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# Classical complement pathway factor alterations in narcolepsy

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## Original Article

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## Abstract

*Objective:* Narcolepsy is a chronic sleep disorder long hypothesised to be an autoimmune disease. Complement-mediated immune mechanisms have not been investigated in detail in narcolepsy. Our aim was to establish the significance of classical pathway activation in narcolepsy. *Methods:* Sera of 42 narcolepsy patients and 26 healthy controls were screened with ELISA to determine the levels of C1q, C3a, C4d and complement component 4 binding protein (C4BP). A home-made ELISA method was developed to detect antibodies to C4BP-alpha (anti-C4BPA). The correlation between complement levels and clinical findings was examined. *Results:* C1q levels were significantly higher in narcolepsy patients while C4d and C4BP levels were significantly lower compared to healthy controls. C3a levels were comparable among patients and controls. Eleven narcolepsy patients showed serum anti-C4BPA levels. Total rapid eye movements (REM) time, sleep onset latency, REM sleep latency, sleep activity, percentage of wakefulness after sleep onset and Epworth sleepiness scale scores were correlated with levels of different complement factors. *Conclusion:* Complement-mediated immune mechanisms might partake in narcolepsy pathogenesis. The precise role of autoantibodies on complement level alterations needs to be investigated. Levels of complement factors and degradation products may potentially be utilised as biomarkers to predict the clinical severity of narcolepsy.

## Significant outcomes

- This study showed increased C1q and decreased C4BP, C4d and C3a levels in narcolepsy, pointing to the fact that complement system factors in narcolepsy might predict disease severity.
- Complement breakdown products may have a negative impact on sleep functions. Especially, C1q may be used as a potential indicator of worsening sleep quality and increased daytime sleepiness.
- The role of other complement system factors in neuroinflammation and sleep physiology needs to be investigated.

## Limitations

- A limitation of our study is the absence of hypocretin level measurements and analysis of putative correlations between hypocretin, C4BP and anti-C4BPA levels.
- Similarly, measurement of autoantibodies that are frequently observed in narcolepsy patients and correlation with complement levels could have provided informative insights.

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## Introduction

Narcolepsy is a chronic sleep disorder characterised by abnormal transition to rapid eye movements (REM) sleep phase and increased daytime sleepiness (Kornum *et al.*, 2017). Hypocretin/orexin deficiency due to loss of hypocretinergic neurons is the main aetiology of the disease (Siegel, 1999; Mahoney *et al.*, 2019). In narcolepsy type 1 (NT1), cataplexy and/or low levels of hypocretin in cerebral spinal fluid must be observed; whereas for narcolepsy type 2 (NT2) diagnosis, cataplexy must be absent (American Academy of Sleep Medicine, 2014).

Both humoral and cellular immune mechanisms seem to play a role in this selective loss of neurons in narcolepsy. Genetic evidences have emphasised the presence of human leukocyte

antigen (HLA)-DQB1*06:02 allele for the differential diagnosis of narcolepsy (Manni *et al.*, 2018). In addition, many mutations in various immunity-related genes have been observed in narcoleptic patients (Singh *et al.*, 2013; Degn *et al.*, 2017; Miyagawa & Tokunaga, 2019; Pedersen *et al.*, 2019; Postiglione *et al.*, 2020). Autoreactive CD4+ and CD8+ T cells were also found to target-specific hypocretinergic neurons in HLA-associated narcolepsy cases (Luo *et al.*, 2018; Pedersen *et al.*, 2019). Recently, the critical importance of HLA characterisation in lower levels of effector memory CD4+ T cells observed in NT1 was mentioned in the literature (Viste *et al.*, 2021). Besides, proinflammatory cytokines, such as interleukin 6 and tumour necrosis factor-alpha, are effective in the sleep-wake cycle and are found at higher levels in patients with narcolepsy compared to healthy controls (Mohammadi *et al.*, 2020). Moreover, increased hypocretin-1 reactive total immunoglobulin (Ig) G and IgM autoantibodies have been detected in NT1 patients' sera (Deloumeau *et al.*, 2010; Nixon *et al.*, 2015). This notion is supported by the presence of a plethora of additional autoantibodies in narcolepsy patients (Kornum, 2020). These findings suggest that different immune system mechanisms may partake in narcolepsy and the disease may have an autoimmune basis.

