Screening for autoantibody targets in post-vaccination narcolepsy using proteome arrays

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Abstract
Narcolepsy type 1 (NT1) is a chronic sleep disorder caused by a specific loss of hypocretin-producing neurons. The incidence of NT1 increased in Sweden, Finland and Norway following Pandemrix®-vaccination, initiated to prevent the 2009 influenza pandemic. The pathogenesis of NT1 is poorly understood, and causal links to vaccination are yet to be clarified. The strong association with Human leukocyte antigen (HLA) DQB1*06:02 suggests an autoimmune pathogenesis, but proposed autoantigens remain controversial. We used a two-step approach to identify autoantigens in patients that acquired NT1 after Pandemrix®-vaccination. Using arrays of more than 9000 full-length human proteins, we screened the sera of 10 patients and 24 healthy subjects for autoantibodies. Reactivity towards previously suggested NT1 autoantigen candidates including Tribbles homolog 2, Prostaglandin D2 receptor, Hypocretin receptor 2 and α-MSH/proopiomelanocortin was not replicated in the protein array screen. By comparing case to control signals, three novel candidate autoantigens were identified in the protein array screen; LOC401464, PARP3 and FAM63B. However, the RBA did not confirm elevated reactivity towards either of these proteins. In summary, three putative autoantigens in NT1 were identified by protein array screening. Autoantibodies against these candidates could not be verified with independent methods. Further studies are warranted to identify hypothetical autoantigens related to the pathogenesis of Pandemrix®-induced NT1.
1 | INTRODUCTION

Narcolepsy type 1 (NT1) is a chronic sleep disorder caused by a specific degeneration of hypocretin neurons in the lateral hypothalamic area. Increased incidence was observed in Finland, Norway and Sweden following Pandemrix®-vaccination (A/California/7/2009 NYMC X-179A, AS03-Adjuvanted H1N1 Influenza A Vaccine) in conjunction with the 2009 influenza pandemic. While the aetiology of NT1 is unknown, indirect evidences support an autoimmune origin, including a strong association with HLA-DQB1*06:02 and with other genes with immune regulatory functions. Pandemrix®-vaccination as a unique and unpredictable environmental trigger, and possibly the virus itself, would be compatible with an autoimmune genesis. Direct proof of an autoimmune aetiology and pathogenesis of NT1 has been lacking as no studies have been able to demonstrate an immune-mediated pathogenesis against the hypocretin neurons. Autoactive T cells towards hypocretin peptides were recently reported, and additional studies are therefore warranted to dissect the possible autoimmunity in NT1.

There are no validated demonstrations of autoantibodies in NT1. Previous studies identified Tribbles homolog 2 as a candidate autoantigen. However, these results were not confirmed in follow-up studies using vaccine-related NT1 patients. The observed cell-degeneration in NT1 was reported to be highly specific to the hypocretin neurons, intermixed melanin-concentrating hormone neurons were reported to be unaffected. This would suggest that hypothetical autoantigens recognized by the immune response after an environmental trigger such as Pandemrix® are specifically expressed by hypocretin cells. Hypocretin proteins have therefore been considered autoantigen candidates. Molecules involved in this neurotransmitter system, including the preprohypocretin and the converting enzyme cleaved peptides hypocretin 1 and hypocretin 2, have been investigated but not been confirmed as relevant immune targets. Autoantibodies directed against the hypocretin receptor 2 (HCRTR2) have been reported and the study further suggested a shared epitope of this receptor with Pandemrix® influenza nucleoprotein, implicating an immunological mechanism involving molecular mimicry. It should be recognized that the HCRTR2 is not expressed exclusively in the hypothalamus, and it is uncertain whether the receptor is expressed on the hypocretin neurons. Follow-up studies have not been able to associate HCRTR2 autoantibodies to NT1. Other suggested autoantigens in NT1 include prostaglandin D2 receptor and the glutamic acid-iso-leucine/α-melanocyte-stimulating hormone, which is a peptide hormone that is cleaved from proopiomelancortin.

Antigen-arrays have previously been used to screen for NT1 autoantigens. A library of protein fragments was screened with sera from NT1 patients and controls. Two new candidates were identified: methyltransferase-like 22 and 5'-nucleotidase cytosolic 1A. Another study identified NRXN1 as a putative candidate. The lack of independent, duplicated studies renders the relevance of current autoantigen candidates uncertain.

