

## ORIGINAL ARTICLE

# High nocturnal sleep fragmentation is associated with low T lymphocyte P2Y<sub>11</sub> protein levels in narcolepsy type 1

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## Abstract

**Study Objectives:** Narcolepsy type 1 (NT1) is associated with hypocretin neuron loss. However, there are still unexplained phenotypic NT1 features. We investigated the associations between clinical and sleep phenotypic characteristics, the NT1-associated P2RY11 polymorphism rs2305795, and P2Y<sub>11</sub> protein levels in T lymphocytes in patients with NT1, their first-degree relatives and unrelated controls.

**Methods:** The P2RY11 SNP was genotyped in 100 patients (90/100 H1N1-(Pandemrix)-vaccinated), 119 related and 123 non-related controls. CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels were quantified using flow cytometry in 167 patients and relatives. Symptoms and sleep recording parameters were also collected.

**Results:** We found an association between NT1 and the rs2305795 A allele (OR = 2, 95% CI (1.3, 3.0),  $p = 0.001$ ). T lymphocyte P2Y<sub>11</sub> protein levels were significantly lower in patients and relatives homozygous for the rs2305795 risk A allele (CD4:  $p = 0.012$ ; CD8:  $p = 0.007$ ). The nocturnal sleep fragmentation index was significantly negatively correlated with patients' P2Y<sub>11</sub> protein levels (CD4:  $p = 0.004$ ; CD8:  $p = 0.006$ ). Mean MSLT sleep latency, REM-sleep latency, and core clinical symptoms were not associated with P2Y<sub>11</sub> protein levels.

**Conclusions:** We confirmed that the P2RY11 polymorphism rs2305795 is associated with NT1 also in a mainly H1N1-(Pandemrix)-vaccinated cohort. We demonstrated that homozygosity for the A risk allele is associated with lower P2Y<sub>11</sub> protein levels. A high level of nocturnal sleep fragmentation was associated with low P2Y<sub>11</sub> levels in patients. This suggests that P2Y<sub>11</sub> has a previously unknown function in sleep-wake stabilization that affects the severity of NT1.

## Statement of Significance

This is the first study to explore associations between clinical and sleep phenotype parameters, the P2RY11 polymorphism rs2305795 genotype, and P2Y<sub>11</sub> protein levels in a large mainly H1N1-(Pandemrix)-vaccinated cohort of NT1 patients and their first-degree relatives. We confirm that the P2RY11 A risk allele predisposes towards NT1 in our cohort. We demonstrate that P2RY11 A homozygote individuals have low T lymphocyte P2Y<sub>11</sub> protein levels. Moreover, specific to NT1, high nocturnal sleep fragmentation is associated with low P2Y<sub>11</sub> protein levels. Our results are the first demonstration of a genetically linked protein in NT1 that is also associated with phenotypic severity. This raises the possibility that P2Y<sub>11</sub> protein has a regulatory role in sleep-wake stability in NT1.

**Key words:** narcolepsy 1; P2RY11; P2Y<sub>11</sub>; sleep fragmentation

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## Introduction

Narcolepsy type 1 (NT1) is a relatively rare chronic neurological sleep disorder, characterized by excessive daytime sleepiness (EDS), early-occurring rapid eye movement (REM) sleep and REM sleep phenomena intruding into wakefulness, such as clinical symptoms like cataplexy (sudden loss of muscle tonus triggered by strong emotions), hypnagogic and hypnopompic hallucinations and sleep paralysis [1]. Fragmented nocturnal sleep (multiple awakenings and changes in sleep stages) is also very prevalent in patients with NT1 [1, 2]. The prevailing hypothesis about NT1 etiology is that there is an autoimmune process targeting the hypocretin (also called orexin) producing neurons causing the loss of approximately 90% of these neurons and, consequently, hypocretin deficiency with low cerebrospinal-fluid hypocretin-1 (CSF hcrt-1) levels [3, 4]. The main genetic risk factor is the human leukocyte antigen (HLA) allele DQB1\*06:02, which is present in 86%–98% of patients [5–7]. The DQB1\*06:02 allele is thought to be a necessary risk factor for NT1 to develop, but it is not sufficient, since 15%–33% of the general population also carries this allele [7–9].

Other HLA genes, but also non-HLA genes involved in immune system responses are associated with NT1, thereby supporting the autoimmune process [10–14]. Streptococcal or viral upper airway infections [15–18], including the 2009 Influenza A (H1N1) pandemic virus strain (A(H1N1)pdm09), are known as environmental triggers. In several European countries, including Norway, a 4.6–13-fold increased risk of NT1 followed the influenza A(H1N1)pdm09 vaccination campaign with Pandemrix [19, 20].

One of the consistently found non-HLA candidate genes is the purinergic receptor P2Y<sub>11</sub> gene (P2RY11). The single nucleotide polymorphism (SNP) rs2305795 in the 3'UTR of P2RY11 has been found to be associated with NT1 across ethnicities in studies conducted mainly in patients whose disease onset occurred before the advent of influenza A(H1N1)pdm09 (pre-H1N1 cohorts) or in (presumably) non-Pandemrix-vaccinated cohorts: Kornum et al. [14], a confirmed pre-H1N1 cohort; Tafti et al. [7], a mainly pre-H1N1 cohort, but in which the time of disease onset and vaccination status is unclear/unspecified in a subcohort in the study; Han et al. [12] and Han et al. [21], cohorts of mainly pre-H1N1 patients but including some post-H1N1 patients, only a few of whom were specified as post-H1N1 infected/vaccinated (but not Pandemrix-vaccinated) patients. The P2RY11 SNP rs2305795 risk allele A was borderline significant in a single, small post-H1N1 NT1 study by Bomfim et al. [22], including 42 H1N1-(Pandemrix)-vaccinated patients.

The gene product, that is, the P2Y<sub>11</sub> surface receptor protein, is moderately expressed in several human tissues and strongly expressed in lymphocytes and in the brain [14, 23–25]. Patients and unrelated controls carrying the NT1-risk-associated P2RY11 SNP rs2305795 A allele had a significantly lower level of CD8 T lymphocyte P2RY11 mRNA expression and significantly shorter cell survival for both CD4 and CD8 T lymphocytes after co-stimulation with ATP and the P2Y<sub>11</sub> agonist NF546 [14]. These findings suggest that P2Y<sub>11</sub> has an immunomodulating effect, although, since P2Y<sub>11</sub> is also highly expressed in brain tissue, the possibility that the effect of P2Y<sub>11</sub> on NT1 is not related to the immune system, but instead to an unknown function of P2Y<sub>11</sub> in the brain, cannot be ruled out. The exact function of P2Y<sub>11</sub> in the central nervous system is not yet known, partly because there is no homologue to the P2RY11 gene in rodents [24].