As one of the humoral mechanisms, complement system is particularly important in many autoimmune metabolic and central nervous system (CNS) diseases such as neuromyelitis optica, autoimmune encephalitis, myasthenia gravis (MG) and systemic lupus erythematosus (SLE) (Dalakas *et al.*, 2020). Anti-complement antibodies have also been shown in MG and SLE (Tüzün *et al.*, 2006; Kallenberg, 2008). It is known that autoantibodies can cause cell damage by complement activation (Alexander *et al.*, 2008). Therefore, changes in peripheral blood complement and complement inhibitor levels, and the presence of serum anti-complement antibodies may be indicators of complement-mediated mechanisms in the CNS. Furthermore, complement factor alterations have been reported in association with sleep dysfunction (Shivshankar *et al.*, 2020). Despite its significance in sleep, the complement system has not been examined in detail in narcolepsy. Normal complement levels in narcolepsy have been mentioned only in a few early studies (Matsuki *et al.*, 1988). However, C4A3, C4B1 polymorphisms and C2 and C4 complement gene variants have been identified for narcolepsy (Matsuki *et al.*, 1988). These variants are also known to localise in between HLA-B and HLA-DR loci, which is close to narcolepsy-associated HLA allele region (Shiina *et al.*, 2009). Moreover, it has been shown that the level of complement factors C3 and C5, and circulating IgG, IgM and IgA increase when sleep deprived (Hui *et al.*, 2007). Besides, C3a levels are known to decline during nocturnal awake time compared to nighttime sleep. In a recent proteomic profiling study, humoral immune response, complement and coagulation cascades were also found to be the main molecular mechanisms for insomnia (Wang *et al.*, 2021). In sum, accumulating evidence suggests that sleep deprivation, daytime sleepiness and sleep quality change various complement factors which may as well explain the pathophysiology of narcolepsy (Hui *et al.*, 2007; Besedovsky *et al.*, 2019; Pak *et al.*, 2020).

Overall, classical complement pathway factors are genetically linked to narcolepsy, and the presence of anti-neuronal antibodies in narcolepsy indicates antibody-dependent classical pathway activation as a potential disease mechanism (Matsuki *et al.*, 1988; Besedovsky *et al.*, 2019). In order to gather more information about the classical complement pathway-mediated immunopathogenesis of narcolepsy, we investigated serum levels of classical pathway complement factors (C1q, C4d) and the classical pathway inhibitor (complement component 4 binding protein, C4BP), which are known to be elevated in serum and accumulated on target tissue in typical antibody and complement-mediated disorders (Segawa *et al.*, 2010; Mihlan *et al.*, 2011). These factors are also activated by acute-phase reactant C-reactive protein and thus might reflect acute inflammation activity (Agrawal *et al.*, 2001; Sjöberg *et al.*, 2006). To assess the common complement pathway activity, we measured levels of C4BP-independent C3-split product C3a, rather than C3b, since C4BP promotes the conversion of C3b to iC3b in the presence of Factor H (Sjöberg *et al.*, 2009; Ramos-Sevillano *et al.*, 2015). Levels of breakdown products were measured since they might more efficiently indicate complement pathway activity (Davies *et al.*, 1988; Lechner *et al.*, 2016). We also sought for potential correlations between complement factor levels and clinical parameters of narcolepsy. Our results suggest that complement pathway activation might be involved in the physiopathology of narcolepsy.

### Aims of the study

The aim of the study was to investigate complement-mediated immune mechanisms in narcolepsy by measuring the serum levels of classical pathway complement factors (C1q, C4d), the classical pathway inhibitor (C4BP) and common complement pathway activity (C3a).

## Material and methods

### Subjects

Consecutive 42 narcolepsy patients (26 men, 32.3 ± 11.8) diagnosed according to the International Classification of Sleep Disorders criteria and whose polysomnography (PSG) and multiple sleep latency test results were compatible with narcolepsy were selected (American Academy of Sleep Medicine, 2005). Eight narcolepsy patients were under modafinil treatment, whereas the remaining patients were not under treatment. Patients with a concomitant disease or other primary sleep disorder were excluded. For healthy control group, 26 age- and gender-matched volunteers (17 men, 31.0 ± 13.2-year-old), who had no infectious disease for the last 3 months, had not used immunosuppressive drugs, had no immunological or neurological disorders, had normal sleep study values and had not been diagnosed with primary sleep disorder were selected. The ethics committee approved the study protocol and all participants signed informed consent forms. Blood samples were collected venously from all participants between 8 and10 a.m. and sera were stored in a −80°C freezer until use.

