The autoimmune hypothesis of narcolepsy

and its unexplored clinical features

M.S. Schinkelshoek

THE AUTOIMMUNE HYPOTHESIS OF NARCOLEPSY AND ITS UNEXPLORED CLINICAL FEATURES

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"Oh sleep! it is a gentle thing,
Beloved from pole to pole!"

Samuel Taylor Coleridge in 'The Rime of the Ancient Mariner'
– part V, 1798

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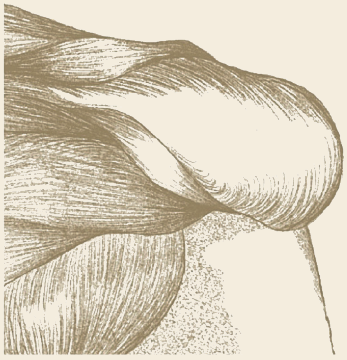
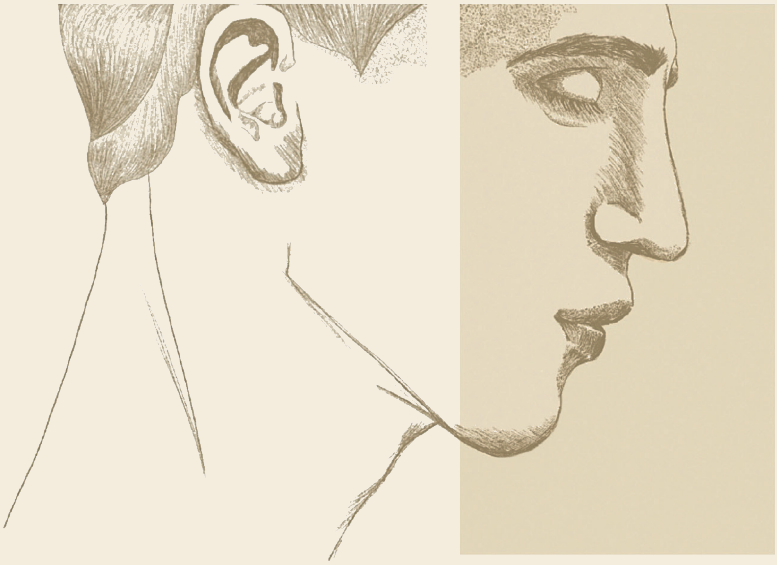




General introduction

General introduction

Narcolepsy is a sleep-wake disorder that can be subdivided in two types, diagnosed according to the criteria outlined in the Third Version of the International Classification of Sleep Disorders (ICSD, 2014). The distinction between type 1 and 2 is made based on the presence of cataplexy and/or hypocretin deficiency. This thesis will focus mainly on narcolepsy type 1 (NT1), since this disease entity is clearly distinct from other hypersomnolence disorders because of the presence of cataplexy and the absence of hypocretin in the cerebrospinal fluid (Table 1). NT1 is characterized by five core symptoms: excessive daytime sleepiness, cataplexy, sleep paralysis, hypnagogic hallucinations and disturbed night sleep. It is a rare disorder with a prevalence of around 1 in 3000 individuals (Wijnans et al., 2013).

Until the 1980s, not much was known about the cause of narcolepsy. During the 1980s the identification of a strong association of a human leukocyte antigen (HLA) molecule with narcolepsy gave researchers a direction where to look for the cause of the disease. This marked the starting point of the interest in the autoimmune hypothesis of narcolepsy.

Table 1. Diagnostic criteria for narcolepsy type 1 according to the Third Version of the International Classification of Sleep Disorders.

Criteria A and B must be met for making the diagnosis of narcolepsy type 1
• A. The patient has daily periods of irrepressible need to sleep or daytime lapses into sleep occurring for at least 3 months.
• B. The presence of one or both of the following:
• 1. Cataplexy and a mean sleep latency of ≤8 minutes and two or more sleep-onset REM periods (SOREMPs) on an multiple sleep latency test (MSLT) performed according to standard techniques. A SOREMP (within 15 minutes of sleep onset) on the preceding nocturnal polysomnogram may replace one of the SOREMPs on the MSLT.
• 2. CSF hypocretin-1 concentration, measured by immunoreactivity, is either ≤110 pg/mL or <1/3 of mean values obtained in normal subjects with the same standardized assay.

Pathophysiology

Discovery of hypocretin and its deficiency in narcolepsy

In 1998, the discovery of a novel peptide signaling system led to the unravelling of the cause of narcolepsy. Two groups separately, but simultaneously, identified two new peptide hormones. The one group called them orexins (Sakurai et al., 1998) after the Greek word for appetite, ὄρεξις, based on the role the hormones supposedly played in the regulation of appetite and metabolism based on their location in the hypothalamus); the other called them hypocretins based on their amino acid sequence that somewhat resembles that of the gut hormone secretin (de Lecea et al., 1998). Hypocretin-1 and hypocretin-2 are peptides that are derived from a common precursor protein called preprohypocretin that is produced solely in a group of around 80,000 neurons that are located in the lateral and posterior hypothalamus, henceforth in this thesis called hypocretin-producing neurons. One year later, in 1999, it was discovered that a mutation in one of the receptors for these hormones, the hypocretin receptor 2, was the cause of narcolepsy in narcoleptic dogs (Lin et al., 1999). Subsequently, the selective loss of hypocretin-producing neurons in the hypothalamus of narcolepsy patients was found to be the cause of narcolepsy in humans (Nishino et al., 2001, Peyron et al., 2000). This discovery directed research in narcolepsy to the question what causes the selective loss of these hypocretin-producing neurons. In addition, the discovery of the hypocretins allowed for using the concentration of hypocretin-1 in the cerebrospinal fluid as a biomarker for narcolepsy (Ripley et al., 2001, Mignot et al., 2002). **Chapter 1** highlights a case that underscores the role of the development of hypocretin deficiency as the cause of NT1 symptoms.

HLA

As mentioned, in the early 1980s, it was found that Japanese narcolepsy patients all carried the HLA class I subtype DR2 (Juji et al., 1984), that is used by antigen-presenting cells of the immune system to present antigens to CD4+ T cells. This strong association was reproduced and it was found that the HLA-DQ subtype DQB1*06:02 is the most frequent subtype in narcolepsy across all ethnic groups (Mignot et al., 1994). This HLA-DQ subtype forms a haplotype with HLA-DQA1*01:02. It was reported that 85-95% of all NT1 patients carry this specific haplotype, compared to a frequency in the general population of

20-30%. In recent studies, the reported association with HLA-DQB1*06:02 in NT1 was found to be closer to an almost perfect association with close to 100% of NT1 patients expressing the HLA-DQB1*06:02 allele (Tafti et al., 2014, van der Heide et al., 2015b). In addition, homozygosity for the HLA-DQB1*06:02 subtype was reported to constitute an even higher risk for the development of NT1 than the presence of one HLA-DQB1*06:02 already does (Pelin et al., 1998). As a result, HLA-DQB1*06:02 has been considered as a genetic factor that is necessary but not sufficient to develop NT1. Apart from the strong association with HLA- DQB1*06:02, there are both positive and negative associations between HLA-DQB1 alleles and NT1: the frequency of HLA-DQB1*03:01 was found to be increased, whereas HLA-DQB1*02:01 (HLA-DQ2), HLA-DQB1*05:01, HLA-DQB1*06:01, HLA-DQB1*06:03 and HLA-DQB1*06:09 were decreased in NT1 patients as compared with healthy controls (Hong et al., 2007, Ollila et al., 2015, Tafti et al., 2014). Apart from HLA-DQ, also positive and negative associations with other HLA subtypes, both HLA class I and class II, such as HLA-DR, HLA-A, HLA-B and HLA-C are described in NT1 (Juvodden et al., 2019b, Lind et al., 2019) albeit not nearly as strong as the association with HLA-DQB1*06:02.

2009 H1N1 influenza A pandemic

The 2009 H1N1 influenza A pandemic brought about a new surge in interest in the autoimmune hypothesis of NT1. This influenza strain, also called Mexican or swine flu, infected and killed many people around the world. The emergence of the pandemic was shortly followed by vaccination programs that had to be established in a shorter time and on a bigger scale than ever before. One year later, an increase in the incidence of NT1 was observed. In several (Northern) European countries, but also in China, a steep increase in narcolepsy incidence compared to the decade before the pandemic was described (Dauvilliers et al., 2013, Feltelius et al., 2015, Lind et al., 2014, Partinen et al., 2012, Han et al., 2011). The role that influenza vaccines played in this increased incidence of NT1 was discussed and investigated widely. Only Pandemrix, a vaccine predominantly used in Scandinavian countries, was suggested to constitute a small, but significant, risk factor for the development of NT1 in these countries (Sarkanen et al., 2018).

Following these epidemiological and laboratory reports, a discussion on the existence of a post-H1N1 NT1 variant has arisen. Several recent Scandinavian

studies have sparked the discussion whether different immunological mechanisms may be involved in post-H1N1 NT1 cases (Juvodden et al., 2019a, Lind et al., 2019). These results would test the hypothesis that sporadic and post-H1N1 NT1 should be regarded as separate entities based on different immunological mechanisms leading to the disease. **Chapter 2** adds new information to this discussion.

Association of NT1 with other infections

Also other infections are associated with the development of NT1. Most notably, the finding of elevated anti-streptococcal antibody titres in recent-onset NT1 patients suggested an association with other upper airway infections than only H1N1 influenza, particularly β-hemolytic streptococcal infections (Aran et al., 2009). Several other studies report associations of NT1 with streptococcal infections (Lopes et al., 2015, Natarajan et al., 2013), flu or other respiratory tract infections (Picchioni et al., 2007), tick-borne encephalitis virus vaccination (Hidalgo et al., 2016) or proxies of infections in the patient, such as month of diagnosis (Han et al., 2011). In **Chapter 1**, we describe a case that developed NT1 shortly after a gastrointestinal infection. The exact role of infections other than H1N1 influenza remains to be elucidated. This is partly due to the fact that NT1 is a rare disease, but also because documentation on the presence of especially viral infections is poor, because many people do not consult a doctor for these infections.

The autoimmune hypothesis of narcolepsy

Figure 1 provides an overview of the autoimmune hypothesis of narcolepsy. The hypothesis is that antigens from outside the body (e.g. H1N1 influenza, *Streptococcus* species, vaccines) trigger the immune system. In people susceptible for developing NT1, these antigens are presented to CD4+ T cells by HLA-DQB1*06:02 on antigen-presenting cells. In these people, cross-reactive CD4+ T cells are present that are able to mount an immune response to both the foreign peptide and the hypocretin peptide which closely resembles this foreign peptide. When activated, these cross-reactive CD4+ T cells elicit an autoimmune response that via multiple suggested pathways (e.g. hypocretin peptide-specific cytotoxic CD8+ T cells or autoantibodies targeting hypocretin-producing neurons) eventually lead to the destruction of hypocretin-producing neurons in the lateral hypothalamus.

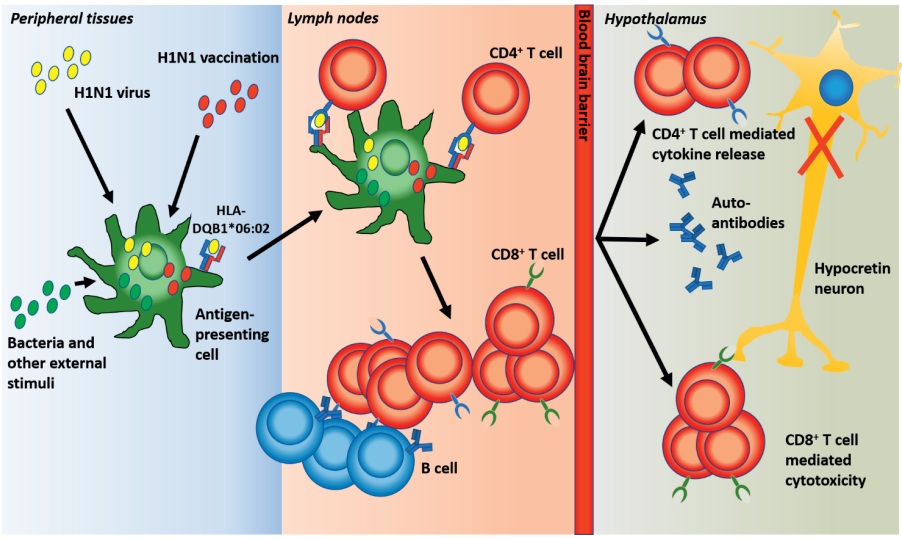


Figure 1. A depiction of the autoimmune hypothesis of narcolepsy, as described in the paragraph above.

Autoreactive immune cells

The hypothesis that cross-reactivity to the 2009 H1N1 influenza virus and hypocretin peptide has led to the development of NT1 in people at risk sparked the search for components of the immune system that are capable of mounting this cross-reactive immune response.

Since many autoimmune disease in the brain are mediated by autoantibodies, these seemed the most promising suspect to target hypocretin-producing neurons, leading to their destruction. However, no studies have been able to find an enrichment of autoantibodies specific for hypocretin or its precursor in NT1 (Giannoccaro et al., 2017, Luo et al., 2017, van der Heide et al., 2015a). Some studies report higher titres of autoantibodies that target antigens moderately specific for hypocretin-producing neurons or specific for peptides in the 2009 H1N1 influenza A virus in NT1 patients (Cvetkovic-Lopes et al., 2010, Lind et al., 2017, Saariaho et al., 2015, Thebault et al., 2015). Another study focusing on the human OX2 receptor reported the presence of a mimic peptide from the influenza nucleoprotein A, used in the 2009 H1N1 influenza A vaccination campaign, that shared protein residues with a fragment of the extracellular part of the OX2 receptor (Ahmed et al., 2015). Furthermore, this study demonstrates that antibodies present in NT1 patient serum cross-react with both the influenza nucleoprotein A and the extracellular part of the OX2 receptor. However, the

results of this study have been disputed by another group claiming that the OX2 receptor is not present on the surface of hypocretin-producing neurons and is therefore not a plausible target antigen for the autoimmune response leading to the development of NT1 (Vassalli et al., 2015).
Reports on T cells recognizing both H1N1 and hypocretin peptides have been published (De la Herran-Arita et al., 2013, Luo et al., 2018), but the first of these studies has subsequently been retracted (De la Herran-Arita and Garcia-Garcia, 2014). In **Chapter 3**, our experiments on cross-reactive T cells are presented. Recent studies have identified autoreactive T cells against hypocretin peptides (Latorre et al., 2018, Pedersen et al., 2019), but these cells were not HLA-DQB1*06:02 restricted. One study showed HLA-DQB1*06:02-restricted autoreactive T cells against hypocretin peptides, but they were found both in NT1 patients and in healthy controls (Jiang et al., 2019).

Genetics

As mentioned before, NT1 is strongly associated with the gene encoding HLA-DQB1*06:02 and other HLA type II genes. Also HLA type I genes, of which the gene product present antigens to cytotoxic CD8+ T cells, are associated with NT1, albeit these associations are considerably weaker than those with HLA type II genes (Ollila et al., 2015, Tafti et al., 2016). In addition to these HLA-associations, genome-wide association and gene sequencing studies have identified several other genes involved in the immune system that are associated with NT1: CTSH, that encodes pro-cathepsin H; P2RY11, that encodes a modulator of the autoimmune response to infection, p2Y purinoreceptor 11; and TNFSF4, that encodes tumor necrosis factor ligand superfamily member 4 (Faraco et al., 2013, Kornum et al., 2011, Tafti et al., 2014, Han et al., 2013). A recent study was the first to RNA sequence hypocretin-producing neurons in late embryonic mice, identifying the transcription of genes that distinguish hypocretin-producing neurons from adjacent melanin-concentrating hormone-producing neurons (Seifinejad et al., 2019). This could be a first step to identifying genes that, like hypocretin, are transcribed only in the hypocretin-producing neurons, and are therefore a plausible alternative target for an autoimmune response leading to the destruction of hypocretin-producing neurons leading to NT1.

Composition of the immune system in NT1

Another approach to identifying the components involved in the autoimmune response that destroys the hypocretin-producing neurons is to identify populations of immune cells that are enriched in NT1 patients compared with healthy controls. Preferably, this must be done in a way that is independent of the presumed autoantigen to focus solely on the immune cell populations that are present in NT1 patients and healthy controls. The composition of the immune system can either be tested in peripheral blood mononuclear cells or in cerebrospinal fluid. Several studies have compared the composition of the immune system in NT1 with that of healthy controls using flow cytometry in which a global T cell activation in peripheral blood of NT1 patients (Lecendreux et al., 2017, Moresco et al., 2018) and an even stronger T cell activation was seen in the cerebrospinal fluid compared with healthy controls (Moresco et al., 2018). In **Chapter 4**, we apply mass cytometry, a technique that is able to delineate immune cell subsets in the peripheral blood with unprecedented resolution, to assess the differences in immune cell composition between NT1 patients with recent symptom onset and HLA-DQB1*06:02-matched healthy controls.

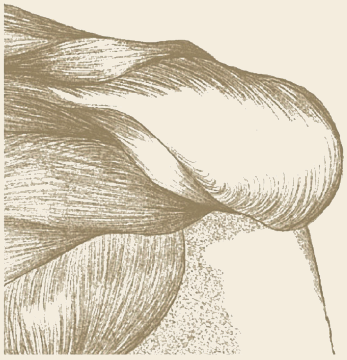
Clinical features of NT1

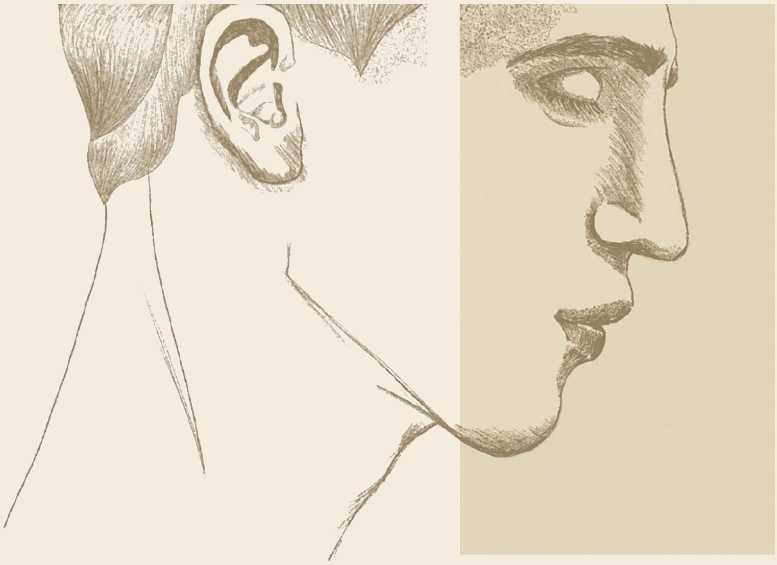
NT1 is traditionally characterized by its four core symptoms: excessive daytime sleepiness, cataplexy, sleep paralysis, hypnagogic hallucinations. Disturbed nocturnal sleep is generally considered to be the fifth core symptom (Black et al., 2017, Bassetti et al., 2019, Kornum et al., 2017b). Although this summary of core symptoms is widespread and acceptable for many sleep specialists, it frequently does not describe the burden of the disorder for a NT1 patient very well. A better picture of the disorder can be generated by focusing on the everyday life of a NT1 patient. With the development of the disorder, frequently in adolescence or shortly after, patients experience an inability to stay awake and concentrated for longer periods of time, interfering with social interaction, education and professional life throughout the years. This is often combined with the inability to stay asleep during the night. In addition, partial expressions of (non-)REM sleep phenomena during the day, such as automatic behavior (non-REM), cataplexy, hypnagogic hallucinations and sleep paralysis (REM), further complicate effective participation in societal life. Also, comorbidities and symptoms such as mood and anxiety disorders (Lopez et al., 2017, Ruoff et al., 2017), autonomic disturbances (Plazzi et al., 2011), apathy, fatigue (Nordstrand

et al., 2019b) and weight gain (Fronczek et al., 2008, Poli et al., 2009), that are frequently reported in NT1 add to the difficulties these patients experience. In **Chapter 5 and 6**, two of these frequently overlooked symptoms in NT1 patients are described.

Outline of the thesis

As can be deducted from the Chapter topics, the focus of this thesis is two-fold. The lion share is about unravelling the autoimmune hypothesis of narcolepsy, which comprises a case report that links the disappearance of hypocretin-1 in cerebrospinal fluid to the emergence of NT1 symptoms (**Chapter 1**), a study on the role of HLA (**Chapter 2**) and cross-reactive T cells (**Chapter 3**) in the autoimmune response leading to NT1 and a study on which immune cells are unique to NT1 patients (**Chapter 4**). The second part of this thesis focuses on symptoms of NT1 that are highly relevant in NT1 patient's everyday life: weight gain (**Chapter 5**) and daytime sleep state misperception (**Chapter 6**).





Chapter 1

The development of hypocretin deficiency in narcolepsy type 1 can be swift and closely linked to symptom onset: clues from a singular case

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Introduction

Narcolepsy type 1 (NT1) is associated with a selective and irreversible loss of >90% of neurons in the lateral hypothalamus that produce the sleep-wake regulating neuropeptides hypocretin-1 and -2 (Thannickal et al., 2000, Peyron et al., 2000). The hypocretin-1 concentration in the cerebrospinal fluid (CSF) serves as a biomarker for the presence or absence of these hypocretin-producing neurons (Nishino et al., 2000b). Therefore, this measurement is part of the diagnostic criteria for NT1 and can be performed as an alternative to the frequently used polysomnography with multiple sleep latency test when there is a clinical suspicion of NT1.
Several case reports show that shortly after narcoleptic symptoms arise the hypocretin-producing neuronal loss is severe enough to lead to very low or undetectable CSF hypocretin-1 concentrations (Kubota et al., 2003, Kanbayashi et al., 2002). However, there is hardly any information about how long it may take before the critical number of cells is lost or how long it takes before severe hypocretin-1 deficiency leads to clinical symptoms. We describe a case that may shed (more) light on this lack of knowledge.

Report of case

In May 2017, we diagnosed a 17-year old Dutch girl with NT1 at the outpatient clinic of the Sleep-Wake Centre SEIN in Heemstede, the Netherlands. She had previously been healthy except for a hospital admission in September 2016 because of high fever, and a suspicion of a viral meningitis. Because of this suspicion, a lumbar puncture was performed. Analysis of the CSF showed a normal cell count and normal protein and glucose levels, excluding a viral meningitis. According to the hospital routine procedures, excess CSF was stored for later use, if necessary. Eventually the fever was attributed to a PCR-confirmed enteroviral gastroenteritis. Since there were no respiratory or dermatologic symptoms, the clinical suspicion of an infection with *Streptococcus pyogenes* was low and further diagnostic testing was not performed.
Within weeks after her dismissal from the hospital in September 2016 she developed severe excessive daytime sleepiness. In December 2016 typical cataplectic attacks (knee-buckling and later general atonia with falls triggered

by laughter and being angry) started to occur. These attacks lasted several seconds, and up to several minutes when the trigger remained present. She also experienced hypnagogic hallucinations, disrupted nocturnal sleep, sleep paralysis and gained 7 kilograms in several months.
When she visited our institution in March 2017, she had high scores on both the Epworth Sleepiness Scale (ESS; 19/24) and the Fatigue Severity Scale (FSS; 6/7). HLA-typing showed HLA-DQB1*06:02 positivity. Sleep investigation results were also supportive for the diagnosis of NT1: a polysomnography with a sleep onset REM period (SOREMP) was followed by a mean sleep latency on MSLT of 2.9 minutes with SOREMPs in 3 out of 5 naps.

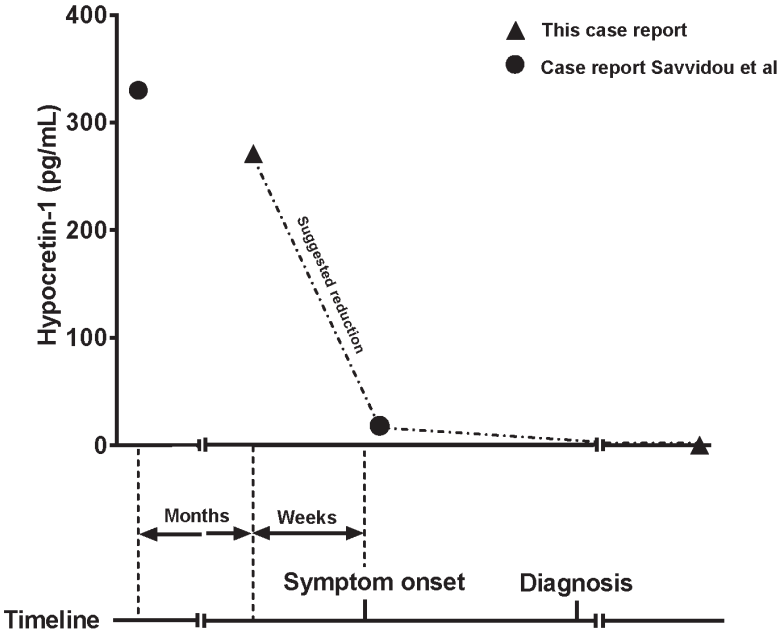


Figure 1.1. This figure visualizes the suggested steep and swift decrease in cerebrospinal fluid hypocretin-1 concentrations that is closely linked to symptom onset, based on this case report combined with previous reports (Savvidou et al., 2013, Kanbayashi et al., 2002, Kubota et al., 2003). Hypocretin-1 concentration was in the normal range only weeks before symptom onset.

We were allowed to use the previously stored CSF for hypocretin-1 measurement. A hypocretin-1 concentration of 271 pg/mL was detected. Hypocretin-1 concentrations were measured in duplicate with a iodine-125 hypocretin-1 radioimmunoassay (Phoenix Pharmaceuticals, Mountain View, CA, USA). This

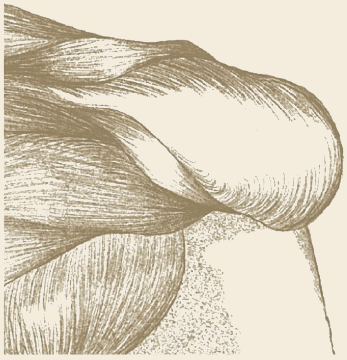
assay has a detection limit of 50 pg/mL and an intra-assay variability of <5%. To adjust for inter-assay variability to previously reported values, Stanford reference CSF samples were included in the assay (Ripley et al., 2001).
Because of this finding, a second lumbar puncture was performed in March 2018, after informed consent, that showed an undetectable hypocretin-1 level in the CSF, which confirmed the diagnosis of NT1. Hypocretin-1 measurements in both CSF samples of this patient were repeated in duplicate in one assay, to rule out inter-assay variability as an explanation for the observed difference between both samples. In line with the earlier measurements, hypocretin-1 levels before and after symptom onset were 272 and 0 pg/mL, respectively.

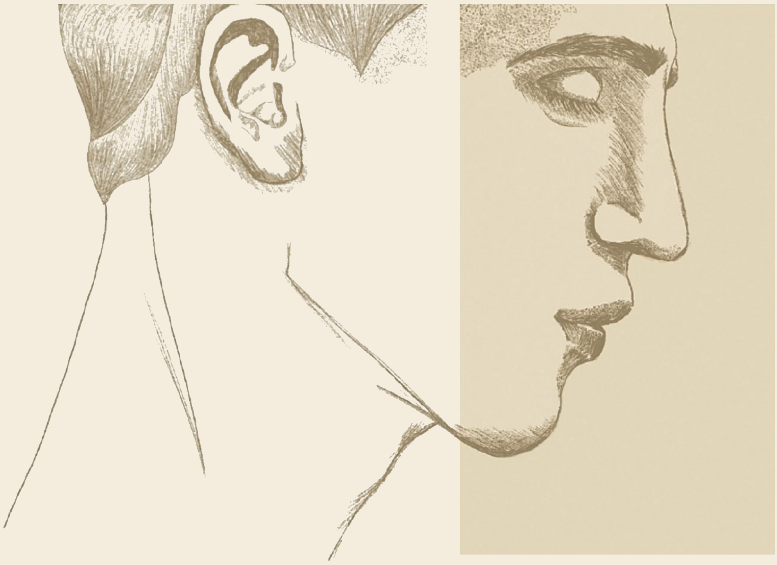
Discussion

The disease phenotype and sleep laboratory findings in this patient are characteristic of sporadic pediatric NT1 (Postiglione et al., 2018). Interestingly, the first NT1 symptoms arose shortly after a normal hypocretin-1 level was measured in the CSF of this patient. This adds to the knowledge from previous case reports that hypocretin-1 deficiency could already be measured within weeks after symptom onset (Kubota et al., 2003, Kanbayashi et al., 2002). Another case report also shows both a hypocretin-1 measurement prior to and several months after symptom onset (Savvidou et al., 2013). Since the first measurement was more than a year before symptom onset, it remained largely unclear how long it took for the hypocretin-producing neurons to disappear in that case. The short interval between hypocretin-1 measurement and symptom onset in this case suggests that the decrease in hypocretin-1 concentration from normal to undetectable levels may be a process lasting only a few weeks or even less.
The onset of symptoms can be gradually in NT1, but also subacute, as in this case. The latter course of symptom onset is described particularly in pediatric (Postiglione et al., 2018) and post-H1N1 influenza narcolepsy cases (Sarkanen et al., 2016). This pediatric patient was not vaccinated in the 2009 H1N1 vaccination campaign. There was also no indication that other environmental triggers presumed to be linked to NT1 symptom onset (such as *Streptococcus pyogenes* (Ambati et al., 2015)) were present in this patient. However, the enteroviral infection that led to the patient's admission to a hospital for several days might constitute an environmental trigger, that has not been described

previously. This case thereby supports the theorem that multiple different environmental triggers are able to cause the presumed autoimmune reaction ultimately leading to the destruction of hypocretin-producing neurons to occur in genetically predisposed individuals.

This case combined with earlier reports suggests that the presumed autoimmune reaction resulting in the onset of NT1 can be swift and closely linked to symptom onset (Figure 1.1). Thereby, it stresses the importance of performing experiments on the autoimmune hypothesis of NT1 shortly after symptom onset. Additionally, this case highlights the importance of focusing future research on the final common pathophysiological pathway of NT1 to be able to identify individuals at-risk for developing the disease, rather than addressing the diversity of environmental triggers, of which we might only have identified the top of the iceberg.





Chapter 2

HLA associations in narcolepsy type 1 persist after the 2009 H1N1 pandemic

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Abstract

We aimed to compare HLA-DQB1-associations in narcolepsy type 1 patients with disease onset before and after the 2009 H1N1 pandemic in a large Dutch cohort. 525 narcolepsy type 1 patients and 1272 HLA-DQB1*06:02-positive healthy controls were included, because of the discussion that has arisen on the existence of sporadic and post-H1N1 narcolepsy type 1. HLA-DQB1-associations in pre- and post-H1N1 narcolepsy type 1 patients were compared. The associations between HLA-DQB1 alleles and narcolepsy type 1 were not significantly different between pre- and post-H1N1 narcolepsy type 1 patients. Both HLA-DQB1-associations with pre- and -post H1N1 narcolepsy type 1 reported in recent smaller studies were replicated. Our findings combine the results of studies in pre- and post-H1N1 narcolepsy type 1 and argue against considering post-H1N1 narcolepsy type 1 as a different entity.