It is still an ongoing discussion whether NT1 with onset after the influenza A(H1N1)pdm09 and Pandemrix vaccination campaign (post-H1N1 NT1) is the same entity as pre-H1N1 NT1, but based on similar HLA predispositions most genetic evidences point towards it being the same disease. However, apart from the non-significant indications from the single small study by Bomfim et al. [22] it is currently not known whether post-H1N1 NT1 patients have a similar association with the non-HLA P2RY11 SNP rs2305795 risk allele as do pre-H1N1 NT1 patients [7, 12, 14, 21, 22]. Moreover, no previous study has explored the P2Y<sub>11</sub> protein levels in CD4 and CD8 T lymphocytes, clinical narcolepsy symptoms and sleep parameters in a large cohort of patients with NT1 and their first-degree relatives. Increased risk of narcolepsy in first-degree relatives has been reported in previous pre-H1N1 studies. One early review estimated that 1%–2% of first-degree relatives develop narcolepsy [26] and two subsequent studies identified symptoms sufficient to justify narcolepsy diagnoses (NT1 or narcolepsy type 2 [NT2]) in 10%–12% of the relatives of NT1 and NT2 patients [27, 28]. These two studies focused on clinical symptoms and sleep characteristics [27], and clinical symptoms in first-degree relatives [28], respectively. Wing et al. [27] described the presence of a “narcolepsy spectrum” (defined as mean multiple sleep latency test sleep latency [MSLT-SL] ≤ 8 min and occurrence of sleep onset REM-sleep periods [SOREMPs]) in 40% of first-degree relatives of presumably mainly pre-H1N1 NT1 and NT2 patients (the study was submitted in early January 2011, and the time of disease onsets and whether the study subjects were H1N1-vaccinated were not specified). This raises the possibility of the existence of narcolepsy subphenotypes in first-degree relatives.

NT1 core symptoms have generally been described as the classical tetrad consisting of EDS, cataplexy, hypnagogic hallucinations, and sleep paralysis, but now sleep fragmentation is recognized as a fifth core characteristic of the disease [1]. The sleep-wake and REM sleep instability that is characteristic of NT1 manifests in sleep recordings as short sleep latency (SL), nocturnal sleep fragmentation and short REM-SL. These sleep phenotype characteristics emerge in parallel with the gradual loss of hypocretin producing neurons in mouse models [29, 30]. Even though this pentad of symptoms is characteristic of NT1, not all patients experience all five symptoms to the same degree [31–33]. It is currently not known what causes the phenotypic variation/subphenotypes, and the extent to which first-degree relatives of post-H1N1 patients with NT1 have these symptoms and sleep features has not yet been fully ascertained [31].

In this study, we aim to dissect the roles of genetic variation in P2RY11 as a risk factor in post-H1N1 NT1 and of variation of P2Y<sub>11</sub> protein levels as a possible contributor to the phenotypic variation seen in patients with NT1 and their first-degree relatives. Our surprising finding of an association between low P2Y<sub>11</sub> protein levels and high nocturnal sleep fragmentation in NT1, but not with other sleep and clinical characteristics, offers new insight into the differential regulation of the NT1 phenotype and its severity.

## Methods

### Participants

Between February 2015 and November 2018, 107 patients with NT1 and their first-degree relatives (i.e. siblings and parents) of

Norwegian origin were consecutively included at the Norwegian Center of Expertise for Neurodevelopmental Disorders and Hypersomnias (NevSom). Thirteen patients were included after previous publications by our group [31, 32, 34–38].

The main inclusion criterion for patients was an NT1 diagnosis according to ICDSD-3 [1] with disease onset after autumn 2009, that is, after the 2009 H1N1 pandemic and Pandemrix vaccination campaign. Notably, our cohort therefore consisted of mainly H1N1-(Pandemrix)-vaccinated patients, but also included a few non-vaccinated patients with disease onset after the pandemic reached Norway in autumn 2009. To avoid inclusion bias, we included both H1N1-(Pandemrix)-vaccinated and non-vaccinated NT1 patients as the 2009 influenza A (H1N1) re-occurred all the following years as seasonal influenza and specific data on H1N1-infections were not available. We therefore cannot exclude that non-vaccinated patients could have been exposed to the influenza A (H1N1) virus. First-degree relatives in the 5–69 age range were included along with the patients. Collective exclusion criteria were the presence of a medical or mental disorder that made it difficult to complete their inclusion. The study was approved by the Regional Ethics Committee (REK 2014#450/2014#451). Written informed consent was obtained from all participants before inclusion (parents signed consent on behalf of their children). All patients were unmedicated with drugs influencing sleep or cataplexy 13–14 days before polysomnography (PSG) and MSLT recordings (one patient continued his lamotrigine medication due to comorbid epilepsy; another only stopped taking modafinil 9 days before inclusion). No medication modifications were requested in first-degree relatives, but two relatives who used medication that affects sleep (i.e. amitriptyline and citalopram) were excluded from the statistical analyses of sleep.

The already reported genotype data for rs2305795 from 123 healthy controls of Norwegian origin recruited through the Norwegian Bone Marrow Donor Register [39] was used for genotype comparisons with the NT1 patients (flow chart: Figure 1, box A).

The subcohort of individuals included for comparisons of T lymphocyte P2Y<sub>11</sub> protein levels between patients and their related controls was selected to be as homogenous as possible. Hence, in the included families the patient's disease onsets ranged from October 2009 to December 2013. Also, they had at least one sibling and/or both parents included (flow chart: Figure 1, box B and Table 1).

Figure 1 flow chart gives an overview of the patients and controls included in the analyses.

## Procedures at inclusion

Patients and first-degree relatives were clinically and neurologically examined, and all were diagnostically evaluated by the same experienced neurologist/sleep expert (S.K.H.). Disease onset was verified to be after the H1N1-(Pandemrix)-vaccination or after autumn 2009 for those who were not vaccinated. All patients, but none of the first-degree relatives, met the ICDSD-3 diagnostic NT1 criteria [1]. Fasting blood samples were drawn between 0700 and 0900 for routine parameter tests, genotyping (HLA DQB1\*06:02 and P2RY11 SNP rs2305795) and for P2Y<sub>11</sub> protein analyses of CD4 and CD8 T lymphocytes. H1N1-(Pandemrix)-vaccination status was confirmed in the Norwegian Immunization Registry

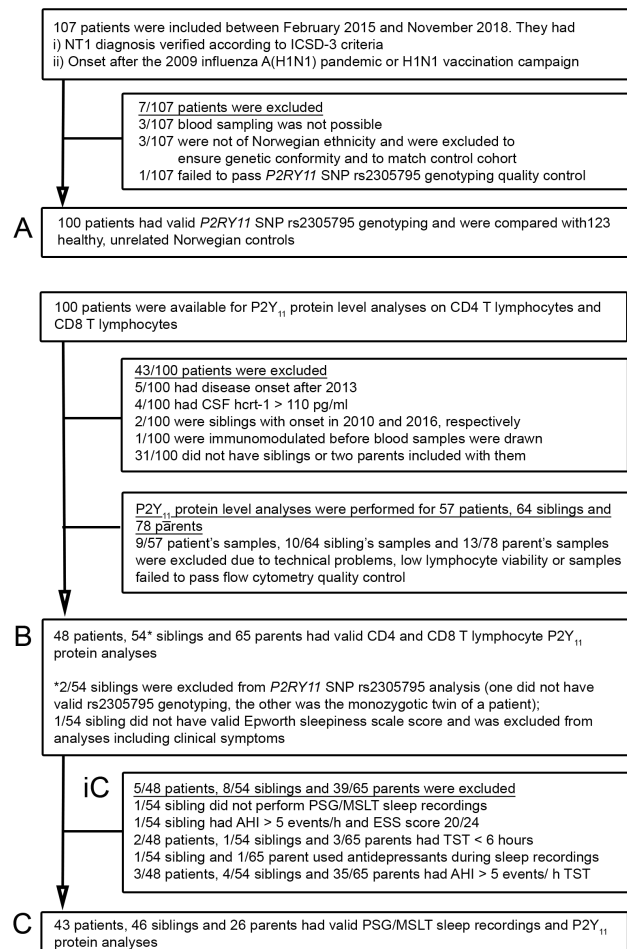


Figure 1. Flow chart over the included patients with NT1 and their first-degree relatives

In Table 1, all data, except the sleep variables are calculated for the cohort defined in box B. Sleep variables are calculated for the cohort defined in box C. Figure 2, A and B consist of patients with NT1 and all first-degree relatives (siblings and parents) defined in box B, and Figure 2, B and C of only the patient and the sibling groups. Figure 3, A and B consist of patients and siblings defined in box C, and Figure 3, C and D consist of only the patient group. Figure 4 shows the four siblings and 35 parents with AHI > 5 events/hour TST defined in box iC. NT1 = narcolepsy type 1; ICDSD-3 = international classification of sleep disorders, third edition; CSF = cerebrospinal fluid; SNP = single nucleotide polymorphism; PSG = polysomnography; MSLT = multiple sleep latency test; AHI = apnea/hypopnea index; ESS = Epworth sleepiness scale; TST = total sleep time.