Protein array technology enables explorative screening of large panels of human proteins. The technique may be used for broad-scale analyses of autoantibody responses and the identification of novel immune targets. We here sought to identify novel autoantigens in NT1 using a two-step approach involving an explorative screen of a panel of more than 9000 full-length human proteins followed by validation studies with targeted radiobinding assays.

2 | MATERIALS AND METHODS

2.1 Study populations

Blood samples were obtained from NT1 patients (n = 31) and their first-degree relatives (FDR) (n = 66) as part of the AMINA-Study (Autoimmune Multiple Sclerosis and Narcolepsy) in collaboration with the Swedish Narcolepsy Association. All patients 31/31 (100%) and 54/66 (82%) of their FDR were vaccinated with Pandemrix® (Tables 1 and 2). Blood samples were obtained during 2015-2017 at healthcare centres in the families’ hometowns.

The protein array screening included in total 10 patient samples; eight close-to-pandemic-collected NT1 patients ascertained in 201220,38 and two samples from the family study (Table 1).

The radiobinding assay included in total 39 patient samples; the same eight close-to-pandemic-collected NT1 patients ascertained in 201220,38 and two samples from the family study (Table 1).

### Table 1: Samples included in the protein array screening

| n | Pandemrix®-vaccinated | Gender | HLA-DQB1*06:02 | Samples collected | Sample type analysed | Range birth years |
|---|-----------------------|--------|---------------|------------------|---------------------|-------------------|
| Narcolepsy Probands | 10 | 10/10 | 6F/4M | 9/10 | 8 samples in 2012 | Sera | 1996-2003 |
| | | 1 sample-HLA Not Available | | | 2 samples in 2015-2016 | |
| Controls | 24 | 0/24 | 9F/15M | Not available | 2006 | Sera | 1946-1987 |
2015-2017 (Table 2). Blood donors as population controls were ascertained from LifeGene (https://lifegene.se/forsk), a total of 188 serum samples were analysed. The controls were matched to the NT1-families in 2:1 ratio based on age, gender and geographical location in Sweden (Table 2). Plasma samples from multiple sclerosis patients (n = 100) were included as neurological disease controls, all recruited for from the neurological clinic at the Skåne University Hospital SUS, Malmö, Sweden. Also included as disease controls were plasma samples from 41 subjects with at least one islet autoantibody, all FDR to type 1 diabetes patients (Table 2).

All study participants gave their written consent to participate in the studies. The Regional Ethical Review Board in Lund, Sweden (dnr 2015/257) approved the study.

### 2.2 Protein array screening

Protein array screening was performed according to the protocol provided by the supplier. Protein arrays (ProtoArray v5.1, PAH05251020, ThermoFisher) were incubated one hour in blocking buffer. Serum samples, 2.5 µl sample in 5 mL washing buffer (diluted 1:2000), were incubated with arrays for 90 minutes. A first washing step, 5x5 minutes in washing buffer, was followed by addition of secondary antibodies; Alexa flour® 647 Goat Anti-human IgG (A21445; ThermoFisher, diluted 1:2000 in washing buffer) and Goat Anti-GST Dylight 550 (#DY550011-13-001, Cayman Chemicals, diluted 1:10 000 in washing buffer), and the arrays were incubated for 90 minutes. A final washing step, 5x5 minutes in washing buffer, followed. Arrays were scanned using Capital LuxScan HT24 (BioCapital). GenePix pro microarray software (v5.1) was used for data acquisition. Cut-offs for positivity were determined as three standard deviations above the mean value for the controls, corresponding to 80 MFI (median fluorescent intensity) for LOC401464, 170 MFI for PARP3 and 174 MFI for FAM63B (Figure 1). Sera from ten Pandemrix®-vaccinated NT1 patients were included; eight samples collected in 2012 and the remaining two samples collected in 2015-2016 (Table 1). Autoantibody reactivity in NT1 sera was compared to reactivity in 24 healthy controls samples; two of which were analysed simultaneously with narcolepsy sera and the remaining 22 analysed at previous occasions.

