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ORIGINAL ARTICLE

Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome

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Abstract

Limited information is available on the identity of antigens targeted by antibodies present in cerebrospinal fluid (CSF) of patients with clinically isolated syndrome (CIS). The aim of this study was to identify novel antigens for CIS and investigate their prognostic potential to predict conversion to multiple sclerosis (MS). We applied serological antigen selection (SAS) to identify antigens interacting with antibodies present in the pooled CSF from four CIS patients, who developed MS. Antibody reactivity towards CIS antigens identified by SAS was tested in CSF and serum from patients with CIS (n = 123/n = 108), MS (n = 65/n = 44), and other (inflammatory) neurological diseases (n = 75/n = 38) as well as in healthy control sera (n = 44). Using SAS, a panel of six novel CIS candidate antigens was identified. CSF antibody reactivity was detected in both CIS and relapsing-remitting

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Abbreviations used: BLAST, basic local alignment search tool; CIS, clinically isolated syndrome; EAE, experimental autoimmune encephalomyelitis; HCFCR1, Host Cell Factor C1 Regulator 1; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MPBS, skimmed milk powder in phosphate-buffered saline; MS, multiple sclerosis; NIND, non-inflammatory neurological disorders; NWM, normal white matter; OIND, other inflammatory neurological diseases; RR, relapsing-remitting; SAS, serological antigen selection; SP, secondary-progressive; TMB, 3,3′,5,5′ tetramethyl-benzidine dihydrochloride.

(RR) MS. Serum reactivity was significantly increased in CIS and RR-MS as compared with controls ($p = 0.03$). For two antigens, the frequency of antibody-positive patients was higher in CIS patients who converted to MS as compared with CIS patients without conversion. We identified novel CIS antigens to which antibody reactivity was primarily detected in CIS and RR-MS as compared to controls. Possible prognostic potential could be demonstrated for two antigens.

Keywords: autoantibody, biomarker, clinically isolated syndrome, multiple sclerosis, serological antigen selection.
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In the majority of multiple sclerosis (MS) patients, MS is preceded by a condition called clinically isolated syndrome (CIS), which is an acute or subacute episode of neurological disturbance due to a single white matter lesion (Miller *et al.* 2005). However, only 30–70% of CIS patients develop MS (Miller *et al.* 2005). An important role of B cells and antibodies has been recognized in CIS and MS. For instance, treatment with the B-cell-depleting monoclonal antibody Rituximab has shown to reduce inflammatory lesions and clinical relapses in relapsing-remitting (RR) MS (Hauser *et al.* 2008). Cerebrospinal fluid (CSF) of both CIS and MS patients is often characterized by the presence of oligoclonal band antibodies (Awad *et al.* 2010) and it has been demonstrated that CSF B cells produce these antibodies in MS (Obermeier *et al.* 2008). At this moment, little is known about the identity of the antigens to which antibodies are directed in CIS and if there are antigens associated with progression to MS. Examples of known antigens for antibodies in CIS are myelin components, such as myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP). Moreover, there are reports indicating an association on such antibody reactivity and conversion to MS (Berger *et al.* 2003; Tomassini *et al.* 2007), although this could not be confirmed by others (Kuhle *et al.* 2007).

Previous studies investigating the antibody response in CIS focused mainly on antigens that have been identified in MS or in other diseases (Berger *et al.* 2003; Tomassini *et al.* 2007; Brettschneider *et al.* 2009; Freedman *et al.* 2009; Lunemann and Ascherio 2009; Otto *et al.* 2011; Szmyrka-Kaczmarek *et al.* 2011). For this reason, we aimed to identify novel antigens for CIS by means of an unbiased technique called serological antigen selection (SAS), which is a high-throughput molecular screening technology based on cDNA phage display (Hufton *et al.* 1999; Govarts *et al.* 2007; Somers *et al.* 2009). Until now, this technique has been used successfully to analyse the autoantibody profile in several diseases, such as MS (Somers *et al.* 2005, 2008; Govarts *et al.* 2009), rheumatoid arthritis (Somers *et al.* 2011), colorectal cancer (Somers *et al.* 2002) and atherosclerosis (Cleutjens *et al.* 2008). To identify novel antigens for CIS, SAS procedures were performed in parallel with two cDNA expression libraries, composed of normal white matter (NWM) and MS plaques, to examine whether primarily normal or diseased brain antigens are targeted by the antibody response in CIS. Subsequently, candidate CIS antigens were identified and antibody reactivity towards these antigens was assessed in CSF and serum from CIS and MS patients and in controls. In addition, the prognostic potential of novel CIS antigens to predict conversion to MS in CIS patients was investigated.

