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RXRA gene variations influence Alzheimer's disease risk and cholesterol metabolism

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Abstract

Cholesterol metabolism is altered in Alzheimer's disease (AD). The nuclear hormone receptor Retinoic X Receptor α (RXRα) is a member of the nuclear ligand-activated transcription factor family. RXRs are key regulators of cholesterol synthesis and thus cholesterol metabolism. We performed a systematic screen for gene variants in the RXRA gene. The effect of these gene variants on the risk of AD was investigated in 405 AD patients (mean age: 74.27 $\pm$ 9.37 years; female 78.6%) and 347 controls (mean age: 73.26 $\pm$ 8.37 years; female 57.2%). Furthermore, the influence of RXRA gene variants on CSF and plasma levels of cholesterol, lathosterol and 24S-hydroxycholesterol were evaluated. One of the identified seven SNPs in RXRA influenced AD risk in our single marker analysis (rs3132293: $P = 0.006$). Haplotype analysis identified a three-marker haplotype (TGC) consisting of rs3118570, rs1536475 and rs3132293, which decreased the risk of AD ($P = 0.009$). The single marker rs3132293 ($P = 0.026$) and the TGC haplotype ($P = 0.026$) influenced CSF lathosterol levels in non-demented controls, and cholesterol levels in the combined sample comprising AD patients and controls (Rs3132293: $P = 0.050$; TGC haplotype: $P = 0.035$). 24S-Hydroxycholesterol CSF and plasma levels were also influenced by rs3132293 (CSF: $P = 0.004$; plasma: $P = 0.001$) and the TGC haplotype (CSF: $P = 0.004$; plasma: $P = 0.002$); this effect was most pronounced in AD patients (rs3132293: CSF: $P = 0.009$, plasma: $P = 0.002$; TGC haplotype: CSF: $P = 0.019$, plasma: $P = 0.005$). Our results suggest that RXRA gene variants might act as risk factor for AD *via* an influence on cerebral cholesterol metabolism.

Keywords: retinoic X receptor α • Alzheimer's disease • association • cholesterol • sequencing

Introduction

Alzheimer's disease (AD) is the most common cause of dementia world-wide. Typical pathological hallmarks are neurofibrillary tangles, amyloid angiopathy, insoluble, extracellular amyloid plaques, mainly consisting of neurotoxic 4 kD amyloid-β peptide (Aβ) and leukoaraiosis, which involves diffuse white matter changes [1]. Research of the last 10 years revealed the involvement of cholesterol metabolism in the pathogenesis of AD. Presence of the apolipoprotein E (APOE) 4 allele is the strongest genetic risk factor for late-onset AD [2]. Cholesterol metabolism is altered in AD patients in that serum levels of cholesterol metabolites such as 24S-hydroxycholesterol and 27-hydroxycholesterol are decreased [3]. Cholesterol depletion or administration of cholesterol lowering 3ß-hydroxy-3ß-methyl-glutaryl-CoA reductase (HMGCoAR) inhibitors, so called statins, inhibits the production of ß-amyloid (Aß) *in vitro* [4] and reduce the levels of Aß *in vivo* and *in vitro* [5]. Statins might reduce the risk of AD [6], even though this therapy is also discussed controversially for AD prevention and treatment [7, 8].

Brain cholesterol is synthesized locally and predominantly, but not entirely independent of blood cholesterol levels [9, 10]. Excess brain cholesterol derived from increased membrane turnover or

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neuronal loss is eliminated *via* cholesterol hydroxylation leading to the formation of 24S-hydroxycholesterol, the major elimination product of cerebral cholesterol.

The nuclear hormone receptor Retinoic X Receptor α (RXRα) is a member of the nuclear ligand-activated transcription factor family and belongs to the steroid hormone receptor super family. RXRs are important regulators of different pathways and are involved in cell proliferation, differentiation, and in glucose, fatty acid and cholesterol metabolism [11]. RXRs can function as homodimers or as heterodimers. Heterodimers of RXRα gene (RXRA) with the nuclear receptors liver X receptor (LXR) or peroxisome proliferator activated receptor (PPAR) have direct influence on cholesterol metabolism in that they influence expression of i.e. Apolipoprotein A1, APOE, ATP-binding cassette transporters (ABC-transporters) and sterol regulatory element-binding protein 1 (SREBP1), which are all key players in cholesterol homeostastis [12–15]. Sequence variations in RXRA might influence gene and protein function, leading to alterations in cholesterol metabolism and by this can influence the risk of AD.