### 2.3 In vitro transcription-translation and radiobinding assay

Complementary deoxyribonucleic acid (cDNA) encoding LOC401464 (accession number XM_379587.2), PARP3 (gene ID: 10 039) and FAM63B (gene ID: 54 629)
were subcloned into pTNT-Vector (Promega, #L561A). $^{35}$S-radiolabeled proteins were obtained through in vitro transcription-translation. Briefly, cDNA was mixed with TnT SP6 Coupled Reticulocyte Lysate System (Promega, #L4600) and $^{35}$S-radiolabeled methionine (PerkinElmer Life Sciences, EasyTag L-(35S) Methionine, #NEG709A). Incubation was set to 90 minutes at 30°C. Separation of in vitro transcribed translated proteins from free methionine was performed with Nap5 columns (GE Healthcare, #17-0853-02). Percent incorporation was 7%, 45% and 14% for LOC401464, PARP3 and FAM63B, respectively. Serum or plasma samples were analysed in duplicates (2.5 µl) and mixed overnight with 60 µl $^{35}$S-LOC401464/PARP3/FAM63B antigen, corresponding to 24 000 counts per minute (cpm). Antibody-bound labelled antigen was separated from free with Protein A-Sepharose (Zymed Laboratories Inc, Protein A-Sepharose 4B Conjugate, #QW10-1090) on filter plates (Millipore, Multiscreen DV filtration plate, #MSDV6B50), as outlined in detail elsewhere. Free radiolabeled proteins were washed through using a vacuum manifold plate washer ELX50/8FMW (BioTek Instruments Inc). Levels of autoantibody bound $^{35}$S-antigen were counted in a Wallac Microbeta Trilux beta counter (Microbeta Trilux, #1450-021; PerkinElmer Life and Analytical Sciences). Rabbit antisera against PARP3 and FAM63B were used as positive controls to convert cpm to in-house arbitrary Units per mL (U/mL) as follows:

**PARP3**: Polyclonal Rabbit/ IgG, Abcam catalogue-number# ab96601 1mg/mL, diluted in human sera in seven steps to produce a standard curve;
- Dilution 1/20 ~ 6400 cpm −125 U/mL, Dilution 1/40 ~ 3000 cpm- 62.5 U/mL,
- Dilution 1/80 ~ 1500 cpm- 31.3 U/mL, Dilution 1/160 ~ 700 cpm −15.6 U/mL,
- Dilution 1/320 ~ 400 cpm −7.8 U/mL, Dilution 1/320 ~ 200 cpm −3.9 U/mL,
- Dilution 1/1280 ~ 100 cpm −1.95 U/mL. An in-house reference serum was found positive for PARP3 (~ 8500 cpm) and included a positive control in all assays.

**FAM63B**: Polyclonal Rabbit/ IgG, Thermo Fisher catalogue-number# PA5-62318 0.1mg/mL, clone# R60977, diluted in human sera in seven steps to produce a standard curve;
Dilution 1/20 ~ 8150 cpm −125 U/mL, Dilution 1/40 ~ 4600 cpm −62.5 U/mL,
Dilution 1/80 ~ 2400 cpm −31.3 U/mL, Dilution 1/160 ~ 1100 cpm −15.6 U/mL,
Dilution 1/320 ~ 550 cpm −7.8 U/mL, Dilution 1/320 ~ 300 cpm −3.9 U/mL,
Dilution 1/1280 ~ 150 cpm −1.95 U/mL.
Due to the absence of LOC401464 antibodies or positive control-samples, levels were presented as cpm.

2.4 | HLA-DQ typing

Association with HLA-DQB1*06:02 were determined using TaqMan® Genotyping with SNP rs9271366 as follows;
GG-homozygous DQB1*06:02,
AG-heterozygous DQB1*06:02,
AA-not-associated to DQB1*06:02

2.5 | Statistics

Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software). Median autoantibody levels between study-groups were compared using Mann-Whitney’s U test or Kruskal-Wallis test. The significance threshold was set to 0.05.

3 | RESULTS

A panel of more than 9000 full-length human proteins was used to perform an explorative screen for novel autoantigens in NT1. The protein arrays were screened with sera from 10 patients with NT1 and 24 population controls. IgG autoantibody reactivity was detected (for full raw antibody array data, see Table S1). FAM63B, LOC401464 and PARP3 were identified as the proteins with the highest signal in patients compared to controls and were selected for validation (Figure 1). With the cut-offs for positivity determined as three standard deviations above the mean value for the controls, elevated signal against LOC401464 and PARP3 was found in five and four out of ten narcolepsy patient sera, respectively, while all healthy controls were negative. FAM63B showed elevated level in two NT1 sera and one control (Figure 1). Tribbles homolog 2, prostaglandin D2 receptor, hypocretin receptor 2 and glutamic acid-isoleucine/α-melanocyte-stimulating receptor 2 were among the proteins identified as novel autoantigens in NT1.
hormone/proopiomelanocortin, all previously suggested candidate autoantigens for NT1 were present in the array. Neither of these antigens generated elevated signals in the protein array screen.

