Autoantibody targets in vaccine-associated narcolepsy

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Abstract

Narcolepsy is a chronic sleep disorder with a yet unknown cause, but the specific loss of hypocretin-producing neurons together with a strong human leukocyte antigen (HLA) association has led to the hypothesis that autoimmune mechanisms might be involved. Here, we describe an extensive effort to profile autoimmunity repertoires in serum with the aim to find disease-related autoantigens. Initially, 57 serum samples from vaccine-associated and sporadic narcolepsy patients and controls were screened for IgG reactivity towards 10 846 fragments of human proteins using planar microarrays. The discovered differential reactivities were verified on suspension bead arrays in the same sample collection followed by further investigation of 14 antigens in 176 independent samples, including 57 narcolepsy patients. Among these 14 antigens, methyltransferase-like 22 (METTL22) and 5'-nucleotidase cytosolic IA (NT5C1A) were recognized at a higher frequency in narcolepsy patients of both sample sets. Upon sequence analysis of the 14 proteins, polymerase family, member 3 (PARP3), acyl-CoA-binding domain containing 7 (ARID4B), glutaminase 2 (GLS2) and cyclin-dependent kinase-like 1 (CDKL1) were found to contain amino acid sequences with homology to proteins found in the H1N1 vaccine. These findings could become useful elements of further clinical assays that aim towards a better phenotypic understanding of narcolepsy and its triggers.

Introduction

Narcolepsy is a rare but chronic disorder affecting the regulation of sleep. This disease is associated with the loss of hypocretin-producing neurons that are among the key elements in control of sleep and wakefulness [1]. The lack of these neurons cause sleep disturbances and excessive daytime sleepiness (EDS) as well as cataplexy, the sudden loss of muscle tone and hallucinations. Although the lack of hypocretin-producing neurons has been shown to cause the disease, the underlying mechanism initially leading to this loss has not yet been identified [2]. A strong association of human leukocyte antigen (HLA) DQB1*0602 in narcolepsy patients, especially in those experiencing cataplexy [3] together with the specific neuronal loss has led to the hypothesis that an autoimmune mechanism might be involved. Furthermore other immunological but non-HLA gene associations have been associated to narcolepsy including the T-cell receptor (TCR), cathepsin H (CTSH), tumour necrosis factor ligand superfamily member 4 (TNFSF4) and P2Y purinoceptor 11 (P2RY11) [4–6].

In several countries, including Sweden, Finland, Norway, France and Ireland, the incidence of narcolepsy was considerably increased after national vaccination campaigns against the H1N1 influenza in 2009 [7–18]. Initial hypotheses of the cause included cross-reactivity reactions and molecular mimicry of vaccine or adjuvant components to hypocretin (HCRT) or other neuronal components [19–21]. Other studies have also reported evidence for B-cell autoimmunity involvement with autoantibodies recognizing tribbles homolog 2 (TRIB2) [22,23], including indications from passive transfer of serum IgG extracts to mice [24]. Similar studies have also suggested other potential targets including alpha-melanocyte-stimulating hormone (αMSH) and neuropeptide-glutamic acid-iso-leucine (NEI) [25,26]. However, this far, no commonly accepted explanation has been presented.

Through microarray technologies it is possible to, in a high throughput fashion, perform broad untargeted screenings of the specificity of autoreactive antibodies [27]. We herein describe the use of human protein fragments to profile the serum IgG repertoire of narcolepsy patients in search for novel disease-related reactivity patterns.
Materials and methods

Sample information

Blood was collected from included participants and the samples prepared as serum or EDTA plasma. In the initial sample set collected in Finland, 57 serum samples were included, as described previously [25]. Among these 57 samples, 39 were from narcolepsy patients (mean age 23, range 8–66 years, 19 males, 19 females and one with missing information) comprised of 20 vaccine-associated, 16 sporadic and three without information on vaccination status. The 18 controls were represented by patients with other neurological diseases (mean age 29, range 13–40 years, seven males and 10 females). The second sample set collected in Sweden consisted of 176 samples, including 59 narcolepsy patients (mean age 24, range 8–74 years, 29 males, 28 females and two with missing information) and 117 controls (mean age 25, range 6–74 years, 52 males, 61 females and four with missing information). Among the 59 narcolepsy patients, 46 were displaying vaccine-associated narcolepsy and four sporadic disease while vaccination status was not available for nine of the patients. The included individuals were recruited as part of the ongoing retrospective case-control study initiated by the Swedish Medical Product Agency during the study period 1 January 2009 until 1 July 2010. Approximately 85% of the participants were vaccinated as part of the Swedish Pandemrix® vaccination campaign of 2009 and the samples were procured 1.5–2 years after the vaccination [28]. The study was approved by the ethical committee at Karolinska Institutet (diary number 2010/1736-31/2 and 2011/734-32). In this collection, controls were represented by both healthy individuals and patients with other neurological diseases.

