

# Widespread white matter changes in post-H1N1 patients with narcolepsy type 1 and first-degree relatives <sup>FREE</sup>

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## Abstract

### Study Objectives

To assess white matter involvement in H1N1-vaccinated hypocretin deficient patients with narcolepsy type 1 (NT1) compared with first-degree relatives (a potential risk group) and healthy controls.

### Methods

We compared four diffusion tensor imaging–based microstructural indices (fractional anisotropy [FA], mean diffusivity [MD], radial diffusivity [RD], and axial diffusivity [AD]) in 57 patients with NT1 (39 females, mean age 21.8 years, 51/57 H1N1-vaccinated, 57/57 *HLA-DQB1\*06:02*-positive, 54/54 hypocretin-deficient), 54 first-degree relatives (29 females, mean age 19.1 years, 37/54 H1N1-vaccinated, 32/54 *HLA-DQB1\*06:02*-positive), and 55 healthy controls (38 females, mean age 22.3 years). We tested for differences between these groups, for parametric effects (controls > first-degree relatives > patients) and associations in patients (cerebrospinal fluid [CSF] hypocretin-1 and disease duration) and first-degree relatives (*HLA-DQB1\*06:02* and H1N1-vaccination). We employed tract-based spatial statistics and used permutation testing and threshold-free cluster enhancement for inference.

### Results

Patients with NT1 had a widespread, bilateral pattern of significantly lower FA compared with first-degree relatives and healthy controls. Additionally, patients with NT1 also exhibited significantly higher RD and lower AD in several focal white matter clusters. The parametric model showed that first-degree relatives had intermediate values. Full sample of patients with NT1 showed no significant associations with disease duration or CSF hypocretin-1.

### Conclusions

Our study suggests widespread abnormal white matter involvement far beyond the already known focal hypothalamic pathology in NT1, possibly reflecting the combined effects of the loss of the widely projecting hypothalamic hypocretin neurons, and/or secondary effects of wake/sleep dysregulation. These findings demonstrate the importance of white matter pathology in NT1.

## Statement of Significance

Although it is well established that narcolepsy type 1 (NT1) is caused by a localized loss of the sleep–wake producing neurons in the hypothalamus, it has been unclear if and how this affects white matter connections. The present study reveals widespread bilateral white matter changes in H1N1-vaccinated patients with NT1 compared with first-degree relatives and healthy controls, suggesting white matter abnormalities in NT1. Furthermore, this is also the first study to explore if first-degree relatives represent an intermediate group in relation to white matter alterations between healthy controls and patients with NT1.

## Introduction

Narcolepsy type 1 (NT1) is a severe, chronic neurological sleep disorder characterized by unstable regulation of sleep/wakefulness and rapid eye movement (REM) sleep, leading to excessive daytime sleepiness/sleep attacks, fragmented night sleep, and early-occurring REM sleep as revealed by polysomnography and/or the multiple sleep latency test (MSLT) [1, 2]. Patients with NT1 have symptoms of REM sleep dysregulation in the form of cataplexy (muscle atonia triggered by emotions), hypnagogic hallucinations, sleep paralysis, and REM sleep without atonia or REM sleep behavior disorder [3]. NT1 is strongly associated with a specific loss of hypothalamic neurons, producing the hypocretin neuropeptides (also called orexins) [4, 5]. Hypocretin 1 and 2 (orexin-A and orexin-B) have been shown to be sleep–wake and tonus regulators [6], and they project widely throughout the brain [7–9].

After the H1N1 mass vaccinations with Pandemrix in 2009/2010, the incidence of narcolepsy increased greater than 10-fold in several European countries, including Norway [10]. Although the exact mechanisms for the loss of the hypocretin-producing neurons are unknown, mounting evidence points to autoimmunity as the explanation [2]. The immune-related *HLA-DQB1\*06:02* allele is carried by 98%–100% of hypocretin-deficient sporadic patients with NT1 [11] and has also been found in 92%–100% of H1N1-vaccinated patients with NT1 [10, 12–14].

It has been speculated that H1N1-vaccinated narcolepsy could be a more severe phenotype than sporadic narcolepsy, and although few differences have been found, an abrupt onset of symptoms [10, 12], a higher frequency of sleep-onset REM periods [14], more frequently disturbed nocturnal sleep, and shorter mean sleep latency [13] have been reported in H1N1-vaccinated narcolepsy.

Moreover, protective human leukocyte antigen (HLA) types have been detected in healthy *HLA-DQB1\*06:02*-carriers [15, 16]. This remarkably strong HLA-allele association implies that CD4<sup>+</sup> T cells play an important role in the development of NT1 [17]. How the immune system is potentially triggered is unknown, but infections and H1N1-vaccinations have both been suggested [2]. Several studies have searched for specific autoantibodies or immune cells but, with a few exceptions, they have been unsuccessful, and there is still no clear evidence of a role of autoantibodies in the development of NT1 [2]. Consequently, it has been suggested that hypocretin neuron destruction may not be a specific immune-mediated destruction, but rather that the hypocretin-producing cells are particularly vulnerable to a more systemic (auto)immune/inflammatory challenge [18]. Reports of increased numbers of histamine neurons might reflect compensatory processes, but it could also be present before the loss of hypocretin-producing cells, as increased levels of histamine have been implicated in the death of neurons in Parkinson disease [19, 20]. The increase of histamine neurons further suggests the possible involvement of additional brain areas/structures in the pathogenesis of NT1.