### Sleep studies

All participants had a whole-night PSG recording using the Embla a10 system (Flaga, Reykjavik, Iceland). Sixteen-channel electroencephalography recordings were used in PSG montage, and electrode placement was performed according to the 10–20 system. Other PSG montages included a left and right electrooculogram, chin electromyogram, left and right tibialis anterior electromyogram, electrocardiogram, nasal pressure, oronasal thermistor, thoracic and abdominal strain gauges, pulse oximetry and synchronised video recording. All recordings started at the subjects' usual bedtime and continued until spontaneous awakening. The following conventional sleep parameters were evaluated: total non-REM/REM time, sleep onset latency, REM sleep latency,

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**Table 1.** Clinical information of NT1 and NT2 cases evaluated in the study

| | NT1 (n = 20) | NT2 (n = 22) | *p* value |
|---|---|---|---|
| Sex (F/M) | 9/11 | 7/15 | 0.575 |
| Age (year, SD) | 32.31 ± 14.33 | 35.5 ± 7.25 | 0.003 |
| Age of disease onset | 19.5 ± 8.83 | 29.4 ± 8.97 | 0.315 |
| BMI (kg/m$^2$) | 24.69 ± 3.4 | 24.47 ± 4.6 | 0.969 |
| REM sleep latency (min) | 57.75 ± 110.77 | 76 ± 80.04 | 0.108 |
| Sleep activity (%) | 86.21 ± 7.96 | 93.66 ± 5.71 | 0.027 |
| Wakefulness (%) | 13.24 ± 8.29 | 5.54 ± 5.28 | 0.088 |
| N1 (%) | 9.16 ± 4.36 | 5.21 ± 2.42 | 0.21 |
| N2 (%) | 43.68 ± 5.92 | 41.89 ± 9.79 | 0.094 |
| N3 (%) | 17.51 ± 6.09 | 25.6 ± 6.71 | 0.765 |
| REM (%) | 16.4 ± 7.15 | 21.75 ± 8.1 | 0.958 |
| Sleep latency (min) | 2 ± 0.76 | 2.25 ± 1.06 | 0.374 |
| SOREM | 2.27 ± 2.04 | 2.8 ± 2.33 | 0.885 |
| ESS | 15.5 ± 3.37 | 15.89 ± 3.53 | 0.654 |
| PSQT | 11.11 ± 5.07 | 6.72 ± 4 | 0.548 |

Parametric results are expressed as mean ± standard deviation.
BMI, body mass index; ESS, Epworth sleepiness scale; F, female; M, male; N, non-REM; PSQT, Pittsburgh sleep quality test; REM, rapid eye movements; SOREM, sleep onset rapid eye movement.

sleep activity, percentage of wakefulness after sleep onset and sleep onset REM. In addition, subjective sleep quality was evaluated by Pittsburgh sleep quality test (PSQT) and daytime excessive sleepiness was tested by Epworth sleepiness scale (ESS). Sleep parameters of NT1 and NT2 cases are reported in Table 1.

### *ELISA studies*

Serum C1q (Thermo Fisher, Waltham, MA, USA), C3a (Thermo Fisher, USA), C4d (Quidel, San Diego, CA, USA), and C4BP (Abbkine, Wuhan, China) levels were determined using commercial ELISA kits, as per manufacturer's recommendations. Plates were read at 450 nm and results were expressed in µg/ml or ng/ml. Anti-C4BPA was investigated by a home-made ELISA using a recombinant C4BPA protein (Novus Biologicals, Centennial, CO, USA). C4BPA (0.4 µg per well) was coated onto 96-well microtiter plates in 0.1 M carbonate/bicarbonate buffer overnight at 4°C. Sera (1:1000) were added and plates were incubated at 37°C for 90 min. After PBS washing, horseradish peroxidase-conjugated anti-human IgG (Abcam, Cambridge, UK) (1:5000) was added and plates were then incubated at 37°C for 90 min, followed by washing and incubation with a chromogen solution in the presence of $H_2O_2$. Plates were read at a wavelength of 450 nm and results were expressed as optical densities. Antibody positivity was defined as two standard deviations above the mean of healthy controls.

### *Statistics*

Parametric variables were compared with Student's *t*-test, whereas categorical variables were compared with Chi-square test. Pearson correlation test was used for correlation analysis. The significance level of the test parameters was accepted as $p < 0.05$ and the confidence interval as 95%. Statistical analyzes and graphs were performed with GraphPad Prism 5 programme.