(SYSVAK). Two patients not found in the SYSVAK claimed with certainty that they had been vaccinated at their workplace and so were included in the vaccinated group. A few patients and relatives reported having had influenza-like symptoms during the winter 2009/2010 or the following years. However, as the general recommendation during the pandemic was to stay at home if feeling ill from influenza, we have no reliable data regarding H1N1-infection rates in the cohort. The patients and relatives completed the Norwegian translation of the Stanford sleep questionnaire [40] and underwent a semi-structured interview about narcolepsy and other sleep disorders. Excessive daytime sleepiness (EDS) was estimated from the self-reported Epworth sleepiness scale (ESS) score. Pathological EDS cut-off was defined as a score of >10/24 that could not be better explained by other medical conditions or drug use [41, 42]. The presence of

**Table 1.** Demographic, clinical, and sleep characteristics, P2RY11 SNP rs2305795 genotype, and T lymphocyte P2Y<sub>11</sub> protein levels of patients with narcolepsy type 1 and their first-degree relatives

	Patients n = 48	Siblings n = 54	Parents n = 65
Gender (F/M)	30/18	25/29	33/32
Age at inclusion, years (median (min-max))	17 (10–62) <sup>a</sup>	19 (6.8–52) <sup>a</sup>	47 (34–61) <sup>b</sup>
HLA DQB1*06:02, yes (% (n))	100 (48)	61 (33)	57 (37)
H1N1-vaccination, yes (% (n))	92 (44)	69 (37)	63 (41)
Disease duration, years (median (min-max))	5.9 (2.4–7.9)		
Days from vaccination to disease onset, (median (IQR))	120 (42, 250) <sup>†</sup>		
CSF hcrt-1, <110 pg/mL (% (n))	100 (42) <sup>‡</sup>		
<b>Symptoms</b>			
Cataplexy/cataplexy-like phenomena*, yes (% (n))	98 (47)	5.6 (3)	0
HH, yes (% (n))	81 (39)	26 (14) <sup>o</sup>	9.2 (6)
SP, yes (% (n))	69 (33)	21 (11) <sup>o</sup>	6.2 (4)
ESS score ≥11/24, yes (% (n))	98 (47)	11 (6) <sup>o</sup>	5.2 (3) <sup>†</sup>
ESS score (mean (±SD))	19 (±3.4)	4.0 (±4.9) <sup>o</sup>	4.0 (±4.9) <sup>†</sup>
Cat or HH or SP or ESS, yes (% (n))	100 (48)	38 (20) <sup>o</sup>	17 (10) <sup>†</sup>
<b>Sleep parameters**</b>			
PSG REM sleep latency, min (median (IQR))	3.0 (1.8, 45) <sup>a</sup>	83 (65, 145) <sup>b</sup>	74 (57, 97)
PSG SFI, events/hour TST (mean (±SD))	14 (±3.9) <sup>a</sup>	11 (±2.8) <sup>b</sup>	14 (±3.1)
MSLT-SL, min (median (IQR))	1.5 (0.80, 3.1) <sup>a</sup>	15 (12, 18) <sup>b</sup>	12 (8.5, 14)
PSG AHI, events/hour TST (median (IQR))	0.36 (0.13, 0.70) <sup>a</sup>	0.35 (0.12, 0.84) <sup>a</sup>	1.3 (0.32, 2.3)
<b>P2RY11 SNP rs2305795 genotypes</b>			
AA, % (n)	46 (22)	38 (20) <sup>†</sup>	42 (27)
GA, % (n)	40 (19)	54 (28) <sup>†</sup>	48 (31)
GG, % (n)	15 (7)	7.7 (4) <sup>†</sup>	11 (7)
<b>P2Y<sub>11</sub> protein levels in CD4 and CD8 T lymphocytes</b>			
P2Y <sub>11</sub> in CD4 T lymphocytes (mean (±SD))	1.03 (±0.30) <sup>a</sup>	1.05 (±0.28) <sup>a</sup>	0.87 (±0.22) <sup>b</sup>
P2Y <sub>11</sub> in CD8 T lymphocytes (mean (±SD))	1.05 (±0.30) <sup>a</sup>	1.05 (±0.30) <sup>a</sup>	0.86 (±0.22) <sup>b</sup>

CSF Hcrt-1 = cerebrospinal fluid hypocretin-1; HH = hypnagogic/hypnopompic hallucinations; SP = sleep paralysis; ESS = Epworth sleepiness scale score; Cat = cataplexy/cataplexy-like phenomena; PSG = polysomnography; REM = rapid eye movement; min = minutes; SFI = sleep fragmentation index; TST = total sleep time; MSLT-SL = multiple sleep latency test; IQR = interquartile range; NREM = non-REM; AHI = apnea/hypopnea index; SNP = single nucleotide polymorphism.

<sup>a</sup>Calculated from 42/48 patients with vaccination date reported in SYSVAK.

<sup>b</sup>Six patients did not have their CSF hcrt-1 measured but had clear-cut cataplexy.

<sup>c</sup>First-degree relatives with cataplexy-like phenomena (defined as rare episodes of muscle weakness associated with laughter, fun, excitement and surprise) did not fulfil the ICSD-3 criteria for narcolepsy.

<sup>d</sup>Calculated from 53/54 siblings (one sibling did not have valid HH, SP and ESS scores).

<sup>e</sup>Calculated from 58/65 parents (seven parents did not have valid ESS scores).

<sup>f</sup>Valid sleep recordings were available from 43/48 patients, 46/54 siblings, and 26/65 parents, and are defined as: unmedicated with drugs affecting sleep two weeks prior to recordings (with two exceptions: one patient with ongoing lamotrigine-treatment due to comorbid epilepsy, and one patient with only 9 days modafinil pause, were included), PSG with ≥6 h total sleep time, and AHI ≤5 events/h TST. Parents were excluded from the sleep parameter comparisons due to known age effects.

<sup>g</sup>Calculated from 52/54 siblings (P2RY11 SNP rs2305795 genotyping failed in one sibling, and the monozygotic twin of a patient was excluded from genotype analysis).

<sup>h</sup>Median or mean of groups having different letters (superscript a or b) were significantly different (linear mixed-effect models, adjusted for age).

clinical features and symptoms was registered. These were defined as at least once in a lifetime, or a more frequent experience of cataplexy (or cataplexy-like phenomena in the case of the relatives), sleep paralysis, and/or hypnagogic hallucinations or an ESS score ≥11/24. In relatives, cataplexy-like phenomena were defined as rare episodes of muscle weakness associated with laughter, excitement, and surprise. One hypocretin-deficient, HLA DQB1\*06:02-positive patient who reported borderline sleepiness (an ESS score of 10), but who, during the interview, described daily problems with sleepiness and difficulties in waking up in the morning, was considered to have NT1. CSF hcrt-1 was measured by the previously described radioimmunoassay method (Phoenix Pharmaceuticals, Belmont, CA, USA) [33]. A low CSF hcrt-1 value was defined as being ≤150 pg/mL.

