay A, Saddoughi SA, Song P, Sultan I, Ponnusamy S, Senkal CE, Snook CF, Arnold HK, Sears RC, Hannun YA, Ogretmen B. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J*. 2009; 23:751–763. [PubMed: 19028839]
- [33]. Mielke MM, Haughey NJ, Bandaru VV, Zetterberg H, Blennow K, Andreasson U, Johnson SC, Gleason CE, Blazel HM, Puglielli L, Sager MA, Asthana S, Carlsson CM. Cerebrospinal fluid sphingolipids, β -amyloid, and tau in adults at risk for Alzheimer's disease. *Neurobiol Aging*. 2014; 35:2486–2494. [PubMed: 24952994]

Fig. 1.

SM (d18:1/18:0) allows to distinguish Alzheimer-like pathology (AD) from samples displaying normal CSF levels. Figure A shows a dot blot of all values of SM (d18:1/18:0) (in nM). The mean is given as a line, and a cut-off value of 546 nM as a dotted line. Note that CSF samples were dichotomized by normal and pathological decreased $A\beta_{42}$ and increased tau and P-tau-181 levels (referring to an AD-like pathology). Figure B shows the probability of dementia correlated to SM (d18:1/18:0) (red) including 95% lower (green) and upper (blue) limits. Figure C gives the receiver operating characteristic (ROC) curve for SM (d18:1/18:0) displaying a sensitivity of 76% and specificity of 66%.

Table 1

Cerebrospinal fluid samples

	Normal	AD-like
<i>n</i>	50	50
Age [year]	75.4±0.7	75.2±0.7 ns
Gender (male/female)	22/28	21/29
Protein [μ g/ml]	461±35	493±39 ns
A β ₄₂ [pg/ml]	767±29	393±48 ***
Tau [pg/ml]	242±17	1005±42 ***
P-tau-181 [pg/ml]	34±2	114±26 ***

Cerebrospinal fluids were collected for routine analysis and frozen at -80°C within 2 days. CSF samples were dichotomized by normal and pathological decreased A β ₄₂ and increased tau and P-tau-181 levels (AD-like pathology). It is internationally well established that AD patients have reduced A β ₄₂ (<500), increased tau (>550), and increased P-tau-181 (>60). All values are given as mean±SEM in pg/ml. Statistical analysis was performed by *t*-test comparison (***) $p < 0.001$ ns - not significant.

Table 2

Altered metabolites in CSF of normal and AD-like samples

		2012 (n = 15)	2011 (n = 20)	2010 (n = 15)	Total (n = 50)
sphingoLip	SM (d18:1/18:0)	<i>p</i> = 0.025	<i>p</i> = 0.001	<i>p</i> = 0.0097	<i>p</i> = 0.00007^a
	SM (d18:1/18:1)				<i>p</i> = 0.0048
	SM(OH)C14:1			<i>p</i> = 0.015	
glyceroPLip	PC aa C30:0		<i>p</i> = 0.04		
	PC aa C32:0	<i>p</i> = 0.026	<i>p</i> = 0.001		<i>p</i> = 0.0001^a
	PC aa C34:1	<i>p</i> = 0.024	<i>p</i> = 0.010	<i>p</i> = 0.046	<i>p</i> = 0.0001^a
	PC aa C34:4			<i>p</i> = 0.046	
	PC aa C36:1		<i>p</i> = 0.04		<i>p</i> = 0.0027
	PC aa C36:6		<i>p</i> = 0.006		
	PC aa C38:4				<i>p</i> = 0.0067
	PC aa C38:5	<i>p</i> = 0.031			<i>p</i> = 0.0142
	PC aa C38:6				<i>p</i> = 0.0086
	PC ae C30:1	<i>p</i> = 0.022			<i>p</i> = 0.0119
	PC ae C34:0		<i>p</i> = 0.006		
	PC ae C38:2			<i>p</i> = 0.009	
	lysoPC a C16:0		<i>p</i> = 0.030		
acylcarnitine	C10:2			<i>p</i> = 0.036	
	C16-OH	<i>p</i> = 0.025			
	C3-DC-M/C5-OH			<i>p</i> = 0.021	<i>p</i> = 0.0044
amino acids	Ala		<i>p</i> = 0.008		
	Gln		<i>p</i> = 0.025		
biogenic amine	Creatinine			<i>p</i> = 0.018	

CSF was collected during the years 2010 and 2012 and frozen at -80°C . A selected number (n) was analyzed using the AbsoluteIDQ[®] kit. Samples were statistically analyzed using *T*-tests. This table shows *p*-values (<0.05 significance) of all altered metabolites divided per year and pooled for all years. Note that only SM (d18:1/18:0) and PC aa C34:1 were both significantly altered in all 3 year groups.