## Results

### *Identification of the levels of complement factors*

Serum C1q levels of narcolepsy patients were significantly higher than those of healthy controls ($p = 0.007$), while C4d ($p = 0.0001$) and C4BP ($p = 0.0012$) levels of narcoleptics were significantly lower (Fig. 1). Narcolepsy patients showed trends towards displaying lower serum C3a levels than healthy controls, however this difference did not attain statistical significance ($p = 0.053$; Fig. 1). To investigate whether antibodies might have decreased C4BP levels and/or employed a neutralising action on C4BP, we measured serum levels of anti-C4BPA by ELISA. Narcolepsy patients showed trends towards exhibiting increased serum levels of C4BPA antibodies as compared to healthy controls ($p = 0.061$; Fig. 1). Moreover, 11 of 42 (26.1%) narcolepsy patients showed anti-C4BPA levels above the threshold level for positivity, while none of the 26 control sera exceeded this threshold ($p = 0.004$ by Chi-square test; Fig. 2). However, C4BPA antibody levels did not significantly correlate with levels of C4BP, C1q, C4d and C3a (not shown). Also, narcolepsy patients with and without C4BPA antibodies showed comparable levels of C4BP ($p = 0.298$), C1q ($p = 0.155$), C4d ($p = 0.411$) and C3a ($p = 0.106$). Likewise, no significant difference was detected between anti-C4BPA antibody positive and negative patients in terms of age, age of disease onset, gender, body mass index, narcolepsy type and sleep parameters measured by the PSG study ($p > 0.05$). No difference was observed between NT1 and NT2 patients in terms of levels of C4BP ($p = 0.831$), C1q ($p = 0.571$), C4d ($p = 0.151$), C3a ($p = 0.259$) and anti-C4BPA ($p = 0.994$; Fig. 3). Similarly, patients with and without modafinil treatment showed comparable levels of all measured complement parameters (not shown).

### *Association of complement factors with clinical features*

Clinical and demographic features of patients and complement factor levels were statistically compared. C1q levels were found to be positively correlated with ESS ($r = 0.317$, $p = 0.048$). On the other hand, C4d levels were positively correlated with REM sleep latency ($r = 0.475$, $p = 0.006$) and percentage of wakefulness after sleep onset ($r = 0.491$, $p = 0.006$), while both C4d and C3a levels negatively correlated with sleep activity ($r = -0.361$, $p = 0.042$; $r = -0.526$, $p = 0.002$, respectively) and total REM time ($r = -0.389$, $p = 0.034$; $r = -0.402$, $p = 0.027$, respectively). The levels C3a were also positively correlated with percentage of wakefulness ($r = 0.533$, $p = 0.002$) and PSQT ($r = 0.451$, $p = 0.005$). No significant correlation was found between anti-C4BPA and C4BP levels and any of the sleep parameters ($p > 0.05$; Fig. 4).

## Discussion

Although the etiopathogenesis of narcolepsy is not fully understood, the autoimmune hypothesis related to narcolepsy has drawn attention for reasons such as strong relationship with the HLA haplotype DQB1*06:02, immunity-related gene polymorphisms, antibodies directed against hypocretin and other antigens expressed by hypocretinergic neurons, and influenza vaccine induced narcolepsy (Jennum *et al.*, 2017; Tanaka *et al.*, 2017; Latorre *et al.*, 2018; Luo *et al.*, 2018; Pedersen *et al.*, 2019; Kornum, 2020; Postiglione *et al.*, 2020). Although these recent neurological investigations have increasingly focused on the role of immunity, there is not yet sufficient data regarding antibody-dependent complement system activation in narcolepsy. Due to

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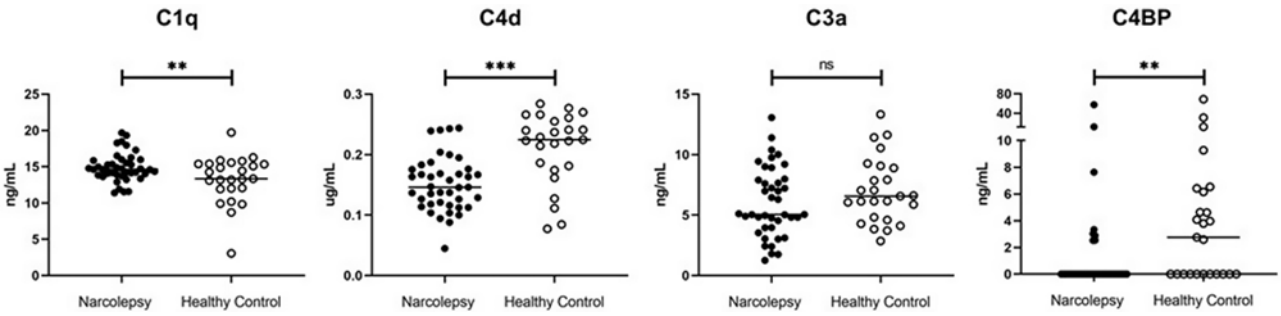



