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CHAPTER 2
HLA DOSAGE EFFECT IN NARCOLEPSY WITH CATAPLEXY

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Narcolepsy with cataplexy is a sleep disorder caused by the loss of hypocretin producing neurons in the hypothalamus. It is tightly associated with a specific HLA allele: HLA-DQB1*06:02. Based on this, an autoimmune process has been hypothesised. A functional HLA-DQ molecule consists of a DQα and a DQβ chain. HLA-DQB1*06:02 (DQβ) has a strong preference for binding to HLA-DQA1*01:02 (DQα), and together they form the functional DQ0602 dimer. A dosage effect would be expected if the HLA-DQ0602 dimer itself is directly involved in the aetiology. An increased expression of the HLA-DQ0602 dimer is expected in individuals homozygous for HLA-DQB1*06:02-DQA1*01:02, but is also hypothesised in individuals heterozygous for HLA-DQB1*06:02 and homozygous for HLA-DQA1*01:02. To study the impact of the expression of the HLA-DQ0602 dimer on narcolepsy susceptibility, 248 Dutch narcolepsy patients and 1272 Dutch control subjects, all of them positive for DQB1*06:02 (heterozygous and homozygous), were HLA-genotyped with attention not only to DQB1 but also to DQA1*01:02.

DQB1*06:02-DQA1*01:02 homozygosity was significantly more often seen in patients compared to controls (OR 2.29) confirming previous observations. More importantly, a significantly higher prevalence of homozygosity for DQA1*01:02 was found in HLA-DQB1*06:02 heterozygous patients compared to controls (OR=2.37, p<0.001). The latter finding clearly supports a direct role of the HLA-DQ molecule in the development of disease.
INTRODUCTION

Narcolepsy is a disorder of the regulation of sleep and wakefulness, resulting in a variety of symptoms such as excessive daytime sleepiness (EDS), cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nocturnal sleep.\textsuperscript{1,2} According to the current classification of sleep disorders, narcolepsy can be divided into narcolepsy with and without cataplexy.\textsuperscript{3}

Narcolepsy with cataplexy is caused by a hypocretin-1 (orexin-A) deficiency,\textsuperscript{4} which is thought to be the consequence of a selective loss of hypocretin producing neurons in the hypothalamus.\textsuperscript{5,6} However, the mechanism behind this loss of hypocretin producing neurons has not been elucidated yet. Several findings point into the direction of an autoimmune cause, although no direct evidence for a role of the immune system has been found yet.

By far the strongest argument for an autoimmune aetiology in narcolepsy is its tight HLA (human leukocyte antigen) association: narcolepsy with cataplexy is the disease with the strongest association with a specific HLA-allele, i.e. HLA-DQB1*06:02.\textsuperscript{7} A functional HLA-DQ molecule originates from the binding of an $\alpha$ chain (DQA1) with a $\beta$ chain (DQB1) (Figure 2.1). Not all $DQ\alpha$ and $DQ\beta$ chains are able to bind to each other (i.e. dimerise). The genes encoding for chains that have affinity to dimerise are often in linkage disequilibrium. Narcolepsy is tightly associated with HLA-DQB1*06:02,\textsuperscript{8-11} which is in linkage disequilibrium with HLA-DQA1*01:02. The opposite is not per se true: HLA-DQA1*01:02 prefers to bind to HLA-DQB1*06:02, but also to the $DQ\beta$ chains DQB1*05:02, DQB1*06:04 and DQB1*06:09.\textsuperscript{12} The $DQ\alpha$ and $DQ\beta$ chain encoded for by this DQB1*06:02-DQA1*01:02 haplotype together form the functional HLA-DQ dimer DQ0602. Worldwide about 85–95% of the narcolepsy with cataplexy patients carry this haplotype, compared to 12–38% of the general population.\textsuperscript{13} For non-familial cases and those with typical cataplexy the association may even exceed the 98%.\textsuperscript{14} Therefore, carrying this haplotype is thought to represent an almost necessary risk factor for the development of narcolepsy, although its mere presence is not sufficient to cause narcolepsy. Results of studies in which exons near the HLA-DQ genes have been sequenced support a direct role for HLA-DQB1*06:02 itself.\textsuperscript{8,14,15} When the HLA-DQ dimer itself is indeed involved in the aetiology, a dosage effect would be expected, meaning that an increased expression of the DQ0602 dimer is associated with a higher susceptibility to the development of narcolepsy. This is expected in individuals homozygous for HLA-DQB1*06:02-DQA1*01:02, but also in individuals heterozygous for HLA-DQB1*06:02 and homozygous for HLA-DQA1*01:02. Similar dosage effects have been described for HLA-DQ molecules associated with diabetes\textsuperscript{16} and celiac disease.\textsuperscript{17,18} If a gene in close linkage with
HLA-DQB1*06:02 is responsible for the increased risk, a dosage effect is only expected in individuals homozygous for HLA-DQB1*06:02, but not in individuals heterozygous for HLA-DQB1*06:02 and homozygous for HLA-DQA1*01:02.