RXRA is located at chromosome 9q34, nearby a putative hot spot for an AD risk gene [16, 17]. RXRA spans about 100 kilo bases and contains 10 exons. The promoter was just recently identified and characterized [18]. Several polymorphisms have been described in single-nucleotide polymorphism (SNP) databanks, but only few have been confirmed in human populations. In general, studies on RXRA polymorphisms are scarce [19] and association studies with AD are lacking. Thus, we performed a systematic screen for RXRA gene variants in AD patients and non-demented probands and investigated, if the detected polymorphisms influence the risk of AD and levels of cholesterol, the cholesterol precursor lathosterol and the cerebral cholesterol elimination product 24S-hydroxycholesterol.

Methods

Probands

AD patients ($n = 405$, mean age: 74.1 $\pm$ 7.9; range: 59–96 years; female: 69.1%) were recruited from the Department of Psychiatry, University of Bonn, Germany, and from the Division of Neuroradiology of the Central Institute of Mental Health, Mannheim, Germany. Patients were diagnosed according to DSM-IV, supported by clinical examination, detailed structured interviews, neuropsychological testing, cognitive screening including mini mental state examination (MMSE) [20] and neuroimaging studies. Healthy controls ($n = 347$, mean age: 72.8 $\pm$ 7.6; range: 64–100 years; female: 53.5%) were recruited with the support of the local Census Bureau and the regional Board of Data Protection (Nordrhein-Westfalen, Germany) and from the Central Institute of Mental Health, Mannheim, Germany. The cognitive status was assessed by neuropsychological testing and structured interviews. All participants of the study gave informed written consent. The study has been approved by the Ethics Committee of the Faculty of Medicine of the University of Bonn and of the institutional ethics committee, Mannheim, Germany.

Table 1 Primers used for the screening of RXRA exonic regions

Exon	Primer sequences	Product sizes
1	F: 5′-CAGACACAAGTAGTTTACATTGTTGG-3′	215 bp
	R: 5′-GAGCGGCAGGAAATGTTTGG-3′	
2	F: 5′- CCTGGCCCGTAGTGAGTGTG-3′	753 bp
	R: 5′-AGGCCACAGCCTCTCTCTGC-3′	
3 + 4	F: 5′- AGCCTCGGTGTCTGTGTGTG-3′	1216 bp
	R: 5′-GCCTTGCCATGCTTCTGTC-3′	
5	F: 5′- TGGGGATGCTGGTGTTGTGG-3′	400 bp
	R: 5′-GCAGCCCCCAGGACCTCTCT-3′	
6	F: 5′-GCCACACCTAATCACCTCTGC-3′	483 bp
	R: 5′-GGAGGTAGGAGTGGGTCCTG-3′	
7	F: 5′-GAACGGGCATTCTCAGGAAC-3′	472 bp
	R: 5′-TCTGAGCCTCCTCCTCATGC-3′	
8	F: 5′-TTGGCTTGGCCCATCTCAGC-3′	391 bp
	R: 5′-CAGAAGGCAGCATGGCCACA-3′	
9	F: 5′-CTGGCCGTGGGTTCCACAGT-3′	459 bp
	R: 5′-GGACCTCCTGCTGCCTGCTC-3′	
10	F: 5′-CCCTTCATCTCCGTCCTCAG-3′	870 bp
	R: 5′-CTGGGCACCAGTATTCAGAGC-3′	

Screening for variations in RXRA

Leukocyte DNA was isolated with the Qiagen® blood isolation kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). PCRs of the exons and flanking intronic regions were performed with primers designed from the sequence of chromosome 9 (GenBank acc.no.: NT_019501) as outlined in Table 1. The resulting amplification products were investigated by cycle sequencing and big dye terminator chemistry with the ABI Prism Genetic Analyzer 310A (PE Biosystems, Weiterstadt, Germany) in 40 probands. This number of probands is sufficient to detect frequent sequence variations (>5% of the population) with a likelihood of >95%.

Genotyping of SNPs

For the determination of all seven identified SNPs in RXRA in the whole study population, genotyping methods performed with restriction fragment length polymorphism (RFLP) or allele-specific PCR (Polymerase chain reaction-CTPP [21]) were established. Details are given in Table 2. As a quality measure samples were randomly analyzed by cycle sequencing with the ABI Prism genetic analyzer 310 A (PE Biosystems). The APOE genotype was studied as described by Hixson and Vernier [22].