In the Norwegian population, the prevalence of *HLA-DQB1\*06:02* is high (28%–33%) and 45%–50% of the population were H1N1-vaccinated, which could be a factor contributing to the high narcolepsy rates after H1N1-vaccinations in Norway [10, 21, 22]. As HLA-types are inherited dominantly, *HLA-DQB1\*06:02*-positivity will be present in at least 50 per cent of first-degree relatives of Norwegian H1N1-vaccinated patients with NT1, approximately 50 per cent of whom will also be H1N1-vaccinated with Pandemrix [10]. It has been reported that first-degree relatives of patients with sporadic (nonvaccinated) narcolepsy have a 10–40 times greater risk of developing narcolepsy [23]. In an Asian study, 12.3 per cent of first-degree relatives of patients with sporadic narcolepsy were found to have narcolepsy, and 39.5 per cent were classified as “narcolepsy spectrum” reflected by abnormal MSLT results of sleep-onset REM or a mean sleep latency of ≤8 min, thus only partially fulfilling the criteria for narcolepsy on MSLT [24]. Similarly, 11 per cent of Italian patients with sporadic narcolepsy had first-degree relatives with narcolepsy symptoms [25]. It is therefore probable that first-degree relatives represent a group vulnerable to developing narcolepsy and associated sleep-related symptoms after H1N1-vaccination.

Diffusion tensor imaging (DTI) is an magnetic resonance imaging (MRI)-based method that is sensitive to the magnitude and direction of water diffusion in brain tissue and has been widely applied to characterize white matter microstructural properties in several brain diseases [26–28]. Conventional DTI indices include fractional anisotropy (FA), reflecting the degree of anisotropic or directional diffusion, and mean diffusivity (MD),

representing the average diffusion regardless of direction. Radial diffusivity (RD) and axial diffusivity (AD) reflect the degree of diffusion perpendicular and parallel to the primary diffusion direction, respectively [29, 30]. Although neurobiological interpretations should be made with caution [29, 31–35], FA has been used extensively as a proxy for white matter coherence and integrity, and its sensitivity to myelination in major white matter tracts with coherent fiber orientation and low fiber dispersion was recently demonstrated in mice using the CLARITY technique [36]. FA alterations have been reported across a range of central nervous system (CNS) disorders and conditions, including schizophrenia [27], dementia [26], and normal brain development and aging [37]. AD and RD represent different components of the diffusion tensor; decreases in AD have been associated with axonal damage, and increases in RD have been linked to myelin damage [29, 31–34].

Although NT1 is strongly associated with a loss of hypothalamic hypocretin-producing neurons, subsequent interactions with other brain regions and their connections might be factors modulating individual risk, clinical expression, and development/maintenance of disease. Four previous DTI studies of sporadic narcolepsy have yielded conflicting results [38–41], probably primarily due to the small sample sizes ( $n = 8–22$  patients with NT1), heterogeneous clinical patient characterizations, and different DTI methods employed. No DTI studies have previously been performed in H1N1-vaccinated patients with NT1 and first-degree relatives.

To test the hypothesis that the risk of developing NT1 is accompanied by white matter abnormalities beyond primary hypothalamic hypocretin neuron loss, we collected DTI data from 57 patients with NT1 (39 females, mean age 21.8 years, 57/57 *HLA-DQB1\*06:02*-positive, 51/57 H1N1-vaccinated, and 54/54 hypocretin deficient (three with unknown hypocretin status), and 55 healthy controls (38 females, mean age 22.3 years). To differentiate between genetic susceptibility and the effect of narcolepsy disease, we also investigated 54 first-degree relatives (29 females, mean age 19.1 years, 32/54 *HLA-DQB1\*0602*-positive, 37/54 H1N1-vaccinated).

We employed tract-based spatial statistics (TBSS) to compare FA, MD, RD, and AD between groups, and tested the hypothesis that the potential increased risk in the first-degree relatives is accompanied by similar yet attenuated white matter abnormalities by testing for associations with a parametric group model in which the first-degree relatives are a group intermediate between the patients and the healthy controls. We tested for associations with disease duration and cerebrospinal fluid (CSF) hypocretin-1 in patients with NT1, and we also explored the effects of *HLA-DQB1\*06:02*-status and H1N1-vaccination in first-degree relatives. To control for family-wise error and to avoid arbitrary cluster-forming thresholds, we used permutation testing and threshold-free cluster enhancement (TFCE) for inference.

## Materials and Methods

### Participants

Table 1 summarizes the demographic and clinical information of the groups. Briefly, 57 patients with NT1 who reported disease onset after the H1N1-vaccinations in 2009/2010, and 54 first-degree relatives, who were referred to the Norwegian Centre of Expertise for Neurodevelopmental Disorders and Hypersomnias (NevSom) for narcolepsy disease and family education/counseling courses, were consecutively included from June 2015 to April 2017. Not all first-degree relatives of narcolepsy type 1 patients were first-degree relatives of patients included in this study, as sometimes patients were excluded, but all these excluded patients had a verified narcolepsy type 1 diagnosis. After thorough evaluation of the patient’s medical history and records, the disease onset was changed for three patients with NT1 prior to the H1N1-vaccinations (all three had typical NT1 phenotypes and were hypocretin-deficient, and *HLA-DQB1\*06:02*-positive with cataplexy; for this reason, they were kept in the study). H1N1-vaccination in Norway involved the use solely of the vaccine Pandemrix. H1N1-vaccination status of patients and first-degree relatives was obtained from the official Norwegian Immunisation Registry (SYSVAK). Two patients who reported having been H1N1-vaccinated in their workplace without being registered in the SYSVAK were also included in the H1N1-vaccinated group. CSF hypocretin-1 measures were available in 53/54 hypocretin-deficient patients. Thirty-three patients with NT1 had CSF hypocretin-1 less than 40, and 20 patients with NT1 had CSF hypocretin-1 values between 40 and 120.