In addition to the narcolepsy sample collections, a set of myositis samples was also analysed. This set consisted of EDTA plasma that was collected as part of a research project on pathogenesis of myositis, and patients were diagnosed according to previously described criteria [29,30]. This study was approved by the local ethics committee and informed consent obtained from all patients.

Planar antigen microarrays

Planar microarrays were generated within the Human Protein Atlas project and contained human protein fragments of approximately 80 amino acids expressed together with a His6- albumin binding protein (His6-ABP) tag [31,32]. These antigens, representing protein sequences with low homology to other human proteins, were printed in batches of 384 and 30 of these batches (11 520 antigens representing 10 846 unique antigens and 7953 proteins) were utilized for an untargeted screening of the autoimmunity repertoire in serum from Finnish narcolepsy patients and controls. Samples were analysed for IgG reactivity as previously described [33] but in short, samples were diluted 1:250 in assay buffer (3% BSA and 5% milk in PBS supplemented with 0.1% Tween-20 and 160 μg/ml His6-ABP tag) before applied to the arrays. After 75 min of incubation and subsequent washing of slides, interacting IgG molecules were detected through an Alexa Fluor 647 conjugated anti-human IgG antibody and reported as median intensity per spot.

Suspension bead arrays

Antigens with higher reactivity in the narcolepsy samples as well as differentiating between vaccine-associated and sporadic disease in the screening were selected and immobilized on magnetic beads (MagPlex, Luminex Corp., Austin, TX) as previously described [27]. In later stages of analysis, 10 fragments representing previously suggested autoimmune targets within narcolepsy (TRIB2, HCRT, HCRTR1 and HCRTR2) were included together with 47 fragments for 22 adenosine-related proteins and immobilized to beads according to the same protocol (see Supplementary Table 1 for antigen sequences). Successful immobilization was evaluated through an anti-His6-ABP antibody (Agrisera) and the beads were combined into an array in suspension. Samples were diluted as for the planar arrays and incubated with beads during 1 h for the narcolepsy sample collections and during 2 h for the myositis samples before beads were washed and interactions cross-linked with 0.2% paraformaldehyde. Detection was mediated through a R-phycocerythrin-conjugated anti-human IgG F(ab)2 reagent (Jackson ImmunoResearch, West Grove, PA) and median fluorescence intensity per sample and bead identity used for data analysis.

Peptide arrays

One sample with high reactivity to several antigens in the first verification was selected and analysed on a whole proteome high-density peptide array containing 2.1 million peptides. The peptides were composed of 12 amino acids with six amino acids overlap and the analysis was performed as previously described [34], except for a few minor adjustments. Plasma was diluted 1:100 in 10 mM TBS (pH 7.4, 0.45% (w/v) NaCl) and alkali soluble casein 0.5% (Novagen, EMD Chemicals, San Diego, CA) and incubated with the peptide array overnight in 4 °C. The incubation was followed by washing for 3 × 10 min in 10 mM TBS supplemented with 0.05% Tween-20 and then incubated with the detection reagent (Alexa Fluor® 647-AffiniPure (Fab)2 fragment Goat Anti-Human IgG, Fcγ Fragment Specific, Jackson ImmunoResearch, West Grove, PA). The array was washed once again and rinsed in dH2O, dried and scanned at 2 μm-resolution using NimbleGen MS200 Scanner (Roche NimbleGen, Madison, WI). The scanned image was aligned and pixel intensities extracted using NimbleScan2 (Roche NimbleGen).

Data analysis

Statistical analysis and selection of antigens were based on a previously described approach [35]. In brief, to select antigens from the screening, the median intensities were batch-wise converted into a binary variable indicating if reactivity was observed or not by applying sample specific cut-offs of median intensity plus 5x the median absolute deviation (MAD). Antigens with five times higher reactivity frequency in the disease group and with reactivity in at least 10% of the narcolepsy patients were selected for further analysis. Similarly, antigens displaying reactivity frequencies five times higher for vaccine-associated narcolepsy patients vs sporadic and vice versa, requiring at least 20% of the samples in the highest group, were also selected for analysing using the
bead arrays. Similarly, for data generated by the bead arrays, a sample specific cut-off of 10xMAD plus the sample median was applied to convert the results into a binary variable for the narcolepsy samples while the corresponding cut-off for the myositis samples was based on 35xMAD plus the sample median. The significance of differences in proportion of reactive samples was evaluated using Fisher’s exact test.