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Table 2 Methods for the determination of RXRA gene variants

Variation	Primer	Method	Enzyme/primer	Alleles, fragments (Bp)
Rs226677, IVS1+33 A/G	F: 5′-CCTGGCCCGTAGTGAGTGTG-3′	RFLP	NlaIV	G-Allel: 217, 100, 78, 73, 59 55, 35, 30, 20, 10
	R: 5′-AGGCCACAGCCTCTCTCTGC-3′			A-Allele: 295, 100, 73, 59 55, 35, 30, 20, 10
Rs1805352, IVS1-46 A/C	F: 5′-AGCCTCGGTGTCTGTGTGTG-3′	CTPP	F-A: 5′-CGTGGGGGACATAGGGAA-3′	A-Allele: 247, 190
	R: 5′-GCAGATGTGCTTGGTGAAGG -3′		R-C: 5′-CGGGGTGTACACCAGGTTTG-3′	C-Allele: 247, 90
Rs3118570, IVS5-99 T/G	F: 5′-GAACGGGCATTCTCAGGAAC-3′	RFLP	HphI	G-allele: 270, 110, 90
	R: 5′-TCTGAGCCTCCTCCTCATGC-3′			T-Allele: 360, 110
Rs1536475, IVS6+70 A/G	F: 5′-GAACGGGCATTCTCAGGAAC-3′	RFLP	AciI	A-Allele: 215, 140, 115
	R: 5′-TCTGAGCCTCCTCCTCATGC-3′			G-Allele: 215, 140, 90, 25
Rs3132293, IVS7-126 T/C	F: 5′-CTGGCCGTGGGTTCCACAGT-3′	CTPP	F-C: 5′-AGGCATGTCCAGCGGCATC-3′	C-Allele: 459, 400
	R: 5′-GGACCTCCTGCTGCCTGCTC-3′		R-T: 5′-GCAGAGCAGGTGGTGGAGGA-3′	T-Allele: 459, 94
IVS8-100	F: 5′-CTCCACCACCTGCTCTGCAC -3′ *	RFLP	DraIII	T-Allele: 350, 30
	R: 5′-GGACCTCCTGCTGCCTGCTC-3′			G-Allele: 380
Rs1805343, IVS8 -27 G/A	F: 5′-CCCTTCATCTCCGTCCTCAG-3′	RFLP	MspI	G-Allele: 385, 228, 200, 65
	R: 5′-CTGGGCACCAGTATTCAGAGC-3′			A-Allele: 385, 280, 200

*The underlined letter represents a mismatch in the primer for the generation of an allele-specific enzyme restriction site.

Analysis of cholesterol, lathosterol and 24S-hydroxycholesterol in CSF and plasma

After an overnight fast, cerebro spinal fluid (CSF) samples from 149 AD patients and 86 non-demented controls were obtained by lumbar puncture. Plasma samples from 289 AD patients and 230 non-demented controls were obtained by venous puncture. AD patients were diagnosed as described above. For CSF collection, non-demented controls referring to the Department of Neurology, University of Bonn, who underwent lumbar puncture during clinical routine diagnosis of neurological disorders other than neurodegenerative or inflammatory diseases of the central nervous system were recruited. Plasma samples were obtained from non-demented controls recruited in the Department of Psychiatry, University of Bonn, Germany, and from the Division of Neuroradiology of the Central Institute of Mental Health, Mannheim, Germany. Patients with symptomatic cardiac disease, renal or hepatic dysfunction, insulin-dependent diabetes mellitus, untreated thyroidal dysfunction, blood–brain-barrier-disturbance, inflammatory disease, other neurodegenerative disorders or alcohol abuses were excluded. The cognitive status of the control group was assessed by MMSE [20]. Samples were frozen in aliquots and stored at –80°C until assay. CSF and plasma concentrations of lathosterol, 24S-hydroxycholesterol and CSF cholesterol were measured by a modified sensitive method performed with combined gas chromatography-mass spectrometry as described previously [23]. Plasma concentrations of cholesterol were measured by standard enzymatic procedures (Boehringer, Mannheim, Germany). Since plasma concentrations of 24S-hydroxycholesterol or lathosterol and cholesterol are highly correlated, plasma levels of 24S-hydroxycholesterol and lathosterol were corrected for plasma cholesterol.

Statistical analysis

Allele frequencies and genotype distribution of RXRA polymorphisms in the different diagnosis groups were compared using X^2-statistics. Logistic regression analysis was used to examine the simultaneous effect of RXRA polymorphisms, the APOE4 allele, age and sex on the risk of AD.

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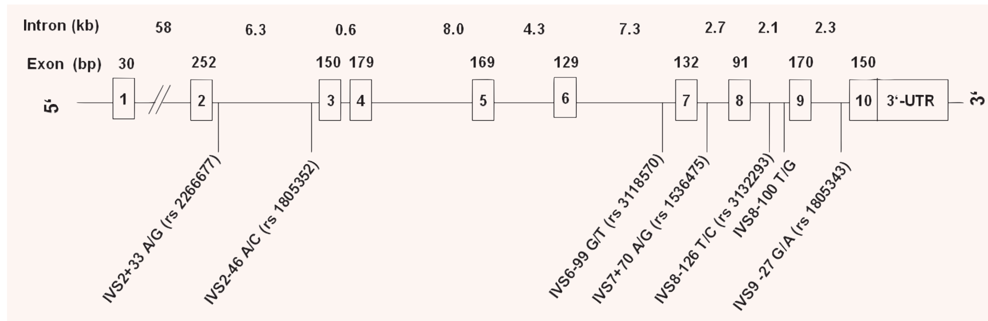

Fig. 1 Schematic representation of the RXRA gene and localization of the identified gene variants.