Previous HLA studies in narcolepsy patients heterozygous for HLA-DQB1*06:02-DQA1*01:02, revealed a role of several accompanying HLA-haplotypes (i.e. located in trans with DQB1*06:02-DQA1*01:02). Heterozygosity with DQB1*03:01, DQA1*06, DQA1*03:03, DRB1*04, DRB1*08, DRB1*11 or DRB1*12 turned out to increase the risk of developing narcolepsy, whereas heterozygosity of DQB1*06:02 with DQB1*06:01,

Figure 2.1 Dimerisation of DQ α and a DQ β chains to a functional HLA-DQ molecule. A functional HLA-DQ molecule is formed by binding of a DQ α (DQA1) and a DQ β (DQB1) chain. Every person carries two DQ α chains and two DQ β chains. All these chains form functional HLA-DQ molecules by (cross)dimerisation, leading to a maximum of 4 different HLA-DQ molecules. (A) Heterozygosity for DQA1*01:02-DQB1*06:02: a maximum of four different DQ haplotypes can be expressed, probably only one of those the predisposing one. (B) Homozygosity DQA1*01:02 - heterozygosity DQB1*06:02: all DQB1*06:02 chains dimerise with the only available DQA1*01:02 chain.
DQB1*06:03, DQB1*05:01 or DQA1*01 (non DQA1*01:02) turned out to decrease the risk. Furthermore, a two to four times increased risk of developing narcolepsy is reported in Caucasians homozygous for DQB1*06:02. To further investigate the role of the DQ0602 dimer in developing narcolepsy, we explored the role of HLA-DQB1*06:02-DQA1*01:02 in combination with the haplotype located in trans with it, with special attention to DQA1*01:02. Increased homozygosity for DQA1*01:02 in patients heterozygous for DQB1*06:02 would support a direct role of the HLA-DQ molecule in the development of narcolepsy.

METHODS

Subjects

248 patients and 1272 control subjects, all positive for DQB1*06:02-DQA1*01:02 (heterozygous and homozygous), were included in this study. All patients were recruited from the sleep clinic of the department of Neurology, Leiden University Medical Centre. They all suffered from narcolepsy with clear-cut cataplexy according to the ICSD-3 criteria. Taking the probable different aetiology of familial narcolepsy into account, identified familial cases were excluded. The DQB1*06:02- DQA1*01:02 positive controls were taken from a panel of randomly selected, healthy unrelated Dutch individuals. All patients and controls were of European ancestry.

HLA genotyping

HLA-DRB and HLA-DQB1 typing was performed with a reversed approach of the PCR-sequence-specific oligonucleotide probe technique described elsewhere. The typing results of some samples with a rare DRB1-DQB1-DQA1 association were confirmed by PCR-SBT, using the SBT Excellerator HLA-DRB and -DQB kit (Genome Products, Utrecht, The Netherlands). Subsequently, all DQB1*06:02 positive patients and controls were selected to examine their dosage of DQA1*01:02. The absence, heterozygosity or homozygosity for DQA1*01:02 was determined with a sybergreen based rt-PCR, using three different DQA1*SSP primer mixes. On every PCR run the specificity of these primer mixes was checked by adding two controls, one control was homozygous for DQA1*01:02 and the other was negative for DQA1*01:02 (homozygous for DQA1*01:03). Furthermore, to confirm the rt-
PCR results, a routinely used DQA1 PCR-SSP technique was applied on 15 samples and no discrepancy with the rt-PCR results was observed.

Statistical analysis

To evaluate statistical significance two-sided Fisher’s exact test was performed. The p values were corrected for multiple comparisons according to the Šidák method.\textsuperscript{25} Odds ratios and corresponding 95% confidence intervals (CI) were calculated according to the Woolf Haldane test.\textsuperscript{26,27} A too large control group could lead to statistically significant differences that are clinically irrelevant. Therefore, corrected p values are standardized (ps) to a sample size of 900 following the method of Good.\textsuperscript{28} This sample size is derived by: the total number of patients plus three times the number of patients as maximum allowed size for the control group, and rounded up.

RESULTS

Analysis of HLA-DQB1*06:02-DQA1*01:02 homozygosity

The relative and absolute distribution of DQB1*06:02-DQA1*01:02 in trans (DQB1*06:02-DQA1*01:02 homozygosity) in DQB1*06:02-DQA1*01:02 positive patients and controls are given in Table 2.1. Homozygosity for HLA-DQB1*06:02-DQA1*01:02 was significantly more prevalent in patients (OR=2.42).

HLA-DQB1 alleles in trans with HLA-DQB1*06:02

In patients and controls, the absolute and relative distributions of HLA-DQB1 alleles located in trans with HLA-DQB1*06:02 were calculated and are demonstrated in Table 2.2. DQB1*03:01/03:04 and DQB1*05:02 were significantly more prevalent on the other haplotype compared to controls (OR 1.99 and 3.25, respectively). On the contrary, DQB1*02 and DQB1*