**Table 1.**  
Demographic and clinical data

	Narcolepsy type 1 ( <i>n</i> = 57)	First-degree relatives ( <i>n</i> = 54)	Healthy controls ( <i>n</i> = 55)
Gender (female), <i>n</i> (%)	39 (68.4)	29 (53.7)	38 (69.1)
Age (years), mean ± <i>SD</i>	21.8 ± 11.0	19.1 ± 8.3	22.3 ± 9.9
Age at disease onset (years), mean ± <i>SD</i>	16.1 ± 10.6	N/A	N/A
Disease duration (years), mean ± <i>SD</i>	5.7 ± 1.5	N/A	N/A
H1N1-vaccinated, <i>n</i> (%)	51 (89.5)	37 (68.5)	N/A
Cataplexy, <i>n</i> (%)	55 (96.5)	8* (14.8)	N/A
<i>HLA-DQB1*06:02</i> -positivity, <i>n</i> (%)	57/57 (100)	32 (59.3)	N/A
CSF hypocretin-1 ≤ 1/3 of level in normal population	54 /57 (3/57-N/A)	N/A	N/A
Hypnagogic hallucinations, <i>n</i> (%)	50 (87.7)	11 (20.4)	N/A
Sleep paralysis, <i>n</i> (%)	39 (68.4)	9 (16.7)	N/A
H1N1-vaccination & <i>HLA-DQB1*06:02</i> -positivity, <i>n</i> (%)	51 (89.5)	22 (40.7)	N/A
H1N1-vaccination & <i>HLA-DQB1*06:02</i> -negativity, <i>n</i> (%)	0 (0)	15 (27.8)	N/A
Unvaccinated & <i>HLA-DQB1*06:02</i> -positivity, <i>n</i> (%)	6 (10.5)	10 (18.5)	N/A
Unvaccinated & <i>HLA-DQB1*06:02</i> -negativity, <i>n</i> (%)	0 (0)	7 (13.0)	N/A

Based on additional health record information after inclusion, the disease onset was changed for three patients with NT1 to being prior to the H1N1-vaccinations (all three had typical NT1 phenotypes, and were hypocretin-deficient, and *HLA-DQB1\*06:02*-positive with cataplexy; for this reason they were kept in the study). \*All first-degree relatives with signs of cataplexy experienced it seldom, but with triggers known to elicit cataplexy, laughter, fun/excitement and surprise.

N/A = not available; SD = standard deviation.

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Patients were taken off all narcolepsy medication for 14 days before MRI-scanning, except for two patients with NT1 who, due to severe cataplexy, were without narcolepsy medication for only 7 days. Exclusion criteria for patients with NT1 and first-degree relatives were as follows: severe neurological, psychiatric, or somatic disorders; previous head injury with loss of consciousness for 10 or 30 min amnesia; metallic implants; neuroradiological findings requiring clinical follow-up; and excessive movement during the MRI-scanning. NT1 is associated with an increased number of comorbidities [2]. We included patients with the following comorbidities: Asperger syndrome (*n* = 1), attention-deficit hyperactivity disorder (ADHD; *n* = 1), migraine (*n* = 6), Tourette syndrome (*n* = 1), anxiety (*n* = 1), depression (*n* = 1), prematurity without severe long-term complications (*n* = 1), kidney disease (*n* = 2), type 2 diabetes (*n* = 1), and hypothyroidism (*n* = 2). In total, there were 15 patients with comorbidity (some patients had more than one comorbidity). We also included the following cases in the first-degree relatives group: Asperger syndrome (*n* = 1), attention-deficit disorder (ADD, *n* = 2), migraine (*n* = 6), dyslexia (*n* = 4), anxiety (*n* = 1), prematurity without severe long-term complications (*n* = 2), kidney disease (*n* = 1), previous encephalitis without long-term complications (*n* = 1), and bipolar type 2 disorder (*n* = 1). In total, there were 16 first-degree relatives with comorbidity (some first-degree relatives had more than one comorbidity).

All patients and 59.3 per cent of the first-degree relatives were *HLA-DQB1\*06:02*- positive. All patients with 54/54 NT1 with a measured hypocretin level were hypocretin-deficient (CSF hypocretin-1 ≤ 1/3 of level in normal population [same as the definition in Table 1]); this measurement had not yet been made in three patients. 89.5 per cent of all patients and 68.5 per cent of first-degree relatives were H1N1-vaccinated. 96.5 per cent of patients with NT1 had cataplexy. 14.8 per cent of first-degree relatives had signs of cataplexy, although they seldom experienced it,

they had triggers known to elicit cataplexy: laughter, fun/excitement and surprise. 87.7 per cent of all patients reported hypnagogic hallucinations and 68.4 per cent experienced sleep paralysis. 20.4 per cent of first-degree relatives also experienced hypnagogic hallucinations and 16.7 per cent had experienced sleep paralysis.

Data from 55 healthy controls matched for age and sex were included from ongoing large-scale studies coordinated by the Norwegian Centre for Mental Disorders Research (NORMENT). Exclusion criteria were as follows: neurological, psychiatric, or somatic disease; severe psychiatric family history; previous head injury with loss of consciousness for 10 or 30 min amnesia; metallic implants; neuroradiological findings indicating ongoing or previous disease or abnormalities and excessive movement during MRI-scanning.

## Standard protocol approvals, registrations, and patient consents

The study was approved by the Norwegian regional committees for medical and health research ethics (REK), and all participants provided written informed consent prior to inclusion.

## Narcolepsy diagnosis

All NT1 diagnoses were made by the same experienced neurologist and sleep medicine expert (Stine Knudsen) according to International Classification of Sleep Disorders (ICSD)-3 criteria [1].

Patients and first-degree relatives underwent clinical consultations, semistructured interviews about narcolepsy and sleep disorders using a Norwegian translation of the validated Stanford Sleep Questionnaire [42], clinical examinations including a neurological examination, collection of routine blood samples, actigraphy, polysomnography, MSLT, and HLA-typing. Measurements of CSF hypocretin-1 levels in patients were also obtained using a slight modification to the method of Phoenix Pharmaceutical St. Joseph, MO, USA. Analysis was carried out in the Hormone Laboratory of Oslo University Hospital. All patients fulfilled the ICSD-3 criteria for narcolepsy after clinical evaluation, polysomnography, MSLT, and hypocretin measurement. No first-degree relatives fulfilled the ICSD-3 criteria for narcolepsy after clinical evaluation, polysomnography, and MSLT.