Haplotype analysis was performed with FAMHAP16 [24, 25], a software program estimating haplotype frequencies using the expectation-maximization (EM) algorithm. Haplotype frequencies in cases and controls were compared with the global likelihood ratio test as implemented in FAMHAP16. To account for multiple testing 10,000 permutations were performed. Linkage Disequilibrium (LD) plot analysis was performed with Haploview [26].

The effect of association of RXRA single SNP and haplotype with lathosterol, 24S-hydroxycholesterol and cholesterol levels was tested by MANOVA using the APOE4 allele and age as covariates. Since it is known from the literature that there are differences in the levels of cholesterol and metabolites between AD patients and controls [3, 27], separate analyses for each diagnosis group were performed. *P*-Values were set at two sided $P < 0.05$.

Results

Single locus analysis

Seven polymorphisms were identified in RXRA in our sample (Fig. 1); none of the identified polymorphisms was located in the exonic region of the gene. The Hardy-Weinberg disequilibrium was verified for AD patients and controls and all RXRA polymorphisms. Allele frequencies and genotype distributions are given in Table 3. Due to a frequency of less than 5% of the minor allele of IVS8-100, this polymorphism was excluded from further analyses. X^2-Statistics revealed differences in genotype distribution between AD patients and controls for SNPs rs1805352, rs3118570, rs1536475 and rs3132293.

We performed a logistic regression analysis including the remaining six RXRA polymorphisms and the co-variates, APOE4 allele, age and sex. Rs3132293 (IVS7-126 T/C) significantly influenced the risk of AD in that carriers of an rs3132293 CC genotype presented with a reduced risk of AD (rs3132293: $X^2 = 7.60$; df = 1; $P = 0.006$; OR = 0.64; 95% CI = 0.46–0.88). As expected, age ($P = 0.037$), female gender ($P = 0.001$) and the presence of the APOE4 allele ($P < 0.001$) were associated with an increased risk of AD. The other RXRA polymorphisms did not influence the risk of AD in this model (carrier of the minor allele *versus* non-carriers: rs226677: $P = 0.34$; rs1805352: $P = 0.96$; rs3118570: $P = 0.39$, rs1536475: $P = 0.71$; rs1805343: $P = 0.42$).

Haplotype analysis

LD plot analysis performed with Haploview revealed high-linkage disequilibrium D' scores between all RXRA polymorphisms investigated in this study (Fig. 2). For haplotype analysis performed with FAMHAP16, only haplotypes with a frequency of more than 5% were included. Famhap16 identified a three-marker haplotype consisting of rs3118570 (IVS5-99 T/G), rs1536475 (IVS6+70 A/G) and rs3132293 (IVS7-126 T/C) to influence the risk of AD ($P = 0.009$, global statistics, 10,000 permutations). Homozygote carriers of the TGC haplotype were significantly less frequent in the group of AD patients compared to controls ($X^2 = 10.09$; df = 1; $P = 0.001$, Tables 4 and 5), suggesting that this haplotype might act as a protective factor of AD.

To consider for co-variates, we performed an additional logistic regression analysis by including the co-variates APOE4 allele, age and gender. Homozygote carriers of the TGC haplotype presented with a reduced risk of AD ($X^2 = 7.32$; df = 1; $P = 0.007$; OR = 0.648; 95% CI = 0.47–0.89). As expected, female gender ($P = 0.001$), age ($P = 0.032$) and the presence of the APOE4 allele ($P < 0.001$) were associated with an increased risk of AD.

CSF parameters

To verify if RXRA single marker, rs3132293 and the RXRA TGC haplotype, which both influenced the risk of AD, might also influence CSF levels of cholesterol, the cholesterol precursor lathosterol and the cerebral cholesterol elimination product 24S-hydroxycholesterol, we performed MANOVAs independently for the RXRA single marker and the haplotype.

