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ORIGINAL PAPER

SORL1 genetic variants and cerebrospinal fluid biomarkers of Alzheimer's disease

Liang-Hao Guo · Christine Westerteicher · Xin-Hui Wang · Martina Kratzer · Amalia Tsolakidou · Meizi Jiang · Timo Grimmer · Simon M. Laws · Panagiotis Alexopoulos · Hideaki Bujo · Alexander Kurz · Robert Perneczky

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Abstract The neuronal sortilin-related receptor with A-type repeats (SORL1, also called LR11 or sorLA) is involved in amyloidogenesis, and the *SORL1* gene is a major risk factor for Alzheimer's disease (AD). We investigated AD-related CSF biomarkers for associations with *SORL1* genetic variants in 105 German patients with mild cognitive impairment (MCI) and AD. The homozygous CC-allele of single nucleotide polymorphism (SNP) 4 was associated with increased Tau concentrations in AD, and the minor alleles of SNP8, SNP9, and SNP10 and the haplotype CGT of these SNPs were associated with increased SORL1 concentrations in MCI. SNP22 and SNP23, and the haplotypes TCT of SNP19-21-23, and TTC of SNP22-23-24 were correlated with decreased Aβ42 levels in AD. These results strengthen the functional role of *SORL1* in AD.

Keywords Amyloid cascade · Biomarker · Mild cognitive impairment · Dementia · Genetic risk

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L.-H. Guo · C. Westerteicher · M. Kratzer · A. Tsolakidou · T. Grimmer · P. Alexopoulos · A. Kurz · R. Perneczky (✉)
Department of Psychiatry and Psychotherapy, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany
e-mail: robert.perneczky@lrz.tum.de

X.-H. Wang
Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

M. Jiang · H. Bujo
Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba, Japan

S. M. Laws
Centre of Excellence for Alzheimer's Disease Research and Care, School of Exercise Biomedical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia

S. M. Laws
Sir James McCusker Alzheimer's Disease Research Unit (Hollywood Private Hospital), Perth, WA, Australia

Introduction

The neuronal sortilin-related receptor with A-type repeats (SORL1, also called LR11 or sorLA) has been linked to protective effects against amyloidogenesis in Alzheimer's disease (AD) [1]. SORL1 seems to be capable of regulating the intracellular trafficking and processing of amyloid precursor protein (APP) by impairing the cleavage of APP through α-secretase, β-secretase (β-site APP-cleaving-enzyme-1, BACE1), and γ-secretase in a way that leads to reduced levels of soluble APP (sAPP) and amyloid beta protein (Aβ), the major component of amyloid plaques [2]. In line with this theory, reduced SORL1 expression has been demonstrated in human brains with amyloid pathology [3]. *SORL1* gene variants can reduce SORL1 expression or function and thereby increase Aβ production as well as AD risk [4]. Recently, multiple single nucleotide polymorphisms (SNP) within the *SORL1* gene have emerged as risk factors for sporadic AD in a variety of populations. Although replications are inconsistent, implicating influences of multi-ethnicity and allelic heterogeneity [4, 5], several independent studies have observed that significant associations were located in 2 distinct regions: the 5′ end and the 3′ end of the *SORL1* gene [4]. So far, only few studies have reported associations of *SORL1* variants with cerebrospinal fluid (CSF) endophenotypes in

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AD [6–8]. In the present study, we have investigated eleven AD risk SNPs in a German sample to evaluate the effect of *SORL1* variants on the CSF levels of Aβ42, total TAU, sAPPα, sAPPβ, and SORL1 protein as well as on the CSF activity of BACE1.

Methods

The study population consisted of 44 Caucasian patients with probable AD according to NINCDS-ADRDA criteria and 61 patients with mild cognitive impairment (MCI) according to the revised International Working Group on MCI consensus criteria recruited from a university-based memory clinic in compliance with standardized guidelines [9, 10]. Written informed consent was obtained according to the 1975 Helsinki Declaration and the study protocol was approved by the ethics committee of the medical faculty at Technische Universität München.

The CSF concentrations of Aβ42, Tau (Innogenetics, Zwijndrecht, Belgium) as well as sAPPα and sAPPβ (Immuno-Biological Laboratories Co. Ltd., Gunma, Japan) were measured by enzyme-linked immunosorbent assay (ELISA) as described previously [11]. BACE1 activity in CSF was determined as the fluorescence signal of europium, which is proportional to the activity of BACE1, by a commercial BACE1 assay kit (Perkin Elmer Inc., Turku, Finland) according to a standard protocol [12, 13]. SORL1 concentration in CSF was quantified by ELISA in the laboratories of Sekisui Medical Co Ltd. (Ryugasaki, Japan) according to published procedures [14]. Genomic DNA was extracted from whole blood, and the apolipoprotein E *(APOE)* genotype was determined by a polymerase chain reaction and restriction enzyme digestion, simultaneously utilizing two distinct restriction enzymes, according to standard procedures.

Five marker SNPs at the 5′ end of the *SORL1* gene, rs661057 (SNP4), rs11600875, rs668387 (SNP8), rs689021 (SNP9), and rs641120 (SNP10), as well as 6 markers at the 3′ end, rs2070045 (SNP19), 21rs18ex26 (SNP21), rs1699102 (SNP22), rs3824968 (SNP23), rs2282649 (SNP24), and rs1010159 (SNP25), were selected from the published data based on their significant association with AD risk in Caucasian populations [4, 5, 7]. The genotypes were determined using TaqMan assays (SNP assays-on-demand) on a StepOne analyzer with StepOne software v2.1 (all assays, machine, and software from Applied Biosystems, Carlsbad, CA, USA).

Deviations from the Hardy–Weinberg equilibrium to exclude population stratification were tested for all 11 *SORL1* SNPs (http://www.oege.org/software/hwe-mr-calc.shtml) [15]. The sample size required to detect a significant difference between carriers and non-carriers with 90% power and a type I error rate of 0.05 was estimated in G-Power v3.1.3 [16] at $N = 14$ per group according to previous results [7] (mean Aβ42 concentration difference between carriers and non-carriers of the *SORL1* SNP23 T-allele of 56.60 ng/L with a shared standard deviation of 41.59 ng/L).