## Polysomnography recordings

All patients and first-degree relatives were evaluated by polysomnography and MSLT according to the International Classification of Sleep Disorders (ICSD)-3 criteria [1]. All polysomnography recordings were preceded by 10–14 days of actigraphy (Philips Actiwatch, Respironics Inc., Murrysville, PA, USA). The polysomnography recordings were made with the SOMNOmedics system (SOMNOmedics GmbH, Randersacker, Germany) using the electrodes F3-A2, C3-A2, O1-A2, F4-A1, C4-A1, and O2-A1, in addition to vertical and horizontal electro-oculography, surface electromyography (EMG) of the submental and tibialis anterior muscles, electrocardiography, nasal air flow, thoracic respiratory effort, and oxygen saturation. EMG impedance was kept below 10 k $\Omega$  (preferably below 5 k $\Omega$ ). Sleep scoring was conducted according to the American Academy of Sleep Medicine (AASM) criteria [1].

## MRI acquisition and analysis

Imaging was conducted with a General Electric Discovery MR750 3T scanner at Oslo University Hospital using a 32-channel head coil. A 2D spin-echo whole-brain echo planar imaging pulse was used with 60 spatially independent diffusion-sensitized gradient directions: repetition time (TR): 8150 ms; echo time (TE): 83.1 ms; b-value: 1000 s/mm<sup>2</sup>; field of view (FOV) 256 × 256 mm<sup>2</sup>; flip angle: 90°; slice thickness: 2 mm; axial slices: 67; and acquisition matrix: 128 × 128. The acquisition time was 8 min and 58 s.

MRI data were processed and analyzed using the Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL) (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki>) [43, 44]. Preprocessing included topup (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/topup>) to correct for susceptibility-induced distortions and eddy (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/eddy>) to correct for subject movements and eddy currents, and replacing slices with signal loss [45], which has been shown to increase the signal-to-noise ratio [26]. Voxel-wise eigenvalues and eigenvectors and FA, MD, AD, and RD maps were computed using dtifit.

We used TBSS for voxel-wise between-subject analyses [46]. FA volumes were aligned to FMRIB58\_FA standard space image by FMRIB's Non-linear Image Registration Tool (FNIRT) [47, 48]. The mean FA skeleton representing the center of tracts common across subjects was thresholded and binarized at FA > 0.2 and applied to the individual FA data. A similar procedure was used for MD, AD, and RD. We calculated mean skeleton values in FA, MD, AD, and RD for the different groups to identify global effects.

# Statistical analysis

We tested for group differences using a general linear model including age and sex in two different analyses (including/excluding the patients and first-degree relatives with comorbidity). We further tested for parametric group differences using a general linear model with one variable coding each subject as 1 (patient), 2 (first-degree relative), or 3 (control), in addition to their age and sex.

In a subanalysis for the patient group (with/without comorbidity), we included CSF hypocretin-1 (continuous and divided into two groups; undetectable  $\leq 40$  and low = 40–120) and disease duration as well as age and sex. In a separate subanalysis, we included *HLA-DQB1\*06:02*-status and H1N1-vaccination status only within the first-degree relative group. Since all patients with narcolepsy were *HLA-DQB1\*06:02*-positive and only six were unvaccinated, and because we had no information about *HLA-DQB1\*06:02*-status or H1N1 vaccination in healthy controls, respectively, none of them were included in the subanalysis.

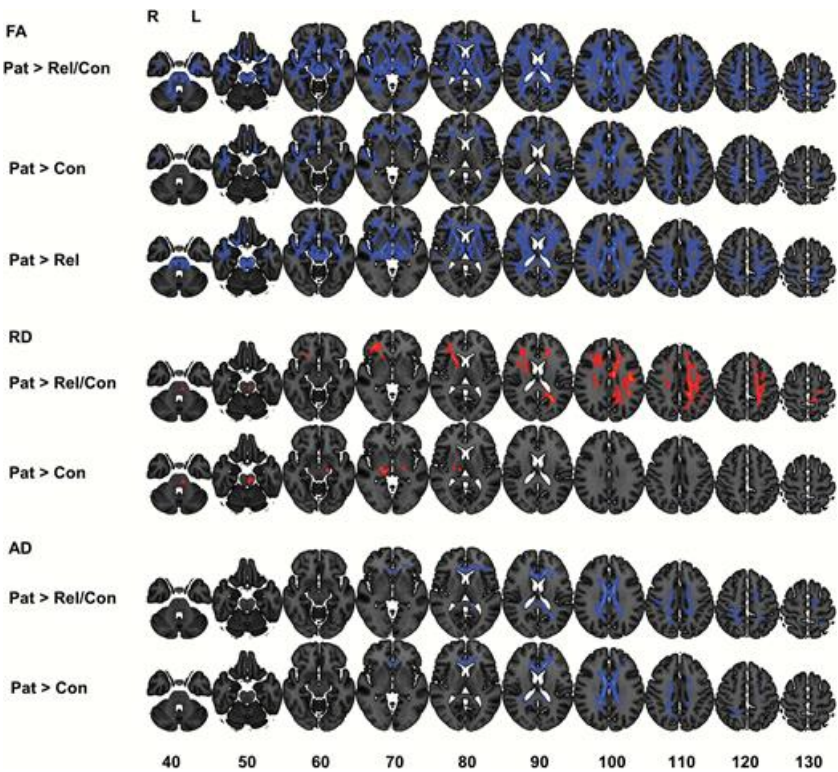
We corrected for multiple testing by running 5000 permutations and TFCE as implemented in Permutation Analysis of Linear Models (PALM) [49, 50]. To control for lack of independence (patients and first-degree relatives from the same family, two patients were related, and siblings within the first-degree relatives group), permutations were constrained between first-degree relatives. Corrected two-tailed values of  $p < 0.05$  were considered statistically significant.