### Sleep recordings

PSG and MSLT recordings were obtained with SOMNOmedic plus system (Domino software, version 2.7.0, SOMNOmedics,

Randersacker, Germany). Measurements were manually scored in 30-s epochs according to AASM scoring criteria [1]. The following variables were recorded during the overnight PSG in accordance with AASM specifications: electroencephalogram (F3-A2, C3-A2, O1-A2, F4-A1, C4-A1 and O2-A1), electrooculogram, chin and leg electromyogram, electrocardiogram, airflow signals, respiratory effort signals, oxygen saturation and body position. MSLT recordings performed the following day consisted of 5 nap opportunities for 30 min at 2-h intervals. Skin-electrode impedances were kept below 10 kΩ (preferably below 5 kΩ). Sleep recordings were considered valid when PSG total sleep time (TST) was ≥6 h, AHI was ≤5 events/h TST, and no medication influencing sleep was used 2 weeks before obtaining the sleep recordings (except for the two patients described above). Siblings and parents with TST ≥6 h and AHI >5 events/h TST constituted a group of individuals having sleep apnea. These were excluded from the primary analysis but used in the secondary analysis of P2Y<sub>11</sub> levels in sleep apnea patients. The calculated sleep fragmentation index comprises all transitions between sleep and wake, and between

the different NREM and REM sleep stages. All participants were requested to wear wrist actigraphs (Philips Actiwatch Spectrum/Spectrum Plus, Respironics Inc., Murrysville, PA) before the sleep recordings. Actigraphy was made to monitor their circadian rhythm and rule out sleep deprivation. Three participants did not receive their actigraph in time to use it, and six used it for less than a week. The others wore them for 7–13 days. Actigraphy was not used as an exclusion criterion.

### HLA DQB1\*06:02 and P2RY11 SNP rs2305795 sequencing

HLA gene amplification was performed using the NGSgo kit (GenDX, Utrecht, The Netherlands). In addition, two custom amplicons targeting the P2RY11 gene region were amplified using 50 ng of DNA, 1.6 U LongRange Enzyme mix (Qiagen), 1× LongRange Buffer (Qiagen), 500 μM of each dNTP and 5 μM of primers. PCR conditions consisted of 95°C initial denaturing for 3 min, followed by 35 cycles at 95°C for 15 s, 65°C for 30 s and 68°C for 6 min, and finally at 68°C for 10 min. The HLA and P2RY11-PPAN amplicons were pooled before preparing the library according to the NGSgo workflow. 2x150-bp paired-end sequencing was carried out in a MiSeq instrument (Illumina, San Diego, USA) using a Miseq Reagent Kit v2 (300 cycles) at the Norwegian Sequencing Centre (<https://www.sequencing.uio.no/>). The HLA genotypes were analyzed using NGSengine software (GenDX), while the SNP genotypes from the P2RY11-PPAN region were obtained using GATK [43] with Burrows–Wheeler Aligner's Smith–Waterman Alignment version 0.7.17 [44] and Picard version 2.20.1 [45].

### CD4 and CD8 T lymphocyte flow-cytometry analyses of P2Y<sub>11</sub> protein levels

Whole blood was separated by Lymphoprep (Axis Shield, Dundee, Scotland) density gradient centrifugation, according to the manufacturer's recommendation. Buffy coat was collected, washed twice with phosphate-buffered saline (PBS), re-suspended in 10% DMSO, and gradually frozen to –80°C before being transferred to liquid nitrogen and stored until use. The samples were divided into 11 subsets, each of which consisted of duplicate samples from 16–23 individuals. The cryopreserved lymphocytes, one subset at a time, were thawed following standard procedures [46], and 3–10 × 10<sup>5</sup> cells were used per test.

Cells were stained with fixable viability dye (FVD) eFluor 506 and Human BDFc Block to allow for the exclusion of dead cells and to reduce nonspecific antibody binding. Fluorophore-conjugated antibodies (CD3-BV421, CD8-PeCy7, CD19-PerCP-Cy5.5, CD56-PE and CD16-APC-Ef780) or isotype controls at optimal concentrations dissolved in PBS were added. Antibody details are given in Table S1. Cells were stained in the dark for 30 min at 4°C, washed twice in PBS + 2% fetal bovine serum, fixed in IC fixation buffer for 45 min at room temperature, and subsequently washed in permeabilization buffer. The conjugated antibodies P2Y<sub>11</sub>-APC and CD4-FITC, or isotype controls at optimal concentrations (Table S1), were dissolved in permeabilization buffer, and cells were incubated for 1 h in the dark at room temperature and subsequently washed twice in permeabilization buffer. Finally, PBS was added. The specificity of the staining for P2Y<sub>11</sub> was confirmed beforehand using transfected HEK cells [47, 48].

Stained cells were analyzed with three laser LRS-Fortessa cell analyzers (BD Biosciences) with software BD FACSDiva v 8.0.1 (BD Biosciences). Appropriate controls (unstained, FVD-stained, and Isotype/Fluorescence Minus One-stained cells) were applied to each analysis. Software FlowJo v 10 (FlowJo LLC, Ashland, OR, USA) was used for further analyses. Normalization was performed to enable analyses across the subsets (means of duplicates were divided by the subset mean). The coefficient of variance in the duplicate samples varied from 0% to 25%. Three samples did not have duplicates or had a coefficient of variance >15%, cells in three samples had too low viability to be analyzed and samples from five individuals were excluded from the analyses due to analyze failure. In addition, one full subset (samples from 21 individuals) were unfortunately lost due to technical problems with the flow cytometer. In total, the excluded samples were from 9 patients, 10 siblings and 13 parents. Gates for the cell compartments (B lymphocytes, T lymphocytes and NK cells) and P2Y<sub>11</sub> protein levels in CD4 T lymphocytes and CD8 T lymphocytes were set by visual inspection guided by isotype gating (Figure S1).

### Statistical analyses

Data were analyzed using R/RStudio (<http://r-project.org/>).

The odds ratio for P2RY11 SNP rs2305795 genotype comparisons between NT1 patients and healthy controls was tested by applying Fisher's exact test.

The first-degree relatives (divided into sibling- and parent groups) served as controls in the analyses investigating the T lymphocyte P2Y<sub>11</sub> protein levels in NT1 patients. To account for the lack of independence between the participants (patients and controls) the linear mixed-effect model from the lme4() package in R was used [49]. In a linear expression, this model calculates both fixed-effect estimates (parameters that do not vary across individuals, such as the confounder age) and random effect estimates (such as the within-participant variation caused by family relatedness in the cohort). Hence, the linear mixed-effect model was used for calculations throughout the paper, except for the analyses consisting of unrelated patients only (then, multiple linear regression model was used). All models were adjusted for the confounder age. The reported effect estimates are β values, 95% confidence intervals and associated two-sided *p* values; the significance level was set at *p* < 0.05. Patients and siblings were not 1:1 age-matched but had a similar age distribution.

We further calculated all study results with and without the non-vaccinated patients, which did not affect the study results, hence only results from the entire cohort are presented.

## Results

### P2RY11 SNP rs2305795 is associated with post-H1N1 NT1

To replicate the previously found association between the P2RY11 SNP rs2305795 and NT1, we compared P2RY11 SNP rs2305795 genotyping data from 100 patients from our post-H1N1 cohort of mainly Pandemrix vaccinated patients (90/100 were vaccinated) with those of 123 unrelated healthy Norwegian controls (flow chart: Figure 1, box A). The rs2305795 A allele was significantly (*p* = 0.001) more frequent among patients (67.5%) than controls

(51.2%), with an odds ratio of 2.0 (95% CI = 1.3 to 3.0). The associations remained significant when the 10 non-vaccinated patients were excluded (OR = 2.0, 95% CI = 1.3 to 3.1,  $p = 0.001$ ).