Introduction

Narcolepsy type 1 (NT1) is a rare disorder of the regulation of sleep and wakefulness with an incidence of 1 per 100,000 person years and prevalence ranging between 20 and 50 per 100,000 individuals (Wijnans et al., 2013). NT1 is characterised by five core symptoms: excessive daytime sleepiness, cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nocturnal sleep. These symptoms arise as a result of the presumed destruction of over 90 percent of hypocretin (Hcrt)-producing neurons in the lateral hypothalamus hypothesized as being caused by an autoimmune response (Bassetti et al., 2019, Kornum et al., 2017b).

95% of NT1 patients carry the *HLA-DQA1*01:02 / DQB1*06:02* haplotype encoding HLA-DQB1*06:02, an HLA-class II molecule expressed on antigen presenting cells (Tafti et al., 2014). HLA-DQB1*06:02 is also present in about 20% of the general European population. As a result, HLA-DQB1*06:02 has been considered as a genetic factor necessary but not sufficient for development of NT1. Apart from the well-known association with HLA-DQB1*06:02, there are positive and negative associations between other HLA-DQB1 alleles and NT1: the frequency of HLA-DQB1*03:01 (HLA-DQ7) was found to be increased, whereas HLA-DQB1*02:01 (HLA-DQ2), HLA-DQB1*05:01, HLA-DQB1*06:01, HLA-DQB1*06:03 and HLA-DQB1*06:09 were decreased in NT1 patients compared with healthy controls (Hong et al., 2007, Ollila et al., 2015, Tafti et al., 2014).

An increase in the incidence of NT1 has been observed in several European countries since the 2009 influenza A(H1N1) pdm09 pandemic, and the subsequent vaccination campaign (Dauvilliers et al., 2013, Feltelius et al., 2015, Lind et al., 2014, Partinen et al., 2012). Recent studies have identified autoreactive T cells against hypocretin peptides (Latorre et al., 2018, Pedersen et al., 2019), but their reactivity was not HLA-DQB1*06:02 restricted. Reports on T cells recognizing both H1N1 and hypocretin peptides show conflicting results (Luo et al., 2018, Schinkelshoek et al., 2019), with detected T cell cross-reactivity in the first study not being replicated in the second.

Following these epidemiological and laboratory reports, a discussion on the existence of a post-H1N1 NT1 variant has arisen, with specifically Scandinavian countries making the distinction between sporadic and post-H1N1 NT1. Several recent Scandinavian studies report HLA-DQ-haplotypes associated specifically with post-H1N1 NT1 (HLA-DQ2, HLA-DQ7) (Juvodden et al.,

2019b, Lind et al., 2019). These reports started a discussion about whether different immunological mechanisms may be involved in post-H1N1 NT1.
Since these studies are small and only address HLA-DQB1-associations in a cohort of post-H1N1 NT1 patients and a healthy control population, our aim is to compare HLA-DQB1-associations in NT1 patients with disease onset before and after the 2009 H1N1 pandemic in a large Dutch cohort to assess whether differences can be seen which would test the hypothesis that sporadic and post-H1N1 NT1 should be regarded as separate entities based on different immunological mechanisms leading to the disease.

Materials and methods

Participants

We included all NT1 patients of European ancestry who, between 2005 and 2019, were HLA-genotyped for clinical care in the laboratory of the Leiden University Medical Center. Patients came from the sleep clinic of the Department of Neurology, Leiden University Medical Center, the Sleep-Wake Centre of Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede and Zwolle, and the Sleep Centre of Kempenhaeghe, Heeze. All patients were diagnosed with either narcolepsy with cataplexy or NT1 according to the International Classification of Sleep Disorders (ICSD-2 or ICSD-3; (ICSD, 2005, ICSD, 2014)). Age at symptom onset was assessed in all patients to separate those with symptom onset in childhood (<16 years at symptom onset) or adulthood, and we also noted whether the symptoms of NT1 started before or after the 2009 H1N1 pandemic to be able to compare HLA-DQB1-associations before and after the H1N1 pandemic. We also included a cohort of HLA-DQB1*06:02 positive non-related healthy individuals from a panel of randomly selected Dutch individuals (van Rooijen et al., 2012) to be able to assess HLA associations in NT1.

HLA typing

HLA genotyping for HLA-DR and HLA-DQ was performed with a reversed approach of the PCR-sequence-specific oligonucleotide probe technique previously described (Verduyn et al., 1993). If a rare DRB1-DQB1 was found, typing results were confirmed by PCR-SBT, using the SBT Excellerator HLA-DRB and -DQB kit (Genome Diagnostics, Utrecht, The Netherlands).

Statistical analysis

Pearson's chi-square tests were used for comparisons. Odds ratios and corresponding 95 % confidence intervals (CI) were calculated using Cochran-Mantel-Haenszel tests (Cochran, 1954, Mantel, 1963, Mantel and Haenszel, 1959). We compared the frequency of HLA-DQB1 alleles between NT1 patients and healthy controls who were HLA-DQB1*06:02 positive. We also compared the frequency of HLA-DQB1 alleles between NT1 patients with disease onset before and after the 2009 H1N1 pandemic. As the increase in NT1 incidence was reported predominantly in children with NT1, we assessed whether onset during childhood was associated with a change in HLA-DQB1-associations after the H1N1 pandemic.

Since one of our aims was specifically to replicate the results of other studies describing HLA-DQB1-associations in post-H1N1 NT1 patients, for these specific analyses corrections for multiple comparisons were not performed. For all other analyses, p-values were corrected for multiple comparisons according to the Šidák-Holm method (Šidák, 1967). Differences with p-values below 0.05 were deemed significant. All analyses were conducted using the IBM SPSS Statistics 25 software package. It must be noted that due to a high degree of linkage disequilibrium in the HLA complex, the tested alleles and loci are not independent.

Table 2.1. Narcolepsy type 1 patient characteristics.

	Pre-H1N1	Post-H1N1
N	397	128
Males	46%	49%
Age at disease onset (years)	19 (3-67)	14 (2-81)
Disease onset during childhood (<16 years)	31%	52%
Hcrt-1 < 110 pg/mL	110/114 (97%)	40/43 (93%)
Hcrt-1 < 200 pg/mL	114/114 (100%)	43/43 (100%)
HLA- DQB1*06:02 positive (n)	396/397 (100%)	129/130 (99%)
H1N1 vaccination (n)	NA	13/29 (45%)
Proven H1N1 infection (n)	NA	1

Data on age at disease onset are shown as median and range. Data are shown as a percentage of patients in which data was available.

H1N1 = H1N1 influenza pandemic in 2009; Hcrt-1 = hypocretin-1; HLA-DQB1*06:02 = human leucocyte antigen molecule encoded by the HLA-DQB1*06:02 allele; NA = not applicable.

Results

Participant characteristics

We included 525 NT1 patients (128 post-H1N1) and 1272 HLA-DQB1*06:02-positive controls. Participant characteristics are shown in Table 2.1. Notably, all but two NT1 patients were DQB1*06:02 positive. 158 patients had undergone a lumbar puncture for Hcrt-1 measurement; over 95% had Hcrt-1 values in the cerebrospinal fluid that were below the cut-off value of 110 pg/mL; all had Hcrt-1 values below 200 pg/mL.

Table 2.2. Differences between HLA-DQB1 alleles in trans with HLA-DQB1*06:02 between NT1 patients compared with HLA-DQB1*06:02-positive healthy controls. P-values are derived from Pearson Chi-Square tests. Pc-values are calculated using the Šidák-Holm correction for multiple comparisons based on k = 13.

	Healthy controls	NT1	OR	95% CI	p-value	pc-value
N	**1272**	**525**	**NA**	**NA**	**NA**	**NA**
DQ2	315 (24.8%)	82 (15.6%)	0.56	0.43-0.73	<0.001	<0.001
DQ4	37 (2.9%)	24 (4.6%)	1.6	0.94-2.7	0.079	0.658
DQ7	216 (17.0%)	130 (24.8%)	1.6	1.3-2.1	<0.001	0.002
DQ8	137 (10.7%)	56 (10.6%)	0.99	0.72-1.4	0.97	1
DQ9	67 (5.3%)	21 (4.0%)	0.75	0.45-1.2	0.251	0.977
DQB1*05:01	187 (14.7%)	35 (6.6%)	0.42	0.28-0.60	<0.001	<0.001
DQB1*05:02	20 (1.6%)	22 (4.2%)	2.7	1.5-5.0	0.001	0.011
DQB1*05:03	36 (2.8%)	18 (3.4%)	1.2	0.69-2.2	0.499	1
DQB1*06:01	5 (0.4%)	2 (0.4%)	0.97	0.19-5.0	0.966	1
DQB1*06:02	91 (7.2%)	85 (16.1%)	2.5	1.8-3.4	<0.001	<0.001
DQB1*06:03	86 (6.8%)	6 (1.1%)	0.16	0.07-0.37	<0.001	<0.001
DQB1*06:04	64 (5.0%)	41 (7.8%)	1.6	1.0-2.4	0.024	0.267
DQB1*06:09	11 (0.9%)	3 (0.6%)	0.66	0.18-2.4	0.516	1

HLA-DQ2/4/7/8/9 = human leucocyte antigen molecule encoded by the HLA-DQB1*02:01/-DQB1*04:01/-DQB1*03:01 or -03:04/-DQB1*03:02 or -03:05/-DQB1*03:03 allele; NT1 = narcolepsy type 1; NA = not applicable; OR = Mantel-Haenszel common odds ratio; pc-value = corrected p-value.

The frequency of HLA-DQB1*06:02 homozygosity is unchanged between pre- and post-H1N1 NT1 patients

The important role of HLA-DQB1*06:02 in NT1 is demonstrated by its presence in almost all pre- and post-H1N1 NT1 patients (396/397 patients vs. 127/128 patients). Homozygosity for HLA-DQB1*06:02 is associated with NT1 with an odds ratio of 2.5 (7.2% in the healthy control population vs 16.2% in NT1 patients; Table 2.2). Furthermore, the percentage of NT1 patients homozygous

for HLA-DQB1*06:02 is unchanged between pre- and post-H1N1 NT1 patients with 16.4% in pre-H1N1 NT1 patients and 15.6% in post-H1N1 NT1 patients (p = 0.79; Table 2.3).

Table 2.3. Differences in distribution of HLA-DQB1 alleles in trans with HLA-DQB1*06:02 between pre- and post-H1N1 NT1 patients. P-values are derived from Pearson Chi-Square tests. Pc-values are calculated using the Šidák-Holm correction for multiple comparisons based on k = 13.

	Pre-H1N1	Post-H1N1	OR	95% CI	p-value	pc-value
N	**397**	**128**	**NA**	**NA**	**NA**	**NA**
DQ2	62 (15.6%)	20 (15.4%)	0.98	0.57-1.7	0.949	1
DQ4	18 (4.5%)	6 (4.6%)	1.0	0.40-2.6	0.969	1
DQ7	95 (23.9%)	35 (27.3%)	1.2	0.76-1.9	0.436	0.999
DQ8	41 (10.3%)	15 (11.5%)	1.1	0.60-2.1	0.697	1
DQ9	19 (4.8%)	2 (1.5%)	0.31	0.07-1.4	0.100	0.747
DQB1*05:01	29 (7.3%)	6 (4.6%)	0.61	0.25-1.5	0.285	0.987
DQB1*05:02	18 (4.5%)	4 (3.1%)	0.67	0.22-2.0	0.520	1
DQB1*05:03	12 (3.0%)	6 (4.7%)	1.6	0.58-4.3	0.368	0.997
DQB1*06:01	2 (0.5%)	0 (0%)	NA	NA	0.417	0.999
DQB1*06:02	65 (16.4%)	20 (15.4%)	0.93	0.54-1.6	0.790	1
DQB1*06:03	5 (1.3%)	1 (0.8%)	0.61	0.07-5.3	0.647	1
DQB1*06:04	28 (7.1%)	13 (10.0%)	1.5	0.74-2.9	0.276	0.985
DQB1*06:09	3 (0.8%)	0 (0.0%)	NA	NA	0.320	0.993

H1N1 = 2009 pandemic (H1N1)pdm09 influenza A strain; HLA-DQ2/4/7/8/9 = human leucocyte antigen molecule encoded by the HLA-DQB1*02:01/-DQB1*04:01/-DQB1*03:01 or -03:04/-DQB1*03:02 or -03:05/-DQB1*03:03 allele; NA = not applicable; NT1 = narcolepsy type 1; OR = Mantel-Haenszel common odds ratio; pc-value = corrected p-value.

Multiple HLA-DQ alleles other than HLA-DQB1*06:02 are associated with NT1

In addition to the association between HLA-DQB1*06:02 and NT1, Table 2.2 gives an overview of other HLA-DQ alleles and their association with NT1. HLA-DQ2, HLA-DQB1*05:01 and HLA-DQB1*06:03 are negatively associated with NT1, while HLA-DQ7 and HLA-DQB1*05:02 are more frequently found in NT1 patients than in healthy controls (Figure 2.1).

No differences in HLA-DQB1 alleles in trans with HLA-DQB1*06:02 between pre- and post-H1N1 NT1 patients

HLA-DQ alleles in trans with HLA-DQB1*06:02 are shown for pre- and post-H1N1 NT1 patients separately in Table 2.3. For all alleles in trans with HLA-DQB1*06:02 no difference was shown between patients with symptom onset before the H1N1 pandemic and those with symptom onset after 2009 (Figure 2.1)

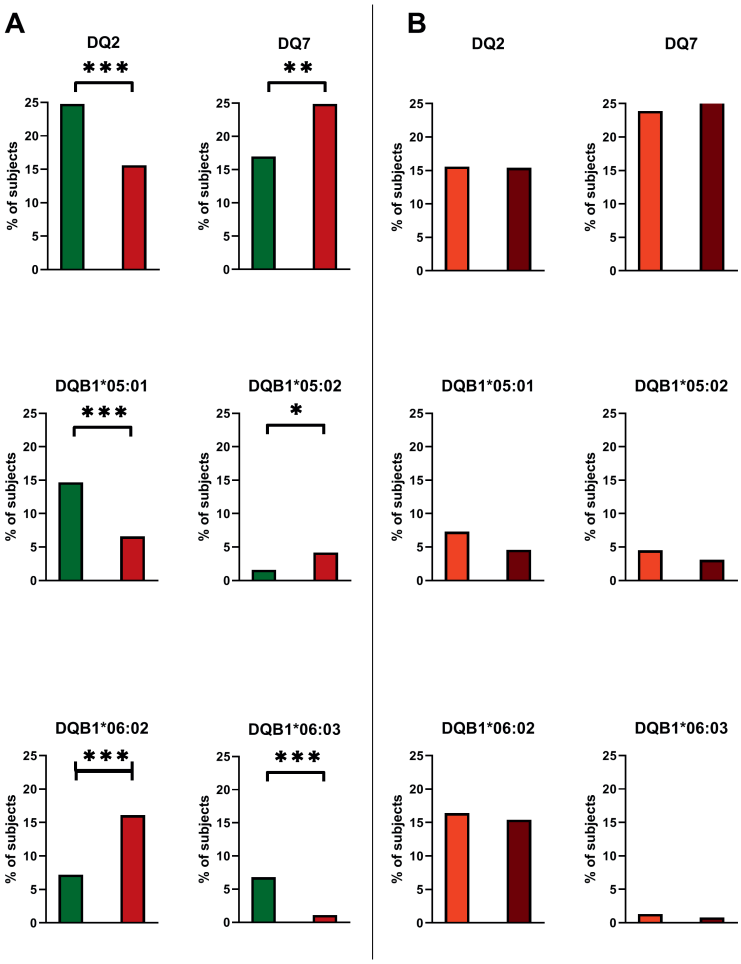


Figure 2.1. A. Visualization of HLA-DQB1 allele frequencies that are significantly different between NT1 patients (red) and HLA-DQB1*06:02-positive healthy controls (green). B. Visualization of HLA-DQB1 allele frequencies that are significantly different between pre- (bright red) and post-H1N1 (dark red) NT1 patients.

HLA-DQ2/4/7/8/9 = human leucocyte antigen molecule encoded by the HLA-DQB1*02:01/-DQB1*04:01/-DQB1*03:01 or -03:04/-DQB1*03:02 or -03:05/-DQB1*03:03 allele; NT1 = narcolepsy type 1; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

HLA-DQB1-associations in post-H1N1 NT1 patients with disease onset during childhood are not different than those in other NT1 patients

No significant changes were demonstrated comparing those with disease onset after H1N1 and onset in childhood with all others with NT1 (Supplementary table 2.1).

Discussion

The associations between HLA-DQB1 alleles and NT1 in this large Dutch cohort have not significantly changed since the H1N1 pandemic in 2009 and the vaccination program following the pandemic. The percentage of NT1 patients that is homozygous for HLA-DQB1*06:02 has remained unchanged and this strongly argues against considering sporadic and H1N1 NT1 to be separate entities with a separate (immune) aetiology.

One study in a Chinese cohort comparing HLA-DQB1-associations in pre- and post-H1N1 NT1 patients found a decrease in the percentage of NT1 patients that was homozygous for HLA-DQB1*06:02 since the 2009 H1N1 pandemic (Han et al., 2013). Differences in HLA-associations in populations from different ethnic background have been reported (Mignot et al., 2001), which may explain these contradictory findings. The positive association between NT1 and HLA-DQ7 and HLA-DQB1*05:02 persist after the H1N1 pandemic. The same goes for negative associations between NT1 and HLA-DQ2, HLA-DQB1*05:01 and HLA-DQB1*06:03.

These findings are in line with those of two recent reports describing these associations in small numbers of post-H1N1 NT1 patients that report positive associations of post-H1N1 with HLA-DQ7 and negative associations with HLA-DQ2 (Lind et al., 2019, Juvodden et al., 2019b). Both these and other studies in NT1 patients before and after the H1N1 pandemic also describe the same associations that we found (Mignot et al., 2001, van der Heide et al., 2015b, Hong et al., 2007, Tafti et al., 2014, Bomfim et al., 2017, Tafti et al., 2016, Han et al., 2013).

The negative association between NT1 and DQB1*06:01 previously reported (Mignot et al., 2001, Hong et al., 2007), were not replicated in the current study. This might be explained by the abundancy of this HLA-DQ allele in the Asian population, compared to the almost absence of this allele in people of European ancestry. Other associations of NT1 (either positive or negative), with HLA-DQ8 (Hong et al., 2007, Bomfim et al., 2017), HLA-DQB1*06:04 (Hong et al., 2007) and HLA-DQB1*06:09 (Tafti et al., 2014, Bomfim et al., 2017) were also not replicated in the current study.

One of the limitations in our study is that we have not been able to distinguish between post-H1N1 NT1 patients and healthy controls who encountered the H1N1 influenza virus and those who did not. We have data on H1N1 exposure and vaccination for only a small subset of all participants included, which can be found in Table 2.1. Given the fact that the H1N1 vaccination coverage in the Netherlands was 30% for the general population and 74% for children (Mereckiene et al., 2012), it is likely that at least a significant percentage of patients with symptom onset after 2009 had encountered the H1N1 virus or were vaccinated against H1N1 during the vaccination campaign. Another factor to consider is the hypothesized post-vaccination risk window, that is defined as a period of two years after the H1N1 vaccination campaign in which the risk of developing NT1 was increased (Sarkanen et al., 2018). Only a small group of participants in the current study were diagnosed in 2010 and 2011, which makes a separate analysis on this group unfeasible. Data on this group is reported in Supplementary table 2.2.

Our findings bridge the findings of studies in sporadic and post-H1N1. In addition to replicating HLA-associations reported in studies focusing on either sporadic NT1 or post-H1N1 NT1 patients, we show that these HLA-DQB1-associations can be found in both groups. Focusing on performing research on the autoimmune hypothesis of NT1 in NT1 patients shortly after symptom onset seems more promising than directing further research to the difference between sporadic and post-H1N1 NT1.

Conclusions

No differences in HLA-associations were found between NT1 patients with symptom onset before and those with symptom onset after the 2009 H1N1 pandemic. The positive association of HLA-DQ7 and negative associations of HLA-DQ2, HLA-DQB1*05:01 and DQB1*06:03 with both pre- and post-H1N1 described in other smaller studies were confirmed in this large Dutch cohort. These results therefore argue against considering sporadic and post-H1N1 NT1 to be separate entities.

Supplementary material

Supplementary table 2.1. Differences in distribution of HLA alleles in trans with HLA-DQB1*06:02 between NT1 patients with symptom onset in childhood after the H1N1 pandemic and the other included NT1 patients. P-values are derived from Pearson Chi-Square tests. Pc-values are calculated using the Šidák-Holm correction for multiple comparisons based on k = 13.

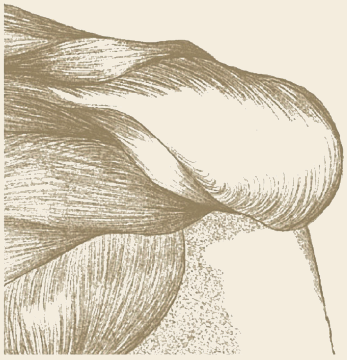
	Post-H1N1 and symptom onset in childhood	Other NT1 patients	OR	95% CI	p-value	pc-value
N	**66**	**459**	**NA**	**NA**	**NA**	**NA**
DQ2	8 (11.9%)	74 (16.1%)	0.71	0.32-1.5	0.382	0.998
DQ4	1 (1.5%)	23 (5.0%)	0.29	0.04-2.2	0.198	0.943
DQ7	22 (33.3%)	108 (23.5%)	1.6	0.93-2.8	0.084	0.682
DQ8	9 (13.4%)	47 (10.2%)	1.1	0.60-2.1	0.425	0.999
DQ9	1 (1.5%)	20 (4.3%)	0.33	0.04-2.5	0.264	0.981
DQB1*05:01	1 (1.5%)	34 (7.4%)	0.19	0.03-1.4	0.070	0.611
DQB1*05:02	2 (3.0%)	20 (4.4%)	0.68	0.16-3.0	0.602	1.000
DQB1*05:03	1 (1.5%)	17 (3.7%)	0.40	0.05-3.1	0.361	0.997
DQB1*06:01	0 (0%)	2 (0.4%)	NA	NA	0.589	1.000
DQB1*06:02	13 (19.7%)	72 (15.7%)	1.3	0.67-2.5	0.435	0.999
DQB1*06:03	1 (1.5%)	5 (1.1%)	1.4	0.16-12.0	0.770	1.000
DQB1*06:04	7 (10.4%)	34 (7.4%)	1.5	0.62-3.4	0.383	0.998
DQB1*06:09	0 (0 %)	3 (0.7%)	NA	NA	0.507	1.000

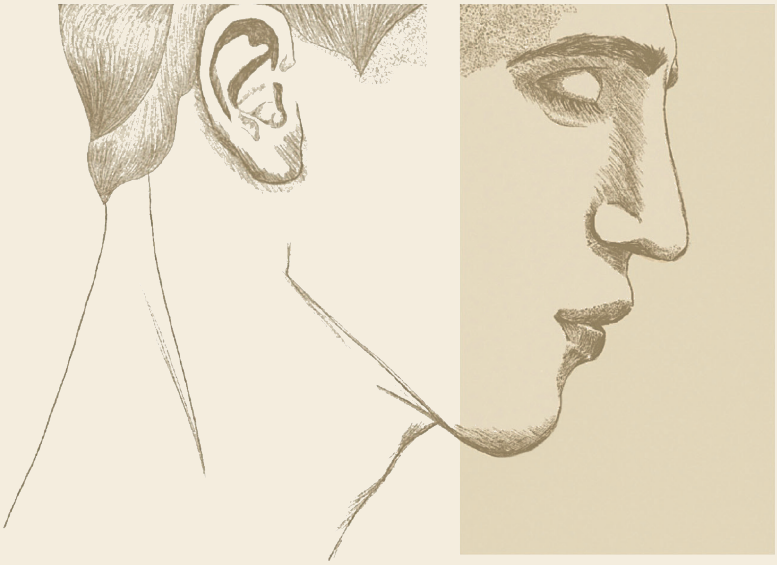
H1N1 = 2009 pandemic (H1N1)pdm09 influenza A strain; HLA-DQ2/4/7/8/9 = human leucocyte antigen molecule encoded by the HLA-DQB1*02:01/- DQB1*04:01/-DQB1*03:01 or -03:04/-DQB1*03:02 or -03:05/- DQB1*03:03 allele; NA = not applicable; NT1 = narcolepsy type 1; OR = Mantel-Haenszel common odds ratio; pc-value = corrected p-value.

Supplementary table 2.2. Distribution of HLA-DQB1 alleles in trans with HLA-DQB1*06:02 of narcolepsy type 1 patients diagnosed in 2010 and 2011.

	NT1 diagnosis in 2010-2011	Adults	Children
N	**47**	**21**	**26**
DQ2	9 (19.1%)	3 (14.3%)	6 (23.1%)
DQ4	2 (4.3%)	1 (4.8%)	1 (3.8%)
DQ7	12 (25.5%)	5 (23.8%)	7 (26.9%)
DQ8	6 (12.8%)	1 (4.8%)	5 (19.2%)
DQ9	0 (0%)	0 (0%)	0 (0%)
DQB1*05:01	5 (10.6%)	4 (19.0%)	1 (3.8%)
DQB1*05:02	0 (0%)	0 (0%)	0 (0%)
DQB1*05:03	2 (4.3%)	2 (9.5%)	0 (0%)
DQB1*06:01	0 (0%)	0 (0%)	0 (0%)
DQB1*06:02	7 (14.9%)	4 (19.0%)	3 (11.5%)
DQB1*06:03	1 (2.1%)	0 (0%)	1 (3.8%)
DQB1*06:04	3 (6.4%)	1 (4.8%)	2 (7.7%)
DQB1*06:09	0 (0%)	0 (0.0%)	0 (0.0%)

HLA-DQ2/4/7/8/9 = human leucocyte antigen molecule encoded by the HLA-DQB1*02:01/-DQB1*04:01/-DQB1*03:01 or -03:04/-DQB1*03:02 or -03:05/-DQB1*03:03 allele; NT1 = narcolepsy type 1.





Chapter 3

H1N1 hemagglutinin-specific HLA-DQ6-restricted CD4+ T cells can be readily detected in narcolepsy type 1 patients and healthy controls

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Abstract

Following the 2009 H1N1 influenza pandemic, an increased risk of narcolepsy type 1 was observed. Homology between an H1N1 hemagglutinin and two hypocretin sequences has been reported.

T cell reactivity to these peptides was assessed in 81 narcolepsy type 1 patients and 19 HLA-DQ6-matched healthy controls.

HLA-DQ6-restricted H1N1 hemagglutinin-specific T cell responses were detected in 28.4% of patients and 15.8% of controls. Despite structural homology between HLA-DQ6-hypocretin and -H1N1 peptide complexes, T cell cross-reactivity was not detected.

These results indicate that it is unlikely that cross-reactivity between H1N1 hemagglutinin and hypocretin peptides presented by HLA-DQ6 is involved in the development of narcolepsy type 1.

Introduction

Narcolepsy type 1 (NT1) is a rare disorder of the regulation of sleep and wakefulness with an incidence of 1 per 100,000 person years and a prevalence ranging between 20-50 per 100,000 individuals (Ohayon et al., 2002, Wijnans et al., 2013). The disorder is characterised by five core symptoms: excessive daytime sleepiness, cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nocturnal sleep. These symptoms arise as a result of the destruction of over 90 percent of hypocretin (Hcrt)-producing neurons in the lateral hypothalamus (Peyron et al., 2000, Thannickal et al., 2000). Unfortunately, causal treatment of the disorder is not yet available.

In pursuit of the disease mechanism, two findings have shifted the focus of narcolepsy research to the hypothesis that the destruction of Hcrt-producing neurons is caused by an autoimmune process. First, 95% of NT1 patients carry the *HLA-DQA1*01:02 / DQB1*06:02* haplotype encoding HLA-DQ6, an HLA-class II molecule expressed on antigen-presenting cells (Juji et al., 1984, Mignot et al., 1997, Tafti et al., 2014), which was later complemented by genome-wide association studies that showed variants within immune system-regulating genes in NT1 patients (Faraco et al., 2013, Han et al., 2013, Hor et al., 2010). Second, an increase in the incidence of NT1 has been observed in several European countries after the 2009 H1N1 influenza pandemic, and the subsequent vaccination campaign (Dauvilliers et al., 2013, Feltelius et al., 2015, Lind et al., 2014, Partinen et al., 2012). Even though there was no wide-spread vaccination campaign in Asian countries, an increased incidence has also been reported in China (Han et al., 2011). This suggests that NT1 might develop as the result of a cross-reactive anti-viral immune response that leads to the destruction of Hcrt-producing neurons. More insight in this reaction could pave the way for causal treatment development in NT1 and potentially also prevention of the disease by identifying individuals at-risk.

Research on the autoimmune reaction has focused on two candidate immune cell types that could drive this reaction leading to NT1: cross-reactive B cells and cross- or autoreactive CD4+ T cells. Four studies (Black et al., 2005, Overeem et al., 2006, Tanaka et al., 2006, van der Heide et al., 2015a) failed to detect autoreactive B cells or autoantibodies to Hcrt or Hcrt-receptors. Another group claimed to have identified autoreactive CD4+ T cells specific for Hcrt (De la Herran-Arita et al., 2013, De la Herran-Arita et al., 2014), but the article was subsequently retracted. While HLA-DR-restricted Hcrt-specific

T cell responses have recently been described (Latorre et al., 2018), this does not explain the strong link with HLA-DQ6. In short, the molecular mechanism underlying the HLA-DQ6 association remains to be determined.
Based on the increased incidence of NT1 after the H1N1 influenza pandemic mentioned above, we aimed to assess whether H1N1 influenza reactive CD4+ T cells are present in NT1 patients. A secondary aim was to assess whether these H1N1 influenza specific CD4+ T cells cross-react with Hcrt. We determined the crystal structure of one earlier identified H1N1 hemagglutinin peptide and two Hcrt peptides bound to the disease-predisposing HLA-DQ6 molecules to assess structural homology. Subsequently, we stimulated peripheral blood mononuclear cells with these peptides to generate specific T cell lines and clones. We then performed proliferation tests on these clones to assess both H1N1- and Hcrt-reactivity and assess HLA-DQ6 restriction, cross-reactivity and T cell receptor sequence of these clones.

Materials and methods

Subjects

Between March 2014 and June 2016, we included all consecutive NT1 patients after informed consent, recruited from the sleep clinic of the department of Neurology, Leiden University Medical Center and the Sleep-Wake Centre of Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede. All patients were diagnosed with NT1 according to the International Classification of Sleep Disorders (ICSD-3; (American Academy of Sleep Medicine, 2014)). Symptom onset in all patients was after the 2009 H1N1 pandemic. Healthy controls were included in the same time period as the NT1 patients and matched for HLA and gender.

Hcrt-1 measurements

CSF samples were drawn for Hcrt-1 measurement in 34 NT1 patients. Hypocretin-1 concentrations were measured in duplicate with an I^{125} hypocretin-1 radioimmunoassay (Phoenix Pharmaceuticals, Mountain View, CA, USA). This assay has an intra-assay variability of <5% and a detection limit of 50 pg/mL. To adjust for inter-assay variability, Stanford reference CSF samples were included in the assay (Ripley et al., 2001, Mignot et al., 2002).