# Results

## Main effects of group on DTI indices

Figure 1 and Table 2 summarize the results of the voxel-wise analysis. Compared with first-degree relatives and healthy controls, patients with NT1 showed significantly lower FA in widespread regions of the brain, comprising 34.2 per cent (53580/156585) of all voxels in the skeleton. The highest proportion of significant voxels was found in the corpus callosum, cingulate gyrus, and uncinate fasciculus, corticospinal tract; the highest absolute number of voxels was found in the superior longitudinal fasciculus and the inferior fronto-occipital fasciculus. There were also significant group differences in subcortical structures like the thalamus, amygdala, and brainstem. Patients showed significantly lower AD in several white matter tracts, including the corpus callosum, superior longitudinal fasciculus and the cingulate gyrus, and higher RD in the corpus callosum, the cingulate gyrus, and several other white matter tracts compared with first-degree relatives and healthy controls. However, there were no significant differences in RD or AD when comparing patients with NT1 with first-degree relatives exclusively.

Figure 1.



Voxel-wise analysis. Patients with NT1 show significantly lower FA and AD, and significantly higher RD when compared with first-degree relatives and healthy controls. There were no significant differences in RD or AD when comparing patients with NT1 with first-degree relatives exclusively. Numbers reflect the z-coordinate in MNI 1-mm space. Only voxels with two-tailed values of  $p < 0.05$ , corrected for multiple comparisons using permutation testing and TFCE, are shown. Pat = patients with NT1; Rel = first-degree relatives; Con = healthy controls. R = right; L = left.

**Table 2.**

Number and proportion of significant voxels in major white matter tracts

White matter tracts	FA	RD	AD
BCC	2045/3183 (64.2)	500/3183 (15.7)	2100/3183 (66.0)
CGL	1724/2767 (62.3)	962/2767 (34.8)	385/2767 (13.9)
UFR	757/1284 (59.0)	34/1284 (2.6)	0/1284(0.0)
GCC	957/1810 (52.9)	51/1810 (2.8)	832/1810 (46.0)
CSTR	2765/5772 (47.9)	165/5772 (2.9)	204/5772 (3.5)
SCC	1216/2573 (47.3)	207/2573 (8.0)	680/2573 (26.4)
CSTL	2518/5536 (45.5)	832/5536 (15.0)	65/5536 (1.2)
UFL	743/1642 (45.2)	19/1642 (1.2)	33/1642 (2.0)
ATRR	3146/6983 (45.1)	645/6983 (9.2)	184/6983(2.6)
IFOFL	2444/5575 (43.8)	103/5575 (1.8)	280/5575 (5.0)
IFOFR	3324/8232 (40.4)	740/8232 (9.0)	89/8232 (1.1)
ATRL	3064/8086 (37.9)	255/8086 (3.2)	292/8086 (3.6)
CGR	682/1812 (37.6)	20/1812 (1.1)	202/1812 (11.1)
FMIN	2608/6951 (37.5)	263/6951 (3.8)	986/6951 (14.2)
SLFL	4062/11288 (36.0)	1299/11288 (11.5)	140/11288 (1.2)
SLFR	3571/10519 (33.9)	126/10519 (1.2)	273/10519 (2.6)
ILFR	1955/5861 (33.4)	0/5861 (0.0)	10/5861 (0.2)
CINGL	441/1406 (31.4)	12/1406 (0.9)	7/1406 (0.5)
ILFL	1849/6337 (29.2)	45/6337 (0.7)	15/6337 (0.2)
SLFTL	53/190 (27.9)	5/190 (2.6)	0/190 (0.0)
CINGR	349/1296 (26.9)	0/1296 (0.0)	34/1296 (2.6)
FMAJ	1548/6293 (24.6)	181/6293 (2.9)	285/6293 (4.5)
SLFTR	126/787 (16.0)	7/787(0.9)	11/787(1.4)

The groups have been compared; FA and AD are significantly lower in patients with NT1 compared with first-degree relatives and healthy controls, whereas RD is significantly higher in patients with NT1 compared with first-degree relatives and healthy controls. There were no significant differences in RD or AD when comparing patients with NT1 with first-degree relatives exclusively. All anatomical regions are based on the Johns Hopkins University (JHU) white matter tractography atlas, except for the corpus callosum, which is based on the ICBM-DTI-81 white matter labels atlas [63–65]. The tractography atlas is probabilistic, but was thresholded to be nonoverlapping so that no voxel would be counted in more than one tract.

R = right; L = left; BCC = body of corpus callosum; GCC = genu of corpus callosum; SCC = splenium of corpus callosum; SLF = superior longitudinal fasciculus; SLFT = superior longitudinal fasciculus (temporal part); ILF = inferior longitudinal fasciculus; ATR = anterior thalamic radiation; IFOF = inferior fronto-occipital fasciculus; UF = uncinate fasciculus; CST = corticospinal tract; Fmin = forceps minor; Fmaj = forceps major; ILF = inferior longitudinal fasciculus; CG = cingulum (cingulate gyrus); UF = uncinate fasciculus; CING = cingulum (hippocampus).

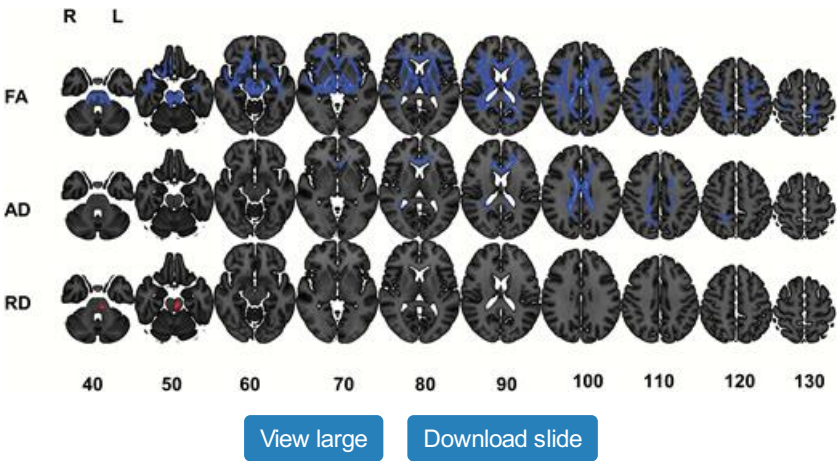
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We also performed the voxel-wise analysis excluding the previously included patients with NT1 and first-degree relatives with comorbidity. The results were significant and similar when comparing the patients with NT1 to the healthy controls. Similar significant differences were also found when comparing patients with NT1 to both first-degree relatives and healthy controls in FA and AD, but not for RD.