For the peptide arrays, signals were filtered and only spots with at least two times the local background intensity were regarded as true signals. Epitope sequences were selected for the corresponding antigen fragments and searched with Blast (BLAST 2.2.30) [36]. Default settings were used in combination with the UniProtKB/SwissProt sequence database with exclusion of metazoan organisms.

To investigate the sequence homology of candidate targets showing differences between sporadic and vaccine-associated narcolepsy, the antigens were computationally divided to 12-mers with 11 aa overlap in order to cover minimal epitopes but also keep specificity [37]. These sequences were used as queries for searches on BLAST against the non-redundant database using default settings and matching against the organism H1N1 (strain: A/California/7/2009) to reveal possible overlapping sequences.

Results
We have performed extensive autoimmunity profiling of the IgG repertoire in serum of narcolepsy patients and healthy controls. Through the use of microarray technologies, reactivity towards thousands of antigens can be analysed using a few microliters of sample material. In an initial screening stage, reactivity towards 10 846 antigens was investigated in 57 patients and controls from Finland. The results were later followed up by analysis of an additional sample set from Sweden including 176 patients and controls. See Figure 1 for an overview of the experimental study setup.

IgG reactivity towards 10 846 antigens
After screening for IgG reactivity in 57 serum samples towards all 11 520 antigens (10 846 unique antigens representing 7953 proteins), the results were combined into sample profiles (Figure 2A). Two narcolepsy samples were removed from further data analysis due to high reactivity towards all antigens, probably due to antibodies reacting with the His6ABP tag. Through application of sample specific cut-off levels, reactivity towards protein fragments was indicated using a binary variable. Summary of the number of reactive antigens in each individual revealed reactivity towards 157–768 (1–7%) of the total number of antigens. This general reactivity was not found to significantly differ between the patients and controls (Figure 2B). However, the same analysis performed according to gender revealed the female narcolepsy patients to have higher reactivity compared to the male patients ($p$ value = 0.004). Although several hundreds of reactive antigens were identified in the screening, the reactivity patterns varied greatly between individuals. The majority of antigens were reactive in only single individuals while only 37 antigens (0.3%) generated reactivity in more than 50% of the samples (Figure 2C).

The selection of antigens for further verification using the bead-based platform was based on binary reactivity frequencies. Antigens with five times higher reactivity frequency in narcolepsy patients compared to controls as well as with the corresponding difference between vaccine-associated and sporadic patients were selected. Additional requirement of reactivity in at least 10% of the narcolepsy patients or 20% in sporadic or vaccine-associated patients respectively, resulted in a final selection of 244 antigens (Figure 3).

Verification of reactivity profiles
In the first verification stage, the same sample material as in the screening (57 samples) was analysed for reactivity towards the reduced set of 244 antigens using the bead-based platform. Based on the results, 14 antigens were selected to show disease-related reactivity (Table 1). These antigens displayed differential reactivity either in the comparison of all narcolepsy patients compared to controls, or when comparing vaccine-associated and sporadic disease (Figures 4 and 5). Reactivity was defined based on definition of sample specific cut-off levels, similar to the procedure for the screening data, and the reactivity frequencies of sample

![Figure 1](image-url)
groups compared. Out of the 14 antigens, two fragments
representing 5'-nucleotidase, cytosolic IA (NT5C1A) and
glutaminase 2 (GLS2) were found to display significantly
higher reactivity in the narcolepsy patients \((p/\epsilon 0.05)\). In
addition, reactivity to KRTAP17-1 was observed in 56% of
sporadic patients while in none of the vaccine-associated
\((p = 0.0001)\). For the other eleven antigens representing
methyltransferase-like 22 (METTL22), translocation protein
SEC63 homolog (SEC63), ATP synthase subunit s-like
protein (ATP5SL), mediator of RNA polymerase II transcrip-
tion subunit 6 (MED6), transmembrane protein 134
(TMEM134), AT-rich interactive domain-containing protein
4B (ARID4B), phosphatidate cytidylyltransferase 1 (CDS1),
histone-lysine N-methyltransferase EHMT2 (EHMT2), neur-
exin-1 (NRXN1), Poly (ADP-ribose) polymerase 3 (PARP3)
and cyclin-dependent kinase-like 1 (CDKL1), no statistical

Figure 2. General reactivity patterns. (A) A representative sample profile displaying reactivity towards 184 of the 10,846 antigens. (B) Comparing the total number of reactive antigens in all samples revealed no difference between narcolepsy patients and controls. However, a higher general reactivity was found for female narcolepsy patients. (C) Highly varying reactivity patterns was observed with the majority of reactive antigens found in only one individual.