Materials and methods

Construction of cDNA phage display libraries

To identify novel antigens for CIS, two cDNA libraries were obtained: a cDNA library derived from active chronic MS plaques, with a primary diversity of 1.0×10^6 recombinants [kindly provided by Dr. M. B. Soares (Soares *et al.* 1994; Becker *et al.* 1997)] and a normal white matter (NWM) cDNA library from normal brain, with a primary diversity of 0.9×10^6 recombinants [obtained from Dr. G.P Owens (Owens *et al.* 1996)]. These libraries were subcloned in our pVI phage display vectors, pSPVIA, pSPVIB and pSPVIC, each representing one of the three different reading frames. The subcloning procedure was performed as described previously (Somers *et al.* 2005).

Patient material

CSF specimens of four CIS patients, all of who developed MS within 2 years after lumbar puncture, were used for SAS procedures (Table 1). These CSF samples were pooled and pre-adsorbed against *E.coli* and phage extracts, as described previously (Somers *et al.* 2005).

Immunoreactivity directed to antigens identified by SAS was assessed in patient and control samples (Table 2). For this reason, paired CSF and serum samples were collected from 105 CIS patients (CIS), 30 RR-MS patients, 11 and 8 patients with non-inflammatory neurological disorders (NIND) and other inflammatory neurological diseases (OIND) respectively. In addition, CSF samples were available from 18 other CIS, 25 RR-MS, 10 Secondary-Progressive

Table 1 Characteristics of CIS patients selected for SAS procedures

CIS patient	Gender[a]	Age	EDSS[b] score
1	F	27	2
2	M	29	1
3	F	35	1
4	M	17	2

[a]F, female; M, male.
[b]EDDS, expanded disability status scale.

Table 2 Characteristics of patients selected for CSF and serum antibody reactivity screening

Patient group	Gender (M/F[a])	Mean age (years) ± SD	Mean disease duration (years)[b] ± SD	EDDS score[c] ± SD
CIS				
CSF (*n* = 123)	27/96	35.9 ± 10.7	0.5 ± 1.4	1.5 ± 1.1
Serum (*n* = 108)	22/86	35.7 ± 10.3	0.5 ± 1.5	1.5 ± 1.1
RR-MS				
CSF (*n* = 55)	10/45	40.8 ± 9.1	8.0 ± 9.0	2.7 ± 1.1
Serum (*n* = 44)	5/39	41.5 ± 8.7	8.9 ± 9.5	2.9 ± 1.1
SP-MS				
CSF (*n* = 10)	2/8	47.2 ± 9.3	14.9 ± 8.1	4.8 ± 1.3
OIND				
CSF (*n* = 28)	15/13	47.4 ± 17.3	NA[d]	NA
Serum (*n* = 14)	8/6	48.9 ± 14.3	NA	NA
NIND				
CSF (*n* = 47)	16/31	47.5 ± 17.8	NA	NA
Serum (*n* = 24)	6/18	44.3 ± 19.1	NA	NA
HC				
Serum (*n* = 44)	15/29	46.1 ± 15.8	NA	NA

[a]M, male; F, female.
[b]Data available on 102/99 (CSF/serum) CIS, 49/43 RR-MS and 10 SP-MS patients.
[c]EDDS, expanded disability disease scale. Data available on 92/79 (CSF/serum) CIS, 24/25 RR-MS and 8 SP-MS patients.
[d]NA, not applicable/not available.

(SP) MS, 36 NIND and 20 OIND patients as well as serum specimens from 3 CIS, 14 RR-MS, 13 NIND, 6 OIND patients and 44 healthy controls (HC). MS diagnosis and MS development in CIS patients was established according to McDonald criteria (McDonald *et al.* 2001; Polman *et al.* 2005). In general, CIS patients were clinically followed for at least 2 years after sampling of CSF and serum. This study was approved by the institutional ethics committee and fulfilled guidelines from the declaration of Helsinki. CSF and serum samples were obtained after informed consent.