Patient characteristics were compared between the AD and the MCI groups using parametric tests for normally distributed data in the Predictive Analytics Software package (PASW) v18 (The SPSS Inc., Chicago, IL, USA). Analysis of covariance (ANCOVA) in PASW was used to test for the genotypic or allelic effect of all 11 SNPs of interest on CSF biomarker concentrations, adjusting for age, gender, and *APOE*, which was coded as a dichotomous variable for carriers and non-carriers of the ε4 allele. In addition, three-marker haplotypes of SNP8/SNP9/SNP10, SNP19-21-23, SNP22-23-24, and SNP23-24-25, again selected from the literature according to their linkage disequilibrium (LD) and the significant association with AD risk, were reconstructed and assessed with the Haplo.stats package in R software v2.1 (http://www.r-project.org/). The associations between *SORL1* haplotypic variants and CSF biomarker concentrations were examined in multivariate linear models after adjustment for age, gender, and *APOE* ε4 carrier status. Only genetic frequency higher than 5% was considered. Significance was set at $p < 0.05$. The study was driven by a priori hypotheses; therefore, no correction for multiple comparisons was applied [17] in accordance with similar previous studies [18].

Results

The demographic and clinical characteristics are summarized in Table 1; genotype and allele frequencies are provided in the Supplementary Tables 1 and 2. None of the 11 SNPs showed significant deviation from the Hardy–Weinberg equilibrium in the AD group; in the MCI group, deviation was only observed for SNP21 (Supplementary Table 1). The APOE ε4 allele was associated with lower Aβ42 levels in the MCI group ($p < 0.001$, $N = 61$). The single-marker analysis revealed significant associations between Aβ42 concentrations and the synonymous coding SNP22 and SNP23 at the 5′ end of the gene in the AD group. Carriers of the SNP22 C-allele ($p = 0.04$, $N = 22$) and SNP23 A-allele ($p = 0.04$, $N = 23$) had lower levels of Aβ42 than non-carriers (Supplementary Table 3). In the haplotype analyses, we observed associations of haplotype TCT (frequency 36.9%) of SNP19-21-23 ($p = 0.04$, $N = 38$) and TTC (frequency 24.7%) of SNP22-23-24 ($p = 0.04$, $N = 38$) with decreased CSF Aβ42 levels in the AD group (Fig. 1a). At the 3′ end of the gene, a significant association between the homozygous minor allele CC of

Table 1 Characteristics of the study sample

	AD (N = 44)	MCI (N = 61)	p value
Age at lumbar puncture*	66 (9.6)	65 (8.7)	0.44
Age at onset of symptoms*	6 (8.8)	63 (8.8)	0.62
Men:women	23:21	35:26	0.81
Schooling, years*	13 (2.9)	1 (2.7)	0.91
MMSE score*	23 (3.1)	27 (1.9)	<0.001**
ApoE4 carrier, n (%)	26 (59.1%)	27 (44.3%)	0.55
Aβ42 (ng/L)*	551.8 (233.52)	771.1 (350.84)	<0.001**
TAU (ng/L)*	627.8 (384.24)	383.9 (255.87)	<0.001**
sAPPα (ng/mL)*	287.1 (159.21)	332.2 (166.75)	0.17
sAPPβ (ng/mL)*	897.0 (402.65)	1047.2 (493.75)	0.10
BACE1 (FU/µL)*	8333.06 (2585.76)	9381.67 (3239.94)	0.08
SORL1 (µg/L)*	11.9 (4.69)	11.9 (4.28)	0.95

SNP single nucleotide polymorphism, *CSF* cerebrospinal fluid, *Aβ42* amyloid beta 42, *sAPPα*, *sAPPβ* alpha- and beta-soluble amyloid precursor protein, *BACE1* β-site APP-cleaving-enzyme-1, *SORL1* sortilin-related receptor with A-type repeats, *AD* Alzheimer's disease, *MCI* mild cognitive impairment, *FU* fluorescence units

* Mean (SD), ** significant at $p < 0.05$

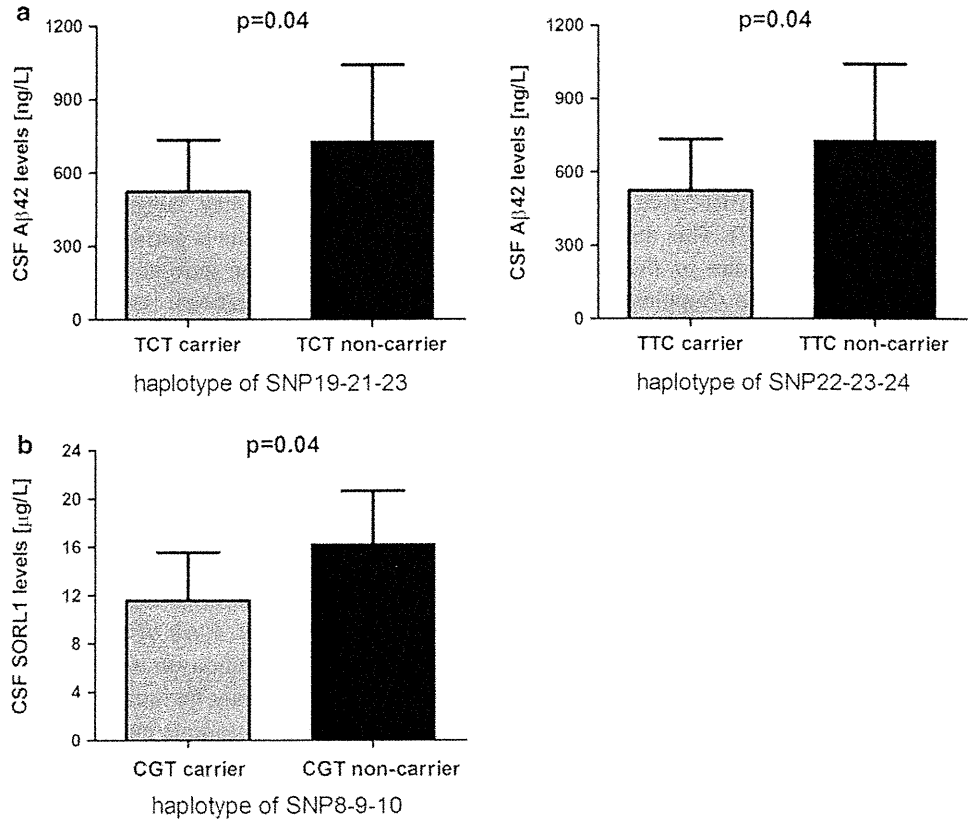

Fig. 1 **a** Effects of SORL1 haplotypes on CSF Aβ42 levels in the AD group; and **b** effects of SORL1 haplotypes on CSF SORL1 levels in the MCI group