Figure 3. Reactivity frequencies and antigen selection. Summary of reactivity frequencies for all 10,846 antigens in the comparison of (A) all narcolepsy patients compared to controls and (B) between vaccine-associated and sporadic disease. Black stars mark the 244 antigens selected for further analysis. Note that antigens with the same reactivity frequency appear as one data point.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein name</th>
<th>Antigen ID</th>
<th>Antigen sequence</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>METTL22</td>
<td>Methyltransferase-like protein</td>
<td>HPRR3160026</td>
<td>Q9BUU2 DEDGDLLDVVRPPRAASDSNPAGPLRDKVHPMIL AQEEDDVGLGEEAGGSPHDIIRIEHTMATPLDEVDG KQV DRESDEKQNDDEAEWQELQSQSRQKERALLEL K S</td>
<td>Narcolepsy vs control Verified in stage II</td>
</tr>
<tr>
<td>SEC63</td>
<td>Translocation protein SEC63</td>
<td>HPRR3720065</td>
<td>QUG8P EDEEEVSDKGDSDEEEETNRDSEQKDGDGSRDRESDEKQNDDEAEWQELQSQSRQKERALLEL K S</td>
<td>Narcolepsy vs control</td>
</tr>
<tr>
<td>NT5C1A</td>
<td>Cytosolic 5'-nucleotidase 1A</td>
<td>HPRR3730197</td>
<td>QBX13 PVWEAALKYDNAPKPKPSKPKPKNVTAIVS</td>
<td>Narcolepsy vs control Verified in stage II</td>
</tr>
<tr>
<td>ATP5SL</td>
<td>ATP synthase subunits-like protein</td>
<td>HPRR3250035</td>
<td>Q9NW81 LQRCHYVDWCLSLRLPLADSLQELSLAGCRIS ERGLACLHHQNLRRISLISPLAVSNPGLTQLVE EMLPLCEVGV DWAELGKSGPEEEQPRDTASPV</td>
<td>Narcolepsy vs control</td>
</tr>
<tr>
<td>MED6</td>
<td>Mediator of RNA polymerase II</td>
<td>HPRR140623</td>
<td>075586 NSGSVLQDSNPFYDRTCNNEVVKMQLRTLT EHLNQMVGEYILHHAQELPFIIRQKQQPSAPA QV IPLADYIIAGYTVQPADLGSVINSRVLTAHVGIQ AFDEAMSYSRCYHPSGKVYWWHFKDBEEQDKVR PKAKRKEEPSSI</td>
<td>Narcolepsy vs control</td>
</tr>
<tr>
<td>TMEM134</td>
<td>Transmembrane protein 134</td>
<td>HPRR3420269</td>
<td>Q9E6X4 QSFIDDAFELSLEDGGPGPSGGVAFGPLHFER RARFEVAGEDKQSRRLQYNL</td>
<td>Narcolepsy vs control</td>
</tr>
<tr>
<td>GLS2</td>
<td>Mitochondrial glutaminase liver isoform</td>
<td>HPRR3890773</td>
<td>Q9U132 ETVKLLQDYQDSYTLSETQAEAAAEALSKENLES MV</td>
<td>Narcolepsy vs control Vaccine-associated vs sporadic Sequence similarity to H1N1</td>
</tr>
<tr>
<td>ARID4B</td>
<td>AT-rich interactive domain-containing protein 4B</td>
<td>HPRR2550094</td>
<td>Q4LE39 VAEEESQSVELEKPPPVNVDSKPIEEKTIVEVND RKAEPSSGNSVLNTPPTEPSVSVTVEGQR QSSVTVESEPLAPN</td>
<td>Vaccine-associated vs sporadic Sequence similarity to H1N1</td>
</tr>
<tr>
<td>KRTAP17-1</td>
<td>Keratin-associated protein 17-1</td>
<td>HPRR3460778</td>
<td>Q9BYP8 DCFCTCTEQNCCCECCECQPCGCAGCCGSCGC</td>
<td>Narcolepsy vs control Vaccine-associated vs sporadic</td>
</tr>
<tr>
<td>CDS1</td>
<td>Phosphatidate cytidylyltransferase 1</td>
<td>HPRR3970178</td>
<td>Q92903 PREAVSPHREGAEAGGGHETESTSDKTDDIDDRTGDNDSRTDSDIEPEPSSDRTPEIIKKALSGLSSR PRSEETLPLKATPDSLAPGSSPSAVTPTVEGDEG ADTPVATPLGIREDENLEGDDGLRGRILLGHAK TKSFFPS</td>
<td>Narcolepsy vs control Epitope identified</td>
</tr>
<tr>
<td>EHMT2</td>
<td>Histone-lysine Nmethyltransferase EHMT2</td>
<td>HPRR3340364</td>
<td>Q96KQ7 PRSEETLPLKATPDSLAPGSSPSAVTPTVEGDEG ADTPVATPLGIREDENLEGDDGLRGRILLGHAK TKSFFPS</td>
<td>Narcolepsy vs control Epitope identified</td>
</tr>
<tr>
<td>NRXN1</td>
<td>Neurexin-1</td>
<td>HPRR3790120</td>
<td>Q9ULB1 TSNLHVTWIDKTITQITATGARNLKLSDKLYIGGY AKETYKSL</td>
<td>Narcolepsy vs control Vaccine-associated vs sporadic</td>
</tr>
<tr>
<td>PARP3</td>
<td>Poly[ADP-ribose] polymerase 3</td>
<td>HPRR4060030</td>
<td>Q9Y6F1 LSKQOJARGFELAELAEALKGPDTDGQSLEELSS HYFTYIPHNPFGSQQPPINSPELLAQAKDMLLVL ADIELAQALQAEVE QAVKTVEEV</td>
<td>Narcolepsy vs control Vaccine-associated vs sporadic Sequence similarity to H1N1</td>
</tr>
<tr>
<td>CDKL1</td>
<td>Cyclin-dependent kinase-like 1</td>
<td>HPRR3870262</td>
<td>Q00532 RHQVQFSTNYFSGKVKPIDPDEDMEPELELKFPNIS YPALGILK</td>
<td>Narcolepsy vs control Vaccine-associated vs sporadic Sequence similarity to H1N1</td>
</tr>
</tbody>
</table>
significance was obtained (Table 2). Nevertheless, their reactivity frequencies indicated differential patterns and they were therefore selected for further evaluation.