Peptides

All peptides used were produced at the Peptide Synthesis Facility of the Department of Immunohematology and Blood Transfusions of the Leiden University Medical Center. Two Hcrt peptides, $Hcrt_{56-68}$ and $Hcrt_{87-99,}$ and one 2009 H1N1 influenza A hemagglutinin (H1N1-HA) peptide, H1N1-$HA_{275-287}$, were selected based on sequence similarity between these peptides and those described in the aforementioned retracted article (De la Herran-Arita et al., 2013, De la Herran-Arita et al., 2014). All peptide sequences can be found in Supplementary Table 3.1. For follow-up experiments, we used processed Hcrt peptides: two truncated peptides lacking the first 4 residues (including histidines on position 59 and 90 of $Hcrt_{56-68}$ and $Hcrt_{87-99}$, respectively), thereby increasing homology between H1N1-$HA_{275-287}$ and the Hcrt peptides. Additionally, the histidine residue on position 59 of the first Hcrt peptide ($Hcrt_{56-68}$) was replaced by an oxo-histidine or alanine residue rendering the two peptides more homologous to H1N1-$HA_{275-287}$. Soluble complexes of HLA-DQ6 containing the peptides H1N1-$HA_{275-287}$, $Hcrt_{56-68}$ and $Hcrt_{87-99}$ were produced essentially as described previously for HLA-DQ2 (Henderson et al., 2007, Petersen et al., 2014). Briefly, the αβ-heterodimer of the HLA-DQ6 extracellular domain was expressed in Hi5 insect cells, with each peptide linked to the N-terminus of the HLA-DQ6 β-chain. The C-termini of the constructs contained an enterokinase cleavable Fos/Jun zippers, and, at the C terminus of the β-chain, a BirA biotinylation sequence followed by a His10-Tag. The complexes were purified via diafiltration, metal affinity, size exclusion and ion exchange chromatography. For crystallisation experiments the Fos/Jun zippers were removed by enterokinase cleavage and ion exchange chromatography.

Crystallisation, data collection, structure determination and refinement

Peptide-HLA-DQ6 complexes were crystallised at 20°C via the hanging drop vapor diffusion method using equal volumes of mother liquor and protein solution at 10 mg/ml in a buffer containing 10mM Tris (pH 8) and 150 mM NaCl. HLA-DQ6-$Hcrt_{56-68}$ and HLA-DQ6-$Hcrt_{87-99}$ were crystallised with mother liquor containing 16-20% PEG4000, 0.1M NaOAc pH4.5-5.0, and HLA-DQ6- $HA_{275-287}$ was crystallised with 23% PEG4000, 0.2M NaI, 0.1M HEPES pH 7. Prior to data collection the crystals were cryoprotected in mother liquor supplemented with 20% glycerol, or 20% ethylene glycol in the case of HLA-DQ6- $Hcrt_{87-99}$, and flash frozen in liquid N_2. X-ray diffraction data was

collected at the mx2 beamline of the Australian Synchrotron using a ADSC Q315r detector and data processing was carried out with XDS (Kabsch, 2010) and Scala (Evans, 2006). The crystal structures were solved by molecular replacement in Phaser (McCoy et al., 2007) using a published HLA-DQ6 structure (PDB code 1UVQ) as search model. The structural models were refined by iterative rounds of model building in Coot (Emsley et al., 2010) and restrained refinement in Phenix (Adams et al., 2010). For further details on data collection and refinement statistics, see Table 3.1.

Table 3.1. Data collection and refinement statistics. HA = hemagglutinin; Hcrt = hypocretin

Wavelength	**HLA-DQ6-Hcrt$_{56-68}$**	**HLA-DQ6-Hcrt$_{87-99}$**	**HLA-DQ6-H1N1-HA$_{275-287}$**
Resolution range	34.52 - 2.0 (2.071 - 2.0)	39.56 - 1.936 (2.005 - 1.936)	40.11 - 1.7 (1.761 - 1.7)
Space group	C 1 2 1	P 2 21 21	P 21 21 21
Unit cell	163.063 72.404 99.277 90 113.763 90	72.538 99.549 148.372 90 90 90	55.764 89.58 115.484 90 90 90
Total reflections	137291 (13803)	160661 (15461)	126644 (12583)
Unique reflections	70012 (6989)	80479 (7782)	64298 (6352)
Multiplicity	2.0 (2.0)	2.0 (2.0)	2.0 (2.0)
Completeness (%)	97.65 (97.90)	99.54 (97.12)	99.84 (99.95)
Mean I/sigma(I)	7.34 (1.86)	9.85 (2.37)	10.47 (2.31)
Wilson B-factor	22.44	23.29	17.15
R-merge	0.06104 (0.3654)	0.03506 (0.2447)	0.04976 (0.3822)
CC1/2	0.995 (0.835)	0.999 (0.948)	0.994 (0.685)
CC*	0.999 (0.954)	1 (0.987)	0.999 (0.902)
R-work	0.1755 (0.2524)	0.1882 (0.2668)	0.1705 (0.2377)
R-free	0.1980 (0.2795)	0.2157 (0.2819)	0.1972 (0.2680)
Number of non-hydrogen atoms	6888	6696	3720
Macromolecules	6044	5990	3083
Ligands	76	90	95
Solvent	768	616	542
Protein residues	751	745	384
RMS (bonds)	0.008	0.009	0.009
RMS (angles)	1.17	1.25	1.46
Ramachandran favored (%)	98.09	97.65	97.61
Ramachandran allowed (%)	1.91	2.35	2.39
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.60	1.21	0.59
Clashscore	5.44	5.58	4.36
Average B-factor	43.49	45.13	26.10
Macromolecules	43.27	44.94	22.61
Ligands	69.59	77.29	77.67
Solvent	42.63	42.29	36.94

Peripheral blood mononuclear cell (PBMC) isolation

Blood was drawn from all patients and healthy controls. PBMCs were extracted using Ficoll-Paque (GE Healthcare, Chicago, USA) gradient reagent. The first experiments were performed on fresh PBMCs, but in the remainder the isolated PBMCs were subsequently frozen in 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint Louis, USA) in fetal calf serum (FCS; Sigma Aldrich, Saint Louis, USA). These samples were stored until use in liquid nitrogen vessels.

Antigen-specific T cell lines

After isolating or thawing of PBMCs of NT1 patients and healthy controls, $1x10^6$ cells were put into culture in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with L-glutamine (Thermo Fisher, Waltham, USA), 10% (pooled) human serum (NHS) and a mixture of the H1N1-$HA_{275-287}$, $Hcrt_{56-68}$ and $Hcrt_{87-99}$ peptides. After every 5 days r-interleukin 2 (20 U/mL final concentration; rIL-2; Novartis, Basel, Switzerland) and interleukin 15 (10 ng/mL final concentration, IL-15; R&D, Minneapolis, USA) were added to each culture (Kooy-Winkelaar and Koning, 2015).

Peptide-specific T cell clone generation

Peptide-specific T cell clones (TCCs) were generated from T cell lines specific for H1N1-$HA_{275-287}$ from 3 NT1 patients by limiting dilution in culture medium containing 10^6 irradiated feeder cells/mL, 20 U/mL rIL-2, 10 ng/mL IL-15 and 1 μg/mL PHA ("feeder mix"; Remel, Lenexa, USA). The cells were stimulated with 20 U/mL rIL-2 and 10 ng/mL in 10% human serum/IMDM after 5 days. After 10 days, growing wells were transferred to 24-well plates and cultured in feeder mix until a confluent layer of cells was formed. For the T cell lines of 9 NT1 patients, streptavidin-PE-HLA-DQ6- H1N1-$HA_{275-287}$ and streptavidin-PE-HLA-DQ6-$Hcrt_{56-68}$ and -$Hcrt_{87-99}$ tetramers were used to directly stain HLA-H1N1-$HA_{275-287}$-, -$Hcrt_{56-68}$- and -$Hcrt_{87-99}$ -specific T cells. Tetramers were produced essentially as described (Ooi et al., 2017). These tetramer-positive CD4+ T cells were sorted by flow cytometry on a FACS-Aria III instrument (BD Biosciences) and expanded as described above. Clones were subsequently generated from the identified H1N1-$HA_{275-287}$-specific and/or $Hcrt_{56-68}$- or $Hcrt_{87-99}$-specific T cells, as described previously (Kooy-Winkelaar and Koning, 2015). All T cell lines used for TCC generation were derived from frozen NT1 patient samples.

Flow cytometry

Peptide-specific T cell lines generated from one patient were incubated for 30 minutes with 11 antibodies for surface staining and subsequently acquired on a LSRII instrument (BD Biosciences). Fluorochrome-labelled antibodies directed against CD3 (clone UCHT1), CD4 (clone SK3), CD5 (clone L17F12), CD7 (clone M-T701), CD14 (clone MφP9), CD27 (clone M-T271), CD28 (clone CD28.2), CD45RA (clone L48) and IgG1 (clone MOPC-21) were from BD Biosciences (San Jose, California, USA), anti-CD8 (clone 3B5) was from Invitrogen (Bleiswijk, the Netherlands) and anti-CD45 (clone HI30) from eBioscience (San Diego, California, USA). Results were analysed using FlowJo V.10 software (Schmitz et al., 2016).

T cell proliferation assay and assays for assessing HLA-DQ6 restriction

Proliferation assays were performed on T cell lines and TCCs in triplicate in 150 μl IMDM supplemented with 10% human serum in 96-well, flat-bottom plates (Corning Life Sciences, Tewksbury, USA) using $1x10^4$ T cells stimulated with $1x10^5$ irradiated *HLA-DQA1*01:02/DQB1*06:02* (HLA-DQ6)-matched allogeneic PBMCs (3,000 RAD) in the presence or absence of either H1N1-$HA_{275-287}$ or $Hcrt_{56-68}$ and $Hcrt_{87-99}$ (10 μg/ml). In HLA-restriction experiments, the *HLA-DQA1*01:02/DQB1*06:02*-matched allogeneic PBMCs were replaced by PBMCs expressing either *HLA-DQA1*03:01/DQB1*03:02* (HLA-DQ8*;* no association with narcolepsy), *HLA-DQA1*01:02/DQB1*06:03* (less frequently found in narcolepsy compared to healthy controls (Tafti et al., 2014)) or a mix of *HLA-DQA1*01:02/DQB1*06:02* and *HLA-DQA1*01:02/DQB1*06:03*. Triplicate wells containing 10^4 T cells supplemented with 20 U/mL rIL-2 and 10 ng/mL IL-15 functioned as a positive control. After 48 h at 37°C, cultures were pulsed with 10 μCi/mL of ^{3}H-thymidine and harvested 18 h later. Proliferation was measured using a MicroBeta Microplate Counter (PerkinElmer, Waltham, MA, USA). A positive response was defined as a stimulation index (SI) of 3 (defined as the mean count in the wells with peptides divided by the mean count in the wells without peptide) (Kooy-Winkelaar and Koning, 2015). Additionally, to confirm HLA restriction of the T cell lines, blocking experiments were performed in which either anti-HLA-class I (W6/32), anti-HLA-DP (B7/21), anti-HLA-DQ (SPV-L3) or anti-HLA-DR (B8.12.2) monoclonal antibodies (mAbs; locally produced) were added to the initial assay.

T cell receptor (TCR) sequencing

TRAV, TRBV and CDR3 gene segment sequences of H1N1-$HA_{275-287}$-specific TCCs were amplified using PCR and a set of specifically designed primers. PCR products rendered in this way were cloned into a Promega pGEM-T Easy vector and subsequently sequenced. The *TRAV* and *TRBV* gene usage and CDR3 sequences for all generated clones were determined using IMGT/V-QUEST (Brochet et al., 2008).

Statistical analysis

Differences at baseline in participant characteristics were calculated with Student's t-tests and Pearson's chi-square test. Pearson's chi-square test was also used for comparing T cell proliferation in NT1 patients as compared to healthy controls. Differences between conditions in the HLA blocking experiments were calculated by one-way ANOVA with a Bonferroni *post hoc* analysis. Differences between P-values below 0.05 were deemed significant. Bonferroni corrections were executed when needed. All analyses were conducted using the IBM SPSS Statistics 23 software package.

Results

Patient characteristics

We included 81 NT1 patients and 19 healthy controls. Patient characteristics are shown in Table 3.2. Notably, all NT1 patients except one were *HLA-DQA1*01:02/DQB1*06:02* (HLA-DQ6) positive. Thirty-four patients had undergone a lumbar puncture for Hcrt-1 measurement; all had Hcrt-1 values in the cerebrospinal fluid that were below the cut-off value of 110 pg/mL based on the ICSD-3 criteria for NT1. NT1 patients were younger than healthy controls, but the distribution of males and females was comparable between the two groups.

Table 3.2. Characteristics of study participants. Data indicate mean ± standard deviation. P-values result from Student's t-tests for the continuous variables and Pearson's chi-square tests for dichotomous variables.

	Narcolepsy type 1	Healthy controls	p-value
N	81	19	
Age (years)	31.6 ± 19.2	55.0 ± 8.4	<0.001
Males (%)	39 (48.1%)	9 (47.4%)	0.951
HLA-DQ6 +	80/81	19/19	0.626
Hypocretin-1 < 110pg/mL	34/34	Not available	Not applicable
H1N1 vaccination (%)	15/47 (32%)	Not available	Not applicable

H1N1-HA$_{275-287}$, Hcrt$_{56-68}$ and Hcrt$_{87-99}$ presented by HLA-DQ6 show structural homology

Based on earlier described peptides of H1N1-HA and hypocretin that are able to bind HLA-DQ6 (De la Herran-Arita et al., 2013, De la Herran-Arita et al., 2014), we investigated the possibility of molecular mimicry between H1N1-HA and Hcrt peptides in the context of HLA-DQ6 presentation. We crystallized and determined the crystal structures of H1N1-HA$_{275-287}$, Hcrt$_{56-68}$ and Hcrt$_{87-99}$ presented by HLA-DQ6 at resolutions of 1.7Å, 2.0Å and 1.95Å, respectively (Figure 3.1). Alignment of the structures revealed that the two Hcrt peptide - HLA-DQ6 complexes, HLA-DQ6-Hcrt$_{56-68}$ and HLA-DQ6-Hcrt$_{87-99}$, were nearly indistinguishable in terms of peptide backbone positioning (Cα rmsd <0.1Å), peptide sidechain conformations and HLA substructure surrounding the peptide. Compared to the two Hcrt complexes, the H1N1-HA$_{275-287}$ peptide was bound to HLA-DQ6 in the expected homologous register and with overall similar backbone positioning (Cα rmsd <0.35Å) and sidechain conformations. Within the 9-mer core of the bound peptides, the most notable differences were observed in the exposed positions p2 and p8 of the peptides (p2-histidine and p8-threonine in the Hcrt complexes, and p2-alanine and p8-isoleucine in the H1N1-HA complex). In addition to the differences in peptide sidechains, we observed a difference in the β-chain helix, which was positioned closer to the peptide in the H1N1-HA complex when compared to the two Hcrt complexes. This homology prompted us to determine whether this could also lead to T cell cross-reactivity to the H1N1-HA and hypocretin peptides in functional experiments.

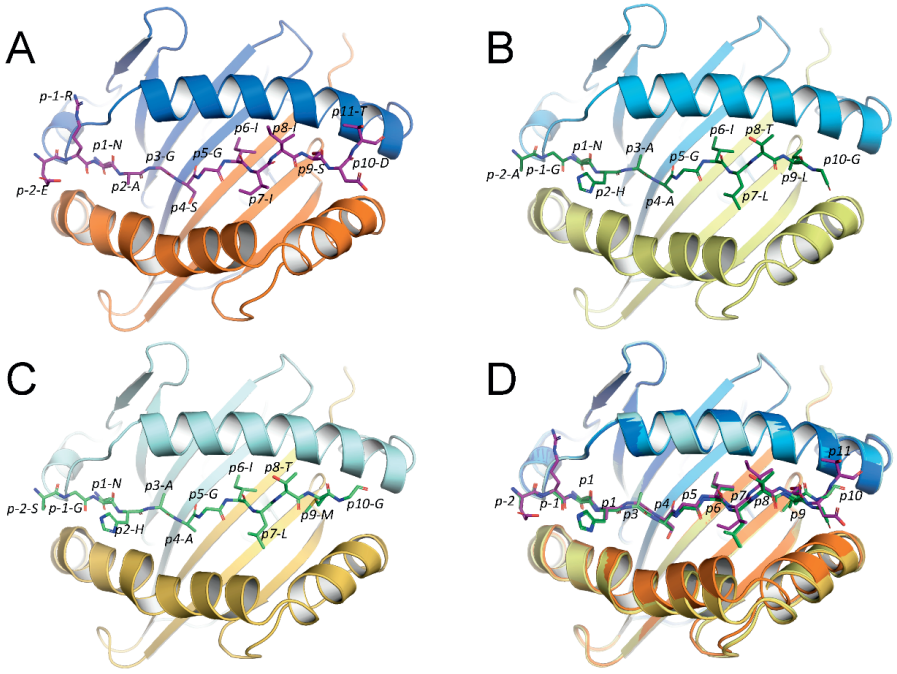


Figure 3.1. HLA-DQ6 presenting H1N1-HA$_{275-287}$, Hcrt$_{56-68}$ and Hcrt$_{87-99}$ peptides show structural homology. A. H1N1-HA$_{275-287}$ peptide. B. Hcrt$_{56-68}$ peptide. C. Hcrt$_{87-99}$ peptide. D. Overlay A., B. and C. HA = hemagglutinin; Hcrt = hypocretin.

HLA-DQ6-H1N1-HA$_{275-287}$-specific CD4+ T cells are readily detectable in NT1 patients and healthy controls, but do not cross-react with HLA-DQ6-Hcrt$_{56-68}$ or -Hcrt$_{87-99}$

T cell lines were generated from PBMCs of NT1 patients and healthy controls by co-culture with a pool of the H1N1-HA$_{275-287}$, Hcrt$_{56-68}$ and Hcrt$_{87-99}$ peptides for 5 days followed by expansion in culture medium containing IL-2 and IL-15. Subsequently, the specificity of these T cell lines against the H1N1-HA$_{275-287}$ peptide was determined by co-culture of the T cell lines with irradiated HLA-DQ6 positive allogeneic PBMCs in the presence or absence of the H1N1-HA$_{275-287}$ peptide. Specific proliferation was measured by determining the incorporation of ^{3}H-thymidine after three days of culture. Proliferation was defined as a stimulation index equal or higher to 3. All T cell lines proliferated in the positive control condition which reinforced our earlier conclusion based on visual assessing T cell lines that they were in good condition. In 28.4% (23/81) of NT1 patients and 15.8% (3/19) of healthy controls, an H1N1-HA$_{275-}$

$_{287}$-specific T cell proliferative response was observed (Figure 3.2). To assess the specificity of the T cell lines against the Hcrt peptides, they were also stimulated with the $Hcrt_{56-68}$ and $Hcrt_{87-99}$ peptides. No reactivity to $Hcrt_{56-68}$ or $Hcrt_{87-99}$ was observed in T cell lines of either NT1 patients or controls (Figure 3.2), indicating the absence of cross-reactivity of H1N1-$HA_{275-287}$-specific T cells with $Hcrt_{56-68}$ and $Hcrt_{87-99}$ in the context of HLA-DQ6. FACS analysis was performed on 13 HLA-DQ6-H1N1-$HA_{275-287}$-specific T cell lines. Analyses revealed a dominance of CD4+ T cells in these T cell lines (Figure 3.3).

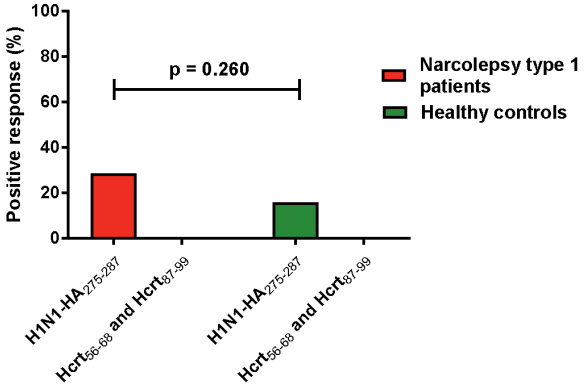


Figure 3.2. Overview of T cell responses to H1N1-$HA_{275-287}$, $Hcrt_{56-68}$ and $Hcrt_{87-99}$ for narcolepsy type 1 patients (red) and healthy controls (green). The P-value results from a Mann-Whitney U-test.

T cell reactivity to H1N1-$HA_{275-287}$ is HLA-DQ6-restricted

T cell clones (TCCs) were generated from the H1N1-$HA_{275-287}$-reactive T cell lines of 12 NT1 patients by either limiting dilution (in T cell lines of 3 NT1 patients) or isolation using streptavidin-PE-HLA-DQ6- H1N1-$HA_{275-287}$ tetramers (in T cell lines of 9 NT1 patients) and tested for reactivity against H1N1-$HA_{275-287}$. In TCCs generated from all H1N1-$HA_{275-287}$-specific CD4+ T cell lines, H1N1-$HA_{275-287}$-specific clonal T cell responses were found (Table 3.3).

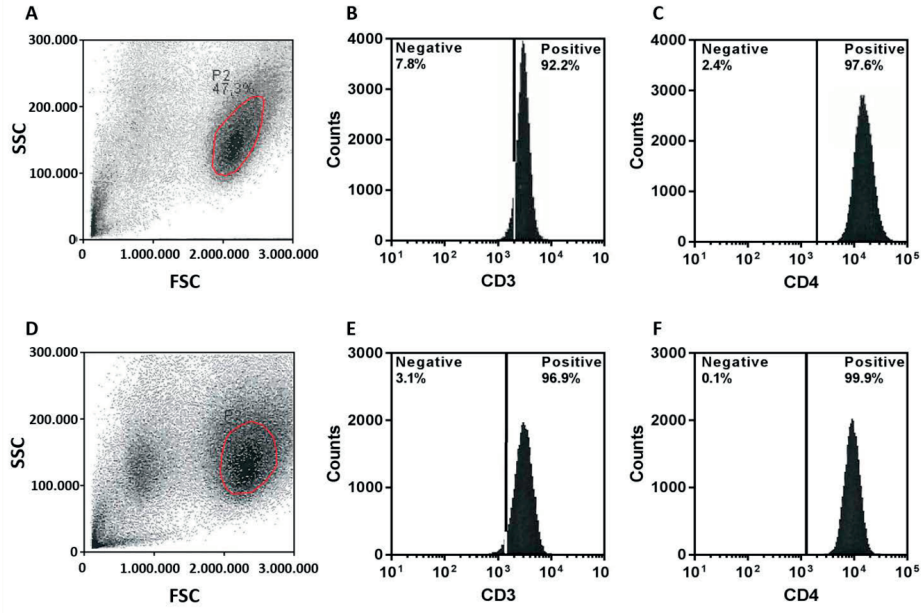


Figure 3.3. H1N1-$HA_{275-287}$-specific T cell lines are predominantly CD4+. These fluorescence-activated cell sorting (FACS) plots show the gating strategy of two T cell lines in different patients (A-C and D-F). Living single cells are selected in the FSC-SSC plot (A and D). In the next plot CD3+ cells are selected (B and E), which show a high percentage of CD4+ cells in the last plot (C and F).

To assess HLA-DQ6 restriction, T cell proliferation experiments were performed with irradiated allogeneic PBMCs of different haplotypes: *HLA-DQA1*01:02/DQB1*06:03*, *HLA-DQA1*03:01/DQB1*03:02* (HLA-DQ8) or a mix of *HLA-DQA1*01:02/DQB1*06:02* and *HLA-DQA1*01:02/DQB1*06:03* next to those expressing *HLA-DQA1*01:02/DQB1*06:02*. T cell proliferation was only observed in the presence of HLA-DQ6-expressing PBMCs and the H1N1-$HA_{275-287}$ peptide. Furthermore, when T cell proliferation experiments were performed with irradiated allogeneic PBMCs expressing HLA-DQ6, an anti-HLA-DQ mAb was able to block T cell proliferation of the generated TCCs. An anti-HLA-class I mAb blocked T cell proliferation considerably less effectively, whereas anti-HLA-DR and –DP mAbs did not affect T cell proliferation (Figure 3.4A-B). These experiments confirm that the detected H1N1-$HA_{275-287}$-specific CD4+ T cell proliferation is HLA-DQ6-restricted.

Table 3.3. H1N1-$HA_{275-287}$-specificity of the generated T cell clones

Subject number	TCCs tested	H1N1-$HA_{275-287}$-specific TCCs
19*	50	1
24	25	25
25	11	11
26	11	11
27*	109	19
29	5	5
32	14	14
35	12	12
38	11	10
39	4	4
47	22	22
50*	130	23

The HLA-DQ6-H1N1-$HA_{275-287}$-specific T cell receptor repertoire in NT1 patients and healthy controls shows no biased expression

Recent reports describe a bias in TCR sequences for recognition of peptide-HLA complexes in CD4+ T cell mediated diseases (Qiao et al., 2014, Petersen et al., 2014). Since we were not able to show significant differences between percentages of NT1 patients and controls with HLA-DQ6-H1N1-$HA_{275-287}$-specific T cells, we searched for differences in the T cell repertoire used by NT1 patients and controls to mount immune responses to this antigen. TCRs expressed by 20 H1N1-$HA_{275-287}$-specific TCCs from 4 NT1 patients and 4 H1N1-$HA_{275-287}$-specific TCCs from 2 healthy controls were sequenced (Table 3.4). 18 TCR sequences were identified in TCCs of NT1 patients; 4 different TCR sequences in TCCs of healthy controls. There was expansion of some clones within a given patient, but these likely arose during the culturing process, with some T cell clones responding better to peptide hence expanding at a greater rate than others subsequently skewing the representative pool. Nevertheless, although only a small sample size was interrogated, no evidence for a biased *TRAV, TRBV* or CDR3 sequence motif was observed across unrelated individuals with NT1 or in healthy controls.

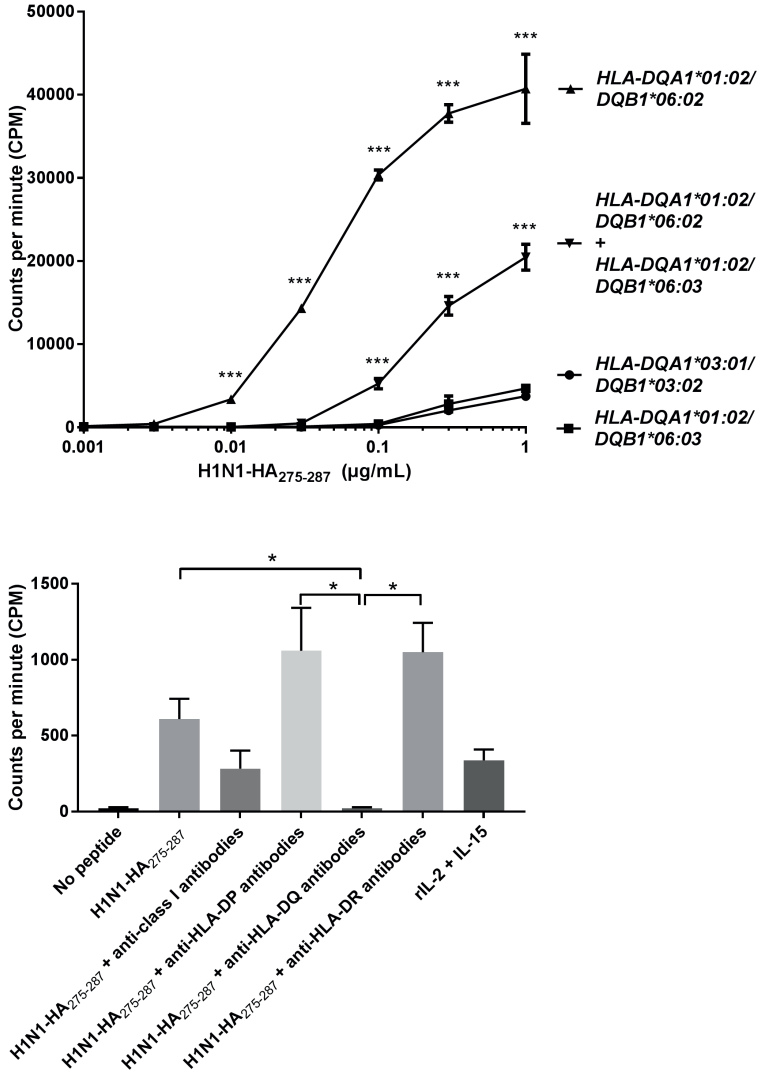


Figure 3.4. A. A H1N1-HA$_{275-287}$ dose-dependent T cell response in a narcolepsy type 1 patient T cell clone (patient number 50, TCC 87) is found only in proliferation experiments performed with irradiated PBMCs that are HLA-DQA1*01:02/DQB1*06:02 (HLA-DQ6)-positive. Experiments were performed in triplicate wells. Significant differences relative to proliferation experiments with irradiated HLA-DQ8 (HLA-DQA1*03:01/DQB1*03:02)-positive PBMCs. B. Anti-HLA-DQ mAb blocks T cell proliferation of the generated TCCs. An anti-HLA-class I mAb blocked T cell proliferation considerably less effectively, whereas anti-HLA-DR and –DP mAbs did not affect T cell proliferation.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$; mAb = monoclonal antibody; PBMCs = peripheral blood mononuclear cells; TCC = T cell clone.

Table 3.4. T cell receptor sequences of 24 (20 narcolepsy type 1 patient, 4 healthy control) H1N1-$HA_{275-287}$-specific T cell clones (TCCs). Green and red values indicate receptor segments that are shared between TCCs of more than 1 narcolepsy type 1 patient or healthy control.