### Demographic, clinical and sleep characteristics of patients with NT1 and their first-degree relatives

Demographic, clinical and sleep characteristics for patients and their first-degree relatives in the final study cohort are presented in the first half of [Table 1](#). An overview of the study cohorts is shown in the flow chart in [Figure 1](#).

Additional PSG and MSLT sleep parameters are presented in [Table S2](#). These are the typical sleep parameter findings for an NT1 cohort as there was no difference in total sleep time between the patients and their age-matched siblings, but the patients had significantly more light sleep (N1) and REM-sleep and a smaller amount of deeper sleep (N2 and N3) than their siblings. Likewise, patients also had significantly more periodic limb movements than their siblings, as expected.

### Age effect on CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels

The second half of [Table 1](#) shows the P2RY11 SNP rs2305795 genotypes and CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels in patients, siblings, and parents.

The mean P2Y<sub>11</sub> protein levels in CD4 and CD8 T lymphocytes were both significantly lower in parents than in patients and siblings (parents vs patients CD4:  $\beta = 0.21$ , 95% CI = 0.033 to 0.40,  $p = 0.023$  and CD8:  $\beta = 0.21$ , 95% CI = 0.024 to 0.40,  $p = 0.029$ ; parents vs siblings CD4:  $\beta = 0.24$ , 95% CI = 0.058 to 0.41,  $p = 0.011$  and CD8  $\beta = 0.22$ , 95% CI = 0.041 to 0.41,  $p = 0.018$ , respectively), whereas no differences were observed between patients and siblings ([Table 1](#)). Moreover, there were no differences between P2Y<sub>11</sub> protein levels in CD4 versus CD8 T lymphocytes.

To analyze whether age affected P2Y<sub>11</sub> protein levels, we included age, P2RY11 SNP rs2305795 genotype, gender and the disease status variable (dichotomous: 48 patients and 117 relatives) as covariates in the linear mixed-effect model. Results showed significant effects of age on P2Y<sub>11</sub> protein levels in both CD4 and CD8 T lymphocytes, i.e., lower levels at higher ages (CD4:  $\beta = -4.5 \times 10^{-3}$ , 95% CI =  $-7.3 \times 10^{-3}$  to  $-1.6 \times 10^{-3}$ ,  $p = 0.002$ ; CD8:  $\beta = -5.3 \times 10^{-3}$ , 95% CI =  $-8.3 \times 10^{-3}$  to  $-2.2 \times 10^{-3}$ ,  $p = 0.001$ ). Individuals homozygous for the P2RY11 SNP rs2305795 A allele had significantly lower P2Y<sub>11</sub> protein levels in both CD4 and CD8 T lymphocyte subsets compared with those that expressed the GA or GG genotype (CD4: 0.11, 95% CI = 0.025 to 0.19,  $p = 0.011$ ; CD8:  $\beta = 0.12$ , 95% CI = 0.034 to 0.20,  $p = 0.047$ ). We did not observe effect from gender or from the disease status variable.

Distributions of B- and T-lymphocyte and natural killer (NK) cells from all participants are presented in [Table S3](#). Among these cell types, parents had lower levels of expression of CD56<sup>high</sup> and CD56<sup>intermediate</sup> NK cell phenotypes and higher fractions of NKT lymphocytes compared with patients. These are established age effects in immune cells. The fraction of T lymphocytes in live mononuclear blood cells was slightly lower in the parent group than in the NT1 patient group (66% and 72%, respectively). No significant differences were observed between NT1 patients and siblings.

### The P2RY11 SNP rs2305795 AA genotype is associated with lower CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels

We next examined whether rs2305795, located in the 3'UTR of the P2RY11 gene, affects T lymphocyte P2Y<sub>11</sub> protein levels, as this has not been investigated before in a large cohort of patients with NT1 and first-degree relatives. Only eleven individuals carried the GG genotype, so they were pooled with those of GA genotype. The joint analysis of patients, siblings, and parents revealed significantly lower P2Y<sub>11</sub> protein levels in CD4 T lymphocytes and CD8 T lymphocytes in individuals homozygous for the NT1 risk allele (P2RY11 SNP rs2305795 AA genotype) than in those who carried the rs2305795 GA or GG genotypes (CD4:  $\beta = 0.11$ , 95% CI = 0.025 to 0.19,  $p = 0.012$ ; CD8:  $\beta = 0.12$ , 95% CI = 0.032 to 0.20,  $p = 0.007$ ; [Figure 2, A and B](#)).

### No association between clinical symptoms and T lymphocyte P2Y<sub>11</sub> protein levels in patients with NT1 and their siblings

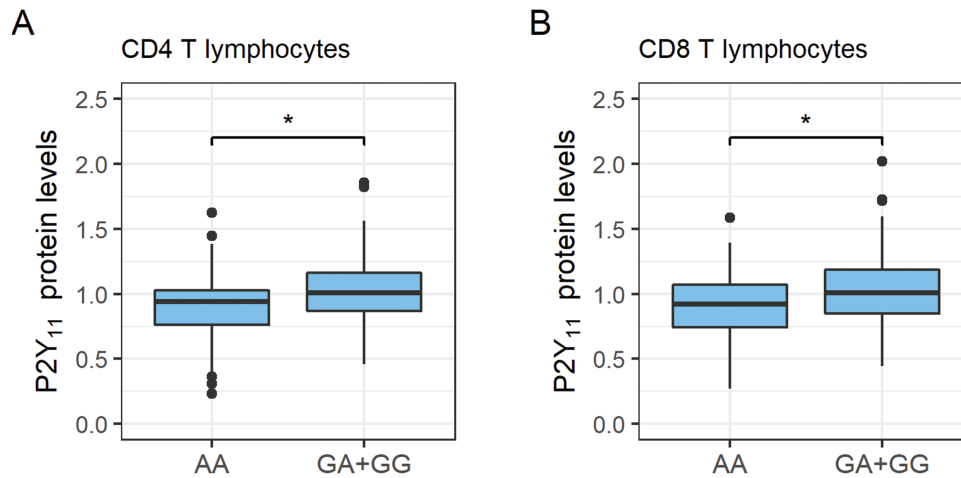
We examined whether T lymphocyte P2Y<sub>11</sub> protein levels differed between patients and their siblings with and without at least one clinical symptom (e.g. either cataplexy/cataplexy-like episodes, hypnagogic hallucinations, sleep paralysis and/or ESS score  $\geq 11/24$ ) ([Figure 2, C and D](#)). In this analysis, we excluded the parents to avoid confounding age effects. Applying the linear mixed-effect model including the presence of at least one clinical symptom, P2RY11 SNP rs2305795 genotypes and age showed that T lymphocyte P2Y<sub>11</sub> protein levels did not differ significantly between patients and their siblings with or without at least one clinical symptom. Within the sibling group, those with at least one clinical symptom had a significantly higher T lymphocyte P2Y<sub>11</sub> protein levels than siblings without any clinical symptoms, when adjusting for P2RY11 SNP rs2305795 genotypes and age (CD4:  $\beta = -0.16$ , 95% CI =  $-0.31$  to  $-0.016$ ,  $p = 0.036$ ; CD8:  $\beta = -0.17$ , 95% CI =  $-0.33$  to  $-0.019$ ,  $p = 0.036$ ). However, after correction for multiple testing (Bonferroni correction for three tests,  $\alpha = 0.017$ ) these results were not significant.