SNP4 and increased Tau levels was observed (p = 0.03, N = 7) in the AD group. No association was found in heterozygous carriers, which points to a strong gene dosage effect (Supplementary Table 4). In the MCI group, at the 3′ end of the gene, SNP8, SNP9, and SNP10 showed significant associations with CSF SORL1 levels in a way that minor allele carriers had increased SORL1 concentrations (SNP8 TT: p = 0.04; SNP9 AA: p = 0.04; SNP10 CC: p = 0.04) (Supplementary Table 5). Again, these associations were driven by the homozygous carriers of the minor alleles of each of the three SNPs. In the haplotype analyses, a significant association between reduced CSF SORL1

levels was found with haplotype CGT (frequency 22.5%) of SNP8-9-10 in the MCI group ($p = 0.04$, $N = 55$) (Fig. 1b). There were no associations between sAPP levels and BACE1 activity with any of the SNPs or haplotypes.

Discussion

SORL1 regulates the intracellular sorting of APP and hinders APP cleavage and thereby Aβ production [1, 2]. The *SORL1* gene has been identified as a major risk factor for sporadic AD [4]. In the present study, associations between *SORL1* genetic variants and CSF levels of Aβ42, Tau, and SORL1 were observed at two distinct gene regions in patients with MCI and probable AD. Associations between *SORL1* genetic variants and CSF sAPPα and sAPPβ concentrations as well as BACE1 activity were not observed.

In the AD group, lower CSF Aβ42 levels were found in carriers of the exonic SNP22 (C-allele) and SNP23 (A-allele), and haplotypes TCT of SNP19-21-23 and TTC of SNP22-23-24 at the 3′ gene end. It has been demonstrated that SNP19 is in strong linkage disequilibrium with SNP22 and SNP23 in various Caucasian cohorts [19]. SNP21, on the other hand, has been reported as AD-related *SORL1* polymorphism in a German cohort [3] and the haplotype TGA of SNP19-21-22 correlated with lower CSF Aβ42 in AD before [7]. This finding was not replicated in our work, probably due to the low frequency of these markers in our sample (Supplemental Tables 1 and 2). In the initial genetic association study [4], the SNP22 C-allele, SNP23 T-allele, and haplotype CTT of SNP22-23-24 were associated with an increased risk for AD. In contrast, in our study, reduced Aβ42 levels were correlated with genotypes and haplotypes consisting of the alternative alleles. This inconsistency suggests that *SORL1* allelic heterogeneity and ethnic variants may also play a role [20]. Since exonic SNPs of the *SORL1* gene are present in the mature mRNA, they could directly alter translation and thus protein levels [21]. Therefore, the 3′ end SNPs, in particular the synonymous coding SNPs, might directly influence the function of the SORL1 protein and thereby alter the CSF levels of Aβ42.

We also found that Tau levels were associated with CC homozygotes of SNP4 in the AD group. The C-allele of SNP4 has been associated with AD among Caucasian populations in multiple independent cohorts and genome-wide association studies before [4, 5, 20, 22–25]. Although the present work is a case-only study that precludes a statement on the association of *SORL1* SNPs with AD risk per se, our data still confirm that the SNP4 C-allele is significantly associated with upregulated CSF Tau levels, which in turn are correlated to neurodegenerative pathology.

SORL1 protein is considered an important regulator of amyloidogenesis since reduced SORL1 levels may lead to dysfunctional retromer trafficking and upregulated cerebral Aβ production [1]. It remains inconclusive how reduced SORL1 protein expression in AD brain is related to alterations of SORL1 in CSF. It has been reported that the expression of SORL1 protein is reduced in brain tissue from patients with sporadic AD [3]. The two published CSF studies are inconsistent in this regard, reporting both decreased [26] and increased [27] SORL1 levels in AD compared with healthy controls. We identified associations between CSF SORL1 concentrations and three AD risk marker SNPs in the MCI group; the homozygous minor allele carriers of the intronic SNP8 (T-allele), SNP9 (A-allele), and SNP10 (A-allele) had increased SORL1 concentrations in CSF. Moreover, the haplotype analysis confirmed that a three-marker haplotype CGT (a combination of the major alleles) of SNP8/SNP9/SNP10 was associated with reduced CSF SORL1 levels in the MCI group. These three SNPs have been confirmed as the most significant AD risk markers within the *SORL1* gene in Caucasian samples in a recent meta-analysis including 11,592 cases and 17,048 controls [28]. The association of three 5′ end SNPs in our study with CSF SORL1 concentrations is consistent with the allelic disease association in this meta-analysis. Since MCI often represents pre-dementia AD, our data may suggest that the influence of *SORL1* genetic variants is particularly relevant in early clinical AD stages.

Our current study extends the existing literature on associations between SORL1 genetic variants and AD biomarkers, thereby supporting the role of SORL1 as an important influence factor on AD pathogenesis. Limitations include the rather small study sample and the lack of longitudinal data as well as neuropathological verification of the diagnoses. Therefore, replication studies with independent larger samples are warranted. We did not aim to replicate the results from previous genetic association studies; neither did we aim to identify new risk SNPs, and no control group was included because of this study design choice. Lack of consistent replication of genetic findings is a common occurrence in the study of complex phenotypes and may be indicative of inadequate power resulting from small sample size and genetic or environmental heterogeneity. The use of CSF biomarkers for genetic studies of AD may provide increased statistical power and important insight into the biological mechanisms by which these variants modulate disease risk. In any study attempting to associate genetic information with pathology, the exact effect of genetic variants on phenotypic variation often remains unclear. On the one hand, the genetic variants may have a direct effect on markers of pathology; on the other hand, neighboring SNPs in LD with the variant tested or other downstream factors may also have an influence.

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Conflict of interest The authors declare that they have no conflict of interest.

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Enhanced Circulating Soluble LR11 in Patients with Diabetic Retinopathy

AQ: au MAO TAKAHASHI, HIDEAKI BUJO, TOMOAKI SHIBA, MEIZI JIANG, TAKATOSHI MAENO, AND KOHJI SHIRAI

• PURPOSE: To investigate the relationship of circulating levels of soluble form of LR11 (sLR11; also called SorLA or SORL1), with the progression of proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus.