Study number	Group	Clone	TRAV	TRAJ	CDR3	TRBV	TRBJ	TRBD	CDR3
19	Patient	4	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
19	Patient	8	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
19	Patient	11	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
27	Patient	128	8-6*01	54*01	CAVTSPPIQGAQKLVF	4-2*01	2-1*01	1*01	CASSQGNGPYNEQFF
27	Patient	161	17*01	34*01	CATDAYNTDKLIF	4-2*01	1-2*01	1*01	CASSQASTGGSNYGYTF
27	Patient	161	21*01	43*01	CAVVHDMRF				
27	Patient	118	25*01	49*01	CAVNTGNQFYF	20-1*01	1-6*01	1*01	CSARVGQDSSPLHF
27	Patient	131	25*01	31*01	CAGGNNNARLMF	20-1*01	2-5*01	1*01	CSAAPGLRPQETQYF
27	Patient	145	13-1*01	37*01	CAPGSGNTGKLIF	12-3*01	2-5*01	2*01	CASSFQDYPQETQY
27	Patient	145	12-1*01	42*01	CVVNDIHYGGSQGNLIF				
27	Patient	125	26-2*01	23*01	CILRSHYNQGGKLIF				
38	Patient	108				4-3*01	2-1*01	1*01	CASSQGGGMGFDEQF
50	Patient	9	17*01	34*01	CATASYNTDKLIF	4-3*01	1-4*01	1*01	CASSRGTAATNEKLF
50	Patient	32	17*01	34*01	CATASYNTDKLIF				
50	Patient	37	19*01	30*01	CALSEDENRDDKIIF	3-1*01	1-6*01	1*01	CASSQSRVSSPLHF
50	Patient	38	17*01	37*01		4-1*01	1-1*01	2*02	CASSQSEGAEAFF
50	Patient	68	12-2*01	26*01	CAVNKGSNYGQNFVF				
50	Patient	80	17*01	34*01	CATSSYNTDKLIF	4-3*01	1-1*01	2*02	CASSSGRGSMNTEAFF
50	Patient	87	17*01	34*01	CATASYNTDKLIF	6-2*01	1-1*01	1*01	CASSAGTGAFF
50	Patient	99	12-2*01	26*01	CAVNKGSNYGQNFVF	20-1*01	1-5*01	1*01	CSAATGTGETFYNQPQHF
102	Control	9	12-2*01/02/03	49*01	CAVHHTNTGNQFYF	19*01/02/03	2-5*01	1*01	CASSRGTGGKETQYF
102	Control	11	26-2*01	3*01	CTQSSASKIIF	4-2*01/4-3*01/03/04	2-5*01	1*01	CASSQASGGLGETQYF
103	Control	1	12-3*01/02	6*01	CAMILSGGSYIPTF	20-1*01/02/03/04/05	1-1*01	1*01	CSAPKNTEAFF
103	Control	2	13-1*01	54*01	CAEGGIQGAQKLVF				

Discussion

HLA-DQ6-H1N1-HA peptide-specific CD4+ T cell responses were readily detected in both NT1 patients and healthy controls, with a higher proportion in the NT1 group. We did not detect HLA-DQ6-Hcrt peptide-specific T cell responses. Our experiments do not support the hypothesis that these Hcrt peptides are implicated in cross-reactivity leading to Hcrt-producing neuronal destruction and, thereby, to NT1.

The HLA-DQ6-H1N1-HA peptide-specific TCCs did show some, but no extensive clustering of T cell receptor sequences in NT1 patients. Larger studies would be required to determine if the H1N1-specific TCR repertoire in NT1 patients differs from controls.

Our findings add to those of others in the field who did show antibody-mediated reactivity to H1N1-HA in NT1 patients. Several groups found H1N1-HA specific antibodies in NT1 patients (Lind et al., 2017, Lind et al., 2014), but H1N1-HA specific T cells have not been described. The lack of reactivity to Hcrt peptides was reported in studies focusing on autoantibodies (Black et al., 2005) and CD4+ T cells (Ramberger et al., 2017, Kornum et al., 2017a). HLA-DR-restricted Hcrt-specific T cell responses have recently been described (Latorre et al., 2018), but that study does not explain the strong link with HLA-DQ6. Interestingly, one other study (Ramberger et al., 2017) reported reactivity to Hcrt-peptide pools in a small minority of NT1 patients, although the peptides used differed from the ones in the current study.

Moreover, in our current study we focused on H1N1-HA and Hcrt peptides that display sequence homology, which does not rule out that other H1N1 peptides are involved. Another possibility is that the key differences between the peptides, a histidine residue at position 59 and 90 of the $Hcrt_{56-68}$ and $Hcrt_{87-99}$ peptides, respectively, as compared with an alanine residue in the corresponding position in the H1N1-$HA_{275-287}$ peptide, may prevent cross-reactive responses. We therefore also tested substitution variants of the Hcrt-peptides in which the histidine residues were replaced by an alanine, but we observed no cross-reactivity to these peptides as well, making it unlikely that posttranslational modification of Hcrt-peptides underlies cross-reactivity (results not shown). In future studies we will therefore be testing T cell responses to peptide pools representing the H1N1 proteome and preprohypocretin.

One of the limitations in our study is that we have not been able to distinguish between NT1 patients and healthy controls that have actually encountered the H1N1 influenza virus and those who have not. We could therefore not be sure whether different exposure to the virus explains the lack of differences in H1N1-HA peptide-specific T cell responses between NT1 patients and healthy controls. However, T cell mediated cross-protective immunity generated by previous H1N1 infections was found to be common in the population (Miller et al., 2010). Another limitation in our study is that we are performing our experiments in blood, while the actual destruction of Hcrt-producing neurons is taking place at the other side of the blood-brain barrier. It was recently described that T cell composition in cerebrospinal fluid differed between NT1 patients and healthy controls (Moresco et al., 2018). Repeating our experiments in cerebrospinal fluid would therefore be a better environment to test for cross-reactive T cells. Additionally, detecting proliferation of a small immune subset that could have driven the destruction of the approximately 80,000 Hcrt-producing neurons (Thannickal et al., 2000) in a vast number of non-proliferating immune cells is technically difficult. The procedure used for peptide-specific TCC generation was also used in previous studies for the isolation of HLA-DQ-restricted gluten-specific T cell clones from patients with celiac disease in which we have determined the affinity of such T cells for the cognate HLA-DQ-gluten complexes. We observed that in cell-free assays the affinity values for some of these HLA-DQ-gluten specific TCRs were comparable with affinity values observed for microbial or non-self TCR–pMHC-II interactions, while others exhibited affinity values in line with those of low-affinity autoreactive TCR–pMHC complexes (Petersen et al., 2014). In cellular assays similar substantial differences were observed (Broughton et al., 2012). Therefore, this indicates that our method allows the identification of both high- and low-affinity T cell clones. However, we cannot exclude the possibility that with our technique we missed the detection of very low frequency T cells. The T cell library method that was performed in a recent publication on T cell reactivity to Hcrt (Latorre et al., 2018) would be an interesting technique to screen for these low frequency clones. Other novel approaches, such as mass cytometry, to address this rare immune subset problem are emerging with techniques that can isolate disease-specific immune subsets with unprecedented detail (van Unen et al., 2017). Repeating our experiments with only those subsets that are specific for NT1, would significantly increase the odds of identifying rare cross-reactive immune cells in NT1 patients should they exist.

Conclusions

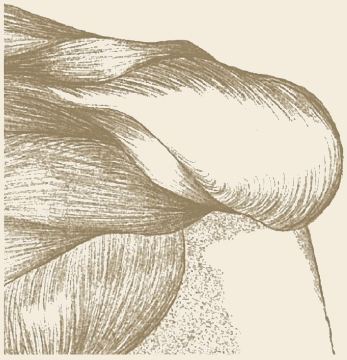
We identified HLA-DQ6-restricted H1N1-HA peptide-specific T cell responses in a subset of NT1 patients and healthy controls. We did not find HLA-DQ6-H1N1-HA peptide-specific T cells cross-reactive to Hcrt-2 peptides. These results indicate that it is unlikely that cross-reactivity between H1N1-HA and Hcrt-2 peptides presented by HLA-DQ6 is involved in the development of NT1.

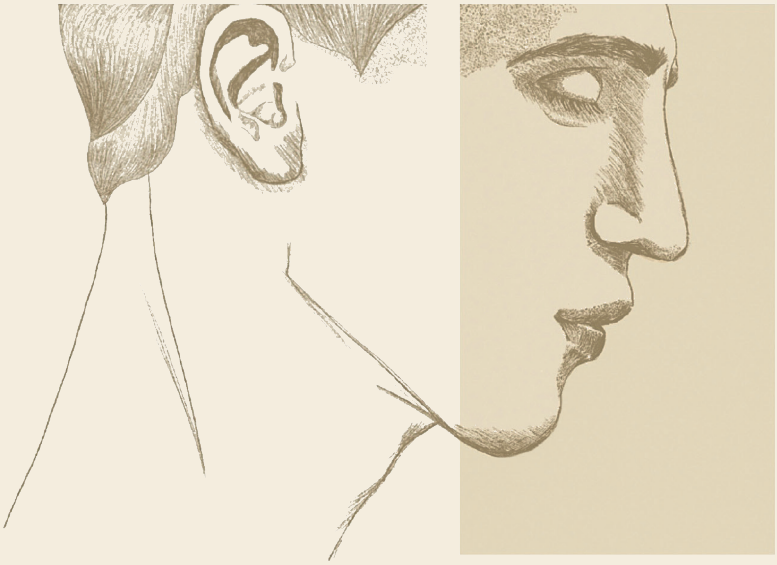
Supplementary material

Supplementary table 3.1. Amino acid sequences of used peptides.

Protein	Fragment	Amino acid sequence
H1N1-HA	H1N1-HA$_{275-287}$	ERNAGSGIIISDT
Hcrt-2	Hcrt$_{56-68}$	AGNHAAGILTLGK
	Hcrt$_{87-99}$	SGNHAAGILTMGR
	Hcrt$_{60-68}$ (truncated)	AAGILTLGK
	Hcrt$_{91-99}$ (truncated)	AAGILTMGR
	Hcrt$_{56-68}$ (substituted amino acid 1)	AGNuAAGILTLGK
	Hcrt$_{56-68}$ (substituted amino acid 2)	AGNAAAGILTLGK

A = alanine; D = aspartic acid; G = glycine; H = histidine; HA = hemagglutinin; Hcrt = hypocretin; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; R = arginine; S = serine; T = threonine; u = oxo-histidine.





Chapter 4

Mass cytometry-based analysis of the immune system in peripheral blood of narcolepsy type 1 patients

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In preparation.

Abstract

Introduction: Even though indirect evidence points strongly to a T cell mediated autoimmune response, the exact key immune cell populations in this response remain elusive. We aimed to identify narcolepsy type 1-specific immune cell populations using mass cytometry, a high-dimensional single-cell analysis technique that permits an in-depth examination of immune cell subtypes together with algorithm-guided analysis approaches.

Methods: Immune cell composition in the peripheral blood of 16 narcolepsy type 1 patients with recent disease onset was compared to 8 HLA-DQ6 (*DQA1*01:02/DQB1*06:02*)-matched healthy controls and 10 narcolepsy type 1 patients longer after disease onset by using mass cytometry.

Results: Populations of memory $CD4^+$ and $CD8^+$ T cells and regulatory $CD4^+$ T cells indicative of an activated phenotype are more frequently found in the peripheral blood of narcolepsy type 1 patients than in healthy HLA-DQB1*06:02-matched controls. Additionally, differences in immune cell composition between narcolepsy type 1 patients with recent disease onset and those longer after disease onset are limited.

Conclusion: These findings point towards multifaceted immune activation in narcolepsy type 1 patients. Performing functional experiments on the immune populations discovered in this study could serve as a starting point for identifying the immune mechanisms driving the autoimmune response leading to the destruction of hypocretin-producing neurons.

Introduction

Narcolepsy type 1 (NT1) is a sleep-wake disorder characterized by a pentad of core symptoms: excessive daytime sleepiness, cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nocturnal sleep. Diagnosis is based on the third edition of the International Classification of Sleep Disorders (ICSD, 2014). Estimations of its prevalence are between 20-50 per 100,000 individuals (Wijnans et al., 2013). NT1 is caused by the destruction of most hypocretin-producing neurons in the lateral hypothalamus, leading to a hypocretin deficiency that can be measured in the cerebrospinal fluid (Peyron et al., 2000, Thannickal et al., 2000). The destruction of the hypocretin-producing neurons is hypothesized to be the result of an autoimmune response. Two discoveries have reinforced this hypothesis. Firstly, it was described that 95% of NT1 patients carry the *HLA-DQA1*01:02 / DQB1*06:02* haplotype that encodes HLA-DQ6, an HLA-class II molecule on human antigen-presenting cells (Juji et al., 1984, Mignot et al., 1997, Tafti et al., 2014). Genome-wide association studies added to this finding by showing associations of NT1 with variants within immune-related genes in NT1 patients (Faraco et al., 2013, Han et al., 2013, Hor et al., 2010). Additionally, the 2009 H1N1 influenza pandemic and the subsequent vaccination campaign was followed by an increase in the incidence of NT1 in several European countries and China (Dauvilliers et al., 2013, Feltelius et al., 2015, Lind et al., 2014, Partinen et al., 2012, Han et al., 2011). These findings shifted the focus of further research on candidate immune cell types driving the autoimmune response leading to the disappearance of hypocretin-producing neurons in NT1.

Studies on the role of B cells failed to detect autoreactive B cells or autoantibodies to hypocretin or its receptors (Black et al., 2005, Overeem et al., 2006, Tanaka et al., 2006, van der Heide et al., 2015a). Studies focusing on the role of autoreactive $CD4^+$ T cells targeting hypocretin show conflicting results. HLA-DQ-restricted (Luo et al., 2018) or HLA-DR-restricted hypocretin-specific $CD4^+$ T cell responses (Latorre et al., 2018) were demonstrated by two groups, while others found HLA-DQ-restricted hypocretin-specific $CD4^+$ T cell responses in both NT1 patients and healthy controls (Jiang et al., 2019, Pedersen et al., 2019). Previously, our group did not show any HLA-DQ-restricted hypocretin-specific $CD4^+$ T cells responses in NT1 patients (Schinkelshoek et al., 2019).

Even though indirect evidence points strongly to a T cell mediated autoimmune response, the exact key immune cell populations in this response remain elusive. We apply another approach to identifying the immune components involved in the autoimmune response that destroys the hypocretin-producing neurons. In this study, we use mass cytometry, a high-dimensional single-cell analysis technique that permits an in-depth examination of immune cell subtypes simultaneously, together with algorithm-guided analysis approaches. This technique has already been shown to be able to separate patients and controls based on their specific immune cell cluster profile (van Unen et al., 2016). We aim to primarily identify populations of immune cells in the peripheral blood that are enriched in NT1 patients with recent disease onset compared with healthy controls.

Materials and methods

Patient samples

NT1 patients were included at the outpatient clinics of the Leiden University Medical Center, Leiden, the Netherlands; Sleep-Wake Centre of Stichting Epilepsie Instellingen Nederland, Heemstede, the Netherlands; Department of Rehabilitation, University of Witten/Herdecke, Witten/Herdecke, Germany; Department of Neurology, Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland. Included patients were grouped based on time from disease onset. Recent disease onset was defined as first symptom onset within 2 years of blood draw. The diagnosis of NT1 was based on the criteria described in the third edition of the International Classification of Sleep Disorders (ICSD, 2014). All patients gave their written informed consent to take part in the research program. We also included non-related healthy individuals of which HLA-DQB1*06:02 status was known. Part of these healthy individuals were participants from a panel of randomly selected Dutch individuals (van Rooijen et al., 2012); all other healthy individuals were recruited in Witten and Bern. All mass cytometry experiments and their initial analysis were performed blinded. This study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol P17.169) and those in Witten (Ethik-Kommission Universität Witten-Herdecke; 211/2017) and Bern (Kantonale Ethikkommission Bern (KEK); ID 2018-01564).

Isolation of peripheral blood mononuclear cells and antibody staining

Peripheral blood mononuclear cells (PBMCs) were cryopreserved using the same protocol in all centres within 24 hours of blood collection. PBMCs were extracted using Ficoll-Paque (GE Healthcare, Chicago, USA) gradient reagent. The isolated PBMCs were subsequently frozen in 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint Louis, USA) in fetal calf serum (FCS; Sigma Aldrich). These samples were stored until use in liquid nitrogen vessels in the participating centres and transported to the Leiden University Medical Center for antibody staining, data acquisition and analysis.

Antibodies

Supplementary table 4.1 lists all heavy metal isotope-tagged monoclonal antibodies that were used. Conjugation of the purified antibodies with heavy metal reporters was performed in-house using the MaxPar X8 Antibody Labeling Kit (Fluidigm, San Francisco, USA) according to the manufacturer's instructions. Optimal labelling concentration was determined by titration for all antibodies.

Antibody Staining and Data Acquisition

PBMCs were thawed according to the standard protocol used at the Department of Immunohematology and Blood Transfusions of the Leiden University Medical Center. After thawing, cells were washed in MaxPar Cell Staining Buffer (CSB; Fluidigm) and incubated with 1 μM Cell-ID intercalator-^{103}Rh in CSB for 15 min at room temperature. Cells were then incubated for 10 min at room temperature with human Fc receptor blocking solution (BioLegend, San Diego, USA) and subsequently stained with the heavy metal isotope-tagged monoclonal antibody mix mentioned in section 2.3 for 45 min at room temperature. After 3 times washing, cells were incubated with 0.125 μM Cell-ID intercalator-Ir (Fluidigm) in MaxPar Fix and Perm buffer (Fluidigm) overnight at 4°C.

The next day, stained cells were washed, resuspended in de-ionized water and acquired on a Helios-upgraded CyTOF2 and Helios mass cytometer 35 (Fluidigm) at an event rate of <500 events/sec in a soluition containing EQ Four Element Calibration Beads (Fluidigm) that were used for normalization for each experiment.

Integrated data analysis

Gating of $CD45^+$ live, single cells from the data derived from single cell acquisition was performed using FlowJo™, version 10. The gating strategy can be found in Supplementary figure 4.1. After sampletagging, hyperbolic ArcSinh transformation with a cofactor of 5, these $CD45^+$ cells were subjected to dimensionality reduction analysis using Cytosplore (Höllt et al., 2016). The overview level of a 6-level hierarchical stochastic neighbor embedding (HSNE) analysis with default perplexity (30) and iterations (1000) was used to identify major immune cell lineages in the merged $CD45^+$ cells of all samples (18.9 x 10^6 cells). Gaussian mean shift (GMS) unsupervised clustering was performed in Cytosplore and an algorithm was used to merge clusters that showed high similarity in ArcSinh5-transformed marker expression. For further zooming into the data, we selected cells based on visible clusters, selecting clusters derived by using the GMS clustering. HSNE analyses were repeated until clusters consisted of a maximum of $0.5x10^6$ landmarks (van Unen et al., 2017). Subsequently, hierarchical clustering on cell frequencies was performed in Matlab using Spearman's rank correlation, as previously described (de Vries et al., 2019). Immune cell clusters that were most strongly correlated based on Spearman's ρ, were assessed for differences in antibody staining. When differences in antibody staining were deemed negligible, the subsets were merged. This process was repeated until differences in antibody staining yielded phenotypically distinct cell clusters. We have not been able to account for technical variation by adding a peripheral blood mononuclear cell (PBMC) reference sample to every mass cytometry experiment. Therefore, batch effects were controlled for in the final set of immune cell clusters.

Statistics

The primary analysis concerned the comparison between the immune cell composition of NT1 patients with recent onset and HLA-DQB1*06:02-matched healthy controls. Secondary analyses were performed between NT1 patients with recent onset and those with onset longer than two years ago. All results are shown as mean ± standard error of the mean (SEM). Differences in relative frequencies of the major immune cell lineages between participant groups were assessed by one-way ANOVA with Bonferroni post-hoc comparisons. For differences between immune cell clusters within major immune cell lineages between participant groups two-tailed Mann-Whitney U tests for unpaired samples were used. Corrections for multiple comparisons were made when

appropriate using the Benjamini-Hochberg procedure. P-values below 0.05 were considered to be statistically significant. All analyses were conducted using the IBM SPSS Statistics 25 software package. Additional analyses were performed to replicate findings of previous studies focusing on the immune cell composition in the peripheral blood of NT1 patients (Hartmann et al., 2016, Lecendreux et al., 2017, Lind et al., 2020, Moresco et al., 2018).

Results

Participant characteristics

Table 4.1 describes participant characteristics. Mass cytometry experiments were performed with PBMCs of NT1 patients and healthy controls. The current cohort consisted of 16 samples of NT1 patients with recent onset of disease (< 2 years), 10 samples of NT1 patients with disease onset more than 2 years before blood sampling, 8 samples of healthy controls that are DQB1*06:02-positive and 5 samples of healthy controls that are HLA-DQB1*06:02 negative.

Table 4.1. Participant characteristics.

	NT1 < 2 years after disease onset	NT1 > 2 years after disease onset	HLA-matched control	Non-HLA-matched control
N	16	10	8	5
Males (n)	7	2	4	1
Time from disease onset (years ± standard deviation)	0.9 ± 0.5	4.1 ± 1.8	NA	NA
Hcrt-1 < 110 pg/mL	6/7	4/5	NA	NA
Hcrt-1 < 200 pg/mL	7/7	5/5	NA	NA
HLA-DQB1*06:02 positive (n)	13/13 (100%)	8/9 (99%)	8/8 (100%)	0/5 (100%)

Hcrt-1 = hypocretin-1; HLA = human leukocyte antigen; NA = not applicable; NT1 = narcolepsy type 1.

Major immune cell lineages

39 immune cell markers were analyzed by mass cytometry in single immune cell suspensions of NT1 patients and healthy controls. The composition of the immune system deciphered using HSNE analysis in Cytosplore on all live, $CD45^+$ cells (a total of 18.9×10^6 cells). Density features of the HSNE-embedded landmarks derived from unsupervised GMS clustering separated seven clusters that corresponded with seven major immune cell lineages: naïve ($CD45RO^-$ $CD3^+CD4^+CD7^+CCR7^+$) and memory ($CD45RO^+CD3^+CD4^+CD7^+$) $CD4^+$

T cells, $CD8^+$ T cells ($CD3^+CD7^+CD8^+$), γδ T cells ($CD3^+CD7^+TCR\gamma\delta^{dim/+}$), B cells ($CD20^+$), $CD3^-CD7^+$ cells innate lymphoid cells (ILC; $CD3^-CD7^+$) and myeloid cell lineages ($CD3^-CD7^-CD11c^+$) (Figure 4.1 and Figure 4.2; Supplementary figure 4.2). The data of each major immune cell lineage was further explored hierarchically by HSNE, as is exemplified in Figure 4.3 for the memory $CD4^+$ T cell compartment, rendering unique immune cell clusters for each major immune cell lineage. Eventually, exploration of all major immune cell lineages identified a total of 185 immune cell clusters, delineated using unsupervised GMS clustering. For merging of highly similar immune cell clusters Spearman's rank correlation was performed, as described in more detail in Materials and methods. 3 clusters containing less than 100 cells were excluded from further analysis.

NT1 with recent disease onset vs HLA-DQB1*06:02-matched controls

Subsequently, the composition of the immune system in the peripheral blood of NT1 patients shortly after disease onset was compared to that of HLA-DQB1*06:02-matched healthy controls. Percentages of $CD45^+$ cells did not differ significantly between NT1 patients with recent disease onset and HLA-DQB1*06:02-matched controls for all major immune cell lineages (Figure 4.2).
More detailed analysis of the immune cell clusters revealed that in the memory $CD4^+$ T cell compartment differences between both participant groups were observed, namely higher frequencies of $CD45RO^+CD4^+$ T cell populations expressing $CD27^+$, $CD127^+$ and/or $CD161^+$ in NT1 patients with recent onset (Figure 4.4 and Supplementary table 4.2). Similarly, also in the $CD8^+$ T cell compartment an immune cell cluster expressing CD27 and CD127 was found more frequently in NT1 patients with recent disease onset (Supplementary figure 4.2).
Regulatory $CD4^+$ T cells, as characterized by the combination of CD4 and CD25 in the absence of CD127, were found more frequently in NT1 patients compared to healthy controls. No differences in effector memory ($CD45RO^+CCR7^-$) or central memory ($CD45RO^+CCR7^+$) $CD4^+$ T cells were found. $CD27^+CD8^+$ T cells were more frequent in NT1 patients compared to healthy controls, albeit not significant, while $CD27^-CD8^+$ T cells were significantly less frequent in the NT1 patient population. In contrast to a recent study, we did not observe an increased frequency of $CD3^-CD56^+$ natural killer cells in NT1 patients (Figure 4.5).

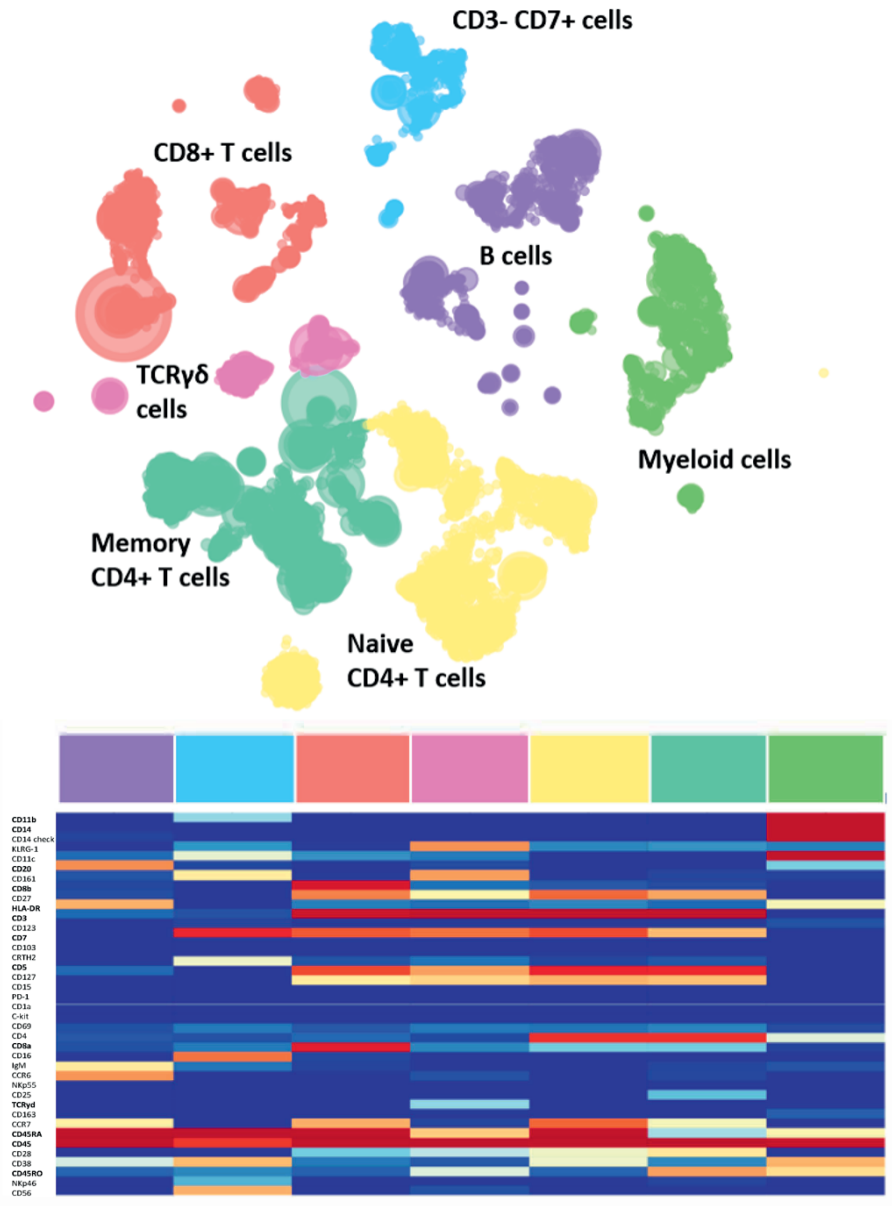


Figure 4.1. Peripheral blood mononuclear cell populations derive from multiple major immune cell lineages. HSNE embedding showing landmarks that represent all live, CD45$^+$ immune cells isolated from participant PBMCs (18.8 x 10^6). Colours in the upper plot delineate the different major immune cell lineages (CD8$^+$ T cells, naïve and memory CD4$^+$ T cells, B cells, CD3$^-$CD7$^+$ cells, myeloid cells and TCRγδ cells). These colours correspond with the colours above the columns in the lower part of the figure. In this plot, rows represent the 39 antibodies identified on immune cells in the different populations. Blue indicates low values of this antibody on the surface on the immune cells in this specific population, while red indicates high values of this antibody on the cell surface. The antibodies that are used to characterize the different immune cell populations derived from HSNE analysis are highlighted in bold.

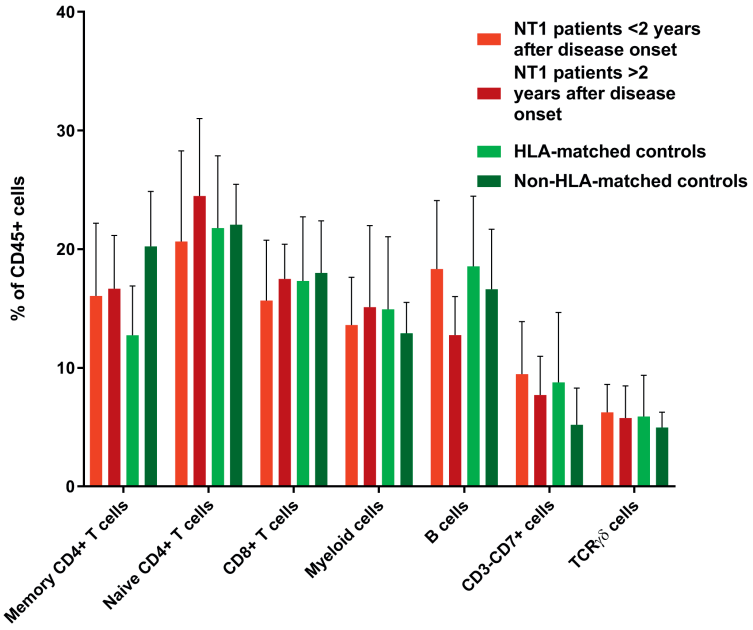


Figure 4.2. Frequencies of major immune cell lineages are comparable between NT1 patients with recent disease onset and HLA- DQB1*06:02-matched healthy controls. Relative frequency of major immune cell lineages in samples from NT1 patients with recent disease onset (bright red), later disease onset (burgundy), HLA-DQB1*06:02-matched healthy controls (bright green) and non-HLA matched controls (dark green) are shown as a percentage of all CD45$^+$ cells per group. Frequencies of none of the major immune cell lineages differed significantly between NT1 patients with recent disease onset compared to either of the other three participant groups.

HLA = human leucocyte antigen; NT1 = narcolepsy type 1; TCR = T cell receptor.

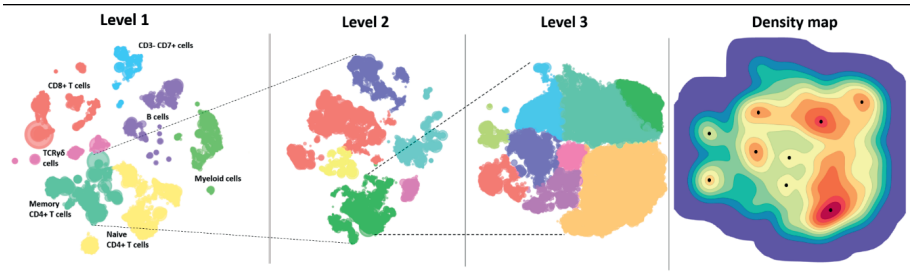


Figure 4.3. Repeated HSNE analyses lead to the identification of distinct memory CD4+ T cell populations. All landmarks are selected and embedded at the next, more detailed levels showing a finer granularity of structures. Each depicted level is the result of a new HSNE analysis on a single population delineated in the former level. This figure shows more detailed levels for the memory CD4+ T cell lineage. Unsupervised GMS clustering was used to identify phenotypically distinct immune cell clusters based on their density features. Black dots indicate the centroids of the identified clusters (as can be seen in the density map on the right).

