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Immunoreactivity to astrocytes in different forms of dementia: High prevalence of autoantibodies to GFAP

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ABSTRACT

Objective: To study the prevalence of autoantibodies to glial and neuronal antigens with a focus on glial acidic fibrillary protein (GFAP) in patients with dementia.
Methods: Sera of 127 patients with different forms of dementia and sera of 82 age-matched patients with various neurological diseases except for dementia, as well as sera from 15 age-matched healthy controls were analyzed for anti-glial or anti-neuronal IgG using 1) primary murine embryonic hippocampus cell cultures, 2) murine brain sections, 3) immunoblotting on mouse brain homogenates and 4) astrocyte cultures. Sera reacting with astrocytes in hippocampus cell cultures were further analyzed using HEK293 cells transfected with human GFAP.
Results: IgG in serum from 45 of 127 (35.5%) patients with dementia but only 8 of 97 (8.2%, $p \leq 0.001$) controls bound to either glial or neuronal structures in cultured murine hippocampus cells. In these cultures antibodies to astrocytes were detected in 35 of 127 (27.5%) of the dementia patients, whereas in controls antibodies to astrocytes were detected in 4 sera only (4.1%, $p \leq 0.001$). Among the sera exhibiting reactivity to astrocytes, 14 of 35 (40%) showed immunoreaction to HEK293 cells transfected with GFAP in dementia patients, representing 11% of all sera. Within the 4 immunoreactive control sera reacting with astrocytes one reacted with GFAP (1.0% of total immunoreactivity, $p = 0.003$).
Conclusions: Autoantibodies to glial epitopes in general and to GFAP in particular are more frequent in patients with dementia than in age-matched controls without dementia, thus indicating the need for further investigations regarding the potential pathophysiological relevance of these antibodies.

1. Introduction

Autoimmune brain diseases caused by pathogenic antibodies gained increasing attention and became one of today's most relevant and growing research fields in neurology and psychiatry. Antibodies are mostly directed against neuronal cell-surface antigens eg. NMDA-, AMPA- and GABA-receptors (Dalmau et al., 2007; Kreye et al., 2016; Lai et al., 2009; Lancaster et al., 2010; Petit-Pedrol et al., 2014) or ion-channel subunits like LGI1 or CASPR2 (Irani et al., 2010). In addition, antibodies against intracellular proteins like synapsin (Piepgras et al., 2015; Höltje et al., 2017), amphiphysin (Folli et al., 1993) or GAD65 (Meinck et al., 2001) have been described. Due to the highly variable clinical presentation, diagnosis can be difficult, especially in cases when autoantibodies known to normally cause acute autoimmune encephalitis mimic neurodegenerative diseases such as atypical dementia (Hansen et al., 2021), Creutzfeldt-Jakob-Disease (Yoo and Hirsch, 2014), rapidly progressive dementia (Li et al., 2019), or frontotemporal dementia (Younes et al., 2018). In 2010, Flanagan et al. established the term "autoimmune dementia" for a subgroup of dementia patients with suspected autoimmune etiology, which improved with immunotherapy (Flanagan et al., 2010). Remarkably, autoantibodies against brain structures also occur in a large number of patients with a correctly diagnosed classic neurodegenerative disorder. The relevance of these antibodies, whether they are a primary cause of the disease or a secondary phenomenon, is unclear (Doss et al., 2014; Giannoccaro et al., 2021; Hansen et al., 2021). We aim to further explore

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Table 1
A) Demographic and dementia type data of the 127 patients analyzed in this study. Data are given in absolute numbers and proportions. AD = Alzheimer's disease; MCI = mild cognitive impairment; FTD = frontotemporal dementia; SAE = subcortical arteriosclerotic encephalopathy; LBD = Lewy body dementia; PD=Parkinson's disease dementia; CAA = cerebral amyloid angiopathy B, C) Demographic data of the two age-matched cohorts used for control. Neuropsychological testing was available for the 15 healthy subjects.

A) Dementia cohort.

Mean ± SD age at blood draw N = 127		Sex		Sex %	Type of dementia		Type of dementia %
73,8 ± 9,4		female	56	44	AD	59	46
female	male	male	71	56	MCI	24	19
73,0 ± 9,3	74,6 ± 9,6				FTD	13	10
					SAE	11	9
					SAE/AD	10	8
					LBD/PD	6	5
					CAA	4	3

B) Charité CSF/serum biobank cohort					C) Healthy subjects				
Mean ± SD age at blood draw N = 82		Sex		Sex %	Mean ± SD age at blood draw N = 15		Sex		Sex %
70, 8 ± 7,7		female	44	53,7	75,8 ± 5,1		female	9	60
female	male	male	38	46,3	female	male	male	6	40
70,8 ± 7,0	70,9 ± 8,5				75,9 ± 5,4	75,7 ± 4,6	Mean MMST ± SD 29 ± 0,6/30		

Table 2
Summary of staining results.

	Dementia cohort	Charité-Biobank cohort	Age-matched control cohort	P-value
number	127	82	15	
Immunoreactivity in primary hippocampal cell culture				
Neurons, n (%)	10 (8)	4 (4.9)	0 (0)	$p = 0.40$ dementia vs. Biobank $p = 0.25$ dementia vs. total controls
Neurons and astrocytes, n (%)	7 (5.5)	1 (1.6)	0 (0)	$p \leq 0.001$ dementia vs. Biobank $p \leq 0.001$ dementia vs. total controls
Astrocytes, n (%)	28 (22)	3 (4.7)	0 (0)	$p \leq 0.001$ dementia vs. Biobank $p \leq 0.001$ dementia vs. total controls
Immunoreactivity with HEK293 cells expressing GFAP, n (%)	14 (11)	1 (1.6)	0 (0)	$p = 0.007$ dementia vs. Biobank $p = 0.003$ dementia vs. total controls

Immunocytochemical findings obtained from stainings of mixed neuronal and glial hippocampal/cortical primary cultures as well as GFAP-transfected HEK293 cells at a dilution of 1:200 (n = 127 dementia patient sera, n = 97 sera from age-matched patients or healthy subjects). Data are presented as absolute numbers and proportions. Statistical significance was verified using Chi-square test.

the hypothesis that autoantibodies against brain structures may also play a role in dementia and associate with a less acute form of autoimmune encephalitis with slowly progressing damage to the brain that could manifest as autoimmune dementia. These autoantibodies may not only target neuronal structures but also antigens in glial cells like astrocytes that are crucial for a healthy and functioning CNS.