**Verification in independent material**

Reactivity to the 14 antigens selected from the screening was also analysed in a second verification stage using an additional independent sample set from Sweden. These samples were composed of 176 individuals, including 59 narcolepsy patients. Two of the antigens, namely METTL22 and NT5C1A, were successfully verified with higher reactivity also in the Swedish narcolepsy patients (Figure 6).

Figure 4. Antigens with differential reactivity. Reactivity intensities from the first verification stage for 14 antigens selected based on differential reactivity in comparison either between narcolepsy patients and controls or vaccine-associated and sporadic disease. Red stars indicate samples defined as reactive.

METTL22 was found reactive in 23% \( (n=9) \) of the Finnish narcolepsy patients while only 6% \( (n=1) \) of the corresponding controls and in the Swedish material, the frequencies were 24 and 15% \( (n=14 \text{ and } n=18) \) respectively. For the second antigen, NT5C1A, 21% \( (n=8) \) of the narcolepsy patients were reactive while 0% \( (n=0) \) of the controls and for the Swedish samples, 20% \( (n=12) \) of the patients were reactive and 15% \( (n=17) \) of the controls. The differential reactivity of KRTAP17-1 between sporadic and vaccine-associated patients as seen in the initial analysis were among the Swedish patients found reactive in 28% \( (n=10) \) of those with vaccine-associated disease and 25% \( (n=1) \) of the sporadic patients. Also, although not significantly different, reactivity
to NT5C1A, ARID4B and CDKL1 were concordantly higher and CDS1 concordantly lower in the vaccine-associated patients (Supplementary Figure 1).

**Further investigation of reactivity to NT5C1A**

For the two antigens found with higher reactivity in narcolepsy patients in both the Finnish and Swedish sample collections, additional fragments were included in a third round of verification analysis. The fragment for METTL22 used for initial analysis represented a central part of the protein (amino acid 117–186, HPRR3160026) while the additional fragment was from a more C-terminal region (amino acid 245–319, HPRR3160025). For NT5C1A, the initial fragment (HPRR3730197) mapped to amino acid 25–95 on the N-terminal side and the second fragment (HPRR3730198) to amino acid 329–364 on the C-terminal end. For METTL22, the second fragment revealed reactivity in only three of the Swedish control samples while in none of the other groups. The additional antigen for NT5C1A on the other hand showed reactivity in all sample groups but not with higher frequency in the narcolepsy patients. Interestingly, samples displaying high intensity reactivities showed concordant reactivity to both NT5C1A fragments (Supplementary Figure 2). To investigate the presence of NT5C1A antibodies in a different disease context, a set of