In vitro transcription-translation and RBA were used to validate autoantibody reactivity towards FAM63B, LOC401464 and PARP3 (Figure 2). Included were an extended cohort of NT1 patients (n = 39), the patients FDR (n = 66), multiple sclerosis patients (n = 100), FDR of type 1 diabetes patients (n = 41) and population controls (n = 188). Autoantibody levels towards all three candidates were low and did not differ between patients and controls; FAM63B (P = .175), LOC401464 (P = .778), PARP3 (P = .132) (Figure 2). Plasma from FDR to type 1 diabetes patients were analysed against GADA, IAA, IA-2A, ZnT8A and study-subjects were found positive to at least one islet autoantibody (Table 2).

4 | DISCUSSION

The present study represents a significant effort to uncover autoantibodies in individuals with Pandemrix®-induced NT1. We hypothesized that the use of a previously tested protein array that has been useful to detect autoantigens such as glutamate decarboxylase 2 and transglutaminase 435,36 to name but a few, would offer a unique opportunity to identify candidate autoantigens in NT1 triggered by Pandemrix®. The major finding is that the protein array may result in a signal on solid phase that was not reproducible in the subsequent RBA with the candidate autoantigen generated by in vitro transcription-translation. The advantage with the RBA is the detection of conformation-dependent autoantibodies. This type of autoantibodies is known to predict type 1 diabetes (insulin, IA-2 and ZnT8) and Addison’s disease (21-hydroxylase)—none of these autoantibodies are detected in solid-phase assays.40-42

Autoantigens in NT1 have, despite numerous studies and suggested candidates, not been established, in a way that would survive replication and future standardization workshops (IDW, DASP, IASP, Addison standardization). It remains unclear whether autoantigens are involved in the pathogenesis of NT1. However, there are different explanations to consider why studies may have failed to establish reproducible autoantibodies in NT1. The number of hypocretin neurons in the human brain has been estimated to around 70,000.1 An autoimmune response against this limited cell population should perhaps not be expected to generate a strong systemic immune response. It is possible that autoantibody titres decrease with time, which would make retrospective investigations as the current study, less successful. The limited knowledge of protein expression in the hypocretin neurons poses another challenge to the study of autoantibody responses in NT1, making candidate antigen-based approaches a less feasible option.

FAM63B, LOC401464 and PARP3 autoantibodies could not be confirmed in the validation assays (Figure 2), and hence, their relevance as immune targets in narcolepsy could not be verified. PARP3 is an enzyme with regulatory functions of processes including DNA damage responses34,44 and has been reported as a negative regulator of immunoglobulin class switch recombination.45 Protein expression is found in all major organs, including the brain.46 Notably, PARP3 has previously been found to share sequence homology with influenza nuclear export protein (A/California/7/2009) in the Pandemrix®-vaccine.33 Information about LOC401464 is limited. Function and expression of this putative protein are not known. FAM63B (Mindy-2) is a deubiquitinating enzyme47 found in the cerebellum and in the pineal body.48 Epigenetic alterations to FAM63B have been associated with schizophrenia and bipolar disorder.49,50

Methodological differences between the two autoantibody assays must be considered as explanations to why the herein identified candidate antigens could not be verified, including differences in protein folding, post-translational modification and immobilization of the antigen. These aspects are also relevant to a broader discussion on the difficulties in confirming suggested candidate autoantigens in NT1.

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AUTHOR CONTRIBUTIONS

AL, DE and NL preformed the protein array analysis. AR, ÅL and HEL developed the radiobinding assay. AL, OA and AR preformed RBA analysis. LP, ÅL, OK, HEL and NL designed the overall study. AL, DE and NL wrote the first version of the manuscript. All co-authors contributed to the analysis of data and in writing the final manuscript. All co-authors approved the final version of the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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