SAS procedures

SAS procedures were performed with NWM and MS cDNA phage display libraries and characterized as described previously (Somers *et al.* 2005, 2008). In brief, four consecutive selection rounds were performed in parallel with both cDNA libraries on pooled CSF from CIS patients, to selectively enrich for cDNA products with high affinity for CIS Immunoglobulin G (IgG). Immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μg/mL (first round) or 10 μg/mL (subsequent rounds) rabbit anti-human IgG antibody (Dako, Glostrup, Denmark) in coating buffer (0.1 M sodium hydrogen carbonate pH 9.6). Next, immunotubes were blocked with 2% (w/v) skimmed milk powder in phosphate-buffered saline (MPBS). Meanwhile, 10^{12}–10^{13} phage particles were pre-incubated with the pooled pre-adsorbed CSF (diluted 1 : 15) in 2% MPBS for 1.5 h at 22°C, while shaking. The pre-incubation mixture was then transferred to the immunotube and incubated for 30 min while shaking at 22°C, followed by 2 h of stationary incubation. After extensive washing with 0.1% PBS Tween20 (PBST) and PBS, bound phage were eluted with 100 mM triethylamine and neutralized with 1 M Tris-HCl pH 7.4. To characterize the output from each selection round, eluted phage were used for infection of *E.coli* TG1 bacteria. Subsequently, a colony PCR was performed on phage-infected bacteria with vector primers binding adjacent to the cDNA insert, followed by restriction digestion of the PCR product. In this way, enriched cDNA products representing identical cDNA clones could be selected and identified by sequencing. The basic local alignment search tool (BLAST) of NCBI was used to compare nucleotide and amino acid sequences of the identified clones with public nucleotide and protein databases (http://blast.ncbi.nlm.nih.gov).

Phage ELISA

Phage ELISA was used for (i) a pilot screening to select for the most promising enriched candidate targets and for (ii) a screening for antibody reactivity in CSF and serum towards these selected antigens.

Ninety-six-well flat-bottom plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 10 μg/mL anti-M13 antibody (GE Healthcare, Diegem, Belgium) in coating buffer. Plates were blocked with 5% MPBS for 2 h at 37°C, followed by three times washing with 0.1% PBS Tween20 and once with PBS. In each well, 100 μL polyethylene glycol-purified phage displaying the candidate antigen (7×10^{11} colony-forming units/mL) or corresponding empty phage (used as negative control), were added and incubated for 1 h at 37°C and 30 min at 22°C, while shaking. Washing steps were repeated and 100 μL diluted CSF or serum was added (1 h at 37°C and 30 min shaking at 22°C). CSF was diluted 1 : 5 and serum 1 : 100 with a similar range of IgG concentration for patients and controls (CSF: 1–10 mg/dL, serum: 600–1600 mg/dL). Plates were washed, and the amount of bound IgG was detected with a 1 : 2000 dilution of horse radish peroxidase-labelled goat

anti-human IgG antibody pre-adsorbed against mouse IgG (Life Technologies, Bleiswijk, the Netherlands). Colour development was performed by addition of 100 μL 3,3′,5,5′ tetramethyl-benzidine dihydrochloride (TMB) solution (Perbio Science, Erembodegem, Belgium). The colour reaction was stopped with 50 μL H_2SO_4 and plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland).

Peptide ELISA and competition assays

Synthetic peptides (GL Biochem, Shanghai, China) corresponding to the peptides displayed on phage were used to confirm immunoreactivity detected with the phage ELISA assay. Based on phage ELISA results, an antibody-positive and an antibody-negative serum sample were selected for phage/peptide competition assays. For the competition assay, a standard phage ELISA was performed, after pre-incubation of antibody-positive and antibody-negative serum samples with increasing amounts of corresponding peptide (0–100 μg/mL).

Antibody reactivity was additionally measured in a solid-phase peptide ELISA by coating specific or a random irrelevant peptide (WTKTPDGNFQLGGTEP), as described previously with minor modifications (Somers *et al.* 2011). Briefly, 96-well flat-bottom plates (Greiner Bio-One) were coated with specific or irrelevant peptide in coating buffer for 2 h at 37°C. Plates were blocked with 2% MPBS. Subsequently, 100 μL of a phage ELISA antibody-positive or antibody-negative sample (diluted 1 : 100) was added and incubated at 22°C for 2 h, while shaking. The amount of bound IgG was detected with rabbit anti-human IgG horse radish peroxidase-labelled antibody (1 : 2000; Dako). Colour development was performed by addition of 100 μL TMB and stopped with 50 μL H_2SO_4. Plates were read at 450 nm in a Tecan plate reader.

Statistics

Statistical analyses were performed using Graph Pad Prism 5 software (GraphPad software, La Jolla, California, USA). Phage ELISA data were analysed with the Kruskall–Wallis test, followed by Dunn's multiple comparison test. Putative associations between antibody reactivity and disease were analysed using the Fisher's exact test. Associations between immunoreactivity and clinical parameters were analysed with Fisher's exact test. For categorical and demographical variables, an unpaired two-tailed Student's *t*-test or Mann–Whitney test was used, when appropriate. For all statistical tests, a *p*-value lower than 0.05 was considered statistically significant.