MANOVAs in the whole sample comprising AD patients and controls detected that CSF levels of 24S-hydroxycholesterol and cholesterol were lower in carriers of the rs3132293 C allele (24S-hydroxycholesterol: $P = 0.004$; cholesterol: $P = 0.050$; Table 6) or the TGC haplotype (24S-hydroxycholesterol: $P = 0.004$; cholesterol: $P = 0.035$; Table 6), while levels of lathosterol were not altered ($P = 0.2$). In both analyses presence of the APOE4 allele influenced 24S-hydroxycholesterol levels ($P < 0.001$) but not those of the other cholesterol parameters ($P > 0.5$). Age influenced levels of cholesterol and 24S-hydroxycholesterol (each $P < 0.001$),

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Table 3 Genotype distribution and allele frequencies of RXRA polymorphisms in AD patients and controls

Diagnosis	*n*	Genotypes [%]			Allele frequencies		χ^2-test		
IVS1+33 A/G, rs2266677		**AA**	**AG**	**GG**	**A**	**G**	χ^2	**df**	***P***
Controls	347	217 (62.5)	116 (33.4)	14 (4.1)	0.79	0.21	0.373	2	0.83
AD	405	246 (60.7)	140 (34.6)	19 (4.7)	0.78	0.22			
IVS1-46 A/C, rs1805352		**AA**	**AC**	**CC**	**A**	**C**	χ^2	**df**	***P***
Controls	347	175 (50.4)	128 (36.9)	44 (12.7)	0.69	0.31	6.36	2	0.042*
AD	405	179 (44.2)	186 (46.0)	40 (9.8)	0.67	0.33			
IVS5-99 T/G, rs3118570		**TT**	**TG**	**GG**	**T**	**G**	χ^2	**df**	***P***
Controls	347	241 (69.5)	90 (25.9)	16 (4.6)	0.82	0.18	8.61	2	0.014*
AD	405	261 (64.4)	136 (33.6)	8 (2.0)	0.81	0.19			
IVS6+70 A/G, rs1536475		**GG**	**GA**	**AA**	**G**	**A**	χ^2	**df**	***P***
Controls	347	246 (70.9)	82 (23.6)	19 (5.5)	0.83	0.17	6.54	2	0.038*
AD	405	257 (63.5)	130 (32.1)	18 (4.4)	0.80	0.20			
IVS7-126 T/C, rs3132293		**CC**	**CT**	**TT**	**C**	**T**	χ^2	**df**	***P***
Controls	347	216 (62.2)	107 (30.8)	24 (7.0)	0.78	0.22	13.60	2	0.001*
AD	405	203 (50.1)	177 (43.7)	25 (6.2)	0.72	0.28			
IVS8-100 T/G		**TT**	**TG**	**GG**	**T**	**G**	χ^2	**df**	***P***
Controls	347	333 (96.0)	5 (1.4)	9 (2.6)	0.97	0.03	1.48	2	0.48
AD	405	392 (96.8)	8 (2.0)	5 (1.2)	0.98	0.02			
IVS8 -27 G/A, rs1805343		**AA**	**AG**	**GG**	**A**	**G**	χ^2	**df**	***P***
Controls	347	166 (47.8)	129 (37.2)	52 (15.0)	0.66	0.34	4.96	2	0.084
AD	405	162 (40.0)	179 (44.2)	64 (15.8)	0.62	0.38			

*p-values < 0.05.

Table 4 RXRA haplotypes in AD patients and controls

Haplotype			Controls (n = 346)		AD-patients (n = 400)		Single values			Global statistics		
rs3118570	**Rs1536475**	**rs3132293**	***n***	**%**	***n***	**%**	χ^2	**df**	***P***	χ^2	**df**	***P***
T	G	C	266	74.9	282	69.0	8.18	1	0.004*	13.20	4	0.004*
G	A	T	56	15.8	71	17.4	0.96	1	0.33			
T	G	T	22	6.1	39	9.5	5.82	1	0.02*			
T	A	C	2	0.6	8	1.9	4.91	1	0.03*			

*p-values < 0.05.

Table 5 Distribution of RXRA TGC haplotype including SNPs rs3118570, rs1536475 and rs3132293 in AD patients and controls

Diagnosis	*n*	TGC Homocygotes		Carrier versus non-carrier		
		Carrier	**Non-carrier**	χ^2	**df**	***P***
Controls	347	145 (41.7)	202 (58.3)	7.14	1	0.001*
AD	405	216 (53.3)	189 (46.7)			

*p-values < 0.05.

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Table 6 Influence of single marker rs3132293 and the three-marker TGC haplotype on CSF lathosterol, 24S-hydroxycholesterol and cholesterol levels in AD patients and controls