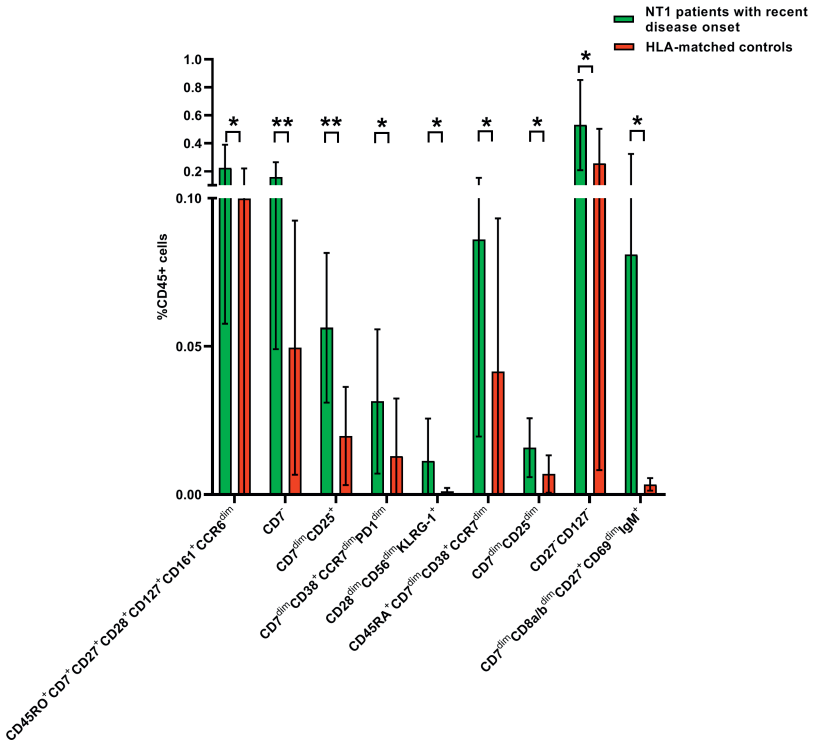


Figure 4.4. Immune cell populations within the CD4+ T cell major immune cell lineages that are significantly different between NT1 patients with recent disease onset and HLA-matched healthy controls. Frequencies are depicted as a percentage of CD45+ cells. Antibody profiles of the clusters in the figure itself are described in comparison with the immune cell cluster on the far left to highlight the differences between the clusters. The complete antibody profile of the depicted clusters is as follows (from left to right):

Cluster 10: CD45RO+CD3+CD4+CD5+CD7+CD27+CD28+CD127+CD161+CCR6dim
Cluster 9: CD45RO+CD3+CD4+CD5+CD7-CD27+CD28+CD127+CD161+CCR6dim
Cluster 13: CD45RO+CD3+CD4+CD5+CD7dimCD25+CD27+CD28+CD161dimCCR6dim
Cluster 17: CD45RO+CD3+CD4+CD5+CD7dimCD27+CD28+CD38+CCR7dimPD1dim
Cluster 25: CD45RO+CD3+CD4+CD5+CD7+CD27+CD28dimCD56dimCD127+CD161+CCR6dim KLRG1+
Cluster 30: CD45RA+CD3+CD4+CD5+CD7dimCD27+CD28+CD38+CD161+CCR7dim
Cluster 32: CD45RO+CD3+CD4+CD5+CD7dimCD25dimCD28+
Cluster 33: CD45RO+CD3+CD4+CD5+CD28+CD161+CCR6dim
Cluster 36: CD45RO+CD3+CD4+CD5+CD7dimCD8a/bdimCD27+CD28+CD69dimCD127+CCR7dim IgM+.

Information on significantly different clusters within other major immune cell lineages including their antibody profile can be found in Supplementary table 4.2. Statistical testing was performed using two-tailed Mann-Whitney U tests for unpaired samples with correction for multiple testing (Benjamini-Hochberg procedure).

* = p <0.05; ** = p <0.01. HLA = human leucocyte antigen; NT1 = narcolepsy type 1.

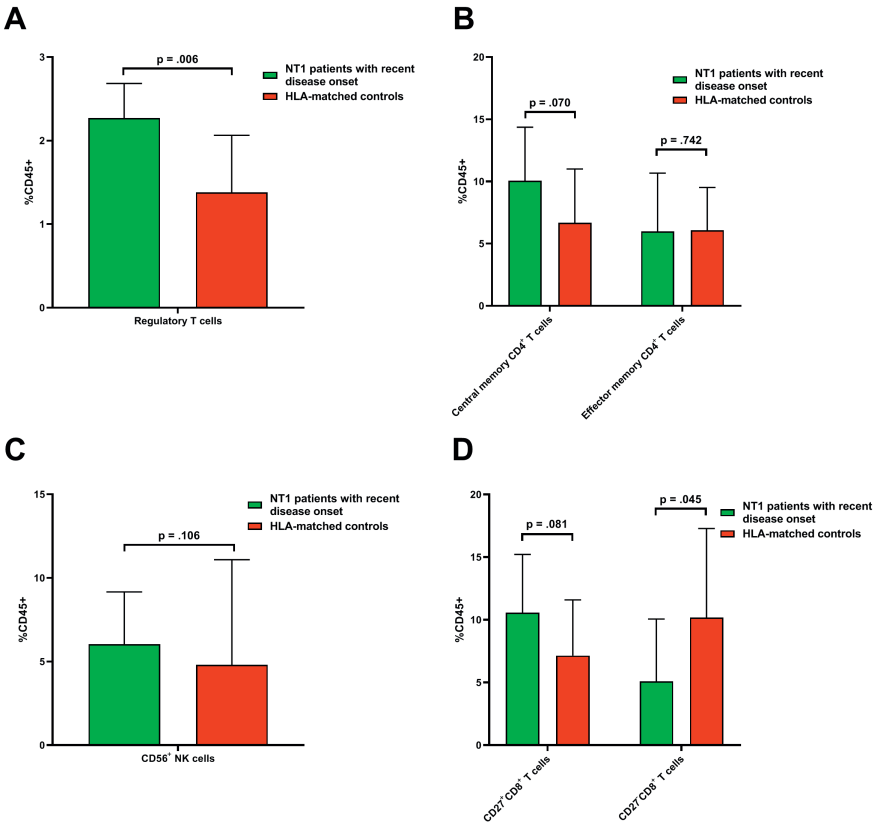


Figure 4.5. Replication of significantly different immune cell populations reported in earlier publications. A. Regulatory CD4+ T cells ($CD4^+CD25^+CD127^-$) were found more frequently in NT1 patients with recent disease onset compared to HLA-matched controls. B. Central and effector memory CD4+ T cell frequencies were similar in both participant groups. C. $CD27^-CD8^+$ T cells were less frequent in NT1 patients with recent disease onset compared to HLA-matched controls. D. The frequency of $CD3^-CD56^+$ natural killer cells was comparable between both participant groups. Statistical testing was performed using two-tailed Mann-Whitney U tests for unpaired samples.
HLA = human leucocyte antigen; NT1 = narcolepsy type 1.

NT1 with disease onset < 2 years vs > 2 years

Differences between NT1 patients within 2 years after disease onset compared with those with onset longer than 2 years before blood was drawn were less pronounced than those between patients with recent onset and controls. However, several immune cell populations within the B cell major immune cell lineage were significantly more frequently found in NT1 patients with recent disease onset. All clusters that were found to be different between these two groups of people with narcolepsy can be found in Supplementary table 4.3.

All NT1 patients vs HLA-matched controls

Since differences between patient groups were less pronounced than those between NT1 patients with recent disease onset and HLA-matched controls, we assessed differences between NT1 patients and HLA-matched controls once again, but this time for all NT1 patients, regardless of time from disease onset. Interestingly, all but one of the immune cell populations described in Figure 4.4 and Supplementary table 4.2 remained significant or differences became even more outspoken (Supplementary table 4.4).

Discussion

We describe an unbiased mass cytometry-based approach to identify immune cell clusters that are enriched in NT1 patients compared to HLA-matched healthy controls. While differences in major immune cell lineages were absent in our sample, we show that several populations of memory $CD4^+$ T cells and one population of $CD8^+$ T cells were more frequently found in NT1 patients than in healthy HLA-DQB1*06:02-matched controls. Additionally, we show that differences in immune cell composition between NT1 patients with recent disease onset and those longer after disease onset were limited. This finding is supported by the fact that most populations discovered in the comparison between NT1 patients with recent disease onset and HLA-matched controls remained significantly different when comparing all NT1 patients with HLA-matched controls.

These results build upon the findings of other studies that have compared the composition of the immune system in NT1 with that of healthy controls using flow cytometry in which a global T cell activation in peripheral blood of NT1 patients (Lecendreux et al., 2017, Moresco et al., 2018) and an even stronger T cell activation was seen in the cerebrospinal fluid compared with healthy controls (Moresco et al., 2018). Moresco et al. further describe more central memory ($CD45RO^+CCR7^+$), more naïve ($CD45RA^+CCR7^+$), less effector memory ($CD45RO^+CCR7^-$) $CD4^+$ T cells and more $CD3^-CD56^+$ cells in NT1 patients. We only replicated a non-significant higher frequency of the latter in recent onset NT1 patients compared to healthy controls. Another study on the immune cell composition in the peripheral blood of NT1

patients (Hartmann et al., 2016) compared PBMCs of healthy donors and NT1 patients with other hypersomnolence disorders by mass cytometry. Of note, the panel of immune cell markers Hartmann et al. used differs from ours as they used antibodies targeting both surface antigens and cytokines, while we used only surface marker antibodies. Moreover, Hartman et al. included mostly NT1 patients several years after disease onset, so that comparisons between results of both studies should be made with caution. However, as our findings indicate, the differences in immune cell composition between NT1 patients with recent and those with later onset might well be negligible. Hartmann et al. reported multifaceted immune activation in primarily the $CD4^+$ and $CD8^+$ T cell compartments. This is partly reflected by our results: several populations of memory $CD4^+$ and $CD8^+$ T cells were found more frequently in NT1 patients compared with HLA-matched healthy controls (Supplementary table 4.2), albeit not corresponding with the population found by Hartmann et al. Since NT1 patients were included longer after disease onset in the study by Hartmann et al, they righteously mention that it remains to be established whether these changes are primarily due to an autoimmune process in NT1. A recent study that compares immune cell composition in Pandemrix®-induced NT1 patients with their healthy siblings by mass cytometry reports a lower frequency of $CD8^+CD27^+$ T cells in patients (Lind et al., 2020). Interestingly, our study shows a non-significant higher frequency of $CD8^+CD27^+$ T cells, that were mostly $CD45RO^-$ and could therefore be considered naïve $CD8^+$ T cells (Hintzen et al., 1994). Additionally, $CD8^+CD27^-$, mostly $CD45RA^+$, T cells were found to be increased in NT1 patients, consistent with terminally differentiated effector $CD8^+$ T cells (TEMRA) (Hamann et al., 1999). Also the increased frequency of regulatory $CD4^+$ T cells, consistent with earlier findings (Lecendreux et al., 2017), points to multifaceted immune activation in NT1 patients.

A limitation to our study is that we were not able to measure cerebrospinal fluid samples in addition to PBMCs. Acquiring cerebrospinal fluid samples close to disease onset is challenging, since many NT1 patients are diagnosed later after disease onset. Also, many of them already meet the criteria for NT1 without the necessity for testing hypocretin-1 in cerebrospinal fluid, which makes a lumbar puncture a redundant invasive procedure. Furthermore, the low amount of cells in cerebrospinal fluid does not allow for performing mass cytometry experiments

reliably. As several studies point out, immune cells crossing the blood-brain barrier are of special interest and technical innovations should make in-depth assessment of the immune cell composition in cerebrospinal fluid possible in the near future.
Additionally, it could be argued that differences between groups might be driven by single patients. We extensively checked our results for outliers. However, variability in the composition of the immune system between patients is high. Validation of our results in other cohorts of NT1 patients is therefore important to confirm the value of our findings.
Our results do not allow for inferring causality. The technique that was used is exploratory and should lead to focused experiments on the role of the identified enriched immune cell populations we identified have in the presumed autoimmune response leading to the destruction of hypocretin-producing neurons. Performing functional experiments on the immune cell populations discovered in this study could serve as a starting point for identifying the mechanisms involved in the autoimmune response leading to the destruction of hypocretin-producing neurons. Assessing immune cell reactivity to antigens of interest (such as hypocretin, H1N1 or other, still unknown antigens) with the identified NT1-specific immune cell populations seems a promising strategy, which allows for diminishing the background noise that other immune cells might have caused in previous experimental antigen-centered research on the autoimmune response leading to NT1.

Conclusions

Populations of memory $CD4^+$ and $CD8^+$ T cells and regulatory $CD4^+$ T cells are more frequently found in the peripheral blood of NT1 patients than in healthy HLA-DQB1*06:02-matched controls. Differences in major immune cell lineages are absent in our sample. Additionally, differences in immune cell composition between NT1 patients with recent disease onset and those longer after disease onset are limited. These findings point towards multifaceted immune activation in NT1 patients. Performing functional experiments on the immune cell populations discovered in this study could serve as a starting point for identifying novel mechanisms driving the autoimmune response leading to the destruction of hypocretin-producing neurons.

Supplementary material

Supplementary table 4.1. Summary of antibodies used for the mass cytometry experiments.

Antibodies	Clone	Source	Identifier
Anti-human CD45	HI30	Fluidigm	Cat# 3089003B
Anti-human CD14	Tük4	ThermoFisher Scientific	Cat# Q10064
Anti-human C-Kit	104D2	Fluidigm	Cat# 3143001B
Anti-human CCR6	G034E3	Fluidigm	Cat# 3141003A
Anti-human CD45RA	HI100	Fluidigm	Cat# 3169008B
Anti-human CD15	W6D3	BioLegend	Cat# 323035
Anti-human CD69	FN50	Fluidigm	Cat# 3144018B
Anti-human CD4	RPA-T4	Fluidigm	Cat# 3145001B
Anti-human CD8a	RPA-T8	Fluidigm	Cat# 3146001B
Anti-human CD1a	HI149	Sony	Cat# 2100510
Anti-human CD16	3G8	Fluidigm	Cat# 3148004B
Anti-human CD25	2A3	Fluidigm	Cat# 3149010B
Anti-human CD5	UCHT2	BioLegend	Cat# 300627
Anti-human CD123	6H6	Fluidigm	Cat# 3151001B
Anti-human TCRγδ	11F2	Fluidigm	Cat# 3152008B
Anti-human CD7	CD7-6B7	Fluidigm	Cat# 3153014B
Anti-human IgM	MHM88	BioLegend	Cat# 314527
Anti-human CD103	Ber-ACT8	BioLegend	Cat# 350202
Anti-human CRTH2	BM16	BioLegend	Cat# 350102
Anti-human CD122	TU27	BioLegend	Cat# 339015
Anti-human CCR7	G043H7	Fluidigm	Cat# 3159003A
Anti-human CD163	GHI/61	Fluidigm	Cat# 3154007A
Anti-human KLRG1	REA261	Miltenyi Biotec	Cat# 120-014-229
Anti-human CD11c	Bu15	Fluidigm	Cat# 3162005B
Anti-human CD20	2H7	BioLegend	Cat# 302343
Anti-human CD161	HP-3G10	Fluidigm	Cat# 3164009B
Anti-human CD127	AO19D5	Fluidigm	Cat# 3165008B
Anti-human CD8b	SIDI8BEE	Ebio	Cat# 15257407
Anti-human CD27	O323	Fluidigm	Cat# 3167002B
Anti-human NKp46	9E2	BioLegend	Cat# 331902
Anti-human NKp44	253415	R&D	Cat# MAB22491
Anti-human CD3	UCHT1	Fluidigm	Cat# 3170001B
Anti-human CD28	CD28.2	BioLegend	Cat# 302937
Anti-human CD38	HIT2	Fluidigm	Cat# 3172007B
Anti-human CD45RO	UCHL1	BioLegend	Cat# 304239
Anti-human HLA-DR	L243	BioLegend	Cat# 307651
Anti-human PD-1	EH 12.2H7	Fluidigm	Cat# 3175008B
Anti-human CD56	NCAM16.2	Fluidigm	Cat# 3176008B
Anti-human CD11b	ICRF44	Fluidigm	Cat# 3209003B

Supplementary table 4.2. All immune cell populations within all major immune cell lineages that were significantly different between NT1 patients with recent disease onset compared with HLA-matched controls. All clusters that were more frequently found in NT1 patients with recent disease onset are green, those more frequently found in HLA-matched controls are red. Statistical testing was performed using two-tailed Mann-Whitney U tests for unpaired samples. Except for cluster 2 and 4 of the naïve $CD4^+$ T cell lineage, cluster 9 and 13 of the memory $CD4^+$ T cell lineage, cluster 14 of the γδ T cell lineage and cluster 9 of the $CD3^-CD7^+$ immune cell lineage ($p<0.01$), all p-values from the analyses shown were between $p = 0.01$ and $p = 0.05$.

Naive $CD4^+$ T cells	Antibody profile
Cluster 2	$CD45RA^+CD3^+CD4^+CD5^+CD7^{dim}CD27^+CD28^+CD38^+CCR7^+$
Cluster 4	$CD45RA^+CD3^+CD4^+CD5^+CD7^{dim}CD27^+CD28^+CD38^+CCR7^+$
Cluster 9	$CD45RA^+CD3^+CD4^+CD5^+CD7^+CD11b^{dim}CD14^{dim}CD11c^{dim}CD127^{dim}CD38^{dim}$

Memory $CD4^+$ T cells	Antibody profile
Cluster 9	$CD45RO^+CD3^+CD4^+CD5^+CD7^-CD27^+CD28^+CD127^+CD161^+CCR6^{dim}$
Cluster 10	$CD45RO^+CD3^+CD4^+CD5^+CD7^+CD27^+CD28^+CD127^+CD161^+CCR6^{dim}$
Cluster 13	$CD45RO^+CD3^+CD4^+CD5^+CD7^{dim}CD25^+CD27^+CD28^+CD161^{dim}CCR6^{dim}$
Cluster 17	$CD45RO^+CD3^+CD4^+CD5^+CD7^{dim}CD27^+CD28^+CD38^+CCR7^{dim}PD1^{dim}$
Cluster 25	$CD45RO^+CD3^+CD4^+CD5^+CD7^+CD27^+CD28^{dim}CD56^{dim}CD127^+CD161^+CCR6^{dim}$ $KLRG1^+$
Cluster 30	$CD45RA^+CD3^+CD4^+CD5^+CD7^{dim}CD27^+CD28^+CD38^+CD161^+CCR7^{dim}$
Cluster 32	$CD45RO^+CD3^+CD4^+CD5^+CD7^{dim}CD25^{dim}CD28^+$
Cluster 33	$CD45RO^+CD3^+CD4^+CD5^+CD28^+CD161^+CCR6^{dim}$
Cluster 36	$CD45RO^+CD3^+CD4^+CD5^+CD7^{dim}CD8a/b^{dim}CD27^+CD28^+CD69^{dim}CD127^+C-CR7^{dim}$ IgM^+

$CD8^+$ T cells	Antibody profile
Cluster 20	$CD45RA^+CD3^+CD5^+CD7^+CD8a^+CD8b^+CD27^+CD127^{dim}CCR7^{dim}$

γδ T cells	Antibody profile
Cluster 4	$CD45RA^+CD3^+CD5^+CD7^+CD25^{dim}CD27^+CD28^{dim}CCR6^{dim}CCR7^{dim}$
Cluster 5	$CD45RA^+CD3^+CD5^+CD7^+CD8a^+CD8b^+CD27^+CD28^+CD103^{dim}CD161^+CCR6^{dim}$ $CCR7^{dim}KLRG1^+$
Cluster 7	$CD45^+CD3^+CD5^{dim}CD7^{dim}CD127^{dim}CD161^+KLRG1^{dim}$
Cluster 9	$CD45RA^+CD3^+CD5^+CD7^+CD27^+CD28^+CD38^+CD127^{dim}CCR7^{dim}TCR\gamma\delta^{dim}$
Cluster 14	$CD45RA^+CD3^+CD5^+CD27^+CD28^+CD38^+CCR7^{dim}$

Myeloid cells	Antibody profile
Cluster 13	$CD45RO^+CD4^{dim}CD11b^+CD11c^+CD14^+CD16^+CD38^+HLA\text{-}DR^{dim}$
Cluster 14	$CD45RO^{dim}CD4^{dim}CD8a^{dim}CD11b^+CD11c^+CD14^+CD16^+CD38^+CD161^{dim}$ $HLA\text{-}DR^{dim}IgM^{dim}$

$CD3^-$ $CD7^+$ cells	Antibody profile
Cluster 1	$CD45RA^+CD3^-CD7^+CD16^+CD28^+CD56^{dim}$
Cluster 9	$CD45RA^+CD3^-CD7^+CD8b^+CD11c^{dim}CD16^+CD56^+CD122^{dim}$
Cluster 10	$CD45RA^+CD3^-CD7^+CD16^+CD27^{dim}CD38^+CD56^+CD122^+CD161^+$ NKp46$^+$

B cells	Antibody profile
Cluster 28	$CD45RA^+CD3^+CD4^+CD5^+CD7^{dim}CD8a^{dim}CD14^{dim}CD16^+CD20^+CD38^{dim}CD127^{dim}$ $CD161^{dim}CCR7^{dim}$
Cluster 30	$CD45RA^+CD1a^{dim}CD4^{dim}CD8a^{dim}CD14^{dim}CD16^+CD20^+CD69^{dim}CD161^+CCR6^+$HLA-$DR^{dim}IgM^+$

Supplementary table 4.3. All immune cell populations within all major immune cell lineages that were significantly different between NT1 patients with recent disease onset compared with those with later disease onset. All clusters that were more frequently found in NT1 patients with recent disease onset are green, those more frequently found in NT1 patients with later disease onset are red. Statistical testing was performed using two-tailed Mann-Whitney U tests for unpaired samples. Except for cluster 35 of the memory $CD4^+$ T cell lineage ($p<0.01$), all p-values from the analyses shown were between $p = 0.01$ and $p = 0.05$.

Naive $CD4^+$ T cells	Antibody profile
Cluster 5	$CD45RA^+CD3^+CD4^+CD5^+CD7^+CD11b^+CD15^{dim}CD27^+CD28^{dim}CD38^+CD$-$127^{dim}CCR7^+$
Cluster 16	$CD45RA^+CD3^+CD4^+CD5^+CD7^+CD27^+CD28^{dim}CD127^+CCR7^+$

Memory $CD4^+$ T cells	Antibody profile
Cluster 1	$CD45RA^+CD3^+CD4^+CD5^+CD7^+CD27^+CD28^{dim}CD127^+CD161^{dim}CCR7^+$
Cluster 10	$CD45RO^+CD3^+CD4^+CD5^+CD7^+CD25^{dim}CD27^+CD127^+CD161^+CCR6^{dim}$
Cluster 35	$CD45RA^+CD3^+CD4^+CD5^+CD7^+CD8a^{dim}CD8b^{dim}CD27^+CD28^{dim}CD$-$38^{dim}CD69^{dim}CD127^{dim}CCR7^+IgM^+$
Cluster 36	$CD45RO^+CD3^+CD4^+CD5^+CD7^{dim}CD8a^{dim}CD8b^{dim}CD27^+CD28^+CD69^{dim}C$-$D127^+CCR7^{dim}IgM^+$

$CD8^+$ T cells	Antibody profile
None.	

γδ T cells	Antibody profile
Cluster 18	$CD45RA^+CD3^+CD4^{dim}CD5^+CD7^+CD8a^{dim}CD8b^+CD27^+CD28^{dim}CD127^+CCR7^+$

Myeloid cells	Antibody profile
Cluster 25	$CD45RA^+CD4^{dim}CD11c^+CD16^+CD20^{dim}CD123^{dim}$HLA-$DR^{dim}$
Cluster 32	$CD45RA^+CD1a^{dim}CD4^{dim}CD8a^{dim}CD8b^+CD16^{dim}CD20^+CD69^{dim}CD123^{dim}C$ $CR6^+CCR7^{dim}C$-kit^{dim}HLA-DR^+IgM^+

$CD3^-$ $CD7^+$ cells	Antibody profile
None.	

B cells	Antibody profile
Cluster 8	$CD45RA^{+}$
Cluster 10	$CD45RA^{+}CD11b^{+}CD15^{+}CD16^{+}$
Cluster 11	$CD45RA^{+}CD11b^{+}CD11c^{dim}CCR6^{dim}IgM^{dim}$
Cluster 12	$CD45^{dim}CD38^{dim}CD69^{+}IgM^{dim}$
Cluster 13	$CD45RA^{+}CD3^{+}CD5^{dim}CD7^{dim}$ $CD11b^{dim}CD11c^{dim}CD14^{dim}$ $CD27^{dim}CD69^{+}$HLA-DR^{dim} IgM^{+}
Cluster 20	$CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{+}CD20^{+}CD27^{+}CD28^{dim}CD38^{dim}CD127^{dim}CCR6^{+}$ $CCR7^{+}$HLA-$DR^{+}IgM^{dim}$

Supplementary table 4.4. Populations within those presented in Supplementary table 4.2 that remained significantly different when tested for all NT1 patients (shortly and longer after disease onset) compared with HLA-matched controls. All clusters that were more frequently found in NT1 patients are green, those more frequently found in HLA-matched controls are red. Statistical testing was performed using two-tailed Mann-Whitney U tests for unpaired samples. To all immune cell clusters that differed with a $p<0.01$ an asterisk is added. To those clusters that differed with a $p<0.001$ two asterisks are added.

Naive $CD4^{+}$ T cells	Antibody profile
Cluster 2★	$CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD27^{+}CD28^{+}CD38^{+}CCR7^{+}$
Cluster 4★	$CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD27^{+}CD28^{+}CD38^{+}CCR7^{+}$
Cluster 9	$CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{+}CD11b^{dim}CD14^{dim}CD11c^{dim}CD127^{dim}CD38^{dim}$

Memory $CD4^{+}$ T cells	Antibody profile
Cluster 9★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{-}CD27^{+}CD28^{+}CD127^{+}CD161^{+}CCR6^{dim}$
Cluster 10★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{+}CD27^{+}CD28^{+}CD127^{+}CD161^{+}CCR6^{dim}$
Cluster 13★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD25^{+}CD27^{+}CD28^{+}CD161^{dim}CCR6^{dim}$
Cluster 17	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD27^{+}CD28^{+}CD38^{+}CCR7^{dim}PD1^{dim}$
Cluster 25★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{+}CD27^{+}CD28^{dim}CD56^{dim}CD127^{+}CD161^{+}CCR6^{dim}$ $KLRG1^{+}$
Cluster 30★	$CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD27^{+}CD28^{+}CD38^{+}CD161^{+}CCR7^{dim}$
Cluster 32★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD25^{dim}CD28^{+}$
Cluster 33★	$CD45RO^{+}CD3^{+}CD4^{+}CD5^{+}CD28^{+}CD161^{+}CCR6^{dim}$

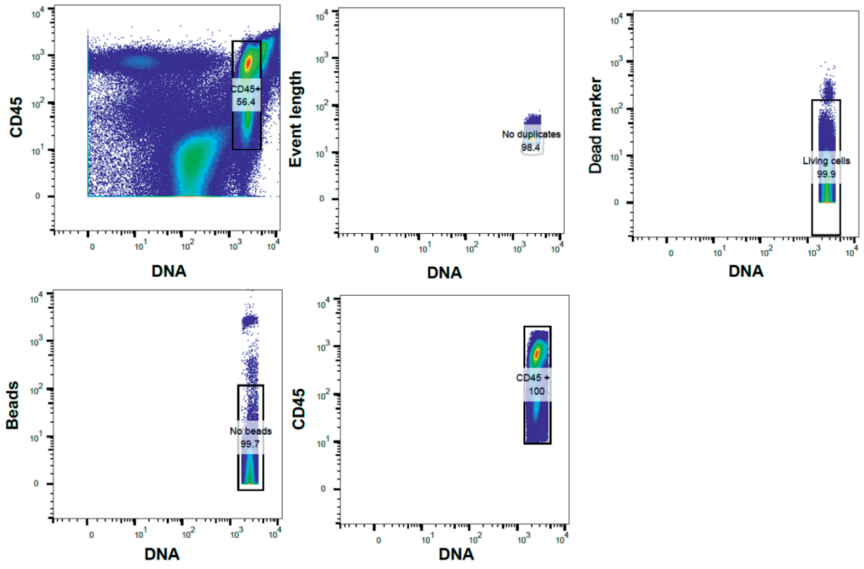
$CD8^{+}$ T cells	Antibody profile
Cluster 20★	$CD45RA^{+}CD3^{+}CD5^{+}CD7^{+}CD8a^{+}CD8b^{+}CD27^{+}CD127^{dim}CCR7^{dim}$

γδ T cells	Antibody profile
Cluster 4★	$CD45RA^{+}CD3^{+}CD5^{+}CD7^{+}CD25^{dim}CD27^{+}CD28^{dim}CCR6^{dim}CCR7^{dim}$
Cluster 5	$CD45RA^{+}CD3^{+}CD5^{+}CD7^{+}CD8a^{+}CD8b^{+}CD27^{+}CD28^{+}CD103^{dim}CD161^{+}CCR6^{dim}$ $CCR7^{dim}KLRG1^{+}$
Cluster 7★	$CD45^{+}CD3^{+}CD5^{dim}CD7^{dim}CD127^{dim}CD161^{+}KLRG1^{dim}$
Cluster 9★	$CD45RA^{+}CD3^{+}CD5^{+}CD7^{+}CD27^{+}CD28^{+}CD38^{+}CD127^{dim}CCR7^{dim}TCR\gamma\delta^{dim}$
Cluster 14★★	$CD45RA^{+}CD3^{+}CD5^{+}CD27^{+}CD28^{+}CD38^{+}CCR7^{dim}$

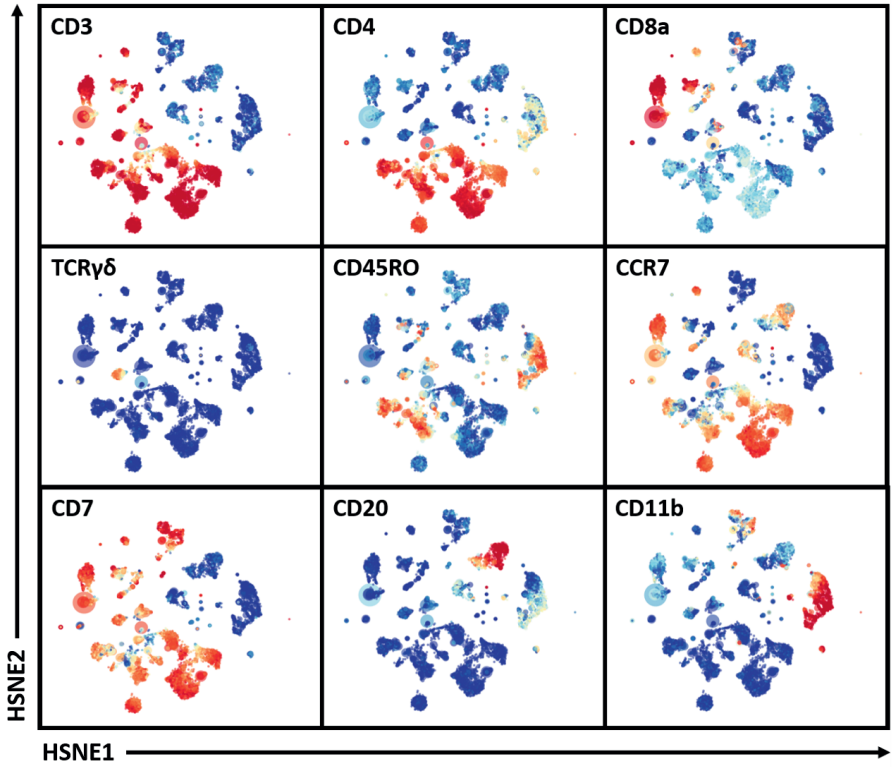
Myeloid cells	Antibody profile
Cluster 13★	CD45RO^{+}CD4^{dim}CD11b^{+}CD11c^{+}CD14^{+}CD16^{+}CD38^{+}HLA-DR^{dim}
Cluster 14	CD45RO^{dim}CD4^{dim}CD8a^{dim}CD11b^{+}CD11c^{+}CD14^{+}CD16^{+}CD38^{+}CD161^{dim} HLA-DR^{dim}IgM^{dim}

CD3^{-} CD7^{+} cells	Antibody profile
Cluster 1	CD45RA^{+}CD3^{-}CD7^{+}CD16^{+}CD28^{+}CD56^{dim}
Cluster 9★	CD45RA^{+}CD3^{-}CD7^{+}CD8b^{+}CD11c^{dim}CD16^{+}CD56^{+}CD122^{dim}
Cluster 10★	CD45RA^{+}CD3^{-}CD7^{+}CD16^{+}CD27^{dim}CD38^{+}CD56^{+}CD122^{+}CD161^{+}NKp46^{+}

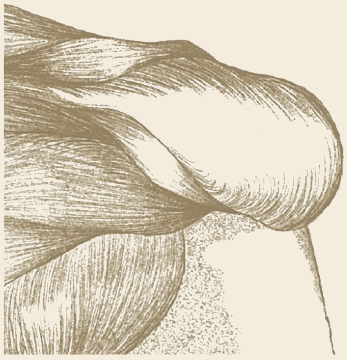
B cells	Antibody profile
Cluster 28	CD45RA^{+}CD3^{+}CD4^{+}CD5^{+}CD7^{dim}CD8a^{dim}CD14^{dim}CD16^{+}CD20^{+}CD38^{dim}CD127^{dim} CD161^{dim}CCR7^{dim}
Cluster 30	CD45RA^{+}CD1a^{dim}CD4^{dim}CD8a^{dim}CD14^{dim}CD16^{+}CD20^{+}CD69^{dim}CD161^{+}CCR6^{+}H-LA- DR^{dim}IgM^{+}

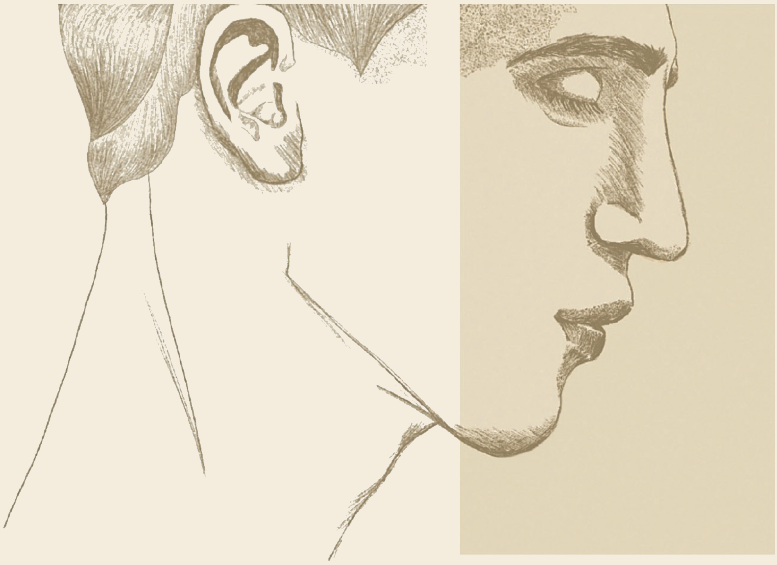


Supplementary figure 4.1. Mass cytometry gating strategy for the identification and selection of single, live CD45+ cells of a representative peripheral blood mononuclear cell sample showing sequential gates with percentages.