**Fig. 1.** Serum C1q, C4d, C3a and complement component 4 binding protein (C4BP) levels of narcolepsy patients and healthy controls. Horizontal lines indicate mean value of each group and lines at the top indicate statistical comparisons between groups. ** indicates $p < 0.01$, *** $p < 0.001$, ns= not significant by Student's *t*-test.

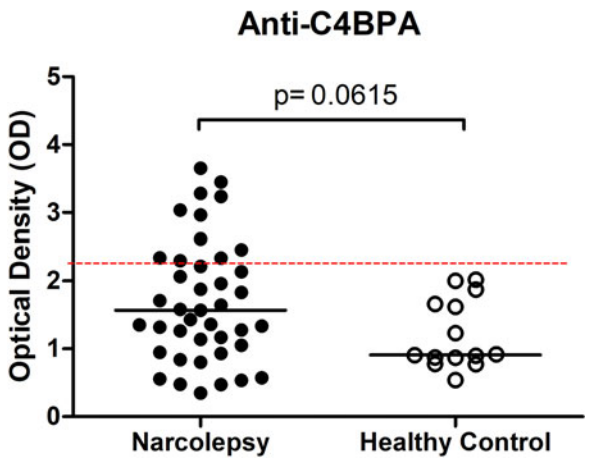



**Fig. 2.** ELISA detection of IgG antibodies directed against complement component 4 binding protein alpha (C4BPA) in sera of narcolepsy patients and healthy controls. The dashed lines represent two standard deviations above the mean of the healthy control samples (cut-off values for positivity). Horizontal lines indicate the mean value of each group.

previously reported association between classical complement pathway gene factors and narcolepsy (Matsuki *et al.*, 1988), in this study, we analysed classical pathway factors and the classical pathway inhibitor C4BP. We primarily looked for degradation products since they are often regarded as typical indicators of complement system activity (Ekdahl *et al.*, 2018).

As an element of innate immunity, complement system is the first line of defense against pathogens and triggers antibody production and T cell-mediated immune response. The main classical pathway element C1q binds to surface IgG and IgM and activates the system. Then, the common pathway factor C3 undergoes enzymatic cleavage in the early complement cascade and transforms into its main proteolytic fragment, C3b. C3b covalently binds to the pathogens or the body's own cells, forms the membrane attack complex resulting in lysis of the pathogens/cells. In order to prevent damage to the body's own healthy cells and tissues by the activated complement system, C4BP inhibits the classical and lectin complement pathways, prevents the formation of the C4bC2a complex known as C3-convertase and cleaves C4b into C4c and C4d fragments. While C4BP inhibits autoimmune processes, it can also lead to infectious conditions by preventing the elimination of pathogens by complement activity (Gordon, 2006; Sjöberg *et al.*, 2009; Abbas *et al.*, 2017).

In our study, we showed for the first time in narcolepsy, to our knowledge, significant alterations in levels of classical pathway factors. However, the complement level profile of narcolepsy patients was not compatible with the profile observed in a typical antibody and classical complement pathway-dependent autoimmune disease such as autoimmune glomerulonephritis (Segawa *et al.*, 2010). In these disorders, autoantibodies lead to full complement activation by recruiting C1q to activate the classic complement pathway, ultimately giving rise to an elevation of C3 and C4 breakdown products and activation of C4BP to regulate the hyperactive complement system. As a result, target tissues typically show deposits of C1q, C4BP, C4d and C3 breakdown products (Segawa *et al.*, 2010; Mihlan *et al.*, 2011). By contrast, narcolepsy patients showed increased C1q but decreased C4BP, C4d and C3a levels. This pattern is congruent with activation of the classical pathway by factors that both stimulate the complement activator C1q and also interact with complement inhibitors and suppress the overall complement pathway activity (Sjöberg *et al.*, 2009). This pattern may be observed in rheumatoid arthritis and SLE patients and putative factors inducing this profile in narcolepsy might be dying cells and extracellular matrix proteins released due to ongoing orexinergic neuron loss (Sjöberg *et al.*, 2009). Reduced C4BP and C4d levels may also be explained by chronic activation and consumption of the classical complement pathway (Bergamaschini *et al.*, 1999). Finally, NT1 patients, who are known to display a high prevalence of anti-neuronal antibodies, did not show an exceptionally higher complement activity than NT2 patients. This results further argue against the involvement of antibodies in complement level alterations observed in our study.