• DESIGN: Cross-sectional study.

• METHODS: Fifty-four patients with type 2 diabetes mellitus were divided into 2 sex- and age-matched groups: one with PDR (n = 29) and the other with nonproliferative diabetic retinopathy (n = 25). The serum sLR11 levels were measured with an immunodetection system followed by chemifluorescence quantification.

• RESULTS: The serum sLR11 levels were higher in the PDR group than in the nonproliferative diabetic retinopathy group (5.8 ± 1.2 U vs 3.7 ± 1.3 U; P < .01). A multivariate regression analysis showed that circulating sLR11 is a factor contributing to the prediction of PDR independent of other classical risk factors, and an area under the receiver operating characteristic curve analysis revealed that the sensitivity and the specificity were equivalent to or more than those of other factors. Among the classical risk factors for PDR, glycosylated hemoglobin levels showed the highest correlation coefficient (P < .01) for the sLR11 concentrations.

• CONCLUSIONS: Serum sLR11 concentration may reflect the progression of PDR in patients with type 2 diabetes mellitus. sLR11, released from immature vascular cells and indicating the development of atherosclerosis, is expected to be a novel candidate biomarker indicating diabetic retinopathy in patients with type 2 diabetes mellitus. (Am J Ophthalmol 2012;xx:xxx.

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From the Cardiovascular Center, Toho University, Chiba, Japan (M.T.); the Department of Ophthalmology, Sakura Hospital Medical Center, Toho University, Chiba, Japan (T.S., T.M.); the Department of Internal Medicine, Toho University, Chiba, Japan (K.S.); and the Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan (H.B., M.J.)

Inquiries to Mao Takahashi, Department of Cardiovascular Center, Sakura Hospital, Medical Center, Toho University, 564-1 Shimoshizu, Sakura-shi, Chiba Prefecture 285-8741, Japan; e-mail: takahashi-04@sakura.med.toho-u.ac.jp

SUSTAINED HYPERGLYCEMIA, EVEN IN THE ABSENCE of other risk factors, can increase the risk of microvascular complications.[1] Given the substantial quality-of-life burden that diabetic retinopathy can confer, the ability to detect early retinal vascular abnormalities sensitively in patients with diabetes mellitus is desirable. The detection of such markers of pathologic cell function in combination with treatment of hyperglycemia is needed.

LR11 (also called SorLA or SORL1), an LDL-receptor AQ: 1 family member, has been identified as a molecule expressed in intimal smooth muscle cells in the development of atherosclerosis and endothelial cells under the condition of dyslipidemia.[2,3] The released soluble form of LR11 (sLR11) promotes pathologic infiltration of macrophages into the damaged vessels.[2] We have shown that the circulating sLR11 levels were increased in patients with coronary artery disease[4] and dyslipidemic subjects with carotid atherosclerosis.[5] A multivariate analysis in these independent studies in patients with atherosclerosis indicated that the sLR11 levels were correlated distinctly with the glycemic level among the classical risk factors for atherosclerosis.[4,5]

Diabetic retinopathy mainly is caused by diffuse endothelial damage at the microvascular level. However, the interesting observations are that the retinopathy is tightly associated with increased cardiovascular mortality,[6–8] reduced coronary reactivity,[9] and poorer prognosis of coronary revascularization procedures.[10,11] Thus, high glucose levels may change the phenotype of endothelial cells as well as that of arterial smooth muscle cells; the pathologic cell phenotype in microvessels of the retina possibly is detected by the circulating sLR11 released from the damaged cells. In this analysis, we investigated the significance of circulating sLR11 with regard to proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus. The factors contributing to the elevation of the serum sLR11 also were analyzed.

METHODS

• STUDY POPULATION: The subjects consisted of 56 consecutive Japanese patients with type 2 diabetes mellitus seeking treatment at the Department of Laboratory Vascular Function, Toho University Sakura Medical Center, who had already given blood samples. PDR was defined

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TABLE 1. Comparison of Type 2 Diabetes Mellitus Patient Background Factors between Nonproliferative Diabetic Retinopathy and Proliferative Diabetic Retinopathy

	NPDR Group	PDR Group	*P* Value
No.	25	29	—
Male (%)	68.2	69	.95
Age (y)	66.0 ± 8.6	62.4 ± 9.7	.15
Duration of diabetes (y)	11.4 ± 7.8	11.9 ± 7.8	.71
Body mass index (kg/m^2)	23.8 ± 4.0	25.8 ± 3.7	.06
Hypertension (%)	63.6	58.6	.72
Dyslipidemia (%)	64.0	44.8	.16
eGFR (mL/minute per 1.73m^2)	60.2 ± 15.3	58.2 ± 28.0	.72
HbA1c (%)	6.5 ± 0.8	7.0 ± 1.4	.10
Fasting blood sugar (mg/dL)	124.6 ± 33.1	132.1 ± 38.2	.63
Total cholesterol (mg/dL)	183.8 ± 34.8	202.6 ± 40.7	.12
LDL cholesterol (mg/dL)	111.7 ± 30.6	124.1 ± 33.3	.26
HDL cholesterol (mg/dL)	47.7 ± 16.4	49.4 ± 10.6	.58
Triglyceride (mg/dL)	122.4 ± 43.1	122.7 ± 52.7	.93
Medications			
Insulin therapy (%)	13.6	65.5	< .0001
Administration of statin (%)	45.5	24.1	.11
Administration of ACE-I or ARB (%)	54.5	41.4	.43

ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor antagonist; eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; HDL = ; LDL = ; NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy.

The data are presented as mean ± standard deviation or number of subjects (%). The unpaired *t* test was used for continuous variables, and the chi-square test was used for categorized variables.

according to the international clinical classification of diabetic retinopathy as neovascularization in the retina.[12] Vitreous surgeries had been performed to treat macular edema (n = 7), vitreous hemorrhage (n = 13), traction retinal detachment (n = 5), or neovascular glaucoma (n = 4). None of the nonproliferative diabetic retinopathy (NPDR) cases had retinal neovascularization. Patients with chronic heart disease with an ejection fraction of less than 50% or chronic renal failure with serum creatinine of more than 1.3 mg/dL were excluded from the study analysis.