Results

Discovery of novel candidate antigens for CIS

Two cDNA phage display libraries suitable for SAS were constructed: one cDNA library was derived from chronic active MS plaques; the other library was generated from NWM derived from healthy brain. In this way, we were able to investigate whether primarily normal or diseased brain antigens are targeted by the antibody response in CIS. To identify CIS-associated antigens, four consecutive selection rounds were performed in parallel for the MS and NWM cDNA phage display libraries on pooled CSF from four CIS patients, who developed MS within 2 years after lumbar puncture. After characterization of the output from the selection rounds, 22 enriched cDNA clones were identified representing novel CIS candidate antigens. Seventeen enriched cDNA clones were obtained from selections on the NWM library, while five clones were obtained from selection on the cDNA library from chronic active MS plaques. One candidate clone was identified in selection outputs from both cDNA libraries.

Of the 22 enriched cDNA clones, the most promising cDNA clones were selected based on antibody reactivity in CSF and serum. To this end, a pilot screening was performed on individual CSF and serum samples from CIS patients used for SAS. For 6 of the 22 clones, named UH-CIS1–6 (University Hasselt, CIS, clone number), CSF and serum immunoreactivity in at least one of four CIS patients used for the SAS procedure could be detected, confirming specific selection by CIS autoantibodies in CSF and serum. On the other hand, antibody reactivity towards these clones was absent in pooled serum from HC (n = 4), NIND (n = 5) and OIND (n = 5) patients. Apart from antibody reactivity in CIS patients, antibody reactivity towards clone UH-CIS3 could be confirmed in pooled serum from five randomly selected MS patients (data not shown). These six clones were further used for a large-scale screening for antibody reactivity in CSF and serum from patients and controls.

The selected candidate antigens, clones UH-CIS1–6, encode for peptides with a size of 6–123 amino acids. Subsequently, these peptides were compared to public protein databases using BLAST analysis. One of the candidate antigens, UH-CIS2 encoded part of Host Cell Factor C1 Regulator 1, which has not been associated with CIS until now (Table 2). UH-CIS6 showed homology to several proteins including Bestrophin 1, Peroxisomal Biogenesis Factor 6 and Ring Finger protein 157. The other clones, UH-CIS1, UH-CIS3, UH-CIS4 and UH-CIS5 were translated from 3′ UTR regions or from non-coding reading frames, but did show partial homology to known proteins (Table 3).

CSF and serum antibody reactivity in CIS and RR-MS towards UH-CIS1–6

To investigate whether antibody reactivity directed to UH-CIS1–6 could be detected in a large cohort of CIS and MS patients, a phage ELISA screening was performed on CSF samples from CIS (n = 123), RR-MS (n = 55), SP-MS (n = 10), OIND (n = 28) and NIND (n = 47) patients (Table 2). For all six antigens, antibody reactivity could be confirmed in other CSF samples from CIS patients not used for SAS procedures. A trend for an increase in antibody reactivity towards the panel was observed in the CIS and RR-MS group as compared to the OIND and NIND patients (Table 4). For the whole panel of CIS candidate antigens, no reactivity could be detected in CSF from SP-MS patients.

Table 3 Identity of candidate CIS clones identified from pilot screening

Clone	cDNA library	Translated cDNA product	Size (amino acids)	Homology on protein level (accession number)
UH-CIS1	NWM	ARAKQDSLKKINGE	14	8/10 (80%) ubiquitin carboxyl-terminal hydrolase 8 (P40818.1) 7/8 (88%) myeloid/lymphoid or mixed-lineage leukaemia (trithorax homologue, Drosophila) [Homo sapiens] (Q03164.5)
UH-CIS2	NWM	AAFGAKVAGAYRPEPQEPRLPPVSV SQPASLPSLPREPLRKQFLSEENMA THFSQLSLHNDHPYCSPPMTFSPAL PPLRSPCSELLLWRYPGSLIPEALRLLRL GDTPSPPYPATPAGDIMEL	123	87/88 (99%) host cell factor C1 regulator 1 (XPO1 dependent) (Q9NWW0.1)
UH-CIS3	NWM	RPWFFSCAHSGLSSSLPIP	19	9/14 (64%) basonuclin 1(Q01954.2) 11/22 (50%) NADH dehydrogenase subunit 4 (ABR57675.1)
UH-CIS4	NWM	LLWASWRQSHLATPFTPTCGVASD GADTQGGTGLSLGIKGTESAHTLSV TKEGTAGPV	58	11/30 (57%) sodium channel, voltage-gated, type V, alpha subunit (Q14524.2) 10/25 (40%) poly (ADP-ribose) polymerase 1(P09874.4)
UH-CIS5	NWM	HIGVWD	6	5/5 (100%) G protein-coupled receptor 124 (NP_116166.9) 5/6 (83%) WD repeat domain 36 (NP_644810.1)
UH-CIS6	NWM	MPVVPATWEAETGESLEPGRRRLQ	24	19/22 (86%) bestrophin 1 (AAC64344.1) 19/24 (79%) peroxisomal biogenesis factor 6 (EAX04126.1) 19/23 (83%) ring finger protein 157 (AAH04231.2)