Reduced levels and/or attenuated function of C4BP might have led to the reduction in serum levels of C4d, which is produced by cleavage of C4b by the C4BP (Sjöberg *et al.*, 2009). C4BP also interacts with C3b (Blom *et al.*, 2003; Sjöberg *et al.*, 2009; Ramos-Sevillano *et al.*, 2015) and thus to avoid the confounding effect of reduced C4BP, we assessed the common complement pathway activity via C3a level measurement. Although C3a is generally regarded as an anaphylatoxin with pro-inflammatory action, emerging anti-inflammatory impact of C3a is currently under scrutiny (Coulthard & Woodruff, 2015). The correlation analysis showed that narcolepsy patients with higher C3a levels were more likely to have reduced total REM-sleep time, higher duration of wakefulness during sleep and worse sleep quality, as measured by PSQT scores. Likewise, patients with higher C4d levels were more likely to show increased REM sleep latency, increased wakefulness after sleep onset and decreased total REM time. These results suggest that these complement degradation products may have hazardous action on sleep functions and, presumably, reduction of C3a and C4d levels in narcolepsy might be a compensating measure to combat against sleep dysregulation. Intriguingly,

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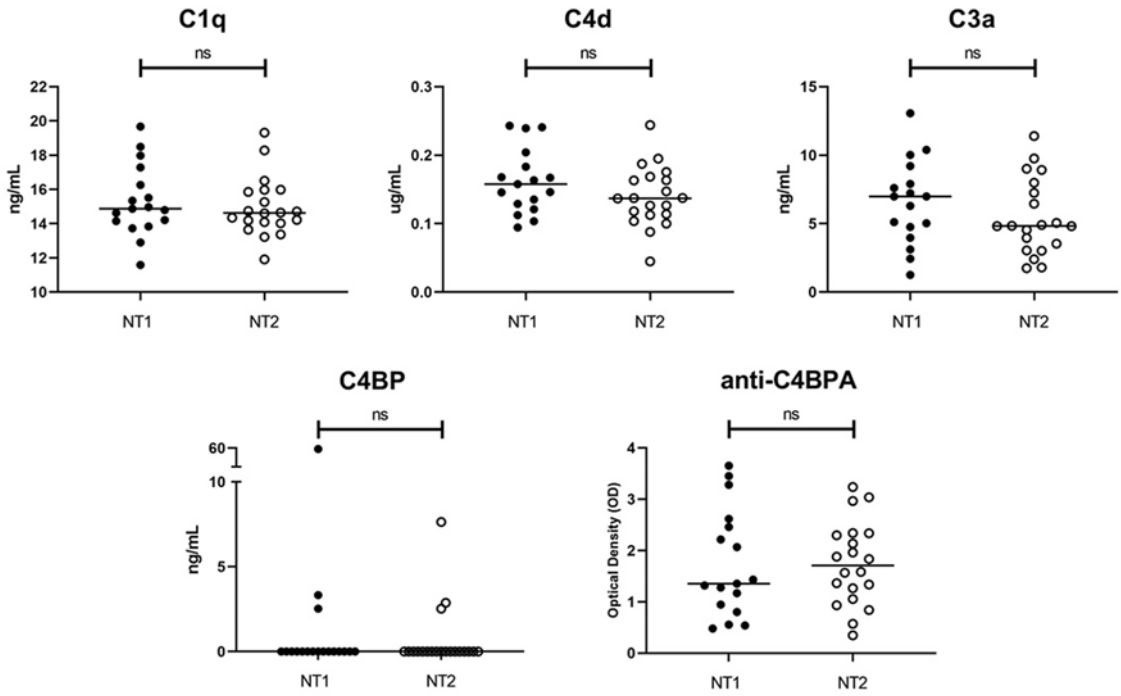



**Fig. 3.** Complement pathway parameters are not significantly different between narcolepsy type 1 (NT1) and narcolepsy type 2 (NT2) patients. Horizontal lines indicate the mean value of each group. *p* value is obtained by comparison of two groups by Student's *t*-test and found non-significant (ns) for all comparisons.

reduced serum levels of C3 and C4 have been linked to longer sleep duration (Hui *et al.*, 2007; Besedovsky *et al.*, 2019; Pak *et al.*, 2020).