Sample	Genotype	*n*	Mean ± SE	F	df	*P*	Mean ± SE	F	df	*P*	Mean ± SE	F	df	*P*
rs3132293			**Lathosterol [μg/dl]**				**24S-Hydroxycholesterol [ng/ml]**				**Cholesterol [mg/dl]**			
All	C-allele carrier	214	0.33 ± 0.02	1.53	1	0.22	2.61 ± 0.07	8.347	1	0.004*	0.49 ± 0.01	3.88	1	0.050*
	Non-carrier	21	0.39 ±0.05				3.27 ± 0.22				0.55 ± 0.03			
Controls	C-allele carrier	77	0.34 ± 0.03	5.15	1	0.026*	2.56 ± 0.12	1.57	1	0.16	0.53 ± 0.02	2.41	1	0.13
	Non-carrier	9	0.54 ± 0.09				3.02 ± 0.32				0.61 ± 0.05			
AD	C-allele carrier	137	0.30 ± 0.02	0.24	1	0.63	2.58 ± 0.09	6.97	1	0.009*	0.44 ± 0.01	1.93	1	0.17
	Non-carrier	12	0.27 ± 0.06				3.4 ± 0.3				0.51 ± 0.04			
Three-marker TGC haplotype			**Lathosterol [μg/dl]**				**24S-Hydroxycholesterol [ng/ml]**				**Cholesterol [mg/dl]**			
All	TGC-haplotype carrier	193	0.32 ± 0.02	0.75	1	0.39	2.57 ± 0.07	8.53	1	0.004*	0.48 ± 0.01	4.49	1	0.035*
	Non-carrier	25	0.35 ± 0.04				3.16 ± 0.19				0.54 ± 0.03			
Controls	TGC-haplotype carrier	67	0.32 ± 0.03	5.19	1	0.026*	2.53 ± 0.12	3.92	1	0.052	0.53 ± 0.02	1.97	1	0.16
	Non-carrier	11	0.49 ± 0.07				3.12 ± 0.29				0.59 ± 0.05			
AD	TGC-haplotype carrier	126	0.30 ± 0.02	0.78	1	0.38	2.54 ± 0.09	5.66	1	0.019*	0.43 ± 0.01	2.90	1	0.09
	Non-carrier	14	0.25 ± 0.05				3.22 ± 0.27				0.49 ± 0.04			

*p-values < 0.05.

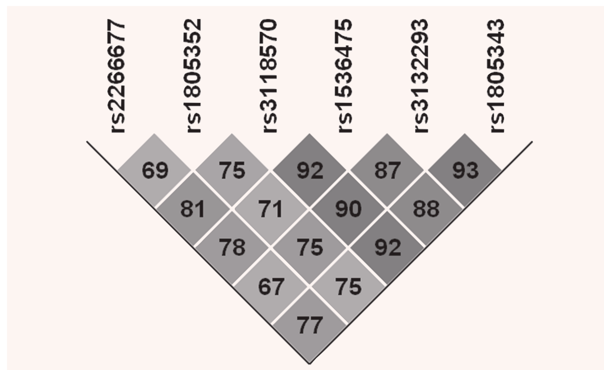

Fig. 2 Linkage Disequilibrium (LD) structure of RXRA gene variations. The number at the intersection of each pair of SNPs represents the pairwise D' values between two SNPs.

but not of lathosterol. Levels of cholesterol and lathosterol were lower in AD patients compared to controls (cholesterol: $P < 0.001$; lathosterol: $P < 0.05$).

In agreement with the literature, we found differences in the levels of cholesterol parameters between AD patients and controls; thus we performed exploratory analyses in the subgroups of AD patients and controls.

In controls levels of lathosterol were lower in carriers of the rs3132293 C allele ($P = 0.026$) or the TGC haplotype ($P = 0.026$). Levels of 24S-hydroxycholesterol and cholesterol were also lower in carriers of the C allele or the TGC haplotype, but this did not reach statistical significance (Table 6).

In AD patients, levels of 24S-hydroxycholesterol were lower in carriers of the rs3132293 C allele ($P = 0.009$) or the TGC haplotype ($P = 0.019$), also cholesterol levels were altered in these patients, but this did not reach significance ($P < 0.17$). We did not find an effect of gene variants on the CSF levels of lathosterol in AD patients.

Plasma parameters

To investigate if RXRA single marker, rs3132293 and the RXRA TGC haplotype also influenced peripheral cholesterol metabolism, we performed MANOVAs independently for the RXRA single marker and the haplotype and investigated their effect on plasma levels of cholesterol, lathosterol/cholesterol and 24S-hydroxycholesterol/cholesterol.

MANOVAs in the whole sample comprising AD patients and controls detected that only levels of 24S-hydroxycholesterol/cholesterol were lower in carriers of the rs3132293 C allele ($P = 0.001$; Table 7) or the TGC haplotype ($P = 0.002$; Table 7). While levels of lathosterol/cholesterol or cholesterol were not altered ($P > 0.2$). Presence of the APOE4 allele influenced levels of all cholesterol parameters ($P < 0.01$), age influenced levels of 24S-hydroxycholesterol/cholesterol ($P = 0.022$) and lathosterol/cholesterol ($P = 0.008$).