• PATIENT DATA ANALYSIS: Blood samples were collected in the morning after an overnight fast. Lipid variables and fasting blood glucose were measured using standard laboratory techniques. The potential risk factors for atherosclerosis were analyzed, including age, sex, body mass index (BMI), smoking, and history of hypertension and dyslipidemia. Hypertension was defined as systolic pressure of more than 140 mm Hg or diastolic pressure of more than 90 mm Hg. Diabetes mellitus was defined as a fasting blood glucose level of more than 126 mg/dL, glycosylated hemoglobin (HbA1c) of more than 5.8%, or both. Dyslipidemia was defined as serum total cholesterol of more than 220 mg/dL and triglycerides of more than 150 mg/dL in the fasting state, or both, and HDL cholesterol of less than 40 mg/dL, or a combination thereof. The serum creatinine level was assayed by an enzymatic method. The estimated glomerular flow rate was estimated using a modified traceable Modification of Diet in Renal equation, as proposed by the Working Group of Japan Chronic Kidney Disease Initiative[13]: estimated glomerular flow rate (mL/minute per 1.73 m^2) = $0.741 \times 175 \times \text{age}^{-0.203} \times \text{serum creatinine}^{-1.154}$ (if female × 0.742).

• MEASUREMENT OF SERUM SLR11: For the analysis of sLR11, fasting blood samples were collected and centrifuged immediately at 4000 g for 10 minutes, and the supernatant immediately was frozen in polypropylene tubes and stored at −80 C until use. Fifty microliters of serum was purified using 39-kDa receptor-associated protein-GST affinity beads (Cosmo Bio). For immunoblotting, equal amounts of protein extracted from pelleted beads were subjected to 10% SDS-PAGE after heating to 95 C for 5 minutes, as described previously[5] under reducing conditions, and were transferred to a nylon membrane. Incubations were carried out with an antibody against LR11 (5-4-30-19-2 at 1:500 dilution),[5] followed by peroxidase-conjugated antimouse immunoglobulin G. The development was performed with the ECL detection reagents (Amersham Pharmacia). The signals were quantified by densitometric scanning using the NIH image software program. The sLR11 levels in each serum sample (50 μL) were determined as an averaged value of 3 quantified signal

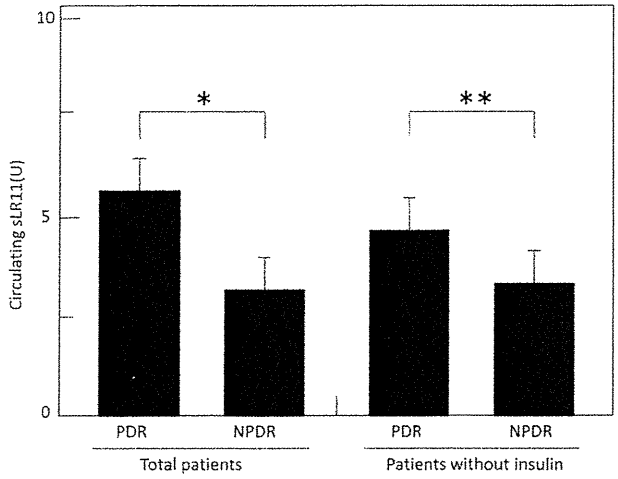

FIGURE 1. Bar graph showing circulating sLR11 levels in the patient groups with proliferative diabetic retinopathy (PDR) or without PDR (NPDR). The sLR11 levels of the total patients and those of the patients without insulin therapy were compared between the PDR and NPDR groups, respectively. Data are expressed as means ± standard deviation. For statistical analysis, the Student *t* tests was used. $*P < .05$, $**P < .01$.

intensities resulting from independent assays using samples with blinded indications and were expressed as a ratio to that of standard serum. The immunologic estimation indicated that the signal of 1 U (in 50 μL serum) corresponded to approximately 50 ng/mL of recombinant sLR11.

• STATISTICAL ANALYSIS: The results are shown as means ± standard deviation or proportion (%) for each index. The statistical analyses were performed using the SPSS Statistical Package for Windows software program version 11.01.1. Comparisons between groups were performed using the Student t test. The data were subjected to a 1-way analysis of variance with the Dunnett multiple comparison of means. A Pearson correlation coefficient analysis was used to assess the associations between measured parameters. Subsequently, multiple linear regression analyses were used to calculate the odds ratio for PDR by controlling for all risk factors. These risk factors were scored as explanatory factors, and the subordinate variable was PDR = 1 and NPDR = 0. The sensitivity and specificity with respect to the presence of PDR were analyzed using a conventional receiver operating characteristic (ROC) curve. P values less than .05 were considered to be statistically significant.

RESULTS

AQ:7 THE PATIENT CHARACTERISTICS ARE SHOWN IN TABLE 1. The age- and gender-matched NPDR and PDR groups comprised 25 and 29 subjects, respectively. There were no

TABLE 2. Results of Multivariate Analysis Investigating Risk Factors for Proliferative Diabetes Retinopathy in Subjects with Type 2 Diabetes Mellitus

	Odds Ratio (95% Confidence Interval)	*P* Values
Age, per 1-y increase	4.12 (0.78 to 0.996)	< .05
Male	0.01 (0.16 to 6.18)	.99
eGFR, per 1-U (mL/minute per 1.73 m^2) increase	0.80 (0.97 - 1.08)	.37
Total cholesterol, per 1-mg/dL increase	1.43 (0.99 to 1.04)	.34
HbA1c, per 1% increase	1.23 (0.24 to 2.04)	.51
sLR11, per 1-U increase	8.50 (1.63 to 12.25)	< .01

eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; sLR11 = soluble form of LR11.