An almost equal sensitivity could be obtained by combining ELISA results for UH-CIS3, UH-CIS4 and UH-CIS6 as compared to the whole panel. Therefore, these clones were selected to further study antibody reactivity in serum of CIS (n = 108), RR-MS (n = 44), OIND (n = 14) and NIND (n = 24) patients, as well as HC (n = 44) (Table 2). Similar to the CSF antibody screening, reactivity could be detected in serum from CIS patients not used for SAS and from RR-MS patients (Table 5). Twenty-eight% of the CIS patients and 23% of the RR-MS patients tested positive for the three markers, although some serum samples from neurological and healthy controls were also antibody positive. This led to a combined sensitivity of 26% and specificity of 87% for CIS and RR-MS. As shown in Table 5, the number of antibody-positive CIS and RR-MS patients for the combined panel (26% vs. 13%, Fisher's exact test: p = 0.03) and UH-CIS6 (21% vs. 7%, Fisher's exact test: p = 0.009) was significantly increased as compared with controls. In addition, a trend for elevated antibody levels towards UH-CIS6 was detected in both CIS and RR-MS (Fig. 1).

To evaluate a putative correlation between CSF and serum antibody reactivity, ELISA results for UH-CIS3, UH-CIS4 and UH-CIS6 from 154 paired CSF and serum samples were compared. Of these 154 CSF samples, 18 CSF samples tested positive for one or more antigens, resulting in a total number of 22 positive ELISA tests. Importantly, 19 of these 22 positive ELISA tests in CSF could be confirmed in serum (data not shown), implicating the presence of a mostly similar antibody repertoire in CSF and peripheral blood.

For both CSF and serum antibody reactivity towards our panel, no significant correlation could be detected between immunoreactivity and gender, age, disease duration,

Table 4 Antibody reactivity in CSF to UH-CIS 1 to 6

Clone	CIS (n = 123)	RR-MS (n = 55)	SP-MS (n = 10)	OIND (n = 28)	NIND (n = 47)
UH-CIS1	4/123[a] (3%)	1/55 (2%)	0/10 (0%)	1/28 (4%)	1/47 (2%)
UH-CIS2	3/123 (2%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	2/47 (4%)
UH-CIS3	8/123 (7%)	10/55 (19%)	0/10 (0%)	0/28 (0%)	3/47 (6%)
UH-CIS4	7/120[b] (6%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	2/47 (4%)
UH-CIS5	2/123 (2%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	0/47 (0%)
UH-CIS6	8/123 (7%)	1/55 (2%)	0/10 (0%)	2/28 (7%)	2/47 (4%)
Total	21/123 (17%)	12/55 (22%)	0/10 (0%)	3/28 (11%)	5/47 (11%)

[a]The cut-off for a positive sample was set at three times the standard deviation (SD) above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for the NIND and OIND samples.

[b]Samples were excluded for analysis when the intra-assay coefficient of variation (CV) > 20% for duplicates.

Table 5 Antibody reactivity in serum to UH-CIS3, UH-CIS4 and UH-CIS6

Clone	CIS (n = 108)	RR-MS (n = 44)	OIND (n = 14)	NIND (n = 24)	HC (n = 44)	Fisher's exact test[b] (p-value)
UH-CIS3	7/108[a] (6%)	4/44 (9%)	0/14 (0%)	0/24 (0%)	4/44 (9%)	0.584
UH-CIS4	4/108 (4%)	1/44 (2%)	0/14 (0%)	1/24 (4%)	1/44 (2%)	1.000
UH-CIS6	25/108 (23%)	7/44 (16%)	0/14 (0%)	3/24 (13%)	3/44 (7%)	0.009
Total	30/108 (28%)	10/44 (23%)	0/14 (0%)	3/24 (13%)	8/44 (16%)	0.030

[a]The cut-off for a positive sample was set at three times the SD above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for HC samples.

[b]Fisher's exact test was used to investigate a putative association between antibody reactivity and disease (CIS and MS versus OIND, NIND and HC).