![Figure 5. Frequencies for differentially reactive antigens. Reactivity frequencies for the 14 antigens displaying differential reactivity in the initial sample set.](image-url)
Table 2. Results for differentially reactive antigens. $p$ values were calculated by Fisher’s exact test and significant differences ($p < 0.05$) are indicated in gray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reactivity [% (N)]</th>
<th>$p$ value</th>
<th>Narcolepsy vs Control</th>
<th>Vaccine-associated vs sporadic</th>
</tr>
</thead>
<tbody>
<tr>
<td>METTL22</td>
<td>23 (9)</td>
<td>20 (4)</td>
<td>31 (5)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>SEC63</td>
<td>23 (9)</td>
<td>25 (5)</td>
<td>19 (3)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>NT5C1A</td>
<td>21 (8)</td>
<td>25 (5)</td>
<td>19 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ATP5SL</td>
<td>18 (7)</td>
<td>10 (2)</td>
<td>25 (4)</td>
<td>0 (0)</td>
</tr>
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<td>MED6</td>
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</tr>
<tr>
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<td>5 (1)</td>
<td>19 (3)</td>
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</tr>
<tr>
<td>GLS2</td>
<td>41 (16)</td>
<td>50 (10)</td>
<td>31 (5)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>ARID4B</td>
<td>18 (7)</td>
<td>30 (6)</td>
<td>6 (1)</td>
<td>22 (4)</td>
</tr>
<tr>
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<td>28 (11)</td>
<td>0 (0)</td>
<td>56 (9)</td>
<td>17 (3)</td>
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<tr>
<td>CDS1</td>
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<td>19 (3)</td>
<td>6 (1)</td>
</tr>
<tr>
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<td>10 (4)</td>
<td>5 (1)</td>
<td>19 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NRXN1</td>
<td>33 (13)</td>
<td>50 (10)</td>
<td>19 (3)</td>
<td>22 (4)</td>
</tr>
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<td>15 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CDKL1</td>
<td>10 (4)</td>
<td>15 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Three narcolepsy patients did not have information on vaccine-associated vs sporadic disease.

Figure 6. Verification in independent samples. The two proteins methyltransferase-like 22 (METTL22), 5'-nucleotidase and cytosolic IA (NT5C1A) were found to have higher reactivity in narcolepsy patients compared to controls in both sample collections. Red stars indicate samples defined as reactive.
samples collected from myositis patients were also analysed. The results revealed higher reactivity in patients with inclusion body myositis (IBM) for both fragments, however only reaching statistical significance for the C-terminal antigen (Supplementary Figure 3).

Exploration of reactivity to TRIB2, HCRT, HCRTR1, HCRTR2 and adenosine-related proteins

As a complement to the antigens selected in this study, we additionally analysed reactivity to protein fragments representing the four mostly discussed potential autoimmune targets within narcolepsy, namely TRIB2, HCRT, HCRTR1 and HCRTR2. The two fragments for TRIB2 showed reactivity in 5 and 8 out of all 233 samples respectively (HPRR270019 corresponding to amino acid 10–149 and HPRR2290062 to amino acid 312–343). For the most C-terminal fragment, frequencies were higher in narcolepsy patients with 2.6% (n = 1) vs 0% (n = 0) in the Finnish sample collection and 5.1% (n = 3) compared to 3.4% (n = 4) controls in the Swedish sample material (Supplementary Figure 4). The hypocretin-related proteins HCRT, HCRTR1 and HCRTR2, were represented by 1, 2 and 4 antigens respectively, and to one of the HCRTR1 fragments (HPRR2090060 corresponding to amino acid 1–48), four narcolepsy samples were reactive. Meanwhile, for the HCRTR2 fragments, reactivity was observed in only one narcoleptic patient and for HCRT, none displayed reactivity in our assay.

To further investigate NT5C1A and its potential role in narcolepsy, 22 adenosine-related proteins were selected, represented by 47 antigens, and reactivity analysed in all samples. None of these antigens revealed any differences in reactivity between the sample groups (data not shown).