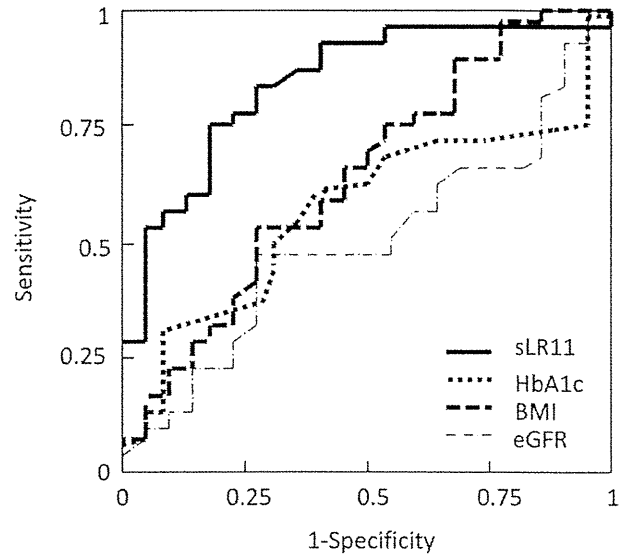

FIGURE 2. Receiver operating characteristic curve for discriminating the probability of type 2 diabetes mellitus patients developing proliferative diabetic retinopathy (PDR) from patients without PDR based on the levels of circulating soluble form of LR11 (sLR11), glycosylated hemoglobin (HbA1c), body mass index (BMI), or estimated glomerular filtration rate (eGFR). The curves show the fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for various cutoff levels of each parameter.

statistically significant differences in BMI, duration of diabetes, frequency of hyperlipidemia or dyslipidemia, or estimated glomerular flow rate between the NPDR and PDR subjects. There were also no statistically significant differences in HbA1c, fasting blood sugar, or lipid concentrations between the NPDR and PDR subjects. Although there was no significant difference in the use frequency of statin, ACE-I, or angiotensin II receptor type 1 blocker AQ: 8 (ARB) between the 2 groups, the frequency of patients

TABLE 3. Area under the Receiver Operating Characteristic Curve Analysis Investigating Cutoff Values for Proliferative Diabetic Retinopathy

Marker	Cutoff	Sensitivity	Specificity	AUC %
sLR11(U)	4.2	0.78	0.77	85
HbA1c (%)	6.5	0.63	0.60	57
BMI (kg/m^2)	24.5	0.59	0.59	64
eGFR (mL/minute per 1.73 m^2)	120.5	0.5	0.46	50

AUC = area under the receiver operating characteristic curve; BMI = body mass index; eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; sLR11 = soluble form of LR11.

TABLE 4. Correlation Analysis of Circulating sLR11 with Various Markers in All Subjects with Type 2 Diabetes Mellitus

	Pearson Correlation Coefficient	*P* Value
Age	−0.07	.63
Male	0.15	.29
Body mass index (kg/m^2)	0.21	.14
HbA1c (%)	0.32	< .01
Fasting blood sugar (mg/dL)	0.19	.17
eGFR (mL/minute per 1.73 m^2)	−0.19	.17
Total cholesterol (mg/dL)	0.27	.05
LDL cholesterol (mg/dL)	0.31	< .05
HDL cholesterol (mg/dL)	−0.10	.51
Triglyceride (mg/dL)	0.25	.07

eGFR, estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; HDL = ; LDL =.

using insulin was significantly higher in the PDR subjects than that in the NPDR subjects.

The circulating sLR11 levels in the NPDR and PDR groups were 3.7 ± 1.8 U and 5.8 ± 2.7 U, respectively (Figure 1), indicating that the sLR11 levels in the PDR group were higher than those in the NPDR group ($P <$ .01). Note that we previously reported that the mean circulating sLR11 levels in 400 dyslipidemic subjects was 3.0 ± 1.0 U.[5] The sLR11 analysis restricted for the patients not treated with insulin showed that the sLR11 levels again were higher in the subjects with PDR (4.8 ± 1.2 U; n = 10) than in those with NPDR (3.7 ± 1.3; n = 12; $P <$ .05). Thus, circulating sLR11 levels were increased in type 2 diabetes mellitus patients with PDR regardless of medication with insulin therapy.

We analyzed the significance of the sLR11 concentration in comparison with other risk factors for PDR, including age, male gender, estimated glomerular flow rate, and the total cholesterol and HbA1c concentrations, in all subjects (Table 2). The multivariate analysis using all variables for PDR showed that the circulating sLR11 level, as well as younger age, strongly associated with PDR independent of other variables.

The ROC curves of the various factors were examined for discriminating the probability of the type 2 diabetes mellitus patients with PDR from the NPDR patients based on the levels of sLR11, the levels of HbA1c, the BMI, or the estimated glomerular filtration rate (Figure 2). The curves showed the fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for various cutoff levels of each parameter. The cutoff level of sLR11 that gave the maximum sensitivity and specificity for PDR was 4.2 U. At the cutoff level, the sensitivity of sLR11 for PDR was 78%, and the specificity was 77%, equivalent to or more than the other classical risk factors, HbA1c, BMI, or estimated glomerular flow rate (Table 3).

Finally, to clarify the correlation between the sLR11 concentration and various clinical parameters in the studied patients, simple regression analyses were performed for the dependent variable (Table 4). The HbA1c levels and LDL cholesterol levels correlated positively with sLR11 ($r = 0.32$, $P < .01$, and $r = .31$, $P < .05$, respectively). No significant correlation was observed between the sLR11 and age, sex, BMI, fasting blood glucose, estimated glomerular flow rate, total cholesterol, HDL cholesterol, or triglyceride.

DISCUSSION

LR11 IS HIGHLY EXPRESSED IN THE ENDOTHELIAL CELLS under the condition of dyslipidemia as well as in the intimal smooth muscle cells migrated from media in the development of atherosclerosis.[2,3] Two recent independent studies for the subjects with dyslipidemia or coronary heart diseases have shown that the concentrations of soluble form, sLR11, were associated with the HbA1c levels in these subjects with different backgrounds.[4,5]

The key cytokines underlying the pathogenesis and development of PDR are similar to those leading to atherosclerosis. The barrier dysfunction of microvessels and retinal ischemia provokes an increase in the ocular levels of inflammatory cytokines and growth factors, including vascular endothelial growth factor, PDGF-BB, and angiotensin II,[14–16] with increased expression of adhesion molecules,[17] all promoting retinal neovascularization. PDGF-BB and angiotensin II trigger the increased expression of LR11 on vascular smooth muscle cells.[2,5] The LR11 expression in endothelial cells is induced under conditions of dyslipidemia, possibly through the activations of combination of cytokines and adhesion molecules.[2,3] Thus, considering that endothelial dysfunction is the first sign of microvascular injury at the organ level[18] and that the progression of diabetic microvascular complications is

modulated by the severity of hyperglycemia through the gradual damages of the endothelium,[19] a high sLR11 concentration in the serum of diabetic patients with PDR may reflect the pathophysiologic endothelial dysfunction associated with diabetes, although the mechanism responsible for the release of sLR11 in circulation remains unresolved.