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Table 7 Influence of single marker rs3132293 and the three-marker TGC haplotype on serum lathosterol, 24S-hydroxycholesterol and cholesterol levels in AD patients and controls

Sample	Genotype	*n*	Mean ± SE	F	df	*P*	Mean ± SE	F	df	*P*	Mean ± SE	F	df	*P*
	rs3132293		**Lathosterol/Cholesterol [µg/mg]**				**24S-Hydroxycholesterol/Cholesterol[ng/mg]**				**[spanname="12to15"]Cholesterol [mg/dl]**			
All	C-allele carrier	512	1.14 ± 0.02	0.01	1	0.95	36.27 ± 0.44	12.0	1	0.001*	237.6 ± 2.1	0.09	1	0.76
	Non-carrier	28	1.14 ± 0.1				29.64 ± 1.87				240.3 ± 8.7			
Controls	C-allele carrier	231	1.18 ± 0.03	0.31	1	0.58	36.77 ± 0.76	2.75	1	0.099	238.5 ± 3.4	1.43	1	0.23
	Non-carrier	12	1.26 ± 0.14				31.58 ± 3.09				255.0 ± 13.6			
AD	C-allele carrier	281	1.11 ± 0.03	0.29	1	0.59	35.43 ± 0.56	10.32	1	0.001*	237.7 ± 2.8	0.48	1	0.49
	Non-carrier	16	1.04 ± 0.13				27.96 ± 2.26				229.6 ± 11.4			
	Three-marker TGC haplotype		**Lathosterol/Cholesterol [µg/mg]**				**24S-Hydroxycholesterol/Cholesterol [ng/ml]**				**[spanname="12to15"]Cholesterol [mg/dl]**			
All	TGC-haplotype carrier	482	1.14 ± 0.02	0.49	1	0.48	36.29 ± 0.45	9.49	1	0.002*	238.9 ± 2.1	0.06	1	0.81
	Non-carrier	37	1.08 ± 0.08				31.13 ± 1.62				237.1 ± 7.5			
Controls	TGC-haplotype carrier	214	1.17 ± 0.04	0.12	1	0.73	36.76 ± 0.78	2.69	1	0.102	239.9 ± 3.3	0.73	1	0.39
	Non-carrier	16	1.22 ± 0.12				32.28 ± 2.67				249.9 ± 11.5			
AD	TGC-haplotype carrier	268	1.12 ± 0.03	1.27	1	0.26	35.56 ± 0.54	8.12	1	0.005*	238.3 ± 2.8	1.06	1	0.30
	Non-carrier	21	0.99 ± 0.12				29.69 ± 1.98				227.7 ± 9.9			

*p-values < 0.05.

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We also performed separate analyses in the subgroups of AD patients and controls. In controls, levels of 24S-hydroxycholesterol/cholesterol were higher in carriers of the C allele or the TGC haplotype, but this did not reach statistical significance (Table 7). Plasma cholesterol and lathosterol/cholesterol levels were neither influenced by the C allele nor by the TGC haplotype in non-demented persons (Table 7).

In AD patients, levels of 24S-hydroxycholesterol/cholesterol were higher in carriers of the rs3132293 C allele ($P = 0.001$) or the TGC haplotype (0.005). Levels of cholesterol or lathosterol/cholesterol were not influenced by any RXRA gene variant (Table 7).

Discussion

Alterations of cholesterol metabolism contribute to AD risk and pathology [3, 28]. RXRA is a key regulator of cholesterol metabolism and the gene is located next a region on chromosome 9 comprising increased LOD scores in AD linkage analyses [17, 29], making RXRA an ideal candidate for an AD risk gene.

We screened exons and flanking introns of RXRA and detected six polymorphisms with a frequency of >5% of the minor allele, which were included in our analyses. We selected only gene variants with a frequency of >5% for our study, and by this followed the common disease-common variant hypothesis [30, 31]. One might argue, that due to an other hypothesis, the common disease-multiple rare variants hypothesis, also multiple gene variants presenting with a low frequency might act as risk factors of AD. However, since it was the aim of our study to investigate the effect of one specific gene we focussed only on common variants in RXRA.

One RXRA polymorphism rs3132293 influenced AD risk in the single marker analysis, in that carriers of the CC genotype presented with a reduced risk of AD. Haplotype analysis identified a three-marker haplotype TGC, combining rs3118570, rs1536475 and rs3132293, which also reduced the risk of AD. Thus it might be concluded that gene variants in RXRA act as risk factor of AD.

All SNPs included in the identified haplotype are in strong linkage disequilibrium. The single marker rs3132293, which influenced AD risk might act as a taq SNP in our analysis. This also explains why the effects of the rs3132293 single marker and of the TGC haplotype on AD risk and on cholesterol parameters were in comparable ranges.