Supplementary figure 4.2. HSNE embedding showing marker expression patterns of antibodies that are used to differentiate between major immune cell lineages on single, live CD45+ cells. Each dot represents a landmark derived from a total of 18.8 x 10^6 peripheral blood mononuclear cells of all participants. All markers are shown with an expression range of 0-5 (blue indicates a low value, red indicates a high value). HSNE = hierarchical stochastic neighbour embedding.





Chapter 5

Decreased body mass index during treatment with sodium oxybate in narcolepsy type 1

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Abstract

Narcolepsy type 1 patients is characterized by an increase in body weight after disease onset, frequently leading to obesity. It was suggested that this weight gain may be counteracted by treatment with sodium oxybate (SXB). We here provide longitudinal BMI data of patients with narcolepsy type 1 after starting treatment with SXB, compared to patients in whom treatment with modafinil was initiated. 81 individuals with narcolepsy type 1 fulfilled the entry criteria for this retrospective study: 59 had newly started treatment with SXB, and 22 had newly started modafinil. Gender-specific differences between both treatment groups were compared using Student's t-tests and mixed effect modeling. Patients using SXB lost weight with a mean BMI decrease of 2.56 kg/m^2 between the first and last measurement (women; p=0.001) and 0.84 kg/m^2 (men; p=0.006). Patients using modafinil, however, gained weight with a mean BMI increase of 0.57 kg/m^2 (women; p = 0.033) and 0.67 kg/m^2 (men; p=0.122). Medication (p=0.006) and baseline BMI (p=0.032) were predictors for BMI decrease. In conclusion, treatment with SXB is associated with a BMI reduction in narcolepsy type 1, while modafinil treatment is not. This effect is most pronounced in those who already have a higher baseline BMI.

Introduction

Narcolepsy type 1 (NT1) is a chronic neurological disorder characterised by excessive daytime sleepiness, cataplexy, sleep paralysis, hypnagogic hallucinations and disturbed nocturnal sleep (Black et al., 2017). In addition to the classical symptoms, other symptoms have been reported. These include autonomic abnormalities and obesity (Fronczek et al., 2008). Narcolepsy type 1 is caused by a loss of hypothalamic hypocretin (orexin)-producing neurons (Nishino et al., 2001). Hypocretin neurons project throughout the central nervous system to areas known to be important in the control of sleep-wakefulness, but also to areas important in neuroendocrine homeostasis, autonomic regulation and the control of feeding (Willie et al., 2001).

From as early as the 1930s it has been reported that obesity is more prevalent in narcolepsy patients than in healthy controls (Daniels 1934; Wang et al., 2016). Abdominal fat deposition and waist circumference were found to be significantly increased in narcolepsy patients (Kok et al., 2003). who also have a higher prevalence of the metabolic syndrome compared to idiopathic hypersomnia patients (Poli et al., 2009). Also in children NT1 onset was associated with rapid weight gain (Ponziani et al., 2016). Cause for the observed obesity in NT1 has not been elucidated. It is probably not secondary to inactivity or to medication use (Black et al., 2017). Studies of eating habits showed conflicting results regarding caloric intake, the prevalence of eating disorders in NT1 (Fortuyn et al., 2008) and basal metabolic rate. A recent study in children with NT1 showed a lower basal metabolic rate (BMR) closely after disease onset, which restored to normal levels in the following months (Wang et al., 2016). Therefore, it is hypothesized that NT1 induces a change in the individual body mass index (BMI) set point (Dahmen et al., 2009), but the exact mechanism causing this hypothesized change in NT1 patients remains unclear. Management of BMI in NT1 is important, as a higher BMI seems a risk factor for diseases, such as diabetes type 2 and cardiovascular disease (Kok et al., 2003) and predisposes to psychosocial and professional disability (World Health Organization, 2000; Narbro et al., 1996).

Recent observations suggest that pediatric and adult narcolepsy patients lose weight when using sodium oxybate (SXB; Boscolo-Berto et al., 2012; Ponziani et al., 2016). Weight loss in narcolepsy patients with (mean loss of 5.1kg) and without cataplexy (mean loss of 2kg) treated with SXB has been reported (Husain

et al., 2009), while another study showed a 5.2kg weight loss after 3 months of SXB treatment (Donjacour et al., 2014). This has not yet been confirmed and long-term follow-up data is not available. We here provide longitudinal BMI data of NT1 patients after starting treatment with SXB, compared to patients in whom treatment with modafinil was initiated. Our hypothesis is that BMI decreases upon introduction of SXB, while use of modafinil will not affect BMI.

Methods

Subjects

Medical records of consecutive individuals diagnosed with NT1 attending the outpatient clinic of the Sleep-Wake Center Stichting Epilepsie Instellingen Nederland (SEIN) and the outpatient clinic of the Leiden University Medical Centre between 2009 and 2017 were reviewed retrospectively. All individuals included (n=81) fulfilled the criteria for NT1 as formulated in the third edition of the International Classification of Sleep Disorders (ICSD-3, 2014). Only those who initiated treatment with either SXB or modafinil and used it for at least three months were included. Those who had already used one of the medications were excluded. Patients were also excluded if there were no measurements recorded, if they suffered from another disorder that is associated with obesity (e.g. hypothyroid disease) or if they were under the age of 18 years. The follow up period ended prematurely if the SXB or modafinil treatment was discontinued, or when additional medication was started. Relevant co-medication use (antidepressants, methylphenidate, dexamphetamine) was extracted from the records. The decision to prescribe either modafinil or SXB was a clinical decision based on the presence and severity of the various NT1 symptoms. Weight at treatment start did not play a role in this decision.

Study design

This is a retrospective follow-up study in which the above described individuals were followed up to July 31st 2017. Weight, length and medication, assessed at the beginning of each visit to the outpatient clinic were extracted from the records. BMI was calculated by dividing the weight (kg) by the squared length (m^2) for each visit.

Statistical analysis

Differences at baseline in participant characteristics between the SXB and modafinil group were calculated with Mann-Whitney U (age), chi-square (gender, co-medication), and Student's t-tests (baseline BMI, treatment duration). To estimate if the BMI of the study cohort was significantly higher than that of the general Dutch population, data from Statistics Netherlands (Centraal Bureau voor de Statistiek, 2016) were used.

To assess whether statistically significant BMI changes occurred during follow-up, a paired sample Student´s test was performed to compare BMI at baseline with BMI at the last visit for four separate groups (based on gender and treatment). Since this comparison is still subject to factors influencing BMI and there was a single outcome parameter, a linear mixed model with a random slope and a random intercept for each individual was fitted. The outcome was the difference in BMI with respect to baseline. As fixed effects we added medication, gender, baseline BMI and follow-up duration, and also the interactions of medication, gender and baseline BMI with follow-up duration. Normality of the fitted mixed model was subsequently assessed using scatter plots and quantile-quantile plots of the model's residuals. Correlations between medication dose and BMI change for individual patients were assessed using Spearman's rank-order correlation tests. P-values below 0.05 were deemed significant. Bonferroni corrections were executed when needed. All analyses were conducted using the IBM SPSS Statistics 23 software package.

Results

Patient characteristics

59 individuals started treatment with SXB and 22 with modafinil (Table 5.1). Follow-up frequency and duration varied considerably among patients (range 0.14-6.94 years). There were no significant differences found in age and baseline BMI between groups. More males were in the SXB group. Mean duration of treatment for the SXB group was longer than that of the modafinil group.

Table 5.1. Characteristics of narcolepsy type 1 patients. Data indicate mean ± standard deviation.

	SXB	Modafinil	p-value
N	59	22	
Age (years)	34.5 ± 13.4	39.0 ± 19.7	0.332
Males (%)	38 (64%)	6 (27%)	0.005
Baseline BMI (kg/m^2)	28.6 ± 4.3	26.5 ± 5.5	0.070
Follow-up (years)	2.0 ± 1.7	1.2 ± 1.2	0.054*
Male	1.9 ± 1.5	0.9 ± 0.7	0.152*
Female	2.2 ± 1.9	1.3 ± 1.4	0.134*
HLA-DQB1*06:02 +	56/57	18/19	0.408
Hypocretin < 110pg/mL	32/35	6/7	0.638

BMI = body mass index; SXB = sodium oxybate. P-values result from Student's t-tests for the difference between treatment groups. * To adjust for multiple testing, p-value <0.05/4 was considered to be significant.

Co-medication

There were no significant differences between the percentages of patients in the SXB and the modafinil group who received no additional medication (p = 0.209) and patients who used antidepressants (p = 0.094). The amount of patients using methylphenidate or dexamphetamine was higher in the SXB group (p = 0.007; Table 5.2).

Table 5.2. Co-medication of narcolepsy type 1 patients. Data indicate number of patients (%). Antidepressants (N SXB-group/N modafinil-group): clomipramine (7/6), venlafaxine (3/0), imipramine (2/1), citalopram (0/1). Median treatment 0.96 years (range 0.25-19 years). P-values result from Student's t-tests for the difference between treatment groups. SXB = sodium oxybate.

	SXB (N = 59)	Modafinil (N = 22)	p-value
None	23 (39.0)	12 (54.5)	0.209
Antidepressant	12 (20.3)	8 (36.4)	0.167
Methylphenidate/dexamphetamine	24 (40.7)	2 (9.1)	0.007

BMI in NT1 patients is higher than mean BMI in Dutch people above 18 years old

The mean BMI of the patient cohort differed significantly from that of a representative sample of Dutch people above 18 years old. In men, the mean BMI of our study population was significantly higher than the mean BMI of Dutch males above 18 years old (mean ± standard error of the mean (SEM) 27.9 ± 0.58 kg/m^2 vs 25.3 kg/m^2; p < 0.001). Likewise, women in our patient groups had a significantly higher BMI than the average for Dutch women above 18 years old (mean ± SEM 28.2 ± 0.93 kg/m^2 vs 25.8 kg/m^2; p < 0.001).

BMI decreases in patient groups between first and last measurement

Four separate groups were constructed based on treatment and gender. Those treated with SXB lost weight during follow-up (-1.58 kg/m^2, SD 2.12 kg/m^2; p < 0.001; Figure 5.1 and Figure 5.2). Women using SXB lost weight with a mean BMI decrease of 2.56 kg/m^2 (SD 2.20 kg/m^2; p = 0.001) which corresponds to an average weight loss of 7.1 kg; men with a mean BMI decrease of 0.84 kg/m^2 (SD 1.71 kg/m^2; p = 0.006) which corresponds to 2.8 kg. Patients using modafinil, however, gained weight (0.60 kg/m^2, SD 0.91 kg/m^2; p = 0.005). A mean BMI increase of 0.57 kg/m^2 (SD 0.96 kg/m^2; 1.6 kg) was found in women (p = 0.033), while a mean BMI increase of 0.67 (SD 0.88 kg/m^2; 2.3 kg) was observed in men (p = 0.122). After adjustment for multiple testing BMI decrease in women using modafinil failed to reach statistical significance.

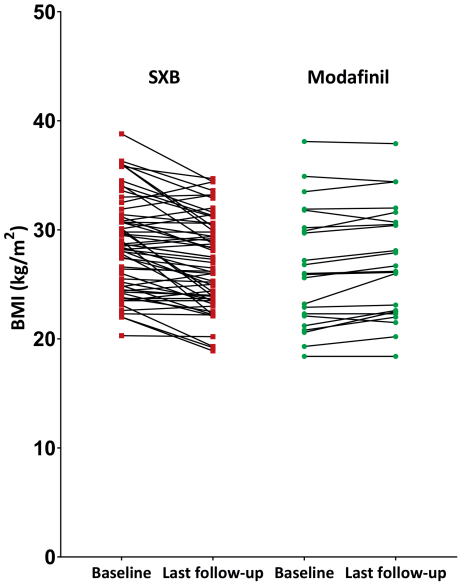


Figure 5.1. Body mass index (BMI) values at baseline and at last follow-up of patients in whom treatment with sodium oxybate (SXB; red, N = 59) and patients in whom treatment with modafinil (green, N = 22) was newly started. Each data point represents one patient at either baseline or follow-up. Individual values per patient are linked.

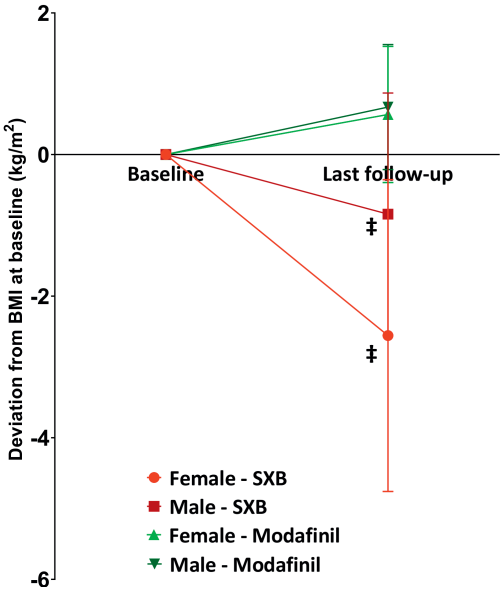


Figure 5.2. Patients treated with sodium oxybate (SXB) showed a decrease in body mass index (BMI) during follow-up while patients treated with modafinil did not. P-values are derived from Student's paired t-tests comparing normalized BMI values at baseline and at last follow-up. Mean follow-up for SXB and modafinil was 2.0 ± 1.7 vs 1.2 ± 1.2 ($p = 0.054$).

⋆ $p < 0.05$ † $p < 0.01$ ‡ $p < 0.001$

BMI at baseline and medication type influence BMI difference over time

Patients using SXB showed a decrease in BMI that was larger than the BMI deviation of modafinil users ($F(1,69.396) = 8.180$, $p = 0.006$). A higher baseline BMI was found to predict a more pronounced BMI decrease ($F(1,26.040) = 5.137$, $p = 0.032$). The effect gender had on BMI deviations was found not to be significant ($F(1,72.923) = 3.464$, $p = 0.067$). An overview of these and all other main and interaction effects can be found in Table 5.3. Mean values of BMI differences from baseline for four groups based on gender and medication type are depicted in Figure 5.3.

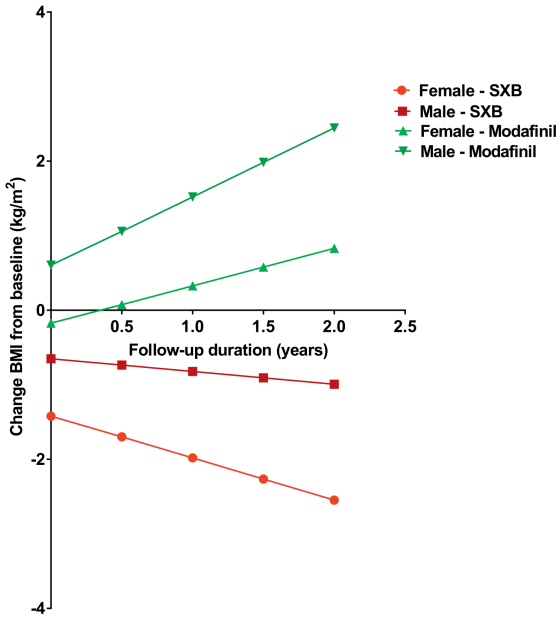


Figure 5.3. Mixed model estimations of the averaged subject in four groups based on gender and medication type. Body mass index (BMI) at baseline is set at 28.0 kg/m^2, the grand mean of our study population. Values for different groups at Follow-up duration = 0 depicts the average baseline BMI value of all patients in that particular group. SXB = sodium oxybate.

BMI change in patients is not correlated with either modafinil or SXB dose

A higher dose of either modafinil or SXB was not correlated with BMI change in our patient cohort. Spearman's rho was 0.188 in modafinil-treated patients ($p = 0.402$) and 0.153 in SXB-treated patients ($p = 0.294$).

BMI decrease in SXB group not explained by methylphenidate or dexamphetamine use

Given the fact that stimulant use is significantly higher in the patient group in which SXB treatment was initiated, a mixed model was fitted on the dataset in which patients using methylphenidate or dexamphetamine were excluded (n = 24 in SXB group, n = 2 in modafinil group; data not shown). Also in this fitted model there was a significant main effect of SXB on BMI ($F(1,40.073) = 16.003$, $p < 0.001$). Additionally, we fitted a mixed model in which dexamphetamine or methylphenidate use was added both as a main effect and as an interaction effect with time (Supplementary Table 5.1). The main effect of dexamphetamine

or methylphenidate use was significant ($F(1,78.787) = 10.215$, $p = 0.002$). However, there still remained a highly significant main effect of SXB on BMI ($F(1,66.799) = 14.916$, $p < 0.001$).

Discussion

Our findings (1) confirm that SXB reduces BMI in NT1 patients and (2) suggest that this is a long lasting effect. Even when accounting for baseline BMI, gender, treatment duration and the interactions between them, the mixed model we fitted statistically significantly demonstrates a BMI decrease in the SXB group, in contrast to a BMI increase in the modafinil group. The BMI decrease in the SXB group is seen in both males and females but is more pronounced in women. Patients using modafinil gain rather than lose weight. Another interesting finding is that a higher BMI at baseline predicts a more pronounced decrease in BMI during medication use over time. This suggests that BMI decrease constitutes an additional beneficial effect of SXB for NT1 patients, especially for those with a higher BMI at baseline. A correlation between medication dose and BMI change is not found in our cohort.
Our findings are in line with a previous report (Husain et al., 2009) on weight loss amongst patients treated with SXB. Patients with NT1 and -2 were assessed, yet without a control group; and data on age, gender or BMI of the smaller cohort were lacking.
Narcolepsy patients have a higher prevalence of obesity than the general population. Indeed, the mean baseline BMI of our cohort was significantly higher than that of a representative sample of Dutch people older than 18 years old.

The exact mechanism by which SXB leads to weight loss is unclear, though several theories exist. It is known (Donjacour et al., 2011) that SXB leads to a consistent increase in nocturnal growth hormone (GH) secretion and that SXB strengthens the temporal relation between GH secretion and slow wave sleep. GH is a potent lipolytic agent and a GH deficiency decreases lean body mass while increasing fat mass. It was suggested that SXB could lead to an increase in lipolysis by restoring GH secretion. This hypothesis was tested in a study showing that SXB stimulates lipolysis in NT1 (Donjacour et al.,

2014). Participants in this study lost on average 5.2 kg in three months of SXB treatment, which supports our results (Husain et al., 2009). If weight loss is mediated through this pathway, gender differences in fat metabolism (Williams et al., 2004) could account for the different trends in BMI change between men and women in our study.
Another hypothesis is that BMI decrease results from the effect of SXB on ghrelin and leptin secretion. A comparison between NT1 patients and healthy controls (Donjacour et al., 2013) did not show any differences in ghrelin and leptin secretion after 3 months of SXB treatment. It was speculated there that the weight loss may also be due to an decrease of food intake and an increase of physical activity leading to a negative energy balance secondary to the sleep-promoting effects of SXB.
In addition to these hypotheses, we propose that the fact that patients who start using SXB are required to cease using alcohol might play an additional role in BMI loss in this patient group. Even though the exact relation between alcohol consumption and weight gain is complex, it can be said that alcohol consumption leads to weight gain (Suter et al., 2005).

The effects of SXB on BMI were compared with modafinil, a commonly prescribed therapy for narcolepsy. The decision to treat an individual with either SXB or modafinil is not always clear-cut. SXB is more often prescribed when cataplexy and disturbed nocturnal sleep are the most invalidating symptoms (Bosch et al., 2012; Boscolo-Berto et al., 2012) while modafinil is prescribed in those suffering most from excessive daytime sleepiness (Guilleminault and Cao, 2011). If cataplexy would have an effect on weight it could therefore lead to a selection bias in our study. There is no data on the relation between cataplexy and BMI. We have also no reason to expect other confounding parameters to be present than the ones we accounted for in our analysis. Earlier studies on the effect of modafinil in NT1 showed no significant BMI changes in those treated with it (Moldofsky et al., 2000; US Modafinil in Narcolepsy Multicenter Study Group, 1998). These studies had a shorter follow up time compared to our study.

Our study has a few limitations. Firstly, due to the retrospective nature we were not able to randomize patients and we were dependent on the methods and data collection which was chosen for the individual. The retrospective design

led to incomplete data about symptom severity at treatment initiation and made correlation between treatment effects on BMI and treatment effects on other symptoms impossible. We were, however, able to review the medical records of all patients with NT1 patients who had treatment started at the outpatient clinic, thereby reducing the risk of selection bias. Our cohort is also larger than those presented in earlier studies on weight loss and SXB (Donjacour et al., 2013; Husain et al., 2009); and we only included NT1 patients as diagnosed based on ICSD-3 guidelines.

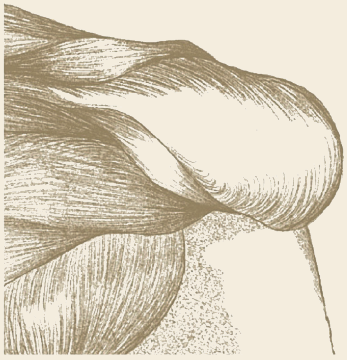
Co-medication constituted a concern in our study. The use of methylphenidate or dexamphetamine was found to be significantly different between groups. Methylphenidate, a stimulant often used in narcolepsy, is known to decrease appetite and dietary fat intake in healthy subjects (Goldfield et al., 2007). In NT1 no such effect has been observed (Kok et al., 2003). It remains unclear whether these findings also lead to a BMI decrease. Given that in our study all patients using these two medications were on a stable dose for at least three months before initiating treatment with SXB or modafinil, it was not likely that this influenced our results in a significant way. We assessed the effect of these stimulants in two ways. Removing individuals using methylphenidate or dexamphetamine from the model still shows a highly significant effect of SXB on BMI. In addition, adding use of dexamphetamine or methylphenidate to the model shows that it leads to a BMI reduction, but does not interfere with the BMI decrease that is demonstrated in the SXB group. Besides stimulants, a non-significant difference in antidepressant use was found between both groups. Results on the effect on BMI of the most frequently used antidepressant clomipramine are contradictory: two groups have reported an increase in appetite and weight gain in patients using clomipramine (Paige et al., 2015; Maina et al., 2004). However, this effect was only shown in patients without narcolepsy and for a higher dose than normally prescribed in NT1. Another group found no BMI changes in narcolepsy patients using clomipramine in patients who were already on a stable dose (Kok et al., 2003).

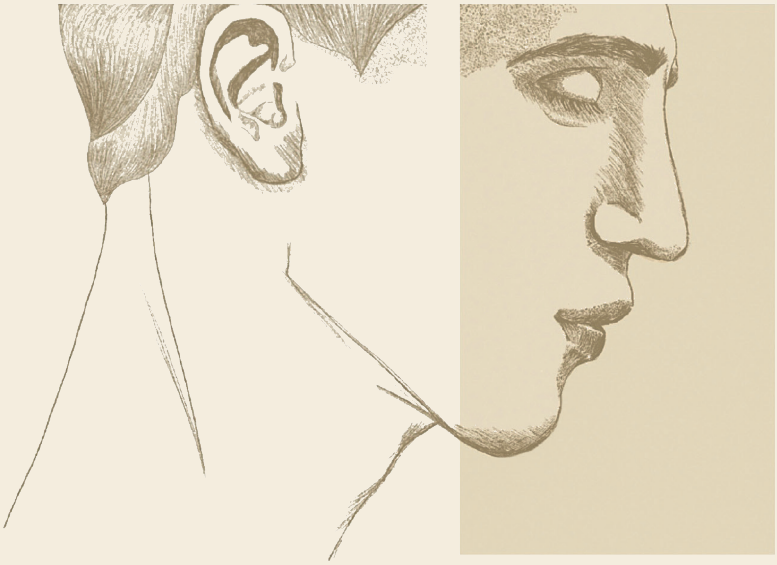
Conclusion

We confirm that treatment with SXB is associated with a decrease in BMI in NT1 patients, while modafinil treatment is not. For the majority of NT1 patients, BMI decrease therefore constitutes an additional beneficial effect of SXB. The weight loss is more pronounced in those with a higher BMI at baseline. Possible weight loss could therefore be another reason to opt for treatment with SXB in NT1, especially in those with a higher BMI. Due to the retrospective nature of this study further prospective and longitudinal studies are needed to confirm our results and further characterize BMI change dynamics.

Acknowledgements

The authors thank P.P. Buijserd-Amesz for her help in acquiring the data and J.W. Sander for critically reviewing this manuscript, specifically concerning the use of the English language.





Chapter 6

Daytime sleep state misperception in a tertiary sleep centre population

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Abstract

Study objectives: Sleep state misperception is common in various sleep disorders, especially in chronic insomnia with a prevalence ranging between 9-50%. Most prior studies used nocturnal polysomnography for the identification of sleep state misperception during nighttime. Our objective was to assess sleep state misperception during daytime in people with sleep disorders with excessive daytime sleepiness.

Methods: In this prospective observational study, we assessed the occurrence of, and factors influencing sleep state misperception in consecutive patients undergoing a routine multiple sleep latency test in a tertiary sleep-wake centre included between 2014 and 2017. Mixed models were applied to assess the influence of patients' clinical data on sleep state perception.

Results: People with narcolepsy type 1 (n=33) and type 2 (n=14), idiopathic hypersomnia (n=56), obstructive sleep apnea (n=31) and insufficient sleep syndrome (n=31) were included. The prevalence of both classical and reverse sleep state misperception did not differ between the sleep disorders (mean 25%, range 8-37%) after correction for sleep stage, sleep onset latency and age. Longer sleep onset latency and reaching only non-REM sleep stage 1 were significant predictors for classical sleep state misperception.

Conclusions: Sleep state misperception is common in people with narcolepsy type 1 and 2, idiopathic hypersomnia, obstructive sleep apnea and insufficient sleep syndrome. Classical sleep state misperception is more frequent in patients with longer sleep onset latencies who only reach non-REM sleep stage 1 during a nap.

Introduction

Sleep state misperception (or paradoxical insomnia) is common in chronic insomnia with a prevalence ranging between 9 and 50% (Bastien et al., 2014, Moon et al., 2015). The International Classification of Sleep Disorders, Second Edition (ICSD-2) defined sleep state misperception as a 'consistent marked mismatch between objective findings from polysomnography (PSG) or actigraphy and subjective self-report or sleep diary', but this concrete phrasing as a mismatch between objective and subjective measures has not returned in the ICSD-3 (ICSD, 2005, ICSD, 2014). Sleep state misperception has also been reported in other sleep disorders, such as obstructive sleep apnea (OSA), and in the general population (Castillo et al., 2014, Choi et al., 2016, Trajanovic et al., 2007). Most performed studies depend on nocturnal polysomnography for identifying sleep state misperception and finding patient-related predictors. Additionally, the focus is mainly on the situation in which subjective sleep time is lower than objective sleep time (classical sleep state misperception), while reverse sleep state misperception is not reported. Interestingly, sleep state misperception during daytime naps has not been well-studied.

Except for one study in OSA (Bishop et al., 1998), studies on sleep state misperception in sleep disorders with excessive daytime sleepiness (EDS) are absent, even though knowledge about sleep state perception seems highly relevant in the assessment of daily functioning for both the patient and the physician. Information about daytime sleep state perception could be an important addition to parameters such as subjective and objective sleepiness for the evaluation of disease severity, treatment decisions and fitness to drive (Liu et al., 2018).