In the present study, the sLR11 levels in the PDR group were increased compared with the NPDR group, regardless of medication with insulin therapy (see Figure 1). The multivariate analysis of all variables showed that the circulating sLR11 level, as well as age, strongly associated with PDR, independent of other variables (see Table 2). The ROC analysis indicated that the sensitivity and specificity of sLR11 is the highest at a cutoff level of 4.2 as a marker of PDR (see Figure 2 and Table 3). Finally, the sLR11 concentration was correlated positively with the HbA1c level (see Table 4), which was consistent with previous observations with subjects with different profiles.[4,5]

Various studies on the pathogenesis of and risk factors for the development of PDR have been conducted, and hypertension and renal failure have been identified as important risk factors, along with poor blood glucose control.[20–23] ROC analyses using the present study subjects showed that the area under the ROC of sLR11 was equivalent to or more than those of the so-far established risk markers (see Table 3). Thus, LR11 may be an additional tool for discriminating patients with a high risk of developing diabetic retinopathy from the increasing population of patients with type 2 diabetes mellitus. Considering the lack of enough data for the role of LR11 in the basic mechanism of PDR, to clarify the clinical significance of LR11 in patients with PDR, further pathophysiologic studies to address the question that sLR11 is a marker or a triggering factor are required.

Thus, one limitation of the present investigation is the lack of information about the sLR11 data in the retina and proliferative membrane in patients with PDR. Second, the data may have been influenced by the continuous use of medication. The sLR11 levels of subjects with insulin therapy were not significantly different from those of subjects without insulin therapy (see Figure 1). In addition, most of the patients had received medication against hypertension with ARBs (see Table 1). Considering the fact that statins and ARBs inhibit the sLR11 expression in cultured cells,[2,5] the circulating sLR11 levels may be modified by these treatments. In this context, there was no significant difference in sLR11 levels between the subject groups with or without use of statin, or ACE-I, or ARB in the PDR subjects (data not shown). Finally, our results were obtained using relatively small sample sets. Clearly, further careful validation studies with larger sample sets to evaluate the effects of sLR11 on microvascular outcomes as primary end points will be required.

In summary, this study presented a novel and potentially clinically relevant new correlation of sLR11 with PDR, thus potentially providing a serum test to indicate patients at greater risk of developing PDR.

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BACE1 activity is related to CSF concentrations of SORL1, soluble amyloid precursor protein and tau

Amalia Tsolakidou[†a], Panagiotis Alexopoulos[†a], Liang-Hao Guo[a], Timo Grimmer[a], Christine Westerteicher[a], Martina Kratzer[a], Meizi Jiang[b], Hideaki Bujo[b], Francesco Roselli[c], Maria Rosaria Leante[c], Paolo Livrea[c], Alexander Kurz[a], and Robert Perneczky[a*]

[a] Department of Psychiatry and Psychotherapy, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 München, Germany

[b] Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

[c] Department of Neurological and Psychiatric Sciences, University of Bari, Piazza G. Cesare 2, 70124 Bari, Italy

[†] These authors contributed equally to this work.

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*Corresponding author:

PD Dr. Robert Perneczky

Klinik und Poliklinik für Psychiatrie und Psychotherapie

Klinikum rechts der Isar der Technischen Universität München

Ismaninger Str. 22, 81675 München, Germany

Telephone: +49 89 4140 4214, Fax: +49 89 4140 4888

Email: robert.perneczky@lrz.tum.de

Abstract

Background: BACE1 activity determines the rate of amyloid precursor protein (APP) cleavage and is therefore the main driver of amyloid β (Aβ) production, which is a pathological hallmark of Alzheimer's disease (AD). **Methods:** The present study explored the correlation between BACE1 activity and cerebrospinal fluid (CSF) markers of APP metabolism and axonal degeneration in 63 patients with mild AD and 12 healthy control subjects. **Results:** In the AD group positive correlations between BACE1 activity and soluble APP (sAPP) β, the APP sorting receptor SORL1 (also known as SorLA or LR11), and tau were detected. BACE1 activity was not associated with $A\beta_{1-42}$ or sAPPα concentrations in the AD group and no associations were found in the control group between BACE1 activity and any of the protein concentrations.

Conclusion: Our results confirm the relevance of BACE1 and SORL1 within the amyloid cascade and also provide a further piece of evidence for the link between amyloid and tau pathology in AD.

Key words: Alzheimer's disease; dementia; biomarker; amyloid cascade; beta-secretase

1. Background

The cerebral pathologic hallmarks of Alzheimer's disease (AD) include the extracellular accumulation of amyloid beta (Aβ) plaques, synaptic and neuronal degeneration, and the presence of tau protein tangles [1]. Aβ plaques mainly consist of the 4-kDa Aβ peptide, which is generated by the enzymatic cleavage of the transmembrane amyloid precursor protein (APP). The first, and rate-limiting, APP cleavage step by the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) [2] results in the production of the N-terminal soluble APP (sAPP) β and a C-99 fragment, which is subsequently cleaved by the γ-secretase complex resulting in Aβ. The alternative processing of APP by the α-secretases precludes the generation of Aβ since the cleavage site lies within the Aβ sequence; sAPPα is a product of this processing way [3]. The relevance of BACE1 for AD is supported by its increased expression and activity in AD brain tissue [4,5] and cerebrospinal fluid (CSF) [6,7].

In addition to the secretases, the sortilin-related receptor with A-type repeats (SORL1, also termed LR11 or sorLA), a member of the apolipoprotein E (APOE) and low-density lipoprotein receptor family [8,9], has emerged as another relevant regulator of APP processing; SORL1 is probably involved in the intracellular sorting of APP and its interactions with the secretases including BACE1 [10]. According to recent evidence SORL1 promotes the retention of APP in subcellular compartments that are less favorable for secretase processing and thereby reduces the extent of proteolytic breakdown into both amyloidogenic and non-amyloidogenic products [11]. In line with this finding, the neuronal expression of SORL1 is dramatically decreased in brains of patients with sporadic AD [12-14]. The large extracellular part of the receptor is released after endoproteolytical cleavage [15] and can therefore be measured in CSF; however, no general consensus has yet been reached regarding the effects of AD on SORL1 concentrations in CSF [16,17].

The aforementioned evidence and theoretical considerations suggest that BACE1 activity should be positively correlated with $A\beta_{1-42}$ and sAPPβ (but not sAPPα), and possibly also with tau as well as SORL1 concentrations in CSF. Some of these assumptions, such as the positive association between BACE1 activity and sAPPβ and tau concentrations, are backed by previous research, whereas others are not [18,19], which warrants replication. Furthermore, the correlation between SORL1, the encoding gene of which is among the strongest known genetic risk factors for sporadic AD [20], and BACE1 in CSF has not been studied so far. The main aim of the present study was to provide evidence in relation to these issues.