When comparing patients with NT1 exclusively to the first-degree relatives, the finding of lower FA in patients with NT1 compared with first-degree relatives was no longer significant. To assess the similarities in the test statistics from the analyses using the full and reduced sample, respectively, we computed the spatial correlation between the t-statistics-map ([Supplementary Figure 1](#)) from the new analysis without comorbidity and the t-statistics-map from the old analysis including comorbidity. The results revealed a spatial correlation 0.8, suggesting a highly similar pattern and direction of effects. Permutation testing revealed no significant differences between first-degree relatives (as a whole group, not divided by HLA-DQB1\*06:02-status) and healthy controls in the voxel-wise analysis that tested for differences specifically between these two groups.

[Figure 2](#) shows the results from the parametric group model. Briefly, in line with the observations reported above, the results revealed a widespread graded pattern suggesting lowest FA and AD values in patients with NT1 and highest values in controls, those of the first-degree relatives being intermediate. All voxels showing this parametric effect of FA overlapped with the significant voxels identified when comparing patients with NT1 with first-degree relatives and healthy controls with 63.4 per cent (33954/53580) voxel overlap. Five thousand one hundred three voxels showed a parametric effect of AD, with 59.1 per cent (4074/6897) voxel overlap with the significant voxels identified when comparing patients with NT1 with first-degree relatives and healthy controls. Eighty-seven voxels showed a parametric effect for RD, with 0.8 per cent (66/7957) voxel overlap with the significant voxels identified when comparing patients with NT1 with first-degree relatives and healthy controls.

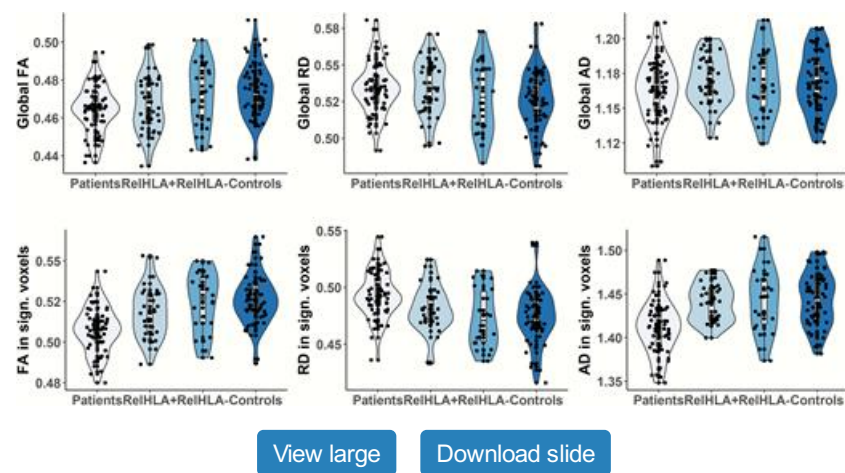
**Figure 2.**



Parametric effects in voxel-wise analysis. Parametric effects suggesting that FA and AD values are lowest in patients with NT1, highest in controls, and intermediate in first-degree relatives. RD parametric effects suggest highest RD values in patients with NT1, lowest in controls, with intermediate values in first-degree relatives. Numbers reflect the z-coordinate in MNI 1 mm space. Only voxels with two-tailed values of  $p < 0.05$ , corrected for multiple comparisons using permutation testing and TFCE, are shown. R = right; L = left.

[Figure 3](#) and [Supplementary Table 1](#) summarize the distributions of DTI values across the groups, measures, and regions (global and within-voxel values identified in the voxel-wise analysis). For global FA, we observed a gradual increase in mean FA from patients with NT1 ( $0.465 \pm 0.012$ ), first-degree relatives *HLA-DQB1*\*06:02-positive ( $0.467 \pm 0.015$ ), first-degree relatives *HLA-DQB1*\*06:02-negative ( $0.473 \pm 0.017$ ), and healthy controls ( $0.474 \pm 0.014$ ), and a similar pattern within the significant voxels. Similar parametric increases in RD and decreases in AD were also observed.

**Figure 3.**



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Distributions of mean DTI values within groups and regions. The violin plots show the distributions for each group (NT1, first-degree relatives divided by *HLA-DQB1\*06:02*-status, and healthy controls) for mean FA, mean  $RD \times 10^3$ , and mean  $AD \times 10^3$  across the brain and within significant voxels identified by permutation testing (shown in [Figure 1](#)).

Voxel-wise analysis performed only within the first-degree relative group ( $n = 54$ ) revealed trends, but no significant differences in DTI indices between *HLA-DQB1\*06:02*-positive ( $n = 32$ ) and *HLA-DQB1\*06:02*-negative ( $n = 22$ ) first-degree relatives. Similarly, we found trends, but no significant differences for H1N1-vaccinated first-degree relatives ( $n = 37$ ) divided by *HLA-DQB1\*06:02*-positivity ( $n = 22$ ) and *HLA-DQB1\*06:02*-negativity ( $n = 15$ ).

Voxel-wise analysis within the NT1 patient group with available CSF hypocretin-1 values ( $n=53$ ) revealed no significant associations between DTI indices and CSF hypocretin-1 value (continuous or divided into two groups), neither in the full nor reduced sample. Also, we found no significant associations between disease duration and any DTI measure in the full sample. The analyses revealed a small cluster of voxels showing significant associations between disease duration and RD and another small cluster of voxels showing significant associations between disease duration and MD, for the reduced sample ([Supplementary Figure 2](#)), indicating lower RD/MD with longer disease duration.