Notably, narcolepsy patients exhibited significantly reduced C4BP levels. Expression analysis in a study reported decreased C4BPA expression in patients with obstructive sleep apnoea syndrome (Jurado-Gamez *et al.*, 2012). In addition, decreased levels of C4BP have been reported to be associated with disease risk for schizophrenia, which can be comorbid with narcolepsy (Wang *et al.*, 2015). These results suggest that C4BP may be overconsumed in narcolepsy thus providing a hint about the inflammatory nature of the disease. To dissect whether C4BP reduction could have been due to neutralising action of antibodies, we developed an assay to measure serum IgG antibodies directed against C4BPA. This protein was selected since the alpha subunit appears to be the more functionally relevant component of the C4BP (Blom *et al.*, 2003). Previous studies have shown that antibodies are developed against immune system components with significantly altered expression and utilisation. As an example, anti-C1q antibodies have been detected in autoimmune diseases characterised with enhanced complement activation such as SLE and MG (Tüzün *et al.*, 2006; Orbai *et al.*, 2015). Also, multiple sclerosis patients show antibodies against switch-associated protein 70, expression of which is enhanced in lymphocytes upon activation (Türkoğlu *et al.*, 2016). These antibodies are often associated with disease activity suggesting a functional role in disease mechanisms (Tüzün *et al.*, 2006; Türkoğlu *et al.*, 2016; Shang *et al.*, 2021). However, no correlation could be established between anti-C4BPA and levels of C4BP and other complement factors arguing against a putative impact of these antibodies on complement system activity.

Alternatively, C4BP might be depleted by microorganisms, which widely utilise this protein for survival (Ermert & Blom, 2016). Bacteria carry IgG-like molecules on their surfaces to avoid complement-mediated elimination (Nordenfelt *et al.*, 2012). Narcolepsy patients might be harbouring similar types of pathogens leading to depletion of C4BP. Another potential explanation of our results could be mild liver injury due to modafinil treatment, since C4BP levels are decreased in liver cirrhosis (Kusada-Funakoshi *et al.*, 1991) and the diminished factors C4BP, C4 and C3 are all produced in liver, whereas elevated C1q is mainly produced by macrophages and dendritic cells (Nagura *et al.*, 1985; Kusada-Funakoshi *et al.*, 1991; Chen *et al.*, 2011). However, no significant difference was observed between patients with and without modafinil treatment in terms of complement factor levels.

C1q level increase was another intriguing finding of our study. Earlier studies have proposed that microglial phagocytosis of hypocretinergic neurons might be the leading cause of the disease (Nadjar *et al.*, 2017). A striking feature of microglial cells is to produce C1q (Stephan *et al.*, 2012). In recent years, C1q has gained attention due to its role in synaptic elimination. The weak synapses are tagged with C1q, and subsequently downstream classical complement cascade is activated for the removal of the synapse (Stephan *et al.*, 2012). For example, brain tissue of schizophrenia patients is typified with disrupted synaptic elimination processes and enhanced C1q production (Druart & Le Magueresse, 2019). It can thus be assumed that microglial activation due to exposure to dying neuronal byproducts might be the reason underlying C1q elevation in narcolepsy. The correlation between C1q and ESS is worth mentioning since it indicates worsening sleep quality and increased daytime sleepiness with increased levels of C1q. Thus, C1q may be used as a potential indicator of disease activity in narcolepsy.

A limitation of our study is absence of hypocretin level measurements and analysis of putative correlations between hypocretin, C4BP and anti-C4BPA levels. Similarly, measurement of autoantibodies that are frequently observed in narcolepsy patients and correlation with complement levels could have provided informative insights.