We extended our analysis to examine the possible associations between T lymphocyte P2Y<sub>11</sub> protein levels and each of the individual clinical symptoms: hypnagogic hallucinations, sleep paralysis, and ESS score. Cataplexy could not be analyzed because only one patient did not exhibit it. Parents were again excluded on the grounds of age. There were no significant differences in P2Y<sub>11</sub> protein levels for any of the individual symptoms tested ([Figure S2](#)).

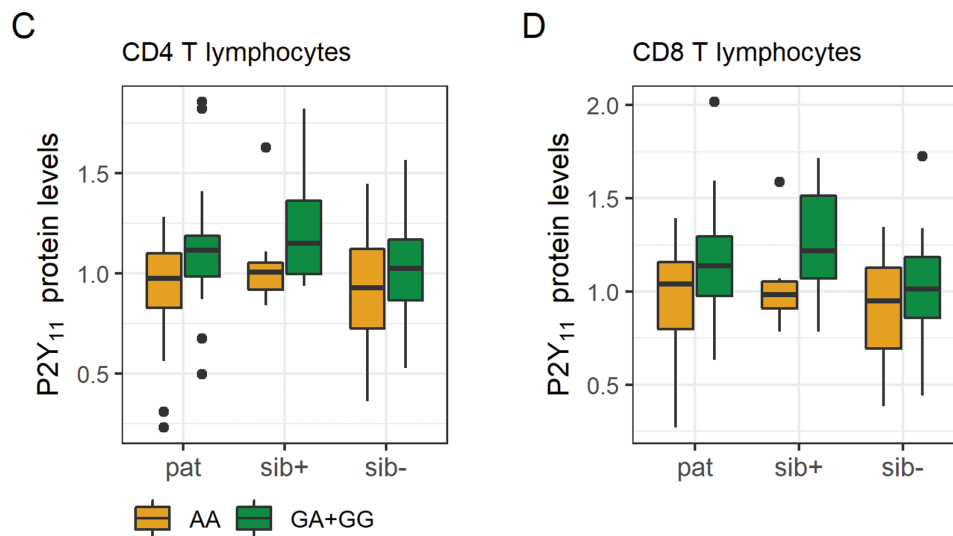
### Nocturnal sleep fragmentation is negatively correlated with T lymphocyte P2Y<sub>11</sub> protein levels in patients with NT1, but not in their siblings

We investigated the possible association between T lymphocyte P2Y<sub>11</sub> protein levels and the core sleep-recording parameters of sleep-wake and REM sleep instability: PSG sleep fragmentation index, PSG REM-SL, and MSLT-SL, in patients and siblings (see flow chart: [Figure 1](#), box C). In patients with NT1, correlation between high sleep fragmentation index and low P2Y<sub>11</sub> protein levels were found in both the CD4 and the CD8 T lymphocyte subsets (CD4:  $\beta = -0.035$ , 95% CI =  $-0.058$  to  $-0.012$ ,  $p = 0.004$ ; CD8:  $\beta = -0.033$ , 95% CI =  $-0.057$  to  $-0.010$ ,

## Patients, siblings and parents



## Patients and siblings with and without symptoms



**Figure 2.** T lymphocyte P2Y<sub>11</sub> protein levels are lower in individuals with the P2RY11 SNP rs2305795 AA genotype.

(A) CD4 T lymphocytes and (B) CD8 T lymphocytes in patients ( $n = 48$ ), siblings ( $n = 52$ ), and parents ( $n = 65$ ) pooled and grouped by P2RY11 SNP rs2305795 AA and GA+GG genotypes (flow chart: [Figure 1](#), box B, two siblings excluded). In both CD4 T lymphocytes and CD8 T lymphocytes P2Y<sub>11</sub> protein levels were significantly lower in individuals homozygous for the rs2305795 A allele (CD4:  $\beta = 0.11$ , 95% CI = 0.025 to 0.19,  $p = 0.012$ ; CD8:  $\beta = 0.12$ , 95% CI = 0.032 to 0.20,  $p = 0.007$ ; linear mixed-effect model adjusted for age).

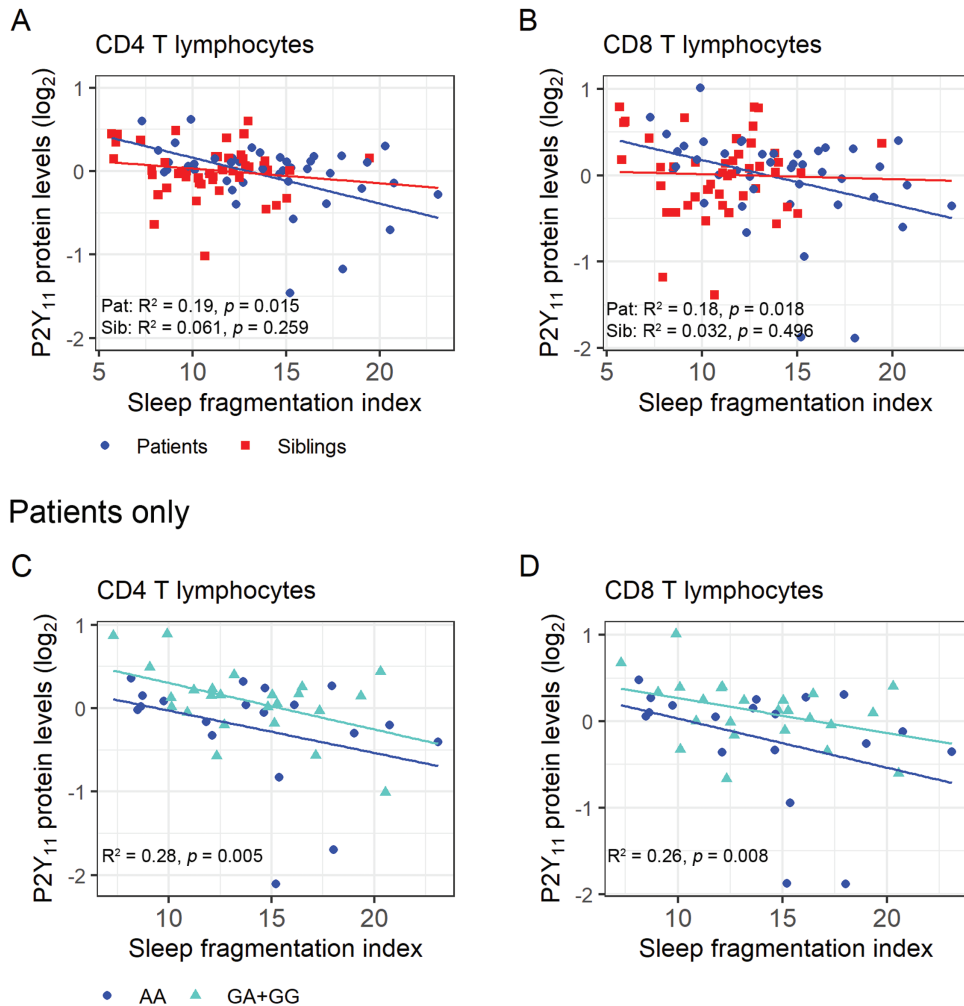
(C and D) T-lymphocyte P2Y<sub>11</sub> protein levels grouped by P2RY11 SNP rs2305795 genotypes for patients ( $n = 48$ ), compared to siblings who had experienced one/more clinical symptoms (Epworth sleepiness scale score  $\geq 11/24$ , hypnagogic hallucinations, sleep paralysis, or cataplexy-like episodes) at least once in their lifetime (sib+,  $n = 20$ ) and siblings without any experience of clinical symptoms (sib-,  $n = 31$ ) in (C) CD4 T lymphocytes and (D) CD8 lymphocytes (flow chart: [Figure 1](#), box B, three siblings excluded). There were no differences between patients and siblings with symptoms or patients and siblings without symptoms (linear mixed-effect model, adjusted for P2RY11 SNP rs2305795 genotype and age).