Glial fibrillary acidic protein (GFAP) is an intracellular intermediate filament protein that is highly expressed in the cytoskeleton of astrocytes. Autoantibodies to GFAP are already known to occur in serum and/or CSF in humans with GFAP astrocytopathy, a disease first described in 2016 as an acute or subacute meningoencephalomyelitis. The diagnosis is made based on the presence of GFAP autoantibodies in CSF/serum (Fang et al., 2016). Main symptoms of the meningoencephalomyelitis include headache, fever, movement disorders, abnormal vision and dysautonomia (Flanagan et al., 2017; Dubey et al., 2018; Kimura et al., 2019; Xiao et al., 2021). The pathogenic role of the GFAP autoantibodies is still not fully understood. GFAP-autoantibodies with unknown relevance were also found in patients with other neurologic diseases, for example traumatic brain injury, glioma or epilepsy (Zhang et al., 2014; Wei et al., 2013; Savas et al., 2021).

Little is known about GFAP-autoantibodies in dementia. In a study investigating clinical characteristics of 19 patients with GFAP-astrocytopathy, 15.8% had symptoms of dementia but together with other more typical symptoms (Long et al., 2018). More research is needed to find out if there is a link between autoantibodies against brain structures and dementia. This led us to the question if specific antigens could be identified in these cases, manifesting in subtypes of autoimmune dementia. Therefore, we screened a cohort of 127 dementia patients for any autoimmune reaction against brain structures, with a special focus on autoantibodies against astrocytes and GFAP.

2. Material and methods

2.1. Patients

Serum samples from 127 patients diagnosed with MCI and various forms of dementia including Alzheimer's disease, frontotemporal dementia, subcortical arteriosclerotic encephalopathy, Lewy body dementia, Parkinson's disease dementia and cerebral amyloid angiopathy based on clinical examination, neuropsychological testing and imaging studies were obtained from the memory clinic of the department of Neurology, Charité – Universitätsmedizin Berlin, and German Center for Neurodegenerative Diseases (DZNE) Berlin, from January 2018 until April 2021 and stored at −80 °C. All patients, irrespective of the form of dementia, were included in the study without any additional exclusion criteria. We used two cohorts of patients as an age-matched control group. The first cohort included 82 patients, older than 60 years with various neurologic diseases. A diagnosis of any form of mild cognitive impairment (MCI) or dementia as well as a diagnosis of neurological

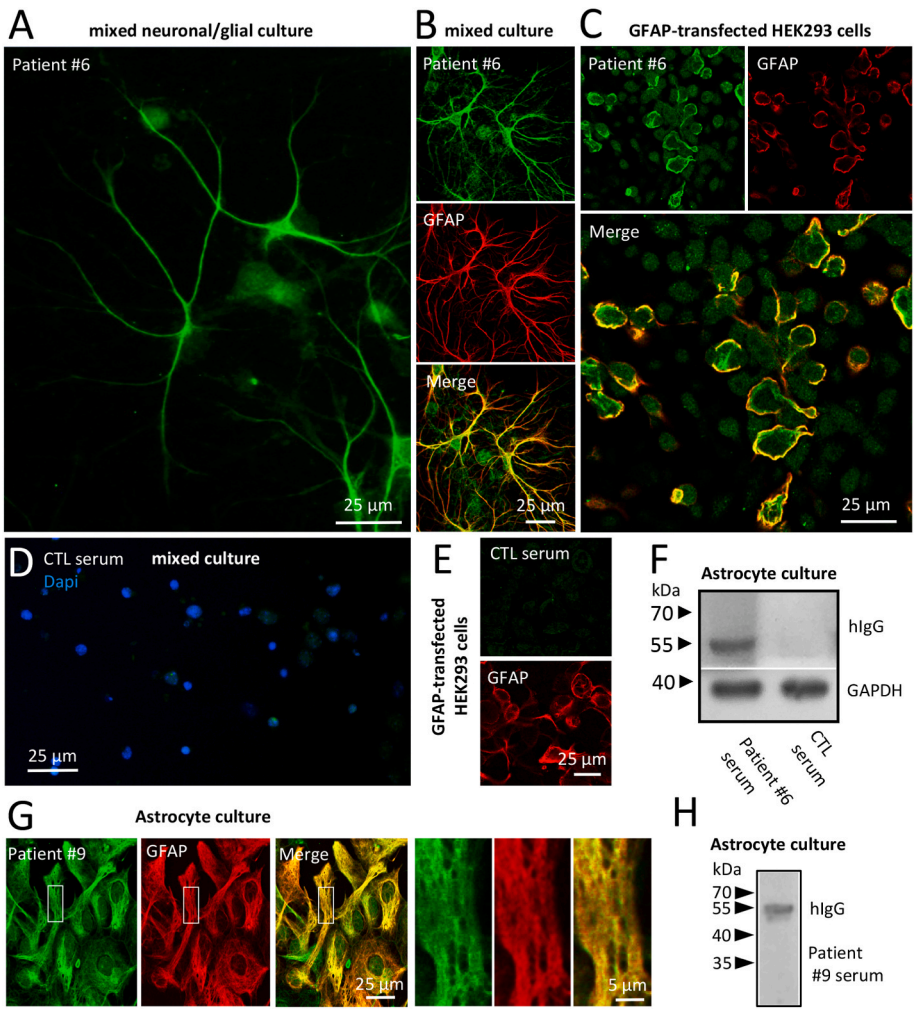

Fig. 1. Serum autoantibodies against glial fibrillary acidic protein (GFAP) in two patients with Alzheimer's disease. A) Patient serum #6 intensely stained large, branched cells that morphologically appeared like astrocytes in mixed embryonic hippocampal/cortical cultures. B) Patient serum antibodies co-localize with GFAP in cultured astrocytes. Cultures were double stained for human IgG and commercial monoclonal GFAP antibody. Both signals stained filament-like structures in stellate astrocytes and showed a high degree of overlap in the soma and astrocytic processes. Confocal imaging C) Patient serum antibodies react with GFAP-transfected HEK293 cells. HEK293 cells were transfected with a human GFAP plasmid. Only transfected cells reacted with patient IgG and signals showed a high degree of overlap with commercial GFAP staining. Confocal imaging D) Mixed embryonic hippocampal/cortical cultures were incubated with a serum from a 73-year old healthy control. No staining occurred. E) Likewise, control serum did not show any immunoreactivity to GFAP-transfected HEK cells. F) Purified astrocyte cultures were subjected to Western blotting and probed with patient serum and an age-matched control serum. For loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. Incubation with patient serum resulted in a single immunoreactive band at around 55 kDa, corresponding to the expected molecular weight of GFAP. Control serum yielded no staining. G) Purified astrocyte cultures were fixed and double stained for binding of IgG from another patient (patient #9) and GFAP, thereby showing a high degree of overlap (see insets). H) In line with this, autoantibodies of patient #9 show a major immunoreactive band at around 55 kDa in Western blots. Reactivity to GFAP was verified using HEK293 cells transfected with human GFAP (not shown).

diseases known to be associated with dementia were used as exclusion criteria. Sera were obtained from the Charité CSF/serum biobank. The second cohort includes sera of 15 individuals, who accompanied patients to the memory clinic. They had no history of any neurologic or psychiatric disease.