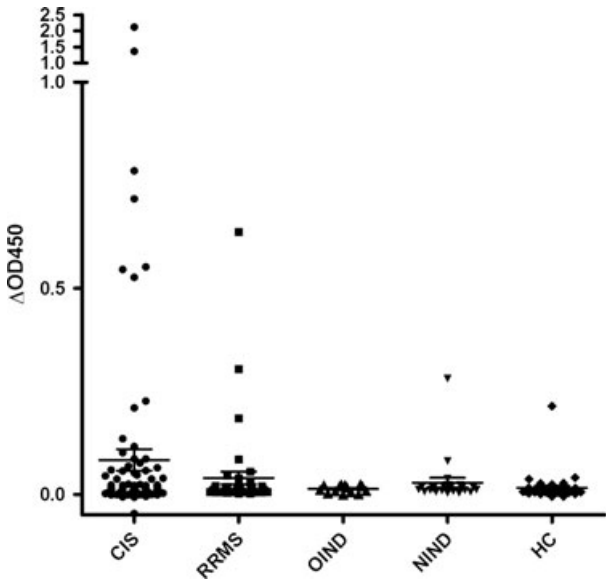

Fig. 1 Increased levels of serum antibody reactivity to UH-CIS6 in clinically isolated syndrome (CIS) and relapsing-remitting multiple sclerosis (RR-MS) patients. Phage ELISA was used to test serum antibody reactivity to UH-CIS6 in patients with CIS and MS and controls. The displayed results are ΔOD values.

Expanded Disability Status Scale (EDSS) or the presence of unique CSF oligoclonal bands. No information on magnetic resonance imaging (MRI) was available for the tested CIS and MS patients.

Synthetic peptides were generated for UH-CIS3 and UH-CIS6. Antibody reactivity towards phage-displayed peptides, UH-CIS3 and UH-CIS6, could be confirmed using these synthetic peptides in solid-phase peptide ELISA and in phage/peptide competition assays (Fig. 2).

Investigation of prognostic potential of UH-CIS1–6 to predict conversion to MS

Antibody reactivity towards the panel of CIS candidate antigens could also be detected in CSF and serum of RR-MS patients. This implies that our panel might have potential prognostic and predictive value for conversion to MS. For this reason, follow-up information concerning MS development in CIS patients was collected. Among the CIS patients tested for CSF and serum antibody reactivity, 59 out of 123 (48%) and 53 out of 108 (49%) CIS patients converted to MS respectively. As shown in Table 6, antibody reactivity towards the panel could be detected in both CSF and serum, irrespective of conversion to MS. A trend for an increase in antibody reactivity towards UH-CIS4 and UH-CIS6 was observed in CIS patients with conversion to MS as compared to CIS patients without MS development. This suggests that antibody reactivity towards UH-CIS4 and UH-CIS6 could be used as a prognostic marker. However, additional CIS patients should be tested to further study the prognostic potential of these antigens.

Discussion

In this study, we used SAS to identify novel CIS antigens. Antibody reactivity towards our panel of six novel antigens could be confirmed in CSF from CIS patients used for SAS procedures, as well as in CSF from other CIS and RR-MS patients. In addition, serum antibody reactivity towards three antigens, UH-CIS3, UHCIS-4 and UH-CIS 6, was significantly increased in CIS and RR-MS patients as compared with (neurological) controls. Furthermore, possible prognostic potential to predict conversion to MS in CIS patients could be demonstrated for two antigens, UH-CIS4 and UH-CIS6.

CIS can be considered either as an isolated event or as an early presentation of MS. Therefore, the antibody response in CIS might be directed to initial antigens involved in pathogenesis. In general, the number of brain lesions and plaques is low and could explain why the antibody response in CIS is preferentially directed towards antigens expressed in the NWM cDNA library. Moreover, another study (Becker *et al.* 1997) demonstrated that the MS cDNA library used in our study contained unique transcripts involved in immune activation, which were absent in two normal brain cDNA libraries. Thus, the antigen repertoire present in MS lesions is