$p = 0.006$ ), whereas no such correlation were detected in the siblings ([Figure 3](#), A and B). Results were still significant when corrected for multiple testing (Bonferroni correction for three tests:  $\alpha = 0.017$ ). There were no significant correlations of P2Y<sub>11</sub> protein levels with MSLT-SL or PSG REM-SL in neither patients nor siblings ([Figure S3](#)).

To determine whether the P2RY11 SNP rs2305795 genotype influenced the relationship between the sleep fragmentation

index and T lymphocyte P2Y<sub>11</sub> protein levels, the AA and GA/GG genotype patient groups were considered separately ([Figure 3](#), C and D). The negative correlation between P2Y<sub>11</sub> protein levels and sleep fragmentation was evident in both CD4 and CD8 T lymphocyte subsets when adjusted for the genotypes and age (CD4:  $\beta = -0.033$ , 95% CI =  $-0.055$  to  $-0.011$ ,  $p = 0.004$ ; CD8:  $\beta = -0.032$ , 95% CI =  $-0.055$  to  $-9.6 \times 10^{-3}$ ,  $p = 0.006$ ).

## Patients and siblings



**Figure 3.** T-lymphocyte P2Y<sub>11</sub> protein levels are associated with sleep fragmentation index in NT1.

P2Y<sub>11</sub> protein levels versus sleep fragmentation index (number of stage changes/hour total sleep time) in (A) CD4 T lymphocytes and (B) CD8 T lymphocytes in patients (n = 43) and siblings (n = 46), respectively (flow chart: [Figure 1](#), box C). The correlation between high sleep fragmentation index and low P2Y<sub>11</sub> protein levels were statistically significant for patients in both CD4 T lymphocytes and CD8 T lymphocytes (CD4:  $\beta = -0.035$ , 95% CI =  $-0.058$  to  $-0.012$ , p = 0.004; CD8:  $\beta = -0.033$ , 95% CI =  $-0.057$  to  $-0.010$ , p = 0.006), but not for siblings.

The P2Y<sub>11</sub> protein levels on (C) CD4 T lymphocytes and (D) CD8 T lymphocytes were subdivided by P2RY11 SNP rs2305795 AA and GA+GG genotypes for the 43 patients. Differences in sleep fragmentation index were conserved in both CD4 T lymphocytes and CD8 T lymphocytes when adjusted for P2RY11 SNP rs2305795 genotypes and age (CD4:  $\beta = -0.033$ , 95% CI =  $-0.055$  to  $-0.011$ , p = 0.004; CD8:  $\beta = -0.032$ , 95% CI =  $-0.055$  to  $-9.6 \times 10^{-3}$ , p = 0.006).

Multiple linear regression models were applied for the cohort of unrelated patients, and linear mixed-effect model for the sibling cohort. Values printed on the figures are R<sup>2</sup> and p value from multiple linear regression models. All models were adjusted for age.

### The nocturnal sleep fragmentation index and T lymphocyte P2Y<sub>11</sub> protein levels in sleep apnea are not correlated

To examine whether sleep fragmentation per se is associated with lower P2Y<sub>11</sub> protein levels, we investigated whether the association between P2Y<sub>11</sub> protein levels and fragmented sleep would also be found in another sleep disorder in which sleep fragmentation is a typical feature. We therefore analyzed this in the available four siblings and 35 parents with sleep apnea (AHI > 5) (flow chart: [Figure 1](#), box iC), of whom 27 were men and 12 were women. Ten out of 39 had an AHI > 15, corresponding to moderate to severe obstructive sleep apnea syndrome. When comparing the sleep fragmentation index with P2Y<sub>11</sub> protein levels in CD4 and CD8 T lymphocytes in

this sleep apnea cohort, no correlation between the two measures was found ([Figure 4](#)). This suggests that sleep fragmentation in general does not affect T lymphocyte P2Y<sub>11</sub> levels or vice versa, but that the observation is specific to NT1.

### Discussion

The association between P2RY11 SNP rs2305795 and NT1 was confirmed in our cohort of mainly H1N1-(Pandemrix)-vaccinated post-H1N1 patients with NT1 compared with unrelated healthy controls. Furthermore, we found significantly lower CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels in the individuals expressing the rs2305795 AA NT1 risk genotype compared with the GA/GG



genotypes, and that P2Y<sub>11</sub> protein levels decreased slightly with age. P2Y<sub>11</sub> protein levels were similar in the patients with NT1 and their siblings with or without clinical symptoms. We noted a significant correlation between the core sleep phenotypic feature of nocturnal sleep fragmentation, and P2Y<sub>11</sub> protein levels in the patients with NT1, but not in first-degree relatives with or without sleep apnea.

The significantly higher frequency of the P2RY11 SNP rs2305795 A allele in our mainly H1N1 vaccinated patient cohort compared with healthy controls is consistent with previous findings in a population of patients of mixed European ancestry with pre-H1N1 NT1 onset [14], and the borderline significant indications of a single small study of 42 H1N1-(Pandemrix)-vaccinated patients [22], is evidence that P2RY11 is a predisposing non-HLA risk gene for NT1, irrespective of H1N1 vaccination status.

We found no differences in the overall distribution of P2Y<sub>11</sub> protein levels between CD4 and CD8 T lymphocytes in patients and siblings when pooled. This is consistent with the findings of Degn et al. [48], but at variance with the findings of another study that reported higher P2Y<sub>11</sub> protein levels in CD8 than in CD4 T lymphocytes [25]. These previous studies were both small, comprising only 11 (four patients and seven first-degree relatives) and eight (healthy donors) cases, respectively [25, 48]. Our data show that there is considerable individual variation in P2Y<sub>11</sub> protein levels, which may well explain this discrepancy. The present study includes 167 individuals (patients, siblings and parents), making our findings substantially stronger than earlier ones. We also find an age effect on P2Y<sub>11</sub> protein levels (lower levels with increasing age). This is the first time the effect of age on P2Y<sub>11</sub> levels has been studied.

We report significantly lower CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels in individuals expressing the rs2305795 AA NT1 risk genotype regardless of the disease state. Kornum et al. [14] compared P2RY11 mRNA levels in patients with NT1 and

healthy controls, and no difference was seen here, which is consistent with our finding. Few studies have investigated P2RY11 mRNA expression in T lymphocytes. Two studies (one consisting of a joint group of 60 patients with NT1 and 56 controls, and the other of 8 healthy blood donors) found significantly higher P2RY11 mRNA expression in CD8 than in CD4 T lymphocytes [14, 25]. The absence of any close correlation between mRNA and protein levels may be caused by, for example, post-transcriptional regulation and degradation [50, 51]. We did not measure mRNA levels in the present study, but instead directly determined the rs2305795 genotype effects on the level of the P2Y<sub>11</sub> protein. It is important to note that the influence of the rs2305795 genotype only explained a small part of the total individual variation in P2Y<sub>11</sub> protein levels.

When comparing patients with NT1 to age-matched siblings, we did not find any differences between blood mononuclear cell levels of CD4 and CD8 T lymphocytes, B lymphocytes, NK or NKT cells. This is consistent with previous reports [52, 53].