The study was approved by the ethics committee of Charité – Universitätsmedizin Berlin. All participants provided written informed consent.

2.2. Immunostaining strategy

The general screening strategy based on the detection of serum autoantibodies by immunostaining on murine neuronal hippocampal/cortical cultures as the primary detection assay. Additionally, purified astrocyte cultures or brain slices were incubated with immunopositive sera. Further characterization involved co-staining with commercial primary and secondary antibodies to identify specific cell types and targeted antigenes. Sera that stained astrocytes, which are also present in primary murine hippocampal culture, indicate the presence of autoantibodies against astrocyte proteins, and were further tested on GFAP-transfected HEK cells.

2.3. Primary murine hippocampal cell culture

We prepared primary cultures of murine primary hippocampal and cortical neurons. First, pregnant SWISS-mice were killed by cervical dislocation to remove the embryos at day E16-17. Embryonic brains were dissected and transferred into a 0.6% PBS-glucose solution. We then isolated the hippocampi and parts of the cortex and collected them in NB-medium containing 10 ml B27, 5 ml penicillin/streptomycin (pen/strep), 1.25 ml L-glutamine and 485 ml Neurobasal Medium. We added N-medium composed of 50 ml fetal calf serum (FCS), 5 ml pen/strep; 5 ml L-glutamine; 10 mM HEPES, 1 mg/ml insulin, 44 mM glucose, 5 ml collagen G, filled up to 500 ml with Dulbecco's Modified Eagle Medium (DMEM), centrifuged at 800 rpm for 2 min at 4 °C, resuspended the pellet in N-medium without collagen, centrifuged again at 800 rpm for 2 min at 4 °C and diluted the cells in NB-starter medium (25 µL Na-glutamate (100 mM)/100 ml NB-media). Coverslips in 24-well plates were incubated with poly-L-lysine solution in PBS (1:20) overnight and then coated with N-medium with collagen. Finally, we removed the medium and added the cell-solution (8×10^4/ml). The cells were incubated for 10–14 days at 37 °C and then used for immunostaining.

2.4. Purified astrocyte culture

For astrocyte cultures, brains from SWISS-mice were dissected between postnatal days P2 and P3. We removed the meninges, transferred the brains into Hank's balanced salt solution (HBSS, Sigma) and suspended them. After centrifugation at 300×*g* for 3 min, the astrocytes were resuspended in HBSS. This step was repeated two more times with smaller pipette diameters. 6-well plates were pretreated with poly-L-lysine (100 µg/ml in PBS) and the cell suspension was added. Astrocytes from ½ whole brain were seeded per single well. We incubated the

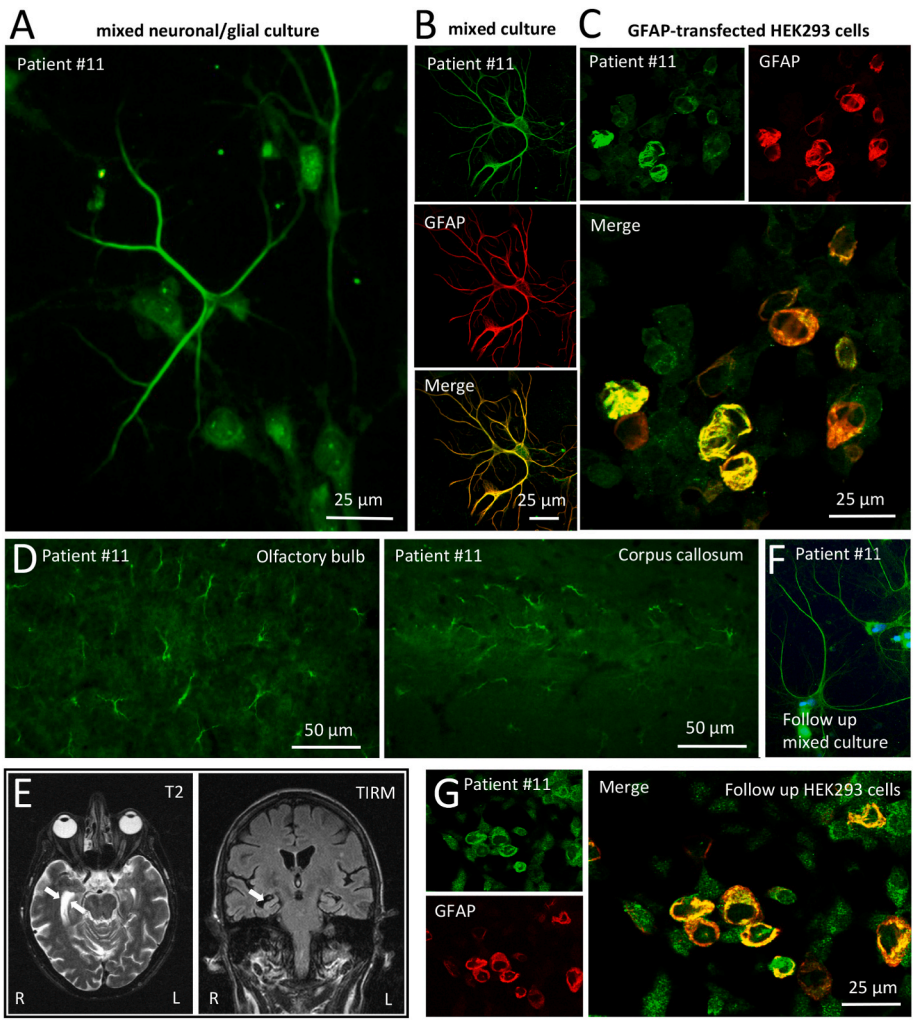

Fig. 2. Follow-up evaluation of serum autoantibodies against glial fibrillary acidic protein (GFAP) in a patient with Alzheimer's disease. A) Patient serum #11 intensely stained large, branched cells that morphologically appeared like astrocytes in mixed embryonic hippocampal/cortical primary cultures. B) Patient serum antibodies co-localize with GFAP in cultured astrocytes. Cultures were double stained for human IgG and GFAP. Both signals stained filament-like structures in stellate astrocytes and showed a high degree of overlap in the soma and astrocytic processes. Confocal imaging C) Patient serum antibodies react with GFAP-transfected HEK293 cells. Only transfected cells reacted with patient IgG and signals showed a high degree of overlap with commercial GFAP staining. Confocal imaging D) Astrocyte staining by patient autoantibodies in brain sections. Patient serum #11 stained astrocytes in various brain areas, being most prominent in the olfactory bulb and *Corpus callosum*. E) Axial and coronal MRIs (T2 and turbo inversion recovery magnitude sequence) of patient #11 demonstrate moderate decrease in brain volume with right mesiotemporal emphasis (arrows). F) Follow up test for the presence of autoantibodies to GFAP in mixed cortical/hippocampal cultures and transfected HEK293 cells. Patient serum was collected again 18 months after first testing and used in the described detection assays. Recurrently, stellate astrocytes were stained in the primary culture and only GFAP-transfected cells HEK293 reacted with patient IgG and both signals showed a high degree of overlap.

cells for 7 days at 37 °C, at 5% CO_2 in DMEM, supplemented with 10% FCS, 100U/ml pen/strep and 2 mM L-glutamine. During this time, the medium was replaced two times and the microglial cells were removed from the astrocytes by shaking the plates. For immunostaining, the cells were collected after 7 days and seeded at a density of 4×10^4 cells per well to 24-well plates which were previously treated with poly-L-lysine (100 µg/ml in PBS). Cells for Western blots remained in culture for 12–14 days.

2.5. GFAP transfected HEK cells

HEK293 cells were transfected with a plasmid encoding for human glial fibrillary acidic protein (GFAP alpha (I), based on a VB900131-8024ppx plasmid backbone, Vector Builder, Chicago, IL, USA) using established transfection procedures applying polyethyleneimine (PEI) as transfection reagent. After a transfection time of 24 h cells were fixed with 4% PFA for 20 min and processed for immunofluorescence as described below. To verify transfection efficiency and for double staining experiments with serum samples, a monoclonal anti-GFAP antibody was used (see 2.7).

2.6. Staining of cultured cells

2.6.1. Hippocampal cell cultures and purified astrocyte cultures

Fixed cells: We removed the medium from the wells, washed the cells with PBS (10%), fixed them with ice-cold methanol at 80% on coverslips for 20 min and incubated them with blocking-solution (0.1% Triton; 5% NGS; 2,0% BSA; PBS) for 1 h at room temperature. We diluted the samples (serum dilution at 1:200) in blocking-solution and incubated the cells with the patient's serum for 24 h at 4 °C. After that, cells were washed with PBS and incubated with the secondary antibody (FITC-conj. goat-a-human IgG, Dianova, #109095003, dilution: 1:100) in secondary-antibody-solution (2% BSA in PBS) for 90 min at room temperature. Again, cells were washed with PBS, stained with DAPI (4′, 6-diamidino-2-phenylindole) for 10 min and mounted with Immu-Mount (Thermo Fisher Scientific, Waltham, MA).

Live cells: Cultured cells were incubated overnight with patient serum at a dilution of 1:200 in culture medium. After removal of the medium and washing with PBS, cells were fixed and treated with secondary antibody as above.

2.6.2. HEK cells

GFAP transfected HEK cells were fixed with 4% PFA dissolved in PBS but otherwise treated as described above.

2.7. Staining of murine brain slices

Perfusion-fixed brains from adult SWISS-mice were dissected, cryoprotected and frozen at −80 °C prior to cutting into 20 µm coronar and sagittal sections including cortex, hippocampus and cerebellum. The slices were washed with PBS and incubated in blocking solution (10% NGS in PBS; 0.3% Triton-X-100) for 30 min at room temperature. The serum samples were diluted (serum dilution at 1:200) in primary-antibody-solution (10% NGS in PBS; 0.3% Triton-X-100; 0.1% NaN_3),

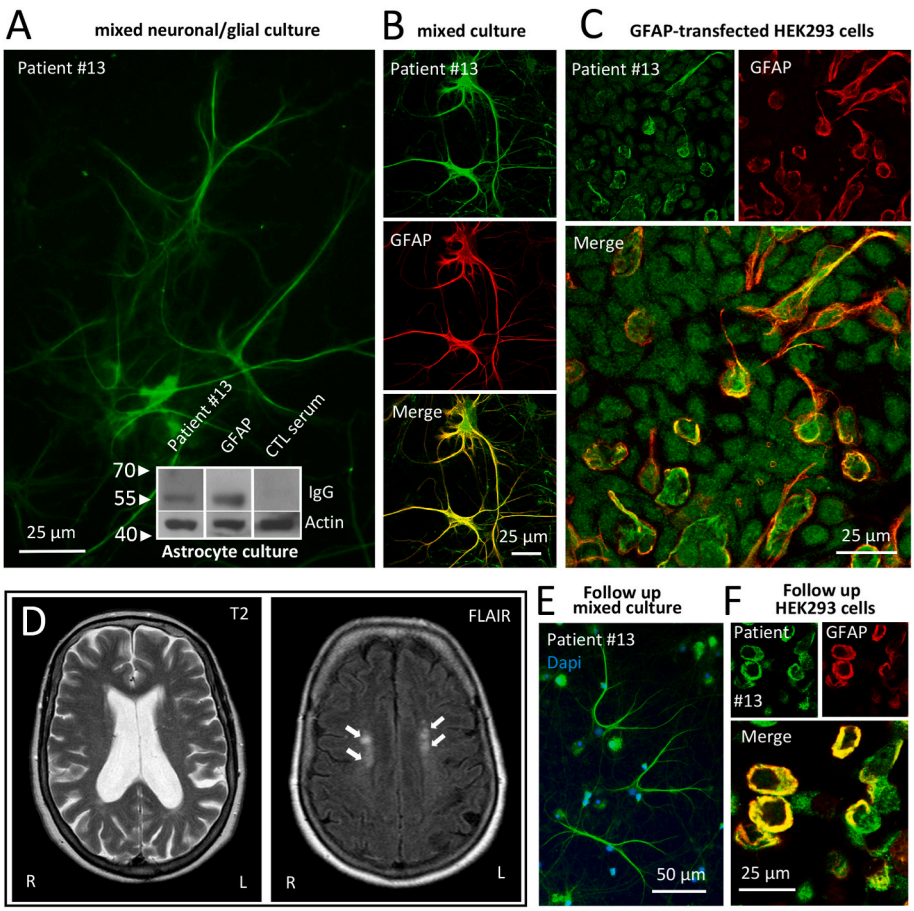

Fig. 3. Follow-up evaluation of serum autoantibodies against glial fibrillary acidic protein (GFAP) in another patient with Alzheimer's disease. A) Patient serum #13 intensely stained large, branched cells that morphologically appeared like astrocytes in mixed embryonic hippocampal/cortical cultures. Inset: Biochemical evaluation showed that incubation with patient serum resulted in a single immunoreactive band at around 55 kDa in purified astrocyte culture corresponding to commercial GFAP staining. Control serum yielded no staining. B) Patient serum antibodies co-localize with GFAP in astrocytes of mixed cultures. Cultures were double stained for human IgG and GFAP. Both signals stained filament-like structures in stellate astrocytes and showed a high degree of overlap in the soma and astrocytic processes. Confocal imaging C) Patient serum antibodies react with GFAP-transfected HEK293 cells. Only transfected cells reacted with patient IgG and signals showed a high degree of overlap with commercial GFAP staining. Confocal imaging D) Axial MRIs (T2 and fluid-attenuated inversion recovery sequence) of patient #13 demonstrate mild to moderate frontoparietal atrophy with corresponding enlargement of the lateral ventricles and periventricular white matter lesions (arrows). E) Follow up test for the presence of autoantibodies to GFAP in mixed cortical/hippocampal cultures and transfected HEK293 cells. Patient serum was collected again 22 months after first testing and used in the described detection assays. Recurrently, stellate astrocytes were stained in the primary culture and only GFAP-transfected cells HEK293 reacted with patient IgG and both signals showed a high degree of overlap.

added to the brain slices and incubated for 24 h at 4 °C. After incubating the slices, we washed them with PBS and incubated them with the secondary antibody (FITC-conj. goat-a-human IgG, Dianova, #109095003, dilution: 1:100) in secondary-antibody-solution (5% NGS in PBS; 0.1% Triton-x-100) for 1 h at room temperature in the dark. The slices were washed with PBS and mounted to the slides.

2.8. Co-staining with commercial antibodies

Co-staining against GFAP was performed on cells and brain slices, following the same protocols as used for staining without commercial antibodies. A commercial primary monoclonal mouse anti-GFAP antibody (Synaptic Systems, Göttingen, Germany, #173011, dilution 1:1000) was applied to cells or brain slices, together with the serum sample. Both secondary antibodies (FITC-conj. goat-a-human IgG, see above) and Alexa-red goat-anti-mouse 594 (MoBiTec, #A11032, dilution 1:1000) were also applied together.

2.9. Screening for co-existing antibodies

Of the sera tested positive for GFAP antibodies 12 were also routinely tested for co-existing antibodies using the standard and research autoimmune diagnostic panels by the Euroimmun AG (Lübeck, Germany). The following antigens were included: Hu, Ri, ANNA-3, Yo, Tr/DNER, Ma/Ta, GAD65, Amphiphysin, Aquaporin4, MOG, NMDA-R, AMPA-R, $GABA_B$-R, LGl1, CASPR2, IGLON5, ZIC4, DPPX, Myelin, CARPVIII, Glycine-R, mGlu-R1, mGlu-R5, $GABA_A$-R, RHO activating GTPase 26, Recoverin, Glu-RD2, Flotillin 1/2, ITPR1, Homer3, Neurochondrin, Neurexin-3-alpha, ERC1, Sez6I2, AP3B2, Contactin1, Neurofascin 155, Neurofascin 186, AT1A3, KCNA2, Dopamin-R2.

2.10. Immunoblots

For immunoblotting, whole brains from adult SWISS-mice or astrocyte cultures grown for 14 days *in vitro* were homogenized (tissue and cells were processed in PBS using a glass-Teflon homogenizer applying 10 strokes at 900 rpm with protease inhibitors added). Homogenates were spun down at 1500×*g* for 10 min, and the resulting supernatant devoid of cell nuclei was diluted in Laemmli buffer and submitted to SDS-PAGE. The membranes containing the transferred proteins were then incubated with patient or control serum at a dilution of 1:200. A mouse monoclonal antibody against GFAP (the same as used for immunofluorescence) served as a positive control. A mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Merck Millipore, Darmstadt, Germany; #MAB374) or a rabbit polyclonal antibody to Actin (Sigma-Aldrich, St. Louis, MO, USA, #A5060) was used as loading control. Horseradish peroxidase coupled goat anti-human kappa light chain secondary IgG (Life Technologies, Carlsbad, CA, USA; #A18859), horse anti-mouse IgG as well as goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; #PI-2000 and #PI-1000) served as secondary antibodies. Immunoreactivity was visualized by using enhanced chemiluminescence (GE Healthcare Europe GmbH, Freiburg, Germany).

2.11. Image acquisition

Images were acquired using either an upright Leica DMLB epifluorescence microscope or a Leica SL confocal microscope.

2.12. Statistics

Statistical significance of differences of frequencies of glial and GFAP

Table 3
Demographical data, dementia type, secondary diagnoses, neuropsychological testing and laboratory findings of 14 GFAP-positive patients verified by transfected HEK293 cells. Means are given and compared to GFAP-negative patients. Laboratory findings were available for 8 GFAP-positive and 53–69 (parameter-depending) GFAP-negative patients.

Patient ID	Age at blood draw	Sex	Type of dementia	Secondary diagnosis	Co-existing Autoantibodies	MMSE	pTau (181)	Total-Tau	Aβ (1–40) [pg/ml]	Aβ (1–42) [pg/ml]	Aβ Ratio	TPC (mg/l)
1	54	m	FTD		negative							
2	73	m	CAA	s/p ICH (2018)	anti-Rho GTPase-activating protein	25/30	91	839.18	11245	502	0.45	608.3
3	79	m	AD	Suspicion of NPH (2017)	negative		62	297.42	29962	537	0.18	809.5
4	61	f	AD	APP gene mutation (c2149 G > A); Epilepsy	negative							301
5	78	m	AD	s/p CRC	negative	7/30	136	1200.97	13213	483	0.37	525.8
6	67	m	AD		anti-myelin	4/30						
7	78	m	AD/SAE	s/p AIS (2012)	n.d.	17/30	31	227.34	7904	557	0.71	410.7
8	79	m	AD	s/p TIA (2017); s/p SAH (2017); RA	negative							
9	82	m	AD	Movement disorder; Lung tumor	negative		136	949	13485	699	0.52	389
10	74	m	AD	HBP, nicotine abuse; T2D	negative	21/30						593.7
11	75	f	AD		n.d.	18/30	73	535.29	17751	575	0.32	306.6
12	78	f	MCI	Suspicion of NPH (2016); organic depression disorder DDX anxiety disorder; s/p CRC	negative	27/30	100	519.8	23683	987	0.42	448.2
13	76	f	AD		negative		54	527	10467	539	0.51	504.6
14	54	f	FTD		unknown surface antigen (this study)							
Mean GFAP +	72±9					17/30 ±8.0	85 ±35	637 ±311	15963 ±6981	610 ±155	0.44 ±0.14	489,7 ±146,9
Mean GFAP –	74±9,4					22/30 ±6.3	81 ±40	617 ±422	15566 ±7682	756 ±361	0.72 ±1.1	508,07 ±211,47

AD = Alzheimer's disease; AIS = androgen insensity syndrome; CAA = cerebral amyloid angiopathy; CRC = colorectal cancer; FTD = frontotemporal dementia; HBP = high blood pressure; ICH = intracerebral hemorrhage; MCI = mild cognitive impairment; NPH = normal pressure hydrocephalus; RA = rheumatoid arthritis; SAE = subcortical arteriosclerotic encephalopathy; SAH = subarachnoid hemorrhage; TIA = transient ischemic attack; T2D = type 2 diabetes; TPC = total protein count.

antibodies between cohorts was assessed using the Chi-square test. P-values ≤0.05 were considered significant.

3. Results

3.1. Frequency of immunoreactivity against glial and neuronal epitopes

Sera from 127 patients diagnosed with different forms of dementia based on clinical examination, neuropsychological testing, and imaging studies (Table 1) were used for initial immunostaining on mixed murine neuronal/glial primary hippocampal/cortical cell culture to screen for antibodies directed against CNS epitopes. The majority of patients were diagnosed with Alzheimer's disease (AD, 46%), followed by mild cognitive impairment (MCI, 19%) and frontotemporal dementia (FTD, 10%). Besides various types of neurons, these cultures always contain a growing population of astrocytes co-cultured with the neurons. Based on previous studies, a serum dilution of 1:200 was applied to fixed and permeabilized cells yielding an optimized signal-to-background ratio. At the chosen experimental conditions, 45 of 127 (35.5%) sera tested positive for IgG autoantibodies, showing various glial or neuronal staining patterns (Table 2). Reactivity was mainly directed against astrocytes (35/127; 27.5%) whereas neuron staining was less frequent (17/127; 13.5%). In addition to the approach using fixed cells we also tested all 127 patient sera on live cultures. However, only 3 sera yielded a signal that was clearly above the background signal (Supplemental Figure 1). Due to this low frequency we decided to focus on fixed cells. To exclude that the observed occurrence of antibodies against CNS epitopes represents the normal proportion found in aged patients a total of 97 serum samples consisting of two cohorts was used as an age-matched control group. Immunostaining results of both control cohorts are given (Table 2). Clinical diagnoses for patients of the control cohort obtained from the Charité CSF/serum biobank (cohort 1) are shown in supplemental Table 1. Age-matched healthy controls had no autoimmune reaction at all, whereas some sera of control cohort 1 were tested positive on hippocampal cell culture. In detail, immunoreactivity was detected in 8 of 97 serum samples (8.2%, p-value of ≤0.001 for dementia cohort vs. total age-matched controls). Of these 8 sera, 3 samples had antibodies against astrocytes only, and 4 samples had anti-neuronal antibodies. One of the samples exhibited immunoreactivity to both astrocytes and neurons (p-value of ≤0.001 for antibodies to astrocytes in dementia cohort vs. total age-matched controls).

In the following, we decided to focus our further investigations on the subgroup of 35 sera obtained from dementia patients that showed autoimmune reactivity against astrocytes in our culture system.

3.2. Frequency of autoantibodies to GFAP

In order to identify targeted antigens, we tested for reactivity against GFAP, a highly abundant intermediate filament protein of the astrocyte cytoskeleton. To this end, HEK293 cells were transfected with cDNA coding for human GFAP and incubated with the 35 patient sera which reacted with astrocytes. Immunocytochemical staining revealed reactivity against GFAP in 14 samples, representing 11% of the total 127 sera, indicating the presence of IgG autoantibodies against this specific antigen. In the control group, only one patient, diagnosed with motor neuron disease, had GFAP autoantibodies, representing 1.0% of the 97 control sera (p-value of 0.003 for antibodies to GFAP in dementia cohort vs. total age-matched controls).

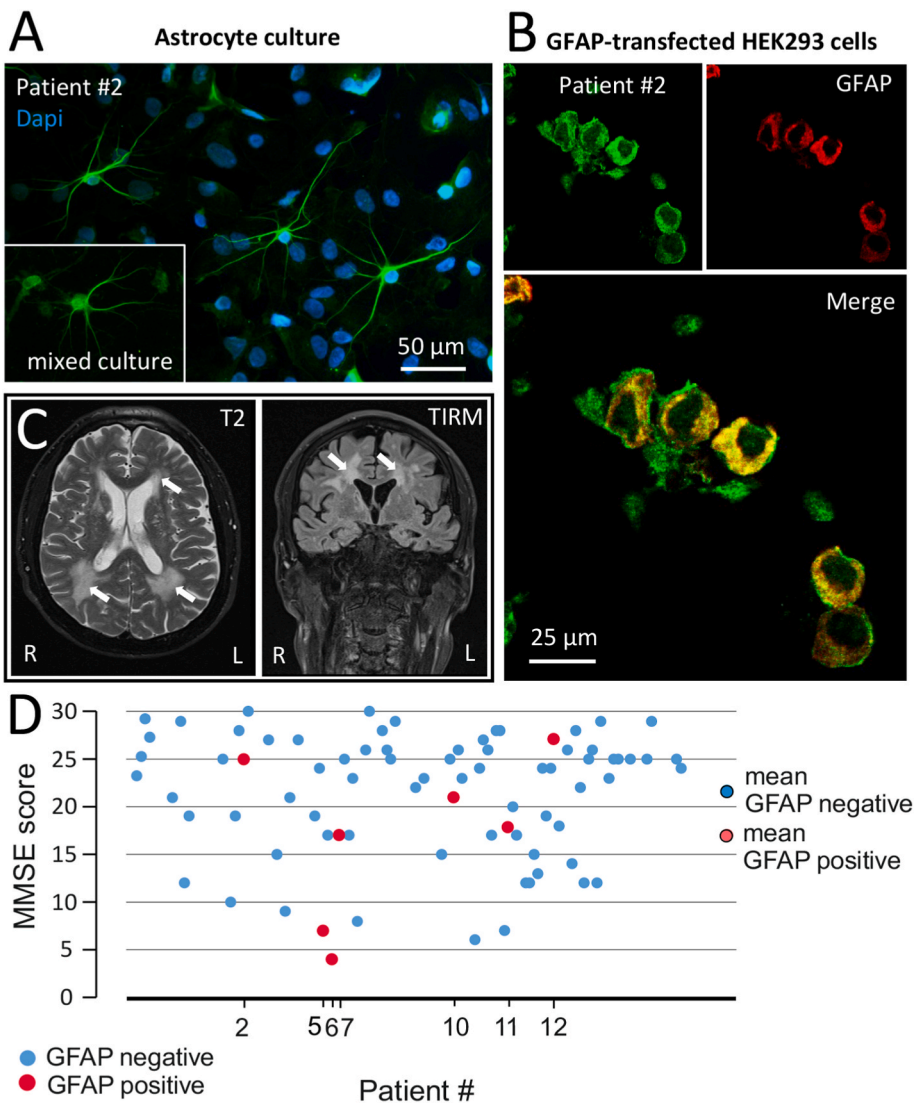

Fig. 4. Serum autoantibodies against glial fibrillary acidic protein (GFAP) in a patient with Cerebral Amyloid Angiopathy. A) Patient serum #2 intensely stained mainly large stellate astrocytes in purified astrocyte cultures. Polygonal flattened cells were only weakly stained. Inset: Patient serum also recognized astrocytes in mixed neuronal/glial cultures. B) Patient serum antibodies react with GFAP-transfected HEK293 cells. Only transfected cells reacted with patient IgG and signals showed a high degree of overlap with commercial GFAP staining. Confocal imaging C) Axial and coronal MRIs (T2 and turbo inversion recovery magnitude sequence) of patient #2 demonstrate marked ubiquitously occurring white matter gliosis (arrows). D) Cognitive screening by Mini-Mental State Examination (MMSE) was available for a total of 79 dementia patients. Blue dots indicate GFAP-negative patient sera, red dots indicate 7 screening scores available for GFAP-positive patients. Mean values are indicated on the right. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Follow-up after 16–22 months was possible for three dementia patients. All three of the sera were still positive for anti-GFAP autoantibodies in HEK cell assays and hippocampal cell culture.

We performed additional experiments, including 1.) Immunoblotting and 2.) Immunohistochemistry to refine our results. Biochemical analysis was performed using Western Blots on purified murine astrocyte cultures and whole brain homogenates to investigate whether serum GFAP antibodies also recognize linear epitopes. Seven of the 14 GFAP-positive sera (#1; #3; #4; #6; #9; #13; #14, all 35 sera that exhibited immunoreactivity to astrocytes were tested) exhibited immunoreactive bands in both or either one of the preparations between 50 and 55 kDa, corresponding to the molecular weight of GFAP. Representative Western blots are shown (Figs. 1 and 3). While all 14 GFAP-positive sera reacted with the typical ramified astrocyte type found when co-cultured with neurons (resembling the *in vivo* morphology) serum #8 also co-localized with GFAP in an intense filamentous staining pattern in flattened, polygonal astrocytes that usually express lower levels of GFAP (Fig. 1G). Two of the 14 sera (#11 and #13) also showed a clear astrocyte staining in fixed murine brain slices, being especially pronounced in the olfactory bulb and *Corpus callosum* (Fig. 2, serum #11). Other immunohistochemical staining procedures, such as the use of unfixed brain sections, were not in the focus of this study.

3.3. Clinical and paraclinical findings

Results for the laboratory testing on serum and CSF are given for GFAP-positive patients and compared with available data from GFAP-negative dementia patients (Table 3). The tests focused on parameters that are part of the standard protocol for the evaluation of a possible dementia diagnosis. Here we highlight the laboratory values for Amyloid-beta and Tau, which usually reach abnormal values in dementia patients. It became evident that most GFAP-positive patients had abnormal values for the majority of investigated parameters. Consequently, all mean values for Tau and Amyloid-beta were abnormal, which means that the values for pTau(181), total tau and total protein count (TPC) were increased and the values for Amyloid-beta (1–42) and beta-Amyloid ratio 42/40 were decreased. In comparison with the GFAP-negative dementia patients, most results were similar in both groups, although Amyloid-beta (1–42) and beta-Amyloid ratio 42/40 were lowered in GFAP-positive patients without reaching statistical significance. Screening for co-existing autoantibodies was performed for 12 of the 14 GFAP positive sera (EUROIMMUN Medizinische Labordiagnostik AG Lübeck, Germany). In two sera (#2 and #6) concomitant autoantibodies were detected against anti-Rho GTPase activating protein and Myelin, respectively (Table 3).

Brain MRIs were available for 11 GFAP positive patients, revealing different findings accompanying the respective diagnosis. Frequently

found pathologies associated with dementia were atrophy (seven patients) and medullary gliosis (six patients). Representative images are given for three patients (Figs. 2–4). Results of neuropsychological testing were available for 7 GFAP-positive patients by Mini-Mental State Examination (MMSE). This test is a relatively broad screening, used to examine a patient's cognitive abilities. A score of 24/30 or higher is considered as normal cognitive function, whereas a lower score suggests any kind of cognitive impairment. The GFAP positive patients had an average score of 17, compared to an average MMSE score of 22 for 72 GFAP negative dementia patients (Fig. 4D).

An illustration of the results for five exemplary patients, their experimental findings and MRI images are presented in Figs. 1–4. Demographic data and clinical characteristics of all GFAP-positive patients are shown in Table 3.

Taken together, our data provide evidence for a higher prevalence of autoantibodies to GFAP in dementia patients compared to age-matched neurological patients without dementia or healthy subjects.

4. Discussion

In the current study, we report on the prevalence of GFAP autoantibodies in dementia patients. We were able to identify GFAP as antibody target in 11% of these patients. The occurrence of these autoantibodies in a significantly higher number than in age-matched healthy controls or patients with various neurologic diseases without dementia raises questions about the relevance of these antibodies. The underlying causes for the generation of autoantibodies to brain antigens are probably not based on a single mechanism but are most likely due to a combination of events. Amongst the factors contributing to the development of autoimmunity are infections, neoplasms, genetic predisposition, or lifestyle factors. Putting the focus on dementia patients, it is assumable that also neurodegeneration plays a major role in the development of autoimmunity in the brain. On the other hand, autoimmunity might be one of many aspects that contribute to dementia. For example, the presence of preexisting or coexisting autoimmune diseases is associated with an increased risk for developing dementia as shown for patients with autoimmune rheumatic diseases (Lin et al., 2018). According to our findings, GFAP antibodies seem to be a hallmark in dementia patients. The obvious question is whether these autoantibodies are pathogenic and might contribute to the course of the disease or represent a secondary phenomenon due to the neurodegeneration. Nevertheless, this might just as well contribute to the disease development. Evidence for the association of autoantibodies to GFAP with dementia exist in the literature. ELISA-testing of sera from patients with Alzheimer's dementia and vascular dementia showed a higher rate of GFAP-autoantibodies in these patients, compared with healthy controls (Tanaka et al., 1989; Mecocci et al., 1992). A few case reports mention patients with GFAP-autoantibodies, presenting with atypical symptoms such as chronic cognitive impairment, rapid progressive dementia or parkinsonism (Natori et al., 2020; Toledano-Illan et al., 2021; Tomczak et al., 2019). Clinical symptoms improved in all patients reported in these studies following immunotherapy. Recently, a case study reported on the association of GFAP autoantibodies in serum with primary progressive aphasia, thereby extending the disease spectrum for these antibodies (Hansen et al., 2022). Mechanistically, animal studies revealed that GFAP astrocytopathy may be caused by GFAP-specific cytotoxic T-cells (Sasaki et al., 2014). Pathologic studies in humans found that CD8(+) T-cells are predominant, but also B-cells, plasma cells and macrophages are involved in the disease (Shu et al., 2018; Yuan et al., 2021; Long et al., 2018). A pooled analysis of 324 cases of classical GFAP astrocytopathy published until 2021 summarizes the clinical features, radiological findings and treatment regimes. The median age of disease onset was 45 years with no sex predominance. 19.8% had neoplasms, most commonly ovarian teratoma. 25.5% had coexisting neuronal autoantibodies, most commonly anti NMDA-R autoantibodies. MRI imaging showed characteristic linear radial enhancement in 43.7%. Treatment consisted of high-dose corticosteroids, intravenous immunoglobulins and plasma exchange. Most patients (86.5%) responded well to acute immunotherapy (Xiao et al., 2021).

Most of the previously described antineuronal autoantibodies that occur in classic autoimmune encephalitis are directed against extracellular antigens. This enables them to directly bind to living cells rendering them more likely pathogenic (Kreye et al., 2016). When focusing on autoimmune gliopathies, there are also known pathogenic autoantibodies that target extracellular proteins, for example anti-Aquaporin 4 or anti-Myelin Oligodendrocyte Glycoprotein autoantibodies in patients with Neuromyelitis optica spectrum disorder (Weinshenker and Wingerchuk, 2017). In contrast to these findings, GFAP is an intracellular antigen. Studies that investigated the pathomechanisms behind GFAP astrocytopathy stated that the disease might be T-cell mediated and the occurring anti-GFAP autoantibodies represent a secondary phenomenon still making them suitable as a disease marker (Fang et al., 2016; Yuan et al., 2021). Nevertheless, the pathomechanisms behind different autoantibodies against intracellular antigens are not fully understood. In fact, there are autoantibodies against intracellular antigens, for example synapsin, that can enter non-permeabilized, living neurons via Fcγ II/III receptor-mediated endocytosis to affect synaptic transmission (Rocchi et al., 2019). A study about GFAP autoantibodies in sera from patients with traumatic brain injury showed that these autoantibodies could enter living astrocytes in rat primary astrocyte culture and cause cell death (Zhang et al., 2014). In vitro-investigations about the pathogenicity of well-known onconeuronal autoantibodies such as Anti-Hu and Anti-Yo which occur in paraneoplastic encephalitis, showed inconsistent results. Some studies revealed that these autoantibodies are able to enter living cells and cause neurotoxicity, whereas others found no evidence or just little evidence for this hypothesis (Greenlee et al., 1993, 2010; Zaborowski and Michalak, 2013). To sum up, considering the existing literature, the pathogenicity of intracellular autoantibodies remains unclear, but the fact that autoantibodies target intracellular antigens does not make it unlikely that they could enter living cells to cause pathogenic effects.

More research is needed to find out whether the detected anti-GFAP antibodies are pathogenic, cause damage *in vivo* and, and if this is the case, contribute to the symptoms of dementia in patients who developed these autoantibodies. Regardless of its pathogenicity, it would also be intriguing to investigate the question whether the GFAP-autoantibodies could serve as a useful clinical marker for the severity and progression of the dementia symptoms as described for anti-NMDA-R autoantibodies in patients with progressive cognitive impairment (Doss et al., 2014). Another aspect that comes along with our finding of GFAP autoantibodies in dementia patients is the question of its relevance for clinical practice. Autoimmune dementia, as described before, is a relatively broad term that is not very well defined. It is generally used for a heterogenic subgroup of dementia patients in which the disease is most likely autoimmune rather than neurodegenerative. Currently, the screening for autoantibodies is not a part of the standard diagnostic process for dementia. However, it has been stated that there are "red flags", for example young age, acute or subacute onset, rapid progressing symptoms, seizures and headaches indicate a possible autoimmune dementia and should lead to testing for autoantibodies (Flanagan et al., 2017). Other features indicating an autoimmune pathogenesis are a fluctuating disease course, early psychosis, known cancer or family history of cancer and autoimmunity. Some symptoms are even strongly suggestive for certain autoantibodies for example Facio-brachial dystonic seizures in patients with LGI1-mediated autoimmune dementia. Taken together, atypical clinical or paraclinical presentation that cannot be attributed to classic causes of dementia, e.g. Alzheimer's disease, vascular dementia, Lewy-body-dementia, frontotemporal dementia or dementia in typical or atypical Parkinson's disease, should raise awareness for a possible autoimmune pathogenesis rather than a primary neurodegenerative process. It is important to improve the clinical diagnostics to identify these patients early on since some of them

improve with immunotherapy (Bastiaansen et al., 2021; Banks et al., 2021). It also needs to be discussed which methods are appropriate to diagnose single patients or even investigate larger cohorts of dementia patients for the presence of autoantibodies, since most of the previously mentioned studies only looked for various specific, already known autoantibodies using standardized screening methods. That is also the case in clinical practice, where samples are sent to specialized laboratories that detect common autoantibodies.

5. Limitations

In our study, there was no phenotype in clinical or paraclinical data specific to GFAP positive patients. This could be due to the relatively small cohort of GFAP positive patients identified. Also, a limitation of our study lies in the fact that no CSF samples were available for testing of autoantibodies to GFAP in this compartment. In classical GFAP encephalopathy, autoantibodies in CSF show higher diagnostic value compared with isolated serum GFAP autoantibodies (Friedrich et al., 2022; Xiao et al., 2021). Therefore, our data cannot be directly interpreted in the context of the classical GFAP encephalopathy manifesting as meningoencephalomyelitis. Rather, GFAP seropositive patients in our cohort did not show characteristic signs in brain imaging such as linear, radial perivascular pattern of enhancement.

In conclusion, given the clinical presentation and age of disease onset our study suggests a different phenotype associated with serum antibodies to GFAP. Altogether, GFAP seropositive patients might represent a subgroup of autoimmune dementia with anti-GFAP antibodies as a hallmark. Further studies will be needed to ascertain the pathogenicity and clinical utility of anti-GFAP antibodies.

Declaration of competing interest

All authors of the manuscript “Immunoreactivity to astrocytes in different forms of dementia: high prevalence of autoantibodies to GFAP” report no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100609.

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