Epitope mapping autoantibodies on peptide microarrays

A sample from one of the narcolepsy patients was selected based on reactivity to several of the 14 antigens (five of 14) and analysed on a whole proteome high-density peptide array. On peptide level, this sample revealed reactive epitopes for two of the 14 antigens, namely EHMT2 and CDS1. For both proteins, epitopes overlapped with the protein fragment regions used in the previous analysis, as shown in Figure 7. The epitope sequences, RGGRILLGHA for EHMT2 and KKALSGLSSR for CDS1, were investigated for sequence similarity to other human as well as bacterial and viral proteins. For CDS1, no identical sequences were found but the protein HIS8 from Listeria monocytogenes, IF-2 from Coxliella burnetii and RS5 from Chlamyphila pneumonia all showed partial sequence identity. Similarly for EHMT2, REXO4 of Saccharomyces cerevisiae and Aspergillus fumigatus, GLMU of Corynebacterium aurimucosum, Y3309 of Desulfovibrio vulgaris and finally LSRA of Escherichia coli and Shigella flexneri were found to contain similar amino acid sequences.

Analysis of antigen sequences in relation to H1N1

Antigens identified with higher reactivity in vaccine-associated narcoleptic patients compared to sporadic cases were computationally divided into sequences of 12 amino acids. BLAST searches of these sequences revealed stretches with high identity to proteins of the H1N1 virus proteome (strain A/California/7/2009). For poly (ADP-ribose) polymerase family, member 3 (PARP3), the peptide QALQAVSEQE mapped to the nuclear export protein and for acyl-CoA-binding domain containing 7 (ARID4B) to the neuraminidase protein with the peptide sequence SCSPSVELE. GLS2, showed sequence similarity with the sequence SKENLES mapping to the nucleocapsid protein of H1N1 and finally CDKL1 mapped, although with lower similarity, to the polymerase PB1 of H1N1 (Table 3).

Discussion

In this study, we performed autoimmunity profiling of serum from individuals with and without narcolepsy in two sample collections from Finland and Sweden. To the best of our knowledge, this is the first broad screening of autoantibody reactivity in blood within the field of narcolepsy that has been performed without a pre-selection of antigens.

Based on the result from the screening of reactivity towards 10 846 antigens, the samples analysed revealed high
inter-individual variation in reactivity profiles in line with what has previously been reported within other disease areas [27]. Even so, the screening resulted in a list of 244 protein fragments indicating differences in reactivity between narcolepsy patients and controls, or similarly, between vaccine-associated and sporadic disease. In the first verification stage, where the same samples were analysed in a bead-based array format, 14 antigens were selected as most interesting based on their reactivity frequencies for the respective sample groups. Although only three of them could be regarded as statistically significant at this stage, we believed that they were all of interest for further evaluation as potential autoimmune targets.

The 14 antigens were further evaluated in an independent sample material. In this analysis, two fragments representing METL22 and NT5C1A were verified as more reactive among the narcolepsy patients compared to controls. METTL22 was just recently annotated on protein level but has been suggested to interact with the chaperones Hsp70, Hsp90 as well as a DNA/RNA binding protein KIN17 involved in DNA replication and DNA damage [38,39]. As indicated by the information available in the Human Protein Atlas (www.proteinatlas.org), METTL22 shows RNA expression in all tissue analysed while for NT5C1A on the other hand, gene expression was found enriched in skeletal and heart muscle but observed also in other tissues including the brain. NT5C1A is a protein involved in nucleoside metabolism and it mainly catalyses the dephosphorylation of AMP to adenosine [40]. It has recently been associated to the autoimmune disease sporadic inclusion body myositis (IBM), where patients were found to have higher levels of anti-NT5C1A autoantibodies [41]. For this reason, reactivity to NT5C1A was also explored in a separate sample material with myositis samples to validate the antigen, where significantly higher reactivity towards the antigen was seen in the IBM group compared to patients with dermatomyositis (DM) in concordance with the previous report. Furthermore, adenosine has been suggested to be involved in sleep homeostasis and the various enzymes in the adenosine generation affect the regulation. It has also been suggested that adenosine can act on adenosine A1 receptors in hypocretin-expressing hypothalamic neurons and thereby possibly regulate hypocretin levels [42,43]. Based on these indications, we extended the analysis of 22 adenosine-related proteins represented by 47 unique antigens. However, and as reported above, neither differential reactivity nor correlation to anti-NT5C1A levels were observed.