A specific and interesting finding in the patients with NT1 was the negative correlation between CD4 and CD8 T lymphocyte P2Y<sub>11</sub> protein levels and nocturnal sleep fragmentation, but the absence of associations between protein levels and other clinical and sleep phenotypic features typical of NT1. Disrupted nocturnal sleep is recognized as a part of the classic pentad of narcolepsy [1, 2, 54–56], but is still a largely unexplained feature. Some sleep-wake neuronal network models propose that the hypocretin deficiency in NT1 causes both unstable daytime wakefulness and fragmented sleep [57], but as hypocretin levels are already very low or practically absent during NREM sleep under normal physiological conditions, it is difficult to understand why hypocretin deficiency in itself should cause the sleep fragmentation in patients with NT1. This suggests that factors other than hypocretin deficiency are also responsible. Our finding that low P2Y<sub>11</sub> protein levels

## No correlation in sleep apnoea

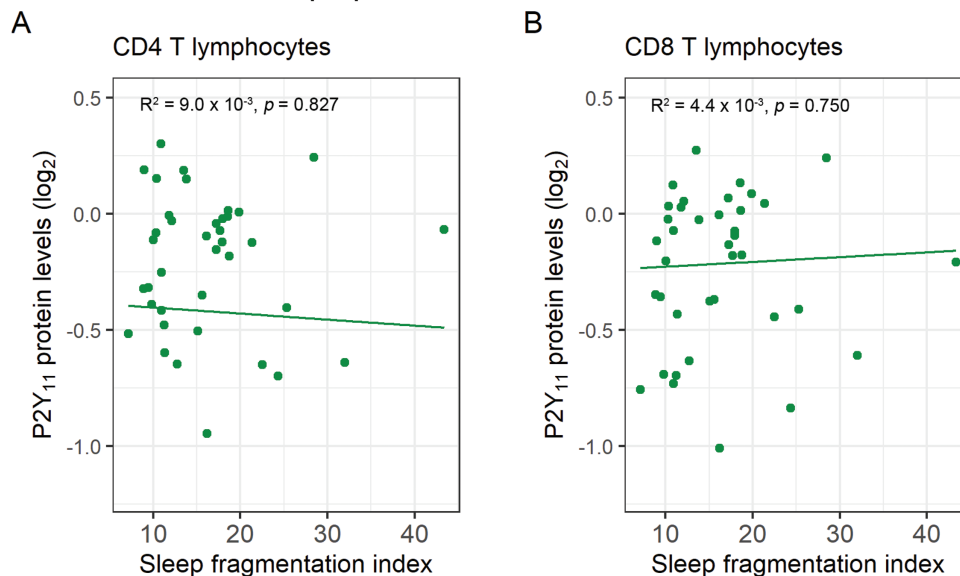


Figure 4: T-lymphocyte P2Y<sub>11</sub> protein levels are not associated with sleep fragmentation index in sleep apnea.

In first-degree relatives with sleep apnea, there were no associations between sleep fragmentation index and P2Y<sub>11</sub> protein levels in neither (A) CD4 T lymphocytes nor (B) CD8 T lymphocytes (linear mixed-effect models, adjusted for age). The figures depict four siblings and 35 parents with AHI >5 events/h total sleep time (flow chart: Figure 1, box iC). Values printed on figures are R<sup>2</sup> and associated p values from multiple linear regression models adjusted for age.

in T lymphocytes are associated with high degrees of sleep fragmentation suggests that P2Y<sub>11</sub> may play a role in phenotypic development and severity during the NT1 disease process. As 38% of siblings and 17% of parents in our cohort had experienced at least one clinical core narcolepsy symptom, this supports that a “narcolepsy spectrum” is also present in the relatives of post-H1N1 NT1 patients, like previously reported in pre-H1N1 cohorts [26–28, 58, 59]. However, the association between T lymphocyte P2Y<sub>11</sub> protein levels and sleep fragmentation index was only found in patients, not in relatives irrespective of the occurrence of clinical symptoms or sleep apnea. This suggests that the effect of P2Y<sub>11</sub> on sleep regulation is specific to narcolepsy. To explore this further we checked, using the UK biobank, if the P2RY11 SNP rs2305795 allele was associated with 19 self-reported sleep phenotypes in healthy individuals. This was not the case (Table S4). Even though this information should be treated with cautions because none of the phenotypes are specific for PSG measured sleep fragmentation, this still supports our observation of no correlation between T lymphocyte P2Y<sub>11</sub> levels and sleep features in the relatives in our study.

A technical limitation of our study is that it was not possible to stain and analyze all T lymphocyte samples at the same time. Instead, we partitioned the samples into 11 subsets and normalized the results by dividing sample means by the subset mean. Another limitation is that we measured P2Y<sub>11</sub> levels in T lymphocytes but not in the brain. As no P2Y<sub>11</sub>-specific tracer for brain imaging exists, it is currently not possible to measure P2Y<sub>11</sub> levels in the human brain. However, as the genetic basis for the P2Y<sub>11</sub> levels will be the same in the two locations, in the interpretation of our data we suggest that differences in P2Y<sub>11</sub> levels in T lymphocytes could be used as a marker of P2Y<sub>11</sub> protein levels in the brain. P2RY11 mRNA is indeed known to be highly expressed in human brain tissue [23], but unknown tissue-specific regulatory mechanisms might also be acting. Indeed, in the public available expression Quantitative Trait Loci (eQTL) data in the GTEx database the biggest effect size of rs2305795 is found on P2RY11 expression in human hypothalamus. In this case, the direction is the opposite of what we observe in the T lymphocytes, namely higher P2RY11 expression with the A variant. Discordant eQTLs, even with opposite allelic effects, in different tissues is commonly seen [60]. Importantly, the data demonstrate that rs2305795 does indeed also affect P2RY11 expression in the brain supporting that variations in protein levels of P2Y<sub>11</sub> can somehow affect hypothalamic and/or brain function and ultimately sleep regulation. Our data do not support nor reject this hypothesis, and further studies are needed to elucidate the role of P2Y<sub>11</sub> in the brain. As the P2RY11 gene does not exist in rodents, no knock-out models exist nor has its role been studied in animal models of NT1. Instead, future studies should include further human genetic studies. These could include the effects of rs2305795 on P2RY11 expression and on the other genes in the synteny. Another important future study would be of the genetic associations with PSG quantified sleep features. Lastly, we did not measure P2Y<sub>11</sub> levels in patients with other central hypersomnias, so we cannot tell whether the correlation between sleep fragmentation and P2Y<sub>11</sub> protein levels occurs only in NT1 individuals with deficient CSF hcrt-1 levels, or whether we might see the same in

patients with narcolepsy type 2, idiopathic hypersomnia or Kleine–Levin syndrome.

## Conclusion

In conclusion, we confirmed a similar association between NT1 and the P2RY11 SNP rs2305795 in our cohort of mainly H1N1-(Pandemrix)-vaccinated post-H1N1 patients with NT1 compared with healthy controls as previously shown in pre-H1N1 cohorts. Our study thereby gives further evidence that, like the HLA-genes, also non-HLA genes predispose to NT1 regardless of H1N1-vaccination status, hence further supporting that pre-H1N1 and post-H1N1 NT1 is most likely the same entity. We also detected that individuals homozygous for the NT1 risk associated P2RY11 SNP rs2305795 A allele had lower levels of P2Y<sub>11</sub> protein in both CD4 and CD8 T lymphocytes. The clinical NT1 phenotypic core symptoms were not associated with P2Y<sub>11</sub> protein levels. However, specifically, we detected a negative correlation between P2Y<sub>11</sub> protein levels and the nocturnal sleep fragmentation index in patients with NT1 but not in first-degree relatives, with or without sleep apnea. Our finding raises the possibility that P2Y<sub>11</sub> has a previously undescribed role in the central regulation of sleep stability that affects disease severity in NT1 specifically and is not related to sleep stability or fragmentation in general.

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