2. Methods

2.1 Participant selection

Sixty-three patients with probable AD with available lumbar CSF samples were identified in the electronic database of the Department of Psychiatry and Psychotherapy at the Technische Universität München. Informed written consent was available for all patients; the study protocol was approved by the ethics committee of the faculty of medicine at the Technische Universität München. The clinical diagnoses had been established by consensus of two experienced clinicians according to NINCDS-ADRDA criteria for probable AD in conjunction with ICD-10 criteria for mild AD dementia. The diagnostic workup included patient and proxy interviews, physical examination, psychometric testing, routine blood sampling, and structural imaging of the brain (MRI or CT). None of the patients showed signs of relevant cerebrovascular disease or any plausible cause for cognitive impairment other than AD. The psychometric assessment was based on the Consortium to Establish a Registry for AD (CERAD) neuropsychological assessment battery, which incorporates the Mini-Mental-State Examination (MMSE). An additional group of

twelve healthy control subjects, recruited at the Department of Neurology at the University of Bari in Italy, was included to explore the associations between the CSF proteins in the absence of any relevant neurodegenerative pathology. The controls had no subjective memory complaints and no history of cognitive impairment. They were independent in their activities of daily living and did not show any signs of a relevant psychiatric or neurological illness.

2.2 CSF sampling and analyses

CSF was collected in sterile polypropylene tubes using atraumatic canulas placed in the L3/L4 or L4/L5 intervertebral space. The CSF was centrifuged for 1800g at 4°C for 10 min immediately after collection to remove cells. Aliquots of the remaining CSF supernatants were stored in polypropylene tubes at -80°C for further processing.

2.3 Determination of $A\beta_{1-42}$, tau, sAPPα and sAPPβ levels

$A\beta_{1-42}$, total tau (Innogenetics, Ghent, Belgium), and sAPPα/sAPPβ (IBL, Gunma, Japan) in CSF were measured in duplicate with commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions as described previously in greater detail (37-39).

2.4 BACE1 activity assay

BACE1 activity was measured using a time-resolved fluorescence activity assay based on SignalClimb technology (TruePoint Perkin Elmer, Turku, Finland) according to optimized manufacturer's instructions (12). The synthetic TruePoint BACE1 substrate is a ten amino acid long peptide with a fluorescent europium (EU) chelate coupled to one end and a quencher of europium fluorescence (QSY7) coupled via lysine to the other end. The hydrolysis of the substrate's protein sequence

CEVNLDAEFK by BACE1 results in a fluorescence signal that is proportional to the activity of BACE1. The fluorescence signal was measured at 37°C in a microplate reader using time-resolved fluorescence (FLUOstar Omega, BMG Labtech, Offenburg, Germany; excitation wavelength: 320 nm, emission wavelength: 615 nm) in black 96-well plates (Perkin Elmer, Turku, Finland) at a final volume of 27 µl, including 10 µl CSF, 2 µl DMSO, and 15 µl BACE1 substrate (0.80 nmol/ml). The continuous measurement of BACE1 activity was started immediately after adding the CSF sample; BACE1 activity was defined as the maximal activity within the first 30 min. Each sample was measured at least four times in order to verify reproducibility. Proteinase inhibitors were added in order to block all non-BACE1 aspartyl protease activity.

2.5. SORL1 concentrations

SORL1 concentrations in CSF were quantified using ELISA in the laboratories of Sekisui Medical Co Ltd. (Ryugasaki, Japan) as described previously (40). Briefly, 10 µl CSF was diluted with 100 µl sample buffer and added to the plate coated with mouse monoclonal antibody M3 (41). After incubating with the biotinylated rat monoclonal antibody R14, the SORL-antibody complex was reacted with horseradish peroxidase-conjugated streptavidin. A standard curve was constructed using purified SORL1 protein. The final absorbance of each sample was determined at 450 nm (40). SORL1 concentrations were only determined in a sub-sample of 40 patients with probable AD and in the entire control group. The first published study using this assay (40) showed that purified SORL1 in CSF was immunologically identical to SORL1 from cell culture, strongly arguing that the ELISA measures the soluble form of the membrane-bound receptor.

2.6 Statistical Analysis

Data were analyzed in the Predictive Analytics Software package (PASW) v18 (The SPSS Inc., Chicago, IL, USA) using two-sided tests. Normal distribution was checked using the Kolmogorov-Smirnov Test. Correlations between BACE1 activity and CSF protein concentrations were investigated using the Pearson's correlation coefficient. P-values were regarded significant at a level of 5 percent; the false discovery rate (FDR; http://sdmproject.com/utilities/?show=FDR), which controls the expected proportion of incorrectly rejected null hypotheses (type-I errors), was used to account for the error in multiple comparisons; i.e. results at $p_{corr} < 0.05$ were regarded significant.

3. Results

The characteristics of the study sample are shown in Table 1. The CSF concentrations of $A\beta_{1-42}$ and tau were in the expected range for this kind of sample (32). The MMSE score range confirmed the mild degree of dementia in the AD group and the lack of any objective cognitive impairment in the control group. In the AD group there was a significant positive correlation after FDR correction for multiple comparisons between BACE1 activity on the one hand and tau ($r = 0.30$, $p_{corr} = 0.04$, $N = 63$) as well as SORL1 concentrations ($r = 0.37$, $p_{corr} = 0.04$, $N = 40$) on the other hand (Figure 1). BACE1 activity was not correlated with sAPPα ($p_{corr} = 0.17$) or $A\beta_{1-42}$ ($p_{corr} = 0.91$) in the AD group. At a more liberal threshold of $p<0.05$ uncorrected for multiple comparisons, BACE1 was also positively correlated with sAPPβ in patients with AD ($r = 0.26$, $p_{uncorr} = 0.05$, $N = 63$). BACE1 activity was not correlated with any of the CSF proteins in the healthy control group (tau: $p_{corr} = 0.96$; $A\beta_{1-42}$: $p_{corr} = 0.75$; sAPPα: $p_{corr} = 0.55$; sAPPβ: $p_{corr} = 0.55$; SORL1: $p_{corr} = 0.90$).