^aFor these three markers (SM d18:1/d18:0, PC aa C32:0, PC aa C34:1) significance is retained after adjustment for multiple testing by means of the Bonferroni method, i.e., $p_{\text{Bonferroni}} = p_{\text{unadjusted}} \times 183 < 0.05$. For all other parameters the Bonferroni corrected *p*-value was >0.05.

Table 3

CSF levels of the most important metabolites altered in normal and AD-like CSF samples

	Normal (<i>n</i> = 50)	AD-like (<i>n</i> = 50)	<i>p</i> -value
SM (d18:1/18:0)	462±138	644±231	0.00007
PC aa C32:0	299±101	401±179	0.0001
PC aa C34:1	1880±564	2498±998	0.0001
PC aa C36:1	264±82	335±142	0.0027
C3-DC-M/C5-OH	22±7	30±15	0.0044
SM (d18:1/18:1)	119±42	159±74	0.0048
PC aa C38:4	245±96	322±196	0.0067
PC aa C38:6	84±38	111±57	0.0086

CSF was collected during the years 2010 and 2012 and frozen at -80°C and analyzed using the Absolute $IQ^{\text{®}}$ kit. This table shows the significantly altered metabolites from 100 CSF samples. Note that SM (d18:1/18:0), PC aa C32:0 and PC aa C34:1 show highly significant increase in CSF. Values are given as mean±SD nM as calculated from the standard curve. Samples were statistically analyzed using *T*-tests.

Table 4

Prediction of dementia by the biomarker SM (d18:1/18:0) – AUC of ROC curve, optimal cut-off value, sensitivity and specificity

Measure	Estimate	95% CI lower bound	95% CI upper bound
Area under ROC curve	0.759	0.665	0.853
Cut-off value [nM] for SM (d18:1/18:0) ^a	546	456	630
Specificity	0.760	0.615	0.865
Bias-corrected ^b	0.740	0.594	0.849
Sensitivity	0.660	0.511	0.784
Bias-corrected ^b	0.660	0.511	0.784
Overall accuracy	0.710	0.563	0.825
Bias-corrected ^b	0.700	0.553	0.817

^a A subject was classified as AD-like if SM (d18:1/18:0) 546 nM, as cognitively intact otherwise.

^b Derived by cross-validation (leave-one-out method).

Table 5

Analysis of unknown CSF samples using SM (d18:1/18:0) (blinded study)

Nr	A β ₄₂	Tau	P-tau-181	SM (d18:1/18:0)	Correct
1	333	587	67	388	no
2	266	588	67	675	yes
3	764	1165	29	494	yes
4	687	263	38	457	yes
5	371	1127	119	801	yes
6	407	990	85	631	yes
7	488	615	99	999	yes
8	261	633	77	1040	yes
9	283	542	84	548	no
10	466	515	53	551	no
11	381	502	34	520	yes
12	761	1125	51	1090	no
13	452	636	68	519	no
14	755	142	27	418	yes
15	168	627	55	509	yes
16	858	1292	42	753	no
17	775	433	49	281	yes
18	573	460	60	672	no
19	1151	435	43	840	no
20	986	1386	35	278	yes
21	435	461	48	588	no
22	421	613	61	564	yes
23	446	677	68	1540	yes
24	372	905	60	649	yes
25	530	208	27	453	yes
26	606	126	14	333	yes
27	854	188	31	586	no
28	618	235	28	320	yes
29	512	365	46	368	yes
30	638	1068	113	775	no
31	484	219	33	309	yes
32	727	553	40	262	yes

CSF samples ($n = 32$) were randomly selected from a pool of samples frozen in the year 2013; the mean age of the subject was 75 ± 1 years; 19 male and 13 females were included. AD-like pathology was diagnosed with the following CSF cut-off levels: A β ₄₂ <500 pg/ml and total tau >550 pg/ml and P-tau-181 >60 pg/ml. The cut-off for SM (d18:1/18:0) for AD-like pathology was defined as >546 nM.