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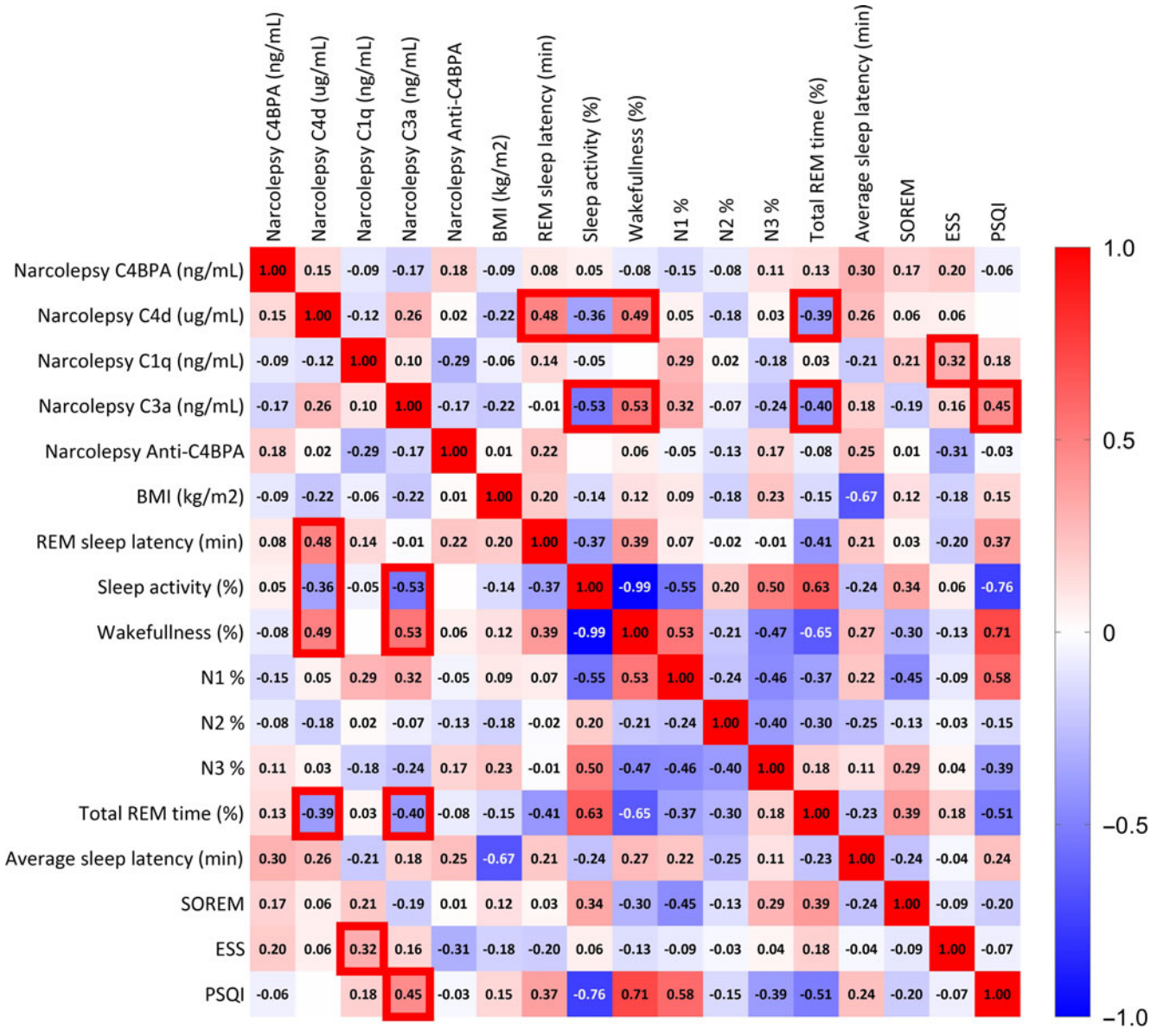



**Fig. 4.** Correlation matrix of complement pathway parameters and clinical features of narcolepsy patients. Positive correlations are represented in red, while negative correlations are represented in blue. The values are expressed as Pearson *r* scores. Darker colours mean higher correlation ($p < 0.05$). The significant correlation scores in line with our hypothesis are shown in red rectangles.

In brief, the complement system is a vastly uncharted field in narcolepsy and our results suggest that the significance of complement factor alterations needs to be further scrutinised. Our results provide reasonable doubt for involvement of the complement system in narcolepsy and also suggest that C1q, C3a and C4d may act as biomarkers in narcolepsy for prediction of disease severity. Therefore, further studies investigating the role of complement system factors in neuroinflammation, and sleep physiology are recommended.

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**Authors contributions.** CİK directed the project. ET was in charge of supervising the project and interpreting the results. DK and GBŞ helped sample selection, performing sleep studies and gathering clinical data of the subjects. DGA and ED were participated in planning the study. VY and HY were involved in planning and performing ELISA studies. HY wrote the manuscript in consultation with all authors providing critical feedback.

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**Conflict of interest.** None.

**Ethical standards.** All procedures comply with the Helsinki Declaration and the ethical standards of Istanbul University, Faculty of Medicine, Clinical Research Ethics Committee.

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