Dreaming is another phenomenon which has not been extensively assessed during daytime naps. Emotions associated with dream perception are thought to be more intense in people with narcolepsy compared to healthy controls. This effect is even more pronounced during daytime naps and is associated with sleep-onset REM periods (SOREMPs (Fosse et al., 2002)).

We hypothesize that sleep state misperception is common in sleep disorders with EDS. Since we consider sleep state misperception in these disorders to be clinically most important during the day, we assessed sleep state perception and factors influencing it, including the occurrence of REM sleep, during the

20-minute nap opportunities of a multiple sleep latency test (MSLT). We also assessed the association between SOREMPs and dream perception.

Methods

Patients

In this prospective observational study, all consecutive patients undergoing a routine multiple sleep latency test between March 2014 and October 2017 in a tertiary sleep-wake centre were assessed for inclusion. All diagnosis were based on ICSD-3 criteria (ICSD, 2005). People with a diagnosis of narcolepsy type 1 (NT1) or 2 (NT2), idiopathic hypersomnia (IH), OSA or insufficient sleep syndrome were included.

Questionnaires

EDS was assessed using the Epworth Sleepiness Scale (ESS), which is validated in NT1 and NT2, IH and OSA (Johns, 1992). Sleep state and dream perception were assessed in the context of clinical care by asking patients after each nap whether they slept and dreamed during that specific nap. No feedback was given to the patient. Two types of sleep state misperception were defined: classical sleep state misperception was present when a patient did not report sleep when sleep was recorded, whereas reverse sleep state misperception was present when a patient did report sleep although sleep was not recorded. Dream perception was not defined as correct or incorrect.

Multiple sleep latency test (MSLT)

The MSLT was performed according to guidelines published before (Littner et al., 2005). It consisted of five sessions of 20 min in which patients had the opportunity to fall asleep. We did not extend these 20 minutes to allow for 15 min sleep after sleep onset. Nap opportunities were given with two hour intervals. The first session started at 09:00 hours. The night before the MSLT took place, a polysomnography was performed.

Statistical analysis

For assessing differences in ESS scores between sleep disorders with EDS, we used one-way ANOVA. Patients were stratified by diagnosis for these analyses.

Post-hoc analyses were performed to assess the differences between all separate groups. Differences in sleep stage sequences between sleep disorders with EDS were analyzed with *Pearson's* chi-squared tests with post-hoc testing to compare sleep disorders one by one. To assess whether other factors that were unevenly distributed amongst patient groups, such as age and sleep onset latency and sleep stages during the nap, might explain sleep state misperception, mixed models were applied to assess the influence of patients' clinical data on sleep state misperception. We included only the nap opportunities in which patients fell asleep and so focused on classical sleep state misperception only. Reverse sleep state misperception was insufficiently present in our dataset to be able to assess factors influencing this type of sleep state misperception. A linear mixed model with a random slope and a random intercept for each individual was fitted. The outcome was classical sleep state misperception. As main effects we added age, sleep disorder (NT1; NT2; IH;OSA; ISS), sleep onset latency, sleep stage reached and the presence of REM to the model. Differences in dream reporting between REM and NREM sleep were analyzed using *Pearson's* chi-squared tests. P-values below 0.05 were deemed significant. Bonferroni corrections were executed when needed. All analyses were conducted using the IBM SPSS Statistics 25 software package.

Results

Patient characteristics

A total of 165 people with 825 nap opportunities were included. People with NT1 and NT2 had a shorter mean sleep latency, had more SOREMPs and reported dreaming more frequently than people with IH, OSA and ISS (Table 6.1). People with NT1 had a shorter REM latency than others who had a SOREMP during the MSLT (3.4 min ± 3.5 vs. 5.7 min ± 3.7; p = 0.001). Scores on the ESS differed significantly between sleep disorders, with people with NT1, NT2, IH and ISS scoring significantly higher than those with OSA (Table 6.2); median ESS of all sleep disorders were well above the cut-off value of 10. Information on polysomnography characteristics can be found in Supplementary table 6.1.

Table 6.1. Baseline and MSLT characteristics. In this table, baseline characteristics and information regarding the MSLT of all patients is summarized. Percentages of dream report are based on all naps, both with and without sleep.

	NT1	NT2	IH	OSA	ISS
N (MSLT)	33	14	56	31	31
Age (years ± SD)	29.9 ± 16.9	35.5 ± 15.3	37.8 ± 15.6	51.0 ± 16.7	35.2 ± 12.0
Gender (% male)	48.5	28.6	45.5	64.3	46.7
BMI (kg/m^2 ± SD)	23.7 ± 3.6	24.4 ± 4.3	25.5 ± 4.7	28.2 ± 4.4	25.7 ± 4.4
Treatment (%)	3 (9%)	2 (14%)	9 (16%)	6 (19%)	2 (7%)
Sedatives	3	0	4	1	2
Stimulants	0	0	0	0	0
Antidepressants	0	0	4	5	2
Melatonin	0	2	2	1	0
MAD	0	0	0	1	0
CPAP	0	0	0	5	0
Naps (% sleep)	93.3	100	86.2	76.6	82.4
Sleep (n)	154	70	244	118	126
No sleep (n)	11	0	39	36	27
Sleep reported (%)	90.3	88.7	64.3	50.0	62.8
SOREMP (%)	58.8	44.3	1.5	2.1	4.7
Dreaming reported (%)	61.0	58.3	20.0	10.0	28.0
MSL (min ± SD)	6.0 ± 5.6	5.7 ± 4.3	9.6 ± 5.9	12.3 ± 5.9	10.9 ± 6.0

BMI = body mass index; CPAP = continuous positive airway pressure; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; MAD = mandibular advancement device; MSL = mean sleep latency; MSLT = multiple sleep latency test; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea; SD = standard deviation; SOREMP = sleep-onset REM period.

Table 6.2. ESS scores. Sleep questionnaire scores of the patients that filled in the Epworth Sleepiness Scale to assess excessive daytime sleepiness.

Diagnosis	ESS	
	Median (range)	Above clinical cut-off
NT1	16 (2-21)	21/23 (91%)
NT2	15 (7-21)	10/11 (91%)
IH	15 (0-24)	36/48 (75%)
OSA	15 (0-24)	9/25 (36%)
ISS	13.5 (5-20)	23/30 (77%)

EDS = excessive daytime sleepiness; ESS = Epworth Sleepiness Scale; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea.

Sleep state misperception is common in sleep disorders

In total, 72 people with sleep disorders had no classic sleep state misperception, 39 people with sleep disorders had 1 nap with classic sleep state misperception, 22 people with sleep disorders had 2 naps with classic sleep state misperception and 28 people with sleep disorders had more than 2 naps with classic sleep

state misperception. 4 patients did not sleep. Classical sleep misperception is common in NT1 and NT2 and even more so in IH, ISS and OSA ($p < 0.001$, Figure 6.1) with 25% of all nap opportunities in which sleep was recorded ($n = 712$), ranging from 7% in NT1 to 39% in OSA. Also reverse sleep state misperception is relatively common in NT1, IH, ISS and OSA with 26% of all nap opportunities in which no sleep was recorded ($n = 113$), ranging from 12% in OSA to 48% in ISS.

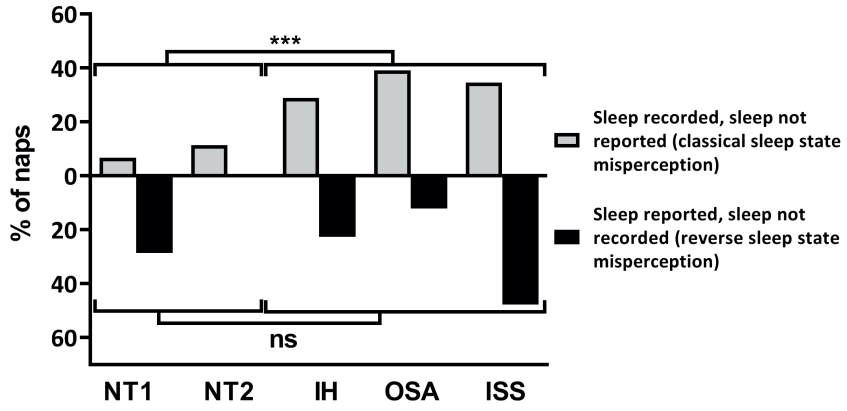

Figure 6.1. Sleep state misperception percentages in MSLT nap opportunities. This figure depicts the percentage of nap opportunities in which patients misperceived sleep per sleep disorder. Nap opportunities in which a patient did not report sleep when sleep was recorded (classical sleep state misperception) are shown in grey; nap opportunities in which a patient reported sleep when no sleep was recorded (reverse sleep state misperception) are shown in black. For classical sleep state misperception, percentages are based on nap opportunities in which sleep was recorded, whereas for reverse sleep state misperception, percentages are based on nap opportunities in which no sleep was recorded. Statistical significance of differences between NT1 and NT2 patients and IH, OSA and ISS patients is shown by the horizontal bars.

*** $p<0.001$; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea.

Long sleep onset latency and only N1 stage sleep predict sleep state misperception

Mixed model analysis took into account other parameters that might have influenced this seemingly strong effect of the sleep diagnosis on classical sleep state misperception during the MSLT. In the mixed model, only nap opportunities were used in which sleep was recorded. A long sleep-onset latency ($\beta = 0.114 \pm 0.033$; $p < 0.001$) and not reaching N2 stage sleep ($\beta = -0.708 \pm 0.329$; $p = 0.043$) were shown to be important predictors for sleep state

misperception during MSLT. Even though the diagnosis NT1 and age were predictors that were not statistically significant, the magnitude of their effect suggests there may be a clinically significant influence of these parameters on sleep state misperception (Table 6.3).

Table 6.3. Linear mixed model of sleep misperception. The linear mixed model of sleep misperception is fitted with a random slope and random intercept per individual for naps in which sleep was recorded. A positive value for B indicates a higher probability of sleep misperception. P-values are based on the parameters in the model. For specific categories within the parameters, such as sleep stage, the confidence interval for the coefficient of each category relative to the other categories is shown.

Parameter	B	95% confidence interval		p-value
		Lower boundary	*Upper boundary*	
Age (per year)	0.014	-0.005	0.032	0.143
Sleep onset latency (per min)	0.114	0.050	0.177	<0.001
Sleep stage reached				0.043
N1	0			
N2	-0.708	-1.352	-0.063	
N3	-0.204	-1.141	0.733	
SOREMP	-0.735	-1.784	0.313	0.169
Diagnosis category				0.273
NT1	0			
NT2	0.455	-0.734	1.643	
IH	1.043	-0.066	2.152	
OSA	1.112	-0.113	2.336	
ISS	1.289	0.097	2.480	
Nap (#)				0.302
1	0			
2	-0.354	-0.901	0.193	
3	-0.227	-0.828	0.374	
4	-0.141	-0.717	0.435	
5	-0.599	-1.217	0.019	

EDS = excessive daytime sleepiness; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea; SOREMP = sleep-onset REM period.

Dream perception is partly dissociated from REM sleep

In 29.3% of naps in which patients had only NREM sleep, patients reported to have dreamed (Table 6.4). The distribution of these findings was different between sleep disorders: when a SOREMP was absent during the nap, people with NT1 and NT2 reported having dreamed more often than those with IH, OSA and ISS. Patient reported not to have dreamed in 25.8% of naps with a SOREMP. Interestingly, in 9 nap opportunities patients (1 NT1, 2 IH, 3 OSA, 3 ISS) reported having dreamed, even though no sleep was recorded.

Direct transition from wake to REM sleep or from wake via N1 to REM sleep is not a highly sensitive measure for NT1 and NT2

People with NT1 and NT2 transited from wake to REM sleep and from N1 to REM sleep significantly more often than those with IH, OSA or ISS when all naps were taken into account ($p < 0.001$ for both sleep stage sequences; Figure 6.2A). However, when only naps with a SOREMP were analysed, transitions from wake to REM sleep and from N1 to REM sleep were not found more often in NT1 and NT2 than in IH, OSA and ISS ($p > 0.05$ for both sleep stage sequences; Figure 6.2B).

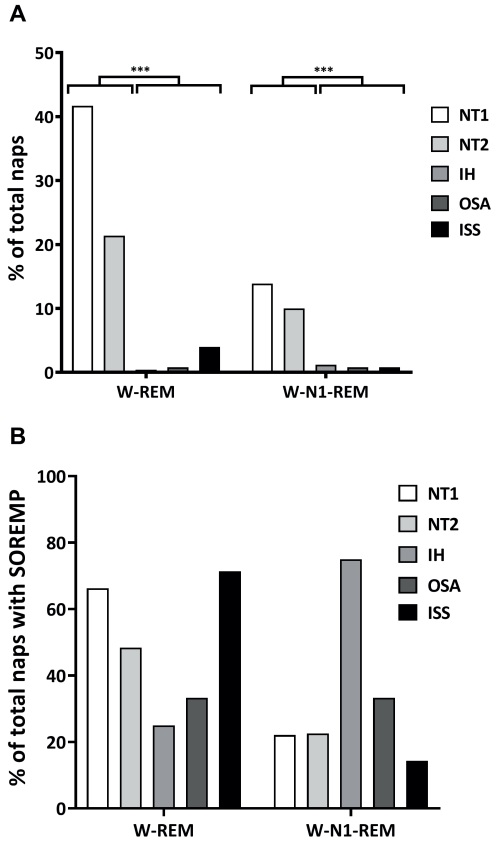

Figure 6.2. Sleep stage sequences in MSLT nap opportunities. A. Sleep stage sequences in all nap opportunities. Wake to REM and wake via N1 to REM sequences are represented for NT1, NT2, IH, OSA and ISS patients as a percentage of the total nap opportunities for that diagnosis. Statistical significance of differences between NT1, NT2 and IH, OSA and ISS patients is shown by the horizontal bars. B. Sleep stage sequences in nap opportunities with a SOREMP. Wake to REM and wake via N1 to REM sequences are represented for NT1, NT2, IH, OSA and ISS patients as a percentage of the nap opportunities with a SOREMP for that diagnosis.

*** $p<0.001$; ns $p>0.05$; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea; W = wake.

Discussion

Sleep state misperception during the short nap opportunities of an MSLT was frequently seen in people with NT1, NT2, IH, OSA and ISS. Classical sleep state misperception was more common than reverse sleep state misperception, mostly due to the fact that in most nap opportunities sleep was recorded.

People with NT1 and NT2 patients misperceived their sleep less than those with IH, OSA and ISS. Multifactorial modelling on nap opportunities in which sleep was recorded, showed that this decreased prevalence of classical sleep state misperception in people with NT1 and NT2 is at least partly due to the fact that they were, on average, younger, had a shorter sleep latency and reached deeper sleep during a nap than those with IH, OSA and ISS. Despite this, a slightly lower percentage of classical sleep state misperception in people with NT1 and NT2 persists, albeit not significant after correction for other factors of influence in a linear mixed model, that could not be explained by the other factors in the model. The fact that sleep state misperception was almost absent in naps with dream reporting (data not shown) and the higher percentage of dream reporting in NT1 and NT2 supports the hypothesis that more intense emotions upon dream perception (Fosse et al., 2002) in people with NT1 and NT2 might explain part of the difference.

The increasing percentage of classical sleep state misperception with age, is in contrast with a study in people with chronic insomnia, where those with sleep state misperception were significantly younger than those without sleep state misperception (Moon et al., 2015). Other studies on sleep state misperception in chronic insomnia find no factors influencing sleep state perception, apart from those dictated by the sleep disorder itself (e.g. total sleep time (Dittoni et al., 2013, Moon et al., 2015)).

The significant percentage of nap opportunities in which people with different sleep disorders misperceived their sleep is in line with previous studies that describe significant poor sleep state perception in a variety of patient groups, such as chronic insomnia (up to 73% sleep state misperception), epilepsy, OSA and even healthy controls (Nam et al., 2016, Goulart et al., 2014, Ng and Bianchi, 2014, Mercer et al., 2002). All studies focused on classical sleep state misperception.

A SOREMP in a nap was not a significant predictor of sleep state misperception in our model. A reason could be the low number of SOREMPs in people with

other sleep disorders than narcolepsy. This may have led to a larger effect of narcolepsy in our model, that would otherwise be assigned to the presence of a SOREMP. A negative effect of selective REM-sleep deprivation on sleep state perception was shown in a study in healthy volunteers (Goulart et al., 2014), indicating a role of REM-sleep in sleep state misperception. Paradoxically, a strong positive correlation between amount of REM-sleep and perceived wakefulness in people with chronic insomnia has been described (Feige et al., 2008).

The fact that longer sleep onset latencies in nap opportunities were associated with more daytime classical sleep state misperception has been reported before in OSA patients (Bishop et al., 1998) and could also have another explanation. Due to the fact that a longer sleep onset latency unequivocally means that sleep duration during that nap was shorter, the increased sleep state misperception could therefore also be due to this shorter sleep duration. Another factor to consider is the limited duration of the nap, which restricts generalization of the relation between sleep onset latency and sleep misperception to these 20 minutes only.

A contribution of circadian factors to sleep state misperception was hypothesized in a PSG study in insomnia (Bianchi et al., 2012). In our study, we did not find a significant effect of nap timing, which argues against a role for circadian factors.

Our study design did not allow for assessing the specific influence of psychiatric comorbidity, personality traits and coping mechanisms on sleep and dream perception, which were earlier described as potential determinants of sleep misperception (Fernandez-Mendoza et al., 2011). Based on the data that we had on psychiatric comorbidity, we did not see any effect on either sleep or dream perception (data not shown).

Medication and device use in our cohort was low, since most MSLTs were performed as part of the diagnostic process before medication was first prescribed. When patients using sedatives, CPAP, MAD and antidepressants were excluded from the analysis, significance of factors influencing sleep state misperception did not change (data not shown). Moreover, stimulants and sedatives were never used on the day of the MSLT. Additionally, we are not aware of any studies showing an effect of medication on sleep state misperception.

Most theories on sleep state misperception focus on the role of increased cognitive arousal and overgeneralization in people suffering from this condition (Takano et al., 2016, Maes et al., 2014, Bonnet and Arand, 1997). However, many

more mechanisms are described, that include a role for selective attention and the influence of transient awakenings (Harvey and Tang, 2012). Unfortunately, we were not able to include the effect of transient awakenings in our model.

Comparison of our findings on reverse sleep state misperception with other studies is challenging, since only few studies mention this type of sleep state misperception, almost exclusively during night time sleep in chronic insomnia (Attarian et al., 2004, Schneider-Helmert and Kumar, 1995, Trajanovic et al., 2007). It was proposed that increased alpha activity during sleep might play a role in reverse sleep misperception (Schneider-Helmert and Kumar, 1995, Schneider-Helmert, 2005).
Notably, dreaming was reported in a considerable amount of naps with only NREM sleep, consistent with extensive (nocturnal PSG) evidence on dreaming in NREM sleep with several studies disputing the paradigm of dreaming being confined to REM sleep (Aserinsky and Kleitman, 1953). Dream recall from NREM sleep stages is reported in 17.9% of NREM naps (both during night and day) in healthy males, while in only 51.2% of REM naps dream experiences were reported (Suzuki et al., 2004, Cavallero et al., 1992), broadly comparable with the percentages we found in this study. When dream perception was assessed only during daylight, it was also shown to be partly dissociated from REM sleep (Chellappa et al., 2009). Our results contribute to the notion that focusing on differences in dream content rather than crude dream perception between NREM and REM dreams might be more worthwhile in understanding the association between dreams and sleep physiology (Solms, 2000).
Our study design did not allow for a distinction between hypnagogic hallucinations, which people with narcolepsy may describe as a dream-like sensation in this context, and 'normal' dreams, since we only asked patients whether they dreamed or not. Theoretically, this might have led to an overestimation of the percentage of naps, both with and without SOREMP, in which patients reported to have dreamed.

Additionally, as reported before (Zhang et al., 2018), people with NT1 in this study have a shorter REM latency than other patients who have a SOREMP during MSLT. Also, the sleep stage sequences wake-REM and wake-N1-REM in the naps of the MSLT were found frequently in people with narcolepsy, in line with earlier findings (Marti et al., 2009, Ferri et al., 2016, Murer et al.,

2017). However, several earlier studies reported these sleep stage sequences in the naps of the MSLT to be highly narcolepsy-specific (Drakatos et al., 2016, Drakatos et al., 2013), while we also observed these transitions in the scarce naps with SOREMPs of people with IH, OSA and ISS.

As the study was performed in a tertiary sleep-wake centre, the makeup of the population is different than would be expected in a secondary sleep-wake centre. The relatively small amount of people with narcolepsy in this study, for example compared with people with idiopathic hypersomnia, is explained by the fact that many people with narcolepsy that are admitted to our tertiary centre have already undergone an MSLT in the referring sleep-wake centre and/or already on medication that cannot be interrupted. Repeating our approach in a secondary sleep-wake centre is therefore warranted to assess the generalizability of our findings.
The fact that research on daytime sleep state misperception is scarce (Bishop et al., 1998) led to a limitation in comparing our results with those of others. Most studies focus specifically on nocturnal sleep state misperception in which different definitions are used to describe the condition. Data is generated using nighttime PSG, while we use daytime data to describe and assess sleep state misperception. To be able to validate our findings, comparisons with other, more extensive, patient groups measured during daytime nap opportunities is needed. These cohort studies could also focus on reverse sleep state misperception. This type of sleep state misperception was scarce in this study and could therefore not be further analyzed using mixed models.

Conclusion

Both classical and reverse sleep state misperception during the short naps of an MSLT is common in NT1, NT2, IH, OSA and ISS. Classical sleep state misperception is more frequent in patients with longer sleep onset latencies who only reach non-REM sleep stage 1 during a nap. The fact that classical sleep state misperception is common in NT1, NT2, IH, OSA and ISS is relevant for patients and sleep physicians when assessing disease severity and fitness to drive and making treatment decisions in sleep disorders with EDS. Notably, dreaming was frequently reported even when REM sleep did not occur.

Acknowledgements

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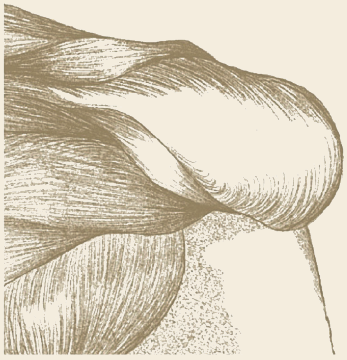
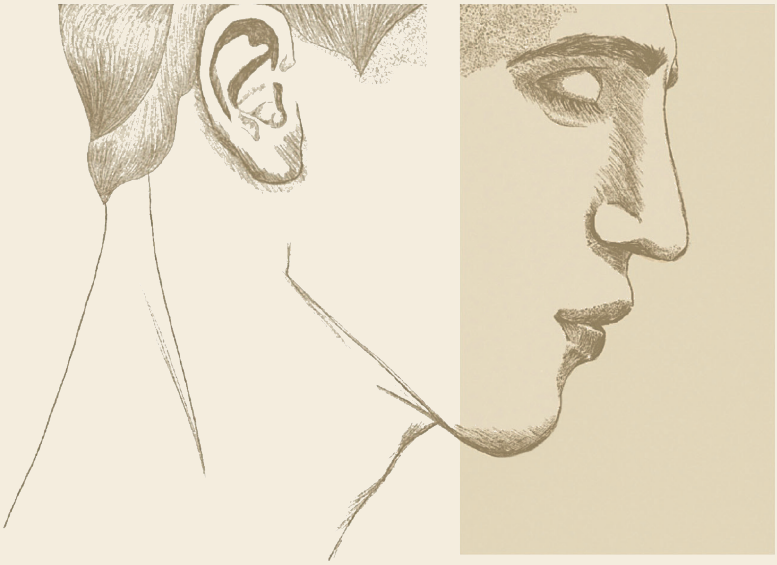
Supplementary material

Supplementary table 6.1. Polysomnography characteristics. In this table, polysomnography characteristics of the night before the MSLT was performed are summarized. All values are presented as median (interquartile range).

	NT1	NT2	IH	OSA	ISS
N	33	14	56	31	31
Total sleep time (h)	7.2 (6.0-8.2)	7.0 (6.2-7.8)	7.0 (6.4-7.7)	6.8 (6.0-7.7)	7.0 (5.3-7.6)
Sleep efficiency (%)	89.8 (82.8-93.9)	88.7 (75.1-96.2)	91.8 (85.0-94.3)	82.5 (74.7-90.8)	93.2 (85.7-95.9)
Sleep latency (min)	5.2 (2.3-9.1)	4.8 (2.9-8.5)	10.7 (5.5-25.4)	15.1 (12.1-26.5)	8.8 (4.3-14.3)
REM sleep latency (min)	10.5 (2.0-125.0)	13.5 (4.5-90)	75.8 (57.5-117.4)	95.5 (64.0-168.0)	88.5 (51.1-110.4)
Wake-index (n/min)	3.4 (2.3-4.5)	2.3 (0.8-3.6)	2.3 (1.6-3.0)	3.0 (2.2-5.2)	2.1 (1.5-2.8)
AHI (n/min)	0.6 (0.1-2.4)	1.0 (0.2-3.5)	1.1 (0.1-3.0)	6.1 (2.2-17.1)	0.5 (0.0-4.0)

AHI = apnea/hypopnea-index; IH = idiopathic hypersomnia; ISS = insufficient sleep syndrome; MSL = mean sleep latency; NT1 = narcolepsy type 1; NT2 = narcolepsy type 2; OSA = obstructive sleep apnea.





Summary, discussion and future perspectives

This thesis falls apart into two parts: part 1 focuses on the autoimmune hypothesis of narcolepsy, while the second part focuses on NT1 symptoms that are frequently neglected, even though they add significantly to the burden of the disorder.

Autoimmune hypothesis of NT1

The same figure that was shown in the introduction of this thesis is depicted here. Figure 1 displays the hypothesis that antigens from outside the body trigger an immune response in people susceptible for developing NT1 that leads to an autoimmune attack on the hypocretin-producing neurons in the hypothalamus. In the Chapters of this thesis, several of the presumed immune mechanisms involved are investigated: **Chapter 1** describes a potential new trigger for the autoimmune response; **Chapter 2** focuses on HLA-DQB1 associations in NT1 before and after the H1N1 influenza pandemic; **Chapter 3** describes the possible role of cross-reactive CD4+ T cells in the autoimmune response leading to NT1; and **Chapter 4** evaluates all immune cells that are depicted in this figure to identify enriched populations in NT1.

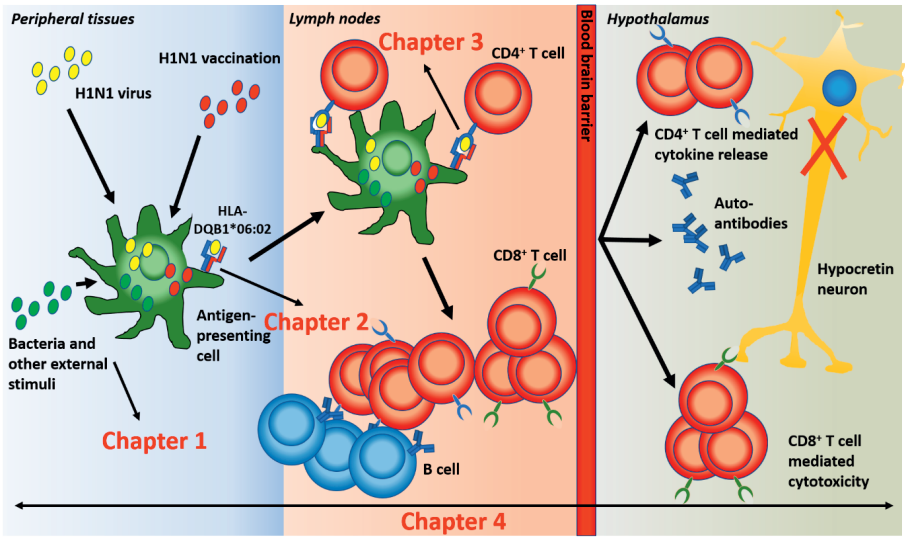


Figure 1. A depiction of the autoimmune hypothesis of narcolepsy and the mechanisms that are investigated in the different Chapters of this thesis.

Chapter 1 describes a NT1 case in which an enteroviral infection may constitute the environmental trigger for the hypocretin-1 deficiency that develops. This has not been described before and highlights the possibility that a variety of infections may trigger the immune system to elicit an autoimmune response targeting the hypocretin-producing neurons. Thereby, this case report stresses the importance of future research focusing on the question how a variety of environmental triggers can lead to a similar outcome: the destruction of hypocretin-producing neurons.

- The short interval between hypocretin-1 measurement and symptom onset in this case suggests that the decrease in hypocretin-1 concentration from normal to undetectable levels may be a process lasting only a few weeks or even less
- Enteroviral infection is a new potential environmental trigger for the autoimmune response leading to NT1

In **Chapter 2,** we describe HLA-DQB1 associations in a cohort of NT1 patients who developed symptoms before or after the H1N1 influenza pandemic. With this approach, we address the postulation of Scandinavian researchers that post-H1N1 NT1 is a separate disease entity. Over the last few years, many studies (Juvodden et al., 2019b, Nordstrand et al., 2019a, Nordstrand et al., 2018, Bomfim et al., 2017, Juvodden et al., 2018, Sarkanen et al., 2016), particularly in Scandinavia, have focused on post-H1N1 only, thereby excluding so called sporadic NT1 patients. We argue, based on the fact that HLA-DQB1 associations have not changed since the H1N1 influenza pandemic, that there is no convincing evidence to justify a distinction between post-H1N1 and sporadic NT1.

- No differences were found in HLA-associations in pre- vs post-H1N1 NT1 patients
- Positive association of pre- and post-H1N1 NT1 with HLA-DQ7 was confirmed
- Negative associations of pre- and post-H1N1 NT1 with HLA-DQ2, -DQB1*05:01, -*06:03 were confirmed
- These results argue against sporadic and post-H1N1 NT1 being separate entities

Chapter 3 describes our study on CD4+ T cells that cross-react with H1N1 influenza and hypocretin peptides. In this study we show that HLA-DQB1*06:02-restricted CD4+ T cell responses against H1N1 influenza peptides are more frequent in NT1 patients than in controls. However, we did not identify any hypocretin peptide-specific CD4+ T cell responses and, thus, no cross-reactivity between H1N1 and hypocretin. This argues against a causal role for these H1N1 peptides in the autoimmune pathogenesis of NT1.

In contrast to our findings, several studies do report the presence of CD4+ and CD8+ T cells that recognize hypocretin (Cogswell et al., 2019, Latorre et al., 2018, Pedersen et al., 2019, Jiang et al., 2019) or show cross-reactivity to hypocretin and H1N1 influenza peptides (Luo et al., 2018). One of the reasons for this discrepancy may be the different techniques that were used. In Chapter 3, we focus specifically on reactivity to H1N1 influenza and hypocretin peptide fragments that were chosen based on their structural resemblance as assessed by crystallography. All other studies used broad peptide pools or peptide libraries, thereby decreasing specificity, but largely increasing the possibility of finding a response.