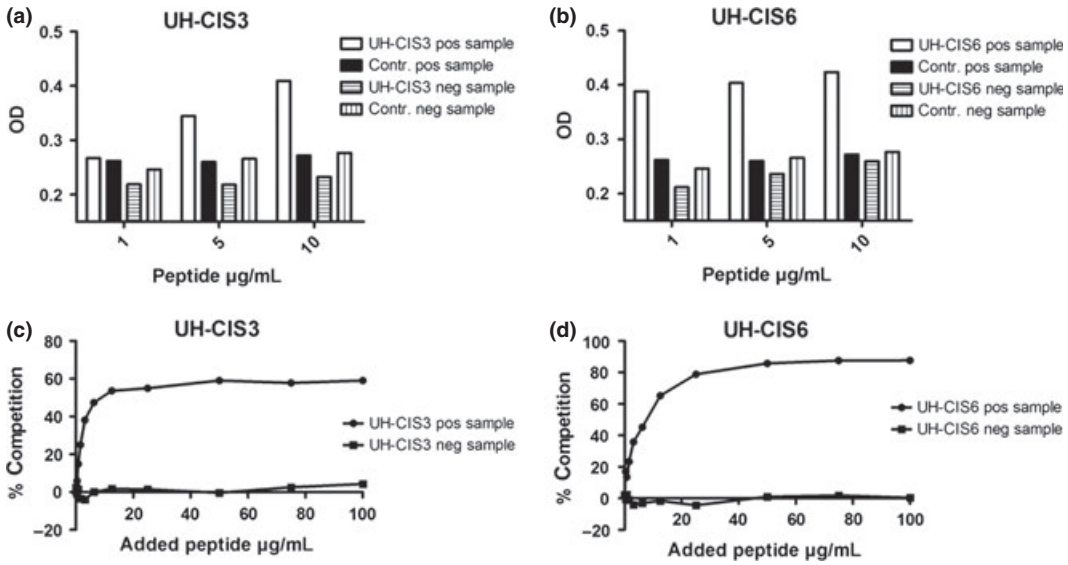

Fig. 2 Antibody reactivity towards UH-CIS3 and UH-CIS6 synthetic peptides. a,b. Solid-phase peptide ELISAs with synthetic peptides corresponding to UH-CIS3 and UH-CIS6 confirms antibody reactivity in positive serum samples based on phage ELISA. c,d. Increasing amounts of UH-CIS3 and 6 synthetic peptides (*X*-axis) result in inhibition (*Y*-axis) of UH-CIS3 and UH-CIS6 phage ELISA derived signals.

Table 6 Antibody reactivity in CSF and serum of CIS patients without and with conversion to MS

	CSF		Serum	
Clone	CIS (n = 64)	CIS-MS (n = 59)	CIS (n = 55)	CIS-MS (n = 53)
UH-CIS1	2/64[a] (3%)	2/59 (3%)	NA[c]	NA
UH-CIS2	1/64 (2%)	2/59 (3%)	NA	NA
UH-CIS3	6/64 (9%)	2/59 (3%)	5/55 (9%)	2/53 (4%)
UH-CIS4	**2/61[b] (3%)**	**5/59 (8%)**	**0/55 (0%)**	**4/53 (8%)**
UH-CIS5	1/64 (2%)	1/59 (2%)	NA	NA
UH-CIS6	**3/64 (5%)**	**5/59 (8%)**	**10/55 (18%)**	**15/53 (28%)**
Total	12/64 (19%)	9/59 (15%)	14/55 (25%)	16/53 (30%)

[a]The cut-off for a positive sample was set at three times the SD above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for the NIND and OIND samples (CSF) and HC (serum).

[b]Samples were excluded for analysis when the %CV > 20% for duplicates.

[c]NA, not applicable.

different as compared with normal brain. In addition, no antibody-positive CSF samples from SP-MS patients were identified for our panel. This might be explained by a restricted antibody response in the progressive phase of MS, which is characterized by neurodegeneration and to a lesser extent by inflammatory processes (Rovaris *et al.* 2006).

CIS and MS are both considered heterogeneous conditions, supported by the presence of a wide spectrum of symptoms in CIS and MS patients. The heterogeneity of the disease is also reflected by the different types of lesions present in MS brain (Lucchinetti *et al.* 2000). Only one of these type lesions, type II, is characterized by the presence of IgG. Therefore, antibodies might play a substantial role in the pathogenesis in only a subset of CIS and MS patients. In addition, because of the fact that phage-displayed proteins are produced by the prokaryotic protein translational machinery, the identification of post-translational modifications is not feasible with phage display systems. Future studies will be performed to investigate whether the addition of such post-translational modifications can increase the sensitivity of our panel. On the other hand, the phage display system used is based on monovalent expression, which allows high-affinity interactions with antibodies.

Zéphir *et al.* (2009) demonstrated that the self-reactive serum IgG repertoire is already distorted in CIS patients and is different from healthy controls. Moreover, they showed that in 80% of CIS patients, this IgG repertoire is similar to MS. Therefore, analysis of the antibody profile in CIS patients might have prognostic potential. For instance, IgG antibodies towards measles, rubella and the varicella zoster virus have been proposed as prognostic marker to predict conversion to MS in CIS patients (Brettschneider *et al.* 2009). In addition, similar results have been obtained for the IgG response towards EBV protein EBNA-1 (Lunemann and Ascherio 2009). On the other hand, conflicting results have been reported for other antibody targets such as MBP, MOG and alpha glucose-based glycans (Berger *et al.* 2003; Kuhle *et al.* 2007; Freedman *et al.* 2009, 2011). For our panel, possible prognostic potential could be demonstrated for two

antigens, UH-CIS4 and UH-CIS6. Although the majority of CIS patients without current conversion were followed for at least 2 years, MS might develop in at least a subset of this group in the future. For example, five CIS patients without MS development who tested positive for UH-CIS4 or UH-CIS6 have unique OCB in the CSF, which has been associated with conversion to MS (Masjuan *et al.* 2006). Therefore, the prognostic value of our panel could increase after a longer period of follow-up. In addition, to further investigate the prognostic potential of our panel, additional CIS patients should be tested.