## Discussion

This is the first DTI study of H1N1-vaccinated patients with NT1 and first-degree relatives, and the largest DTI study performed in NT1. The main findings are the significant and widespread bilateral patterns of lower FA in patients with NT1 compared with first-degree relatives and healthy controls, involving several major white matter tracts and brain areas. FA reflects the degree of anisotropic or directional diffusion, and MD, representing the average diffusion regardless of direction. RD and AD reflect the degree of diffusion perpendicular and parallel to the primary diffusion direction, respectively [29, 30]. Although the neurobiological interpretation of DTI measures should be made with caution (as they are indirect MRI measures), FA is typically used as a proxy for white matter integrity, microstructural organization, and myelination [36] and is often found to be lower across a range of CNS conditions and disorders including schizophrenia [27] and dementia [26]. AD and RD represent different components of the diffusion tensor, and AD decreases have previously been associated with axonal damage and RD increases with myelin damage [29, 32–34]. Our findings of widespread FA decreases can therefore possibly be explained by microstructural differences related to both the loss of axonal and myelin integrity in the white matter tracts of patients with NT1, extending far beyond the previously described specific hypothalamic involvement in NT1. We have not focused on the hypothalamus as TBSS is not an optimal technique for this type of investigation since it is based on a mean FA skeleton thresholded to represent white matter tracts common to all subjects (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/TBSS>).

Four previous DTI studies of patients with sporadic narcolepsy have reported conflicting findings. The first study [38] found lower FA in the midbrain, hypothalamus, and medulla oblongata, and higher FA in the midbrain and pons in eight medicated patients with NT1 compared with 12 controls. The authors speculated that the findings of higher FA could represent compensatory changes. However, the study was of a small, heterogeneous patient group with unknown hypocretin status, in which disease duration varied from 4 to 53 years and also included two *HLA DQB1\*0602*-negative patients, which are quite rare in NT1 [11]. The second study reported lower FA and higher MD in the fronto-orbital cortex and the anterior cingulate in 16 patients with NT1 (10 medicated) compared with 12 controls [39]. Higher MD was also found in the ventral tegmental area, the dorsal raphe nuclei, and the hypothalamus and lower FA in white matter tracts of the inferior frontal and inferior temporal cortices. However, this study was also of a heterogeneous patient group with unknown hypocretin status, and a disease duration of 6 to 59 years. A third study reported a higher apparent diffusion coefficient in the left inferior frontal gyrus and left amygdala, and a lower apparent diffusion coefficient in the left postcentral gyrus in 12

drug-naïve patients with narcolepsy with cataplexy (but with unknown hypocretin status) compared with 12 healthy controls [40]. No significant differences were found between 12 drug-naïve patients with narcolepsy but without cataplexy and the same control group. The fourth study reported lower FA in the bilateral anterior cingulate, fronto-orbital area, frontal lobe, anterior limb of the internal capsule, corpus callosum and the thalamus in 22 drug-naïve patients with NT1 with unknown hypocretin status compared with 26 healthy controls [41].

Our findings, although far more extensive, are in best alignment with those of the study by Park et al., which featured the largest sample size of the four previous studies, suggesting that small sample sizes may have been partially responsible for the previous conflicting DTI results. Since our study is the largest DTI study of NT1, the more global, extensive, bilateral microstructural white matter changes might simply be due to increased power. Moreover, as the onset of disease in most H1N1-vaccinated patients with NT1 occurred close to the time of vaccination, our patient group is very homogeneous, with very similar and relatively short disease duration. The previous studies had mean disease durations of 30.6 years [39], 21.8 years [38], 11.3 years [40], and 11.0 years [41], whereas the mean disease duration was only of 5.7 years in our study. In contrast to previous studies, we also confirmed diagnoses with both hypocretin deficiency and HLA-typing in almost all patients, so we are confident that the sample represents a “hypocretin-deficient” phenotype relatively close to disease onset possibly with ongoing disease activity, not—as was the case in previous studies—a potentially mixed group of the clinically characterized cataplectic phenotype, of which approximately 10 per cent have normal hypocretin levels [11], scanned in a probably more chronic disease stage up to three decades after disease onset.

However, since 89.5 per cent of patients in our study were H1N1-vaccinated, it is also possible that our findings reflect a more full-blown variant of NT1 than that of the exclusively sporadic narcolepsy cohorts examined in previous studies. Although few differences have been found between H1N1-vaccinated narcolepsy and sporadic narcolepsy, there have been reports of higher frequency of sleep-onset REM periods [14], more frequently disturbed nocturnal sleep, shorter mean sleep latency [13], and an abrupt onset of symptoms [10, 12] in H1N1-vaccinated narcolepsy. Furthermore, a GWAS [51] prior and post H1N1 influenza pandemic revealed substantial overlapping associations, but also novel genetic associations in post H1N1 influenza narcolepsy. A different response to early immunomodulation was also seen in case reports of sporadic [52] and H1N1-vaccination narcolepsy [53]. Jointly, these observations support the need for future DTI studies with larger samples of both sporadic narcolepsy and H1N1-vaccinated narcolepsy.

The origin of the observed white matter tracts’ abnormalities in the patients with NT1 remains unknown. We may speculate that the effects probably reflect a combination of (1) a predisposition present prior to the destruction of the hypocretin-producing neurons, (2) initial damage to white matter tracts occurring together with or as a downstream consequence of, the destruction of the hypocretin-producing neurons, and (3) a result of short- or long-term consequences of a secondarily abnormal sleeping pattern [54–56]. The extensive and bilateral white matter changes might be a direct downstream consequence of the loss of the hypocretin-producing neurons, since their projections are widely distributed in the CNS [7–9]. Despite mounting evidence of the existence of immune mechanisms in NT1, no clear evidence has been found of specific autoantibodies or immune cells targeted against specific antigens that are present only on hypocretin neurons. Several studies have found antibodies against tribbles homologue 2 (TRIB2), a protein produced by the hypocretin-producing cells, but also found in other neuron types [2]. Hypocretin-producing cells have also been reported to be particularly vulnerable to systemic inflammatory challenges [18]. In the case of the initial processes leading to the destruction of the hypocretin cells not being specific, the observed white matter changes in our study might reflect part of the initial widespread damage in the brain. Reports of hypothalamic gliosis in NT1 provide no consensus, since two studies detected gliosis [5, 57], whereas a third was unable to detect it [4].