Although both sample collections included a representation of sporadic and vaccine-associated disease, the Swedish material only contained samples from four sporadic patients (7%) compared to 16 (41%) in the Finnish sample set. Although KRTAP17-1, the antigen displaying largest differential reactivity between the disease groups in the Finnish samples (56% in the vaccine-associated and 0% in the sporadic), was found with similar reactivity frequency in the Swedish samples (28 vs 25%), it should be noted that the 25% reactivity among the Swedish sporadic patients corresponds to reactivity in only one individual. This protein could therefore still be of interest in exploring the difference in disease etiology.
Four of the 14 proteins revealed sequence similarity when compared to the H1N1 virus strain used in the Pandemrix® vaccine. One of them, GLS2, is an enzyme that hydrolyses glutamine to glutamate and is thus of importance for neuropsychological functions and homeostasis. Alterations in levels of this neurotransmitter has previously been observed in chronic neurodegenerative disorders [44]. It has also been shown that microglia can disturb the homeostasis of glutamate in neurons by production of more GLS2, thus more glutamate, leading to neurotoxicity [45]. In addition to GLS2, the alignment revealed three other proteins with sequence similarity to viral components including the nuclear capsid, nuclear export and neuraminidase protein as well as the polymerase.

To explore antibody epitopes on the identified antigens, one sample was analysed on a high-density peptide array containing peptides representing the entire human proteome. The selected sample displayed reactivity towards five of the 14 protein fragments, and for two of these five, reactivity was also detected for the corresponding peptides. These results indicate that reactivity towards some of the protein fragments is possibly dependent on the conformational appearance and that the differential representations of a protein as a larger fragment or a peptide generate different reactivity patterns. This observation is in line with previous reports comparing reactivity to full-length protein and peptides [46,47]. For the two proteins for which epitopes were identified, sequence similarity was found to several bacterial proteins. This could either suggest molecular mimicry of bacterial and human proteins or that individuals exposed to various infections are more prone to develop the disease.

As previously mentioned, there are several studies associating genes to the etiology and pathogenesis of narcolepsy and these studies have focused on nucleotide polymorphisms and T-cell mediated-autoimmunity. B-cell involvement in narcolepsy is so far not widely understood and currently the only described potential autoimmune targets are TRIB2, NEI, α-MSH, HCRT, HCRTR1 and HCRTR2. There are several possible explanations as to how antibodies could be involved in the pathobiology of narcolepsy. They could either act as a trigger of disease but not necessarily be instrumental in the hypocretin neuron damage. The antibodies could also be generated as a consequence of immune destruction of hypocretin neurons and thereby represent an epiphenomenon of disease rather than causative factors.

When comparing our findings with previously suggested autoantigens, it is important to keep in mind the distinct differences in protein representation and sub-proteome being investigated and also in technologies used between our study and previous publications. The antigens used here are not full-length proteins, but protein fragments representing certain unique regions of the proteins. This consequently means that they might not cover the same protein sequence as those where reactivity previously has been observed and also that they might form alternative conformations compared to full-length proteins. The two peptides described by Bergman et al [25], NEI and α-MSH are peptides derived from the proteins pro-MCH (PMCH) and pro-opiomelanocortin (POMC) respectively. For these proteins, two fragments for PMCH and one for POMC were included in our initial screening but only one for PMCH included an overlapping sequence with the previously reported NEI peptide (Supplementary Table 1). The serum samples in our study were derived from the same cohort as the tissue-based analysis reported by Bergman et al [25], but we did not observe reactivity towards the PMCH/NEI antigen using the protein fragment. The authors reporting autoantibodies towards components of the hypocretin system (HCRT, HCRTR1 and HCRTR2) based on a radioligand assay state that studies prior to theirs have failed in detecting these due to limitations in presentation of conformational epitopes [48]. Also, Ahmed et al recently discussed that cell-based assays for HCRTR2 detected more autoantibodies than conventional ELISA set-ups and emphasized the importance of a native conformation of HCRTR2 for positive results [21]. Interesting to note in this context is that also in other disease areas, inconsistent results due to alterations in antigen presentation have been observed [49]. For TRIB2 as a potential autoimmune target in narcolepsy, Cvetkovic-Lopes et al reported that anti-TRIB2 titers sharply decrease already during the first years after narcolepsy onset [22]. Our protein fragment cover another part of the protein but the majority of samples used in this study were also collected more than one year after disease onset with the consequence that potentially disease-related autoantibody titers might already have been reduced. These differences in study setup can help to explain the lack of concordant findings between the studies.

In conclusion, we here suggest 14 novel antigens with potential relevance in the context of narcolepsy. Two of the antigens were verified as more reactive in narcolepsy patients in two independent sample materials, although further characterization is required to understand their involvement in disease involvement and progress.

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Declaration of interest

The authors report no conflicts of interest.

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References


**Supplementary material available online**

Supplementary Table 1

Supplementary Figures 1–4