The rs3132293 C-allele and also the TGC haplotype influenced cholesterol metabolism of the brain as measured as reduced CSF levels of lathosterol, 24S-hydroxycholesterol and cholesterol in carriers of these gene variants; however, these effects differed between AD patients and controls. We found RXRA gene variants to influence levels of the cholesterol precursor lathosterol only in non-demented controls. However, influences on 24S-hydroxycholesterol were detected in the whole sample comprising AD patients and controls, but were most pronounced in AD patients. We also found CSF cholesterol levels in carriers of the rs3118570 C allele or the TGC haplotype to be reduced in the whole sample, but statistical analysis of the subgroups of AD patients or controls revealed only marginal effects. Influences of RXRA gene variants on peripheral cholesterol metabolism were also identified by altered levels of 24S-hydroxycholesterol/cholesterol, especially in AD patients, while the other plasma parameters were not changed. Our data suggest, that RXRA gene variants might influence AD risk mainly *via* an influence on cerebral cholesterol metabolism as measured by altered levels of 24S-hydroxycholesterol in CSF and plasma.

24S-Hydroxycholesterol is the major cholesterol elimination product of the brain [32, 33]. The detailed mechanism for the transport of 24S-hydroxycholesterol from brain *via* the blood–brain barrier into the periphery is not fully clear. ABCA1 transporters or organic anion transporting peptide 2 has been shown to be involved in this mechanism [34, 35] and ABCA1 expression is regulated by heterodimers of RXRA with the nuclear receptor LXR [15]. Previous publications report on reduced levels of 24S-hydroxycholesterol in plasma of AD patients compared to controls [3, 36]. In line with this observation, we found AD patients who were non-carrier of the protective RXRA C allele or the TGC haplotype to present with lower plasma levels; however, these probands showed increased CSF levels of 24S-hydroxycholesterol. These findings suggest, that in non-carriers of the protective RXRA gene variants, the elimination of 24S-hydroxycholesterol might be less efficient, possibly *via* a reduced activation of ABCA1 transporters, leading to an accumulation of CSF 24S-hydroxycholesterol.

In non-demented controls, who were non-carriers of the protective RXRA gene variants, we found increased CSF levels of lathosterol, which might be a sign of increased cholesterol *de novo* synthesis. Cholesterol synthesis and thus levels of lathosterol increase if the transcription factor LXR is activated by 24S-hydroxycholesterol [37]. It might be speculated that the higher CSF levels of 24S-hydroxycholesterol in non-demented persons who are homozygote carriers of the RXRA risk genotype might induce an increased synthesis of lathosterol, although the changes in the 24S-hydroxycholesterol levels were only marginally significant in these probands.

The lack of an effect of RXRA gene variants on CSF levels of lathosterol in AD patients might be due to the observation that alterations of cholesterol metabolism are a pathological process in AD [3, 27, 36]. Due to the neurodegenerative processes cholesterol synthesis in the brain of AD patients might be reduced. Comparable diagnosis-specific effects of gene variations on cholesterol metabolism have also been described for the APOE4 allele. It is generally accepted that the APOE4 allele influences plasma cholesterol in non-demented probands and that increased plasma cholesterol levels are observed in carriers of the APOE4 allele [38]. However, in AD patients it has been shown that serum cholesterol levels are not influenced by the presence of the APOE4 allele [39, 40]. It can be assumed that pathological alterations may cover gene effects especially in manifest AD, and thus gene effects might be seen mainly in non-demented probands.

Since studies on gene variations in RXRA are scarce, there is only one study investigating RXRA polymorphisms in psoriasis

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[41], it is unknown, if the identified polymorphisms might influence RXRA function or gene expression. The possible effect of RXRA SNPs on RNA splicing was evaluated using **a**utomated online splice site analysis (https://splice.cmh.edu) [42]. This program revealed that the rs3132293 C allele might cause a five to ninefold increased activity of a SRp40 site, while the rs3118570 T allele might cause a 4–25-fold increased activity of the donor site. It is unknown, if these effect are also present in human beings. Thus, these results have to be considered carefully and putative functions of the SNPs might be speculative. However, these analyses might help to gain a theoretical insight into the putative function of the identified RXRA gene variants.

We cannot exclude, that the relevant polymorphisms are in linkage disequilibrium with another yet unknown gene variant in the RXRA promoter. The promoter of RXRA was recently identified and described [18]; however, this study did not screen for gene variations in this region. It was reported, that the GC base content of the RXRA promoter is especially high, >80% and that there are several shorter regions presenting with 100% GC base content, making amplification of this region extremely difficult. Due to these limitations we were only able to screen a short region of the promoter, and it cannot be excluded, that other functionally relevant variations are located in the other promoter-regions of RXRA.

In conclusion, our results suggest that RXRA gene variants are risk factors of AD in that they influence cholesterol metabolism of the brain. However, the detailed mechanisms will have to be explored in additional studies.

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