Our findings are in line with the hypothesis that widespread white matter changes in NT1 might be a consequence of the loss of the hypocretin-neurons/brain projections or, alternatively, an effect of the instability on maintaining sleep (sleep fragmentation/sleep loss) in narcolepsy. It has been argued that narcolepsy is better characterized as a “dysomnia” than a hypersomnia, since there is no increase in total sleep duration, but rather an inability to maintain wakefulness or sleep for long periods of time [58]. Research suggests that sleep might play an important role in maintaining cell membranes and myelin [59–61], but how the particular abnormal sleeping pattern experienced by patients with NT1 affects the brain is unknown. Although our findings are far more extensive, a study of patients with insomnia reported some overlapping findings [56], in particular decreased FA in the right anterior limb of the internal capsule, right posterior limb of the internal capsule, right anterior corona radiata, right superior corona radiata, right superior longitudinal fasciculus, body of corpus callosum, and right thalamus. The decrease in FA was associated with increases in RD, which the authors took to indicate myelin loss.

We also explored the effect of excluding (15 patients with NT1 and 16 first-degree relatives) comorbidities in both the patients with NT1 and first-degree relatives. The results were significant and similar to the analysis including comorbidity when comparing NT1 with the first-degree and healthy controls, except for in RD. Similar and significant results were also found when comparing patients with NT1 with healthy controls. However, when comparing patients with NT1 with first-degree relatives, there was no longer a significant group difference in FA. This could be due to effects of comorbidity in the patients with NT1/ first-degree relatives or due to the loss of power. The t-statistics maps (Supplementary Figure 1) from the analysis with and without comorbidity showed a spatial correlation of 0.8, suggesting a highly similar pattern and direction of effects.

We found no significant associations between CSF hypocretin-1 (continuous or divided into two groups) and DTI indices in patients with NT1 (with/without comorbidity) with available CSF hypocretin-1 values ( $n = 53$ ). However, a limitation is that the CSF hypocretin-1 was not measured in the same time period as the MRI -scanning was performed, and all the samples were not analyzed with the same RIA-assay, though corrected by internal references as described previously [3, 10]. A floor effect must additionally be taken into consideration, as all patients were hypocretin-deficient (all with low/undetectable CSF hypocretin-1). Furthermore, CSF hypocretin-1 were not available in first-degree relatives or the healthy controls.

We found no significant associations between DTI parameters and disease duration in the full sample of patients with NT1 (with/without comorbidity) with available hypocretin values ( $n = 53$ ), but a few clusters revealed lower RD and MD with longer disease duration in the reduced sample (Supplementary Figure 2).

Another matter of concern is whether predisposed (*HLA-DQB1\*0602*-positive, H1N1-vaccinated) first-degree relatives of patients with NT1 represent a particular risk group. Previous studies carried out on first-degree relatives to patients with sporadic narcolepsy found that first-degree relatives have an increased risk of sporadic narcolepsy and a “narcolepsy spectrum” disorder [23–25]. We found that 14.8 per cent of first-degree relatives had signs of cataplexy elicited by triggers known to elicit cataplexy: laughter, fun/excitement, and surprise, although seldom experienced. Our study revealed a higher percentage of first-degree relatives with sleep paralysis and hypnagogic hallucinations compared with a previous study of healthy participants [62], the higher frequency of sleep paralysis is similar to that noted in another study including first-degree relatives [24].

Regarding DTI findings, in addition to DTI-based white matter microstructural changes in patients with NT1 compared with healthy controls, we provide evidence of a graded pattern of white matter changes in which first-degree relatives (including when divided by *HLA-DQB1\*06:02*-status) present with values intermediate between the DTI values/findings of patients and healthy controls. However, we must emphasize that voxel-wise analysis revealed no significant differences between first-degree relatives (as a whole group) and healthy controls. The sample size did not allow us to stratify first-degree relatives according to *HLA-DQB1\*06:02*-status and H1N1-vaccination status, which are highly relevant measures for risk assessment. This should be explored in future studies. Information about *HLA-DQB1\*06:02*-status and H1N1-vaccination status was not available for the healthy controls, so an analysis for these factors was performed only within the first-degree relative group, which revealed trends, but no significant results were found. Due to small sample sizes and insufficient matching of the age and sex of the group members, we could not perform the combined subanalysis of *HLA-DQB1\*06:02*-positivity  $\pm$  H1N1-vaccination in first-degree relatives. Future large-scale studies are needed to explore associations between sleep-related phenotypes, HLA subtypes, H1N1-vaccination, and immune mechanisms in first-degree relatives and healthy controls, and their joint associations with brain-imaging indices.

In conclusion, we find evidence of widespread white matter brain structure involvement beyond the previously described specific hypothalamic involvement in NT1. The reported white matter changes possibly reflecting a distributed pattern of microstructural alterations, including myelin processes and the integrity and density of axonal membrane in NT1. The extensive findings in our H1N1-vaccinated patient with NT1 cohort partly contrast with previous, smaller studies of sporadic narcolepsy, pointing to either a more full-blown H1N1-vaccination narcolepsy disease variant or greater analytical power. We speculate that the detected widespread white matter changes might be a consequence of the loss of the hypocretin neurons/brain projections, or alternatively an effect of the brain’s instability in maintaining sleep (sleep fragmentation/sleep loss) in narcolepsy. Interestingly, parametric effects of groups indicate that first-degree relatives could be an intermediate group with respect to white matter abnormalities, although no significant differences were found when directly comparing healthy controls and first-degree relatives (not divided by *HLA-DQB1\*06:02*-status). This needs to be explored further in a larger sample of first-degree relatives and extended by analyses of healthy controls with known HLA and H1N1-vaccination status.

## Supplementary material

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Supplementary material is available at *SLEEP* online.

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