Moreover, we validated the supposed specificity of expanded cells in T cell cultures with the H1N1 influenza and hypocretin peptides. We assessed proliferation of T cell clones generated from these T cell cultures, upon a challenge with a hypocretin or H1N1 influenza peptide fragment. This essential step was not performed in two of the aforementioned studies (Luo et al., 2018, Cogswell et al., 2019), thereby rendering conclusions about the specificity of T cell cultures in those latter studies uncertain.

The authors of the first study (Luo et al., 2018) responded to our study in the Journal of Neuroimmunology (Mignot et al., 2019). Firstly, they stress the importance of performing experiments with C-amidated peptides, since they only demonstrated cross-reactivity between hypocretin and H1N1-haemagglutinin peptides when hypocretin peptides were C-amidated. We have unfortunately not been able to repeat our experiments with these peptides to ascertain whether this difference indeed explains the differences between the outcomes of both studies. Secondly, Mignot et al. state that the conclusion that we could not find evidence for molecular mimicry with the peptides tested, was premature, because our sample size would not allow for detecting cross-reactivity to begin with, as we only measured 33 T cell clones. We do not agree with this comment. In our publication, we tested peripheral blood of 81 NT1

patients and 19 HLA-matched healthy controls. In none of our proliferation experiments with T cell lines stimulated with both peptides, we found any proliferative response to the two hypocretin peptides used. Additionally, we isolated H1N1-$HA_{275–287}$-specific T cell clones (157 T cell clones from 12 patients, shown in Table 3.3 in **Chapter 3**) in which we have not been able to detect any reactivity to both hypocretin peptides.

Thirdly, Mignot et al. state that the depth of our TCR sequencing is too low to be able to find any sequences common for NT1. We agree with them on this point, but this was not the primary focus of our project, we elaborate on this limitation of our study in the Discussion section of **Chapter 3**.

Important unsolved issues in studies showing autoreactivity to hypocretin are the identification of hypocretin-specific CD4+ and CD8+ T cells in healthy controls (Jiang et al., 2019, Pedersen et al., 2019) and the finding of HLA-DR-restricted instead of HLA-DQB1*06:02-restricted hypocretin-specific T cells (Latorre et al., 2018), as would have been expected based on the autoimmune hypothesis.

To conclude, the discussed studies add complementary information to the autoimmune hypothesis of narcolepsy. It seems more and more probable that the H1N1 influenza pandemic has a direct link with the development of narcolepsy type 1, but direct evidence of T cells being implicated in the autoimmune destruction of hypocretin-producing neurons is still missing.

- Hypocretin and H1N1-hemagglutinin peptides that are bound to HLA-DQ6 show structural homology
- H1N1-hemagglutinin-specific $CD4^+$ T cells are detected in NT1 patients and controls
- Cross-reactivity between H1N1-hemagglutinin and hypocretin peptides is not observed
- H1N1-hemagglutinin-specific $CD4^+$ T cell reactivity is HLA-DQ6-restricted
- There is no biased expression in the H1N1-hemagglutinin-specific T cell receptor repertoire

The fact that direct evidence for the autoimmune hypothesis of NT1 is lacking, led us to believe that pursuing a different approach to identifying the mechanisms responsible for the destruction of the hypocretin-producing neurons is needed. In **Chapter 4**, we describe our mass cytometry experiments that allow us to compare the immune cell composition in the peripheral blood of NT1 patients with that of healthy HLA-matched controls. This method allows for an in-depth analysis of differences in immune cell composition between both groups. In this way, it is possible to assess the immune system in an unbiased fashion. Our results show several memory $CD4^+$ and $CD8^+$ T cell populations expressing activation markers and regulatory $CD4^+$ T cells indicative of an activated phenotype that are more frequent in NT1 patients with recent disease onset compared with HLA-matched controls. These differences seem to support the autoimmune hypothesis of narcolepsy. However, since differences are mainly found in small populations, they should be regarded with caution and at present can only serve as starting point for identifying novel mechanisms involved in the autoimmune response leading to the destruction of hypocretin-producing neurons. Furthermore, experiments aiming on replicating our findings are very important, since interindividual variability is high and we were only able to replicate a minority of the differences between NT1 patients and healthy controls that were reported previously. Performing experiments on immune cell reactivity to antigens of interest (such as hypocretin, H1N1 or other, still unknown antigens) with the identified NT1-specific immune cell clusters seems a promising strategy, which allows for diminishing the background noise that other immune cells might have caused in experimental research on the autoimmune response leading to NT1 executed until now.

- NT1 patients with recent disease onset have increased frequencies of several memory $CD4^+$ and $CD8^+$ T cell populations compared to HLA-matched controls
- Differences in immune cell composition are small between NT1 patients with recent onset compared to those later after onset
- Identified $CD4^+$ and $CD8^+$ T cell populations seem plausible candidates for identifying mechanisms involved in the autoimmune response leading to the destruction of hypocretin-producing neurons

Future perspectives

The remaining question that this thesis does not touch upon, however, is whether we are searching for an autoimmune response to the right antigen. Thus far, hypocretin has always been regarded as the sole candidate antigen targeted in the autoimmune response in the development of NT1. This seems logical, since hypocretin is not produced in other neurons in the hypothalamus and hypocretin deficiency is strongly linked to the phenotype of the disorder (Lin et al., 1999, Nishino et al., 2000a, Peyron et al., 2000). However, whether other proteins or peptides exist that are relatively specific for hypocretin-producing neurons is largely unknown. Several studies have suggested genes that are relatively specific for hypocretin-producing neurons. However, this data is mostly derived from mouse studies (Liu et al., 2015, Romanov et al., 2017, Cvetkovic-Lopes et al., 2010, Dalal et al., 2013, Mickelsen et al., 2017, Seifinejad et al., 2019). Only one study uses posterior hypothalamus transcriptome data from human post-mortem brains (Honda et al 2010). Further information on the human expression levels of these genes in the hypocretin-producing neurons, or even in the hypothalamus, is lacking. The recent study in which hypocretin-specific CD8+ T cells were identified in healthy controls and NT1 patients was the first to compile data of these studies attempting to identify alternative candidate antigens and also perform experiments to assess reactivity to those antigens (Pedersen et al., 2019). Even though they did not find convincing evidence for autoreactivity, this approach seems promising. However, more reliable data on proteins being produced in hypocretin-producing neurons in humans is a prerequisite for designing experiments assessing immune responses to alternative candidate antigens other than hypocretin in NT1 patients. With the emergence of open access single cell RNA sequencing datasets and *in silico* data analysis methods becoming more and more advanced, identifying these alternative candidate antigens becomes feasible.

Frequently neglected symptoms of NT1

The second part of this thesis focuses on clinical symptoms of NT1. The assumed strong causal and temporal association between the destruction of hypocretin-producing neurons and the appearance of symptoms of NT1 is reinforced in **Chapter 1**. In this Chapter, we describe a case that shows that the relation between the development of hypocretin deficiency and the appearance of NT1 symptoms is causal and can be a process of only weeks. The case is

unique in terms of the short interval between a normal hypocretin-1 value in the cerebrospinal fluid and the first clinical signs of the disease. A second lumbar puncture confirmed the diagnosis of NT1 showing a complete disappearance of hypocretin-1 from the cerebrospinal fluid. In this chapter, we describe the pentad of core symptoms of NT1, but we also highlight the weight gain that the patient experienced.
The weight increase that frequently emerges early in the disease, as in the patient described in Chapter 1 (Daniels, 1934, Wang et al., 2016, Nordstrand et al., 2019b), is one of the most debilitating symptoms. Especially in children who develop NT1 this symptom plays a central role in the burden of the disease (Ponziani et al., 2016).

In **Chapter 5**, we describe a retrospective study on NT1 patients who start using sodium oxybate. Weight loss has been described as a side effect of this medication in one of the hallmark clinical trials on the effect of sodium oxybate in NT1 (The U.S. Xyrem® Multicenter Study Group, 2003) and in our outpatient clinic, patients using sodium oxybate frequently report a decrease in weight when they start using this compound. We compared NT1 patients that started using modafinil with those who started using sodium oxybate and found that sodium oxybate was associated with a mean BMI decrease of 0.84 kg/m^2 in men and 2.56 kg/m^2 in women. NT1 patients that started using modafinil did not significantly lose weight.

Several explanations for both the weight increase when developing NT1 and the subsequent weight loss when initiating sodium oxybate treatment have been proposed. Inactivity (Middelkoop et al., 1995), medication use (Black SW, 2015) and different eating habits (Lammers et al., 1996) as a cause for obesity in NT1 have not been confirmed, even though several symptoms of eating disorders, such as food craving and binge eating were reported in a majority of NT1 patients (Fortuyn et al., 2008). One study reported a lower basal metabolic rate (BMR) close after disease onset in children with NT1, which restored to normal levels in the following months (Wang et al., 2016). Therefore, the effect of NT1 on the individual body mass index (BMI) set point is regarded as the most important cause of the observed obesity. Recent observations suggest that also food-related responses in the cortex are enhanced in NT1 and could potentially contribute to increased food intake leading to obesity, even though this effect on intake has not been shown (van Holst et al., 2018).

Our findings on the effect of sodium oxybate use on body weight are in line with other studies that suggest that NT1 patients lose weight when using sodium oxybate (Boscolo-Berto R, 2012, Husain et al., 2009). Explanations for this treatment effect are lacking. However, recent pre-clinical research suggests that energy-combusting brown adipose tissue may be causally involved in the development of obesity in NT1 (Madden et al., 2012, Sellayah et al., 2011).

- NT1 patients lose weight upon initiating sodium oxybate treatment
- Modafinil use does not lead to a weight decrease in these patients
- The effect of sodium oxybate on weight is more pronounced in women and patients with a high BMI

Chapter 6 contains our findings on daytime sleep-state misperception in NT1 and other sleep disorders. In this prospective study, we assessed the occurrence of, and factors influencing sleep state misperception in several sleep disorders with excessive daytime sleepiness. We looked at both classical and reverse sleep state misperception and found that sleep state misperception is common in people with NT1 and -2, idiopathic hypersomnia, obstructive sleep apnea and insufficient sleep syndrome. Classical sleep state misperception is more frequent in patients with longer sleep onset latencies who only reach non-REM sleep stage 1 during a nap.
Daytime sleep state misperception is a relevant symptom for NT1 patients, since it may influence the capacity to notice daytime naps. In this way, information on the prevalence of daytime sleep state misperception is valuable for both patient and sleep physician when assessing disease severity and medication effect. Based on our findings, this is not only the case for NT1, but also for patients with other, much more prevalent, sleep disorders with excessive daytime sleepiness, such as obstructive sleep apnea and insufficient sleep syndrome. Hence, information on daytime sleep state misperception could also provide valuable information in the assessment of disease severity and treatment effect in other sleep disorders (Liu et al., 2018).

- Daytime sleep state misperception was measured during the short naps of the multiple sleep latency test
- Daytime sleep state misperception is prevalent in NT1 and -2, and other sleep disorders

- A long sleep latency and the occurrence of only NREM stage 1 sleep both predict daytime sleep state misperception
- Patients and physicians should take the presence of daytime sleep state misperception into account when making treatment decisions

Future perspectives

Scientific data on the pentad of NT1 core symptoms is gathered in almost every clinical trial or cohort study that is performed in NT1. Even though the primary endpoint in most studies is either cataplexy frequency or excessive daytime sleepiness, the effect of interventions on hypnagogic hallucinations, sleep paralysis and disturbed night sleep is frequently reported as a secondary endpoint. However, the burden of the disease is only partly dictated by these symptoms. Other symptoms, or neglected expression of core symptoms, such as weight gain, disturbed vigilance, depressive symptoms and sleep state misperception, are adding to the social disability, educational difficulties and mental problems that NT1 patients experience in everyday life. Clinical trials and studies using information derived from prospective clinical databases, that describe the effect of interventions on these other symptoms, are much needed. It will greatly support NT1 patients' futures to be able to treat their symptoms that are not part of the core pentad, based on clinical evidence.

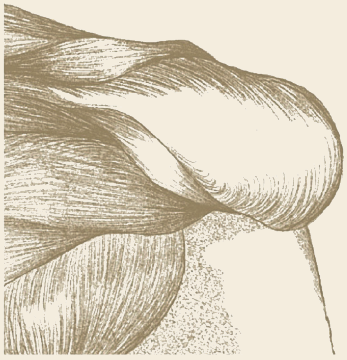
Based on **Chapter 5**, it is clear that weight gain can be influenced by medication, but also that future research should focus on the mechanisms that underlie weight gain in NT1. Investigating the role of brown adipose tissue in both weight gain and subsequent weight loss upon initiation of sodium oxybate treatment seems promising. Many preclinical studies suggest a role for the hypocretin system in brown adipose tissue activation (Madden et al., 2012, Sellayah et al., 2011), but also in the differentiation and development of this tissue (Rogers et al., 2012, Sellayah and Sikder, 2014). However, the first study to assess brown adipose tissue in NT1 patients did not find any differences when its activation was compared to that of healthy controls, but the sample size was small and the protocol did not allow for adequate maximal brown adipose tissue activation measurements (Enevoldsen et al., 2018). Prospective larger studies are needed to evaluate the role of brown adipose tissue in the weight gain accompanying the development of NT1.

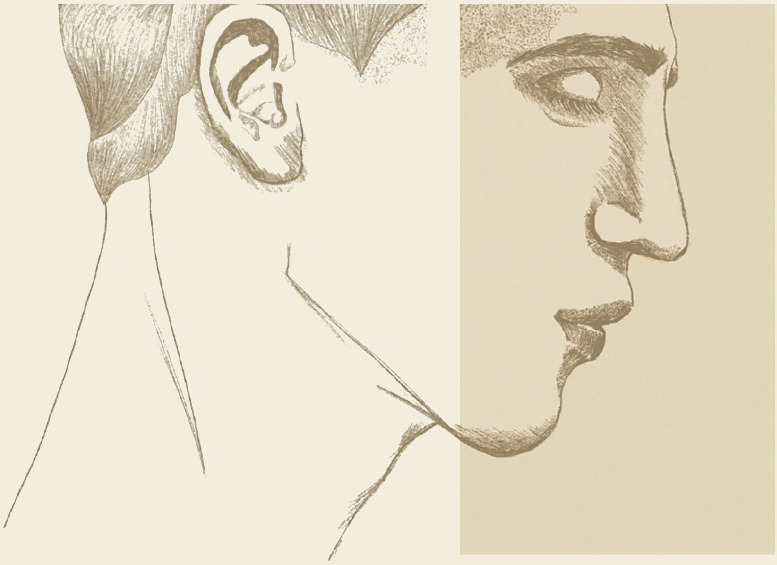
The mechanism that leads to the weight loss that follows the initiation of sodium oxybate treatment in NT1 might also involve brown adipose tissue activation, since the main metabolite of sodium oxybate, succinate, was recently shown to strongly induce brown adipose tissue thermogenesis (Mills et al., 2018).

Chapter 6 on the other hand, shows that symptoms that are not easily recognized, such as sleep state misperception, might influence a patient's ability to assess daytime function adequately and might therefore influence treatment decisions. Being aware of the presence of these symptoms is important for sleep physicians and should always be taken into account when assessing treatment effect. Gathering information on daily functioning from relatives or other people that are close to the patient seems pivotal in adequately assessing the burden of the disease for that patient. This is also highly relevant when performing clinical research, since excessive daytime sleepiness as a primary endpoint is often measured by questionnaires. Developing tools that enable researchers to include symptoms such as sleep state misperception and disturbed vigilance in the outcome parameters of clinical trials could be a first step to acknowledging the diversity of symptoms that make up the burden of NT1.

Overall conclusion

Both basic and clinical research on NT1 have increased rapidly over the past two decades, sparked by the discovery of hypocretin in the late 1990s. However, many questions remain unanswered. A clear understanding on what mechanisms drive the presumed autoimmune response leading to the development of NT1 is pivotal to eventually develop a causal treatment for or prevention of the disorder. This thesis describes several factors that might play a role in the pathophysiological process. At the same time, interest in invalidating consequences beyond the core clinical symptoms slowly but steadily increases, as Chapter 5 and 6 show. For NT1 patients this is arguably even more important than small improvements in preventing daytime sleep and the occurrence of cataplexy.



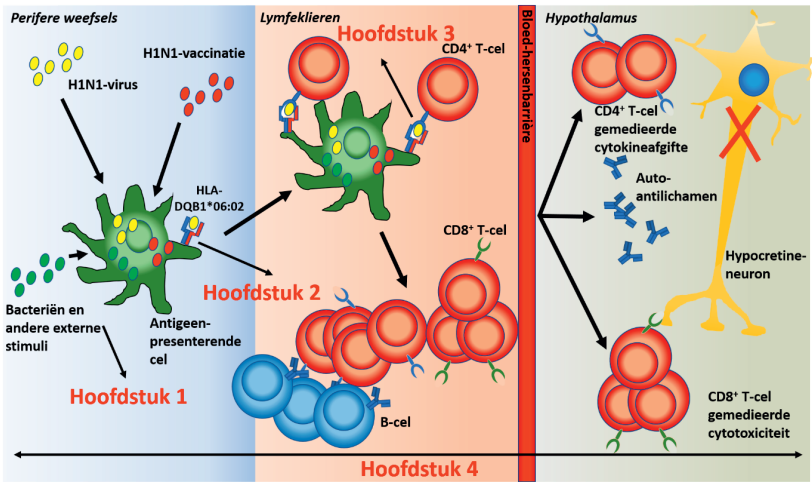


Nederlandse samenvatting

Dit proefschrift valt uiteen in twee delen: deel 1 richt zich op de auto-immuun hypothese van narcolepsie, terwijl het tweede deel symptomen van narcolepsie type 1 beschrijft die vaak onbesproken worden gelaten, terwijl ze significant bijdragen aan de ziektelast.

De auto-immuun hypothese van narcolepsie type 1

De figuur die hier is afgebeeld vat de auto-immuun hypothese van narcolepsie type 1 samen. De hypothese beschrijft dat niet-lichaamseigen antigenen bij mensen die gevoelig zijn voor het ontwikkelen van narcolepsie type 1, een immuunrespons veroorzaken die via verschillende tussenstappen leidt tot het teloor gaan van hypocretine-producerende neuronen in de laterale hypothalamus. De eerste hoofdstukken van dit proefschrift beschrijven enkele van de tussenstappen binnen deze hypothese. **Hoofdstuk 1** beschrijft een nieuwe verwekker die mogelijk tot een auto-immuun respons kan leiden. **Hoofdstuk 2** richt zich op de associaties tussen HLA-genotype en narcolepsie type 1, waarbij de nadruk ligt op verschillen tussen mensen die vóór en ná de Mexicaanse griep pandemie narcolepsie type 1 hebben gekregen. **Hoofdstuk 3** beschrijft de mogelijke rol van kruisreactieve CD4$^+$ T-cellen in de voornoemde auto-immuun respons. **Hoofdstuk 4**, ten slotte, vergelijkt alle immuuncellen in het bloed van mensen met narcolepsie type 1 met die bij gezonde proefpersonen om populaties te vinden die vaker voorkomen bij narcolepsie type 1. Een uitgebreidere beschrijving van de bevindingen per hoofdstuk volgt hierna.



Figuur 1. Een visualisatie van de auto-immuun hypothese van narcolepsie type 1 en de immuunmechanismes die in dat kader worden besproken in de verschillende hoofdstukken van dit proefschrift.

Hoofdstuk 1 beschrijft een casus van een persoon die zowel voorafgaand aan als kort volgend op het ontstaan van klachten passend bij narcolepsie type 1 een lumbaalpunctie heeft ondergaan. De concentratie hypocretine-1 in de liquor verandert tussen de twee meetmomenten van normaal naar ondetecteerbaar laag. De klachten ontstonden bij deze persoon kort na een infectie met een enterovirus, wat insinueert dat dit virus mogelijk ook een rol kan spelen in het activeren van een auto-immuunrespons leidend tot narcolepsie type 1.

In **hoofdstuk 2** worden geen verschillen gevonden tussen HLA-associaties tussen mensen die voor en na de Mexicaanse griep narcolepsie type 1 hebben gekregen. In het hoofdstuk worden eerder beschreven HLA-associaties tussen HLA-DQ7, -DQ2, -DQB1*05:01 en -DQB1*05:03 en narcolepsie type 1 bevestigd. Deze resultaten pleiten tegen de hypothese dat narcolepsie type 1 ontstaan vóór de Mexicaanse griep anders is dan narcolepsie type 1 die daarna is ontstaan.

Hoofdstuk 3 laat zien dat geselecteerde hypocretine- en H1N1-hemagglutininepeptiden als ze gebonden zijn aan HLA-DQ6 structureel homoloog zijn. Verder werden H1N1-hemagglutinine-specifieke $CD4^+$ T-cellen aangetoond, alleen in de aanwezigheid van HLA-DQ6, bij zowel personen met narcolepsie type 1 als gezonde proefpersonen. Kruisreactiviteit tussen H1N1-hemagglutinine- en hypocretinepeptiden werd niet gevonden. Er waren geen aanwijzingen voor de aanwezigheid van voor de ziekte specifieke T-celreceptoren. Deze bevindingen geven dan ook geen direct bewijs voor de auto-immuun hypothese van narcolepsie type 1.

Vanwege het feit dat de eerdere hoofdstukken geen direct bewijs leverden voor de auto-immuun hypothese van narcolepsie type 1, besloten we andere mechanismen te onderzoeken die betrokken zouden kunnen zijn bij het verdwijnen van de hypocretine-producerende neuronen. **Hoofdstuk 4** beschrijft experimenten waarin middels massa cytometrie de samenstelling van het immuunsysteem in het bloed van personen met narcolepsie type 1 is vergeleken met die van gezonde proefpersonen. De gebruikte methode maakt het mogelijk vergelijkingen tot op de individuele immuuncel te maken. De vergelijking wordt verricht door een algoritme, waardoor het risico op bias is geminimaliseerd.

In dit hoofdstuk laten we zien dat verscheidene populaties van geactiveerde $CD4^+$ en $CD8^+$ T-cellen vaker voorkomen bij personen met narcolepsie type 1. Deze bevindingen kunnen dienen als startpunt voor verder analyse van de rol van deze populaties in het auto-immuunproces dat leidt tot het ontstaan van narcolepsie type 1.

Toekomstperspectief

De vraag die in de hoofdstukken van dit proefschrift niet beantwoord wordt is of wij zoeken naar een auto-immuunrespons tegen het juiste antigen. Logischerwijs wordt hypocretine gezien als het antigen waartegen de auto-immuunrespons zich het meest waarschijnlijk richt. Het is echter grotendeels onduidelijk of er niet ook andere antigenen zijn die relatief specifiek zijn voor hypocretine-producerende neuronen. De literatuur hierover betreft tot nog toe met name onderzoeken bij muizen, terwijl slechts één publicatie daadwerkelijk het transcriptoom van de post-mortem humane hypothalamus gebruikt. Het beschikbaar komen van open access single cell RNA sequencing datasets en almaar verbeterende *in silico* data-analyse faciliteert het vergaren van meer informatie over andere kandidaat-antigenen terdege. Het gebruik van deze kennis in immunologische experimenten gericht op het identificeren van autoreactieve immuuncellen lijkt dan ook veelbelovend.

Minder bekende symptomen van narcolepsie type 1

Het tweede deel van dit proefschrift richt zich op klinische symptomen van narcolepsie type 1. De sterke causale en temporele relatie tussen het verdwijnen van hypocretine-producerende neuronen en het ontstaan van symptomen passend bij narcolepsie type 1 wordt onderstreept in **Hoofdstuk 1**. In dit hoofdstuk wordt beschreven dat bij een persoon een eerste lumbaalpunctie een normaal hypocretine-1 toonde. Binnen enkele weken hierna ontwikkelde deze persoon klachten passend bij narcolepsie type 1, wat bevestigd werd met een nieuwe lumbaalpunctie waarbij hypocretine-1 afwezig bleek. We beschrijven in dit hoofdstuk de vijf kernsymptomen van narcolepsie type 1, maar noemen ook de gewichtstoename die erg op de voorgrond stond als symptoom. Dit

symptoom is voor veel mensen met narcolepsie type 1 een belangrijk onderdeel van de ziektelast, met name bij kinderen.

Hoofdstuk 5 beschrijft hiertoe een onderzoek bij mensen met narcolepsie type 1 die beginnen met het gebruik van natriumoxybaat. We vergeleken het effect van dit middel op het gewicht in vergelijking met de gewichtsverandering bij mensen die modafinil begonnen te gebruiken. Natriumoxybaat, een medicament dat gebruikt wordt voor de behandeling van alle kernsymptomen van narcolepsie type 1, bleek tevens voor een significant grotere gewichtsreductie te zorgen dan modafinil. Zowel voor de gewichtstoename als symptoom van narcolepsie type 1 als voor de gewichtsafname door het gebruik van natriumoxybaat is nog niet afdoende verklaring. Preklinisch onderzoek suggereert echter dat er een rol is voor veranderingen in activatie van bruin vetweefsel, verantwoordelijk voor een groot deel van de warmteproductie in het lichaam, in narcolepsie type 1.

Hoofdstuk 6 beschrijft bevindingen over slaapbeleving bij personen met narcolepsie type 1 en andere slaapstoornissen. In dit onderzoek bekeken we in hoeverre mensen met een slaapstoornis in staat waren correct te benoemen of ze al dan niet hadden geslapen tijdens een gepland dutje. Zowel zeggen geslapen te hebben als geen slaap was geregistreerd, als zeggen niet geslapen te hebben als juist wel slaap was geregistreerd kwam veel voor bij mensen met narcolepsie type 1 en 2, idiopathische hypersomnie, obstructief slaapapnoe syndroom en chronische slaapdeprivatie. Het komt frequenter voor bij mensen die later tijdens het dutje in slaap vallen en alleen non-REM slaapstadium 1 bereiken. Deze bevindingen zijn relevant, omdat het bewustzijn van in slaap vallen overdag gebruikt wordt voor het evalueren van ernst van de aandoening en behandeleffect van interventies. In het licht van dit onderzoek moet dus voorzichtig omgegaan worden met de op die manier verkregen informatie.

Toekomstperspectief

De vijf kernsymptomen van narcolepsie type 1, te weten kataplexie, overmatige slaperigheid overdag, hypnagoge hallucinaties, slaapparalyse en verstoorde nachtslaap, worden vaak gebruikt als primaire en secundaire uitkomstmaat bij onderzoek naar de effectiviteit van behandeling. Andere, minder bekende symptomen dragen echter significant bij aan de ziektelast van mensen met narcolepsie type 1. Gewichtstoename, verminderde alertheid, depressieve

klachten en het onvermogen zich bewust te zijn van in slaap vallen zijn enkele van deze klachten die bijdragen aan de sociale handicap, mentale problemen en problemen in onderwijs en opleiding, die mensen met narcolepsie type 1 dagelijks ervaren. Onderzoeken die deze symptomen als uitkomstmaat gebruiken zijn noodzakelijk om te komen tot een evidence-based behandeling van deze onderbelichte symptomen.

Hoofdstuk 5 maakt duidelijk dat gewichtstoename beïnvloed kan worden door medicatie, maar ook dat onderliggende mechanismen nog onvoldoende zijn opgehelderd. Verder onderzoek naar de rol die bruin vetweefsel speelt in zowel gewichtstoename als het door natriumoxybaat geïnduceerde gewichtsverlies lijkt een logische volgende stap. Preklinische onderzoeken suggereren reeds een rol van het hypocretinesysteem bij de activatie van bruin vet. Wij zijn nu bezig met verder preklinisch onderzoek en het opstarten van een klinische trial om een volgende stap te zetten in het ontdekken van de mechanismes achter de gewichtstoename bij het ontstaan van narcolepsie type 1.

Hoofdstuk 6 daarentegen maakt duidelijk dat symptomen die niet zichtbaar zijn, zoals het onvermogen zich bewust te zijn van het in slaap vallen, evaluatie van de ernst van de aandoening en de behandeling danig kunnen bemoeilijken. Onderzoek is noodzakelijk om deze symptomen zichtbaar te maken en zowel patiënten als artsen een handvat te bieden om deze symptomen bespreekbaar te maken in de spreekkamer. Het ontwikkelen van goede meetinstrumenten voor deze symptomen zou hiervoor een noodzakelijke eerste stap zijn.

Conclusie

Zowel preklinisch als klinisch onderzoek naar narcolepsie type 1 heeft zich de afgelopen twee decennia sterk ontwikkeld, aangezwengeld door de ontdekking van de hypocretines in de tweede helft van de jaren ’90. Veel vragen blijven echter onbeantwoord. Een duidelijk begrip van welke immunologische mechanismen leiden tot een auto-immuun respons die de hypocretine-producerende neuronen in de hypothalamus doen verdwijnen is er nog niet. Dit begrip is noodzakelijk om uiteindelijk tot een causale behandeling of zelfs preventie van narcolepsie type 1 te komen. Dit proefschrift beschrijft delen van dit auto-immuunproces en tracht de lezer dichter bij het voornoemd begrip te brengen. Tegelijkertijd pleit dit proefschrift voor aandacht voor onderbelichte, zeer

invaliderende symptomen van narcolepsie type 1, die buiten de kernsymptomen van de aandoening vallen en zodoende amper onderzocht worden. Meer kennis over deze symptomen zal mensen met narcolepsie type 1 in het dagelijks leven waarschijnlijk meer opleveren dan marginale verbeteringen in de behandeling van overmatige slaperigheid overdag en kataplexie.

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Curriculum vitae

Mink Sebastian Schinkelshoek was born in Leiden, the Netherlands, on June 7, 1990 as Ria Bleeker's and Johan Schinkelshoek's first child. Two years later, his brother Job was born. He grew up in Voorhout.

In 2008 he finished secondary school at the Stedelijk Gymnasium Leiden summa cum laude and started studying Medicine at Leiden University in the same year. He soon became interested in Neurology and hence started working as a student-assistant at the Department of Anatomy in his second year, where he assisted in neuro-anatomy dissection courses to first-year medical students for 2 years. He acquired his Bachelor's degree in Medicine in 2011 and meanwhile started a Pre-Master Biomedical Sciences that prepared for the Master program of Biomedical Sciences at the same institute to ascertain whether biomedical research was a venue to be further explored. This Pre-Master and the following Master in Biomedical Sciences convinced him that pursuing a career in neurology combining both clinical work and research was his prime ambition. In 2015, he obtained both Medicine and Biomedical Sciences Master's degrees cum laude. During the last part of his clinical rotations he got in touch with narcolepsy research.

After graduating, he started working as a Neurology resident not in training in the Reinier de Graaf hospital in Delft. Meanwhile, the plan that eventually led to this thesis was forged in the months that he worked in Delft. In November 2016 the PhD on the autoimmune hypothesis started at the Department of Neurology of the Leiden University Medical Center and the Sleep-Wake Centre of Stichting Epilepsie Instellingen Nederland (SEIN) in Heemstede. In 2018, he was awarded the Young Investigator Award at the 7th International Symposium on Narcolepsy in Beverly, MA, USA for his work on the composition of the immune system in narcolepsy type 1. Additionally, he received several travel grants (EAN 2017 and EAN 2019) and won the Best Poster Award at the 2017 European Narcolepsy Day.

Mink Schinkelshoek and his partner Shita Bakker became parents of Ivar Schinkelshoek on 21 September 2019, they live in Voorhout. He is currently working as a resident in training at the Department of Neurology of the Leiden University Medical Center.

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