Although initially identified as CSF antigens, antibody reactivity could be confirmed in the majority of paired serum samples, hereby providing an easily accessible source of antibodies. Whether the antibodies towards our panel are produced in CSF and/or in serum remains elusive, and additional studies are needed to investigate their origin. Previously, abnormal antibody production in MS has been described for both serum and CSF. For instance, it has been shown that CSF B cells produce the CSF oligoclonal immunoglobulin bands in MS patients (Obermeier *et al.* 2008). On the other hand, also an increased number of antibody producing cells have been demonstrated in the bone marrow and peripheral blood of MS patients as compared with healthy controls (Fredrikson *et al.* 1991).

One of the novel CIS candidate antigens was Host Cell Factor C1 Regulator 1 (HCFCR1). This nuclear export factor interacts with HCF-1, a ubiquitously expressed transcription factor, and HCFCR1 is thought to be implicated in HCF-1 transport into the cytoplasm. Here, HCF-1 binds herpes simplex virus protein, VP16. The HCF-1–VP16 complex is then transported to the nucleus, where it acts as a transcription factor to activate herpes simplex virus immediate early genes in herpes-infected cells (Mahajan *et al.* 2002). Interestingly, an intrathecal antibody response towards the herpes simplex virus has been detected in MS (Pohl *et al.* 2010). Other identified CIS antigens from our panel showed homology to proteins that have been linked with MS. For instance, UH-CIS 4 showed homology to the sodium channel Nav1.5. Expression of Nav1.5 has been shown to be up-regulated in reactive astrocytes in MS lesions and could have compensatory effects (Black *et al.* 2010). Inhibition of poly (ADP-ribose) polymerase 1, another protein with similarity to UH-CIS4, has been suggested as a therapy to suppress neuroinflammation in MS (Cavone and Chiarugi 2012). UH-CIS6 displayed high similarity to several proteins, including alternatively expressed isoforms of Bestrophin 1 (19/22 amino acids, 86%) and Peroxisomal Biogenesis Factor 6 (19/24 amino acids, 79%). In addition, mutations in *BEST1* and *PEX6*, genes encoding UH-CIS6 homologues, have been associated with retinopathies (Xiao *et al.* 2010) and several neurological diseases (Steinberg *et al.* 2006). Antibodies towards these described antigens might exert several functions. For example, antibodies towards NaV1.5 could be pathogenic by blocking its proposed compensatory mechanism in MS lesions. On the other hand, antibodies might also have protective roles. For instance, antibodies towards HCFCR1 might lead to a decrease of cytoplasmatic HCF-1, which then indirectly could inhibit viral infection.

As suggested by the BLAST results for UH-CIS6, alternative spliced antigens might be a target for autoimmunity. This is supported by findings reported by Ng *et al.* (2004), who demonstrated that alternative splicing occurred in 100% of 45 randomly selected autoantigens, which was significantly higher than the alternative splicing rate of 42% in 9554 randomly selected gene transcripts. For this reason, it was suggested that alternative splicing could lead to the generation of untolerized epitopes involved in autoimmunity (Ng *et al.* 2004). Of note, alternative splicing has already been described for several known autoantigens, which are associated with MS (Evsyukova *et al.* 2010).

Whether antibodies towards our panel are involved in CIS and MS pathogenesis remains to be elucidated. At least, a subset of known MS-associated autoantibodies has been shown to have pathogenic effects. For instance, oligoclonal IgM against myelin lipids has shown to correlate with disability progression in MS (Villar *et al.* 2005). Coinjection of monoclonal antibodies directed to the axonal antigen neurofascin together with encephalitogenic MOG-specific T cells exacerbated disease in experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Mathey *et al.* 2007). In addition, human serum with high titres of antibodies binding to native MOG has been to shown to enhance demyelination and axonal damage in EAE (Zhou *et al.* 2006).

In summary, we used an unbiased high-throughput technology based on phage display, which led to the identification a panel of novel CIS candidate antigens. Both CSF and serum antibody reactivity towards this panel could be primarily detected in CIS and RR-MS and to a lesser extent in healthy and neurological controls. In the future, experiments will be performed to further characterize these antigens, explore their prognostic potential in more detail and identify their role in CIS and MS pathogenesis.

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Authorship credit

MR designed and performed the experiments, analysed the data and wrote the manuscript. KS and CG designed the experiments and critically evaluated the manuscript. PD,RH, BvW, BdJ, MV, VvP, CS, LV, JA collected the patient material and critically evaluated the manuscript. PS and VS designed the experiments and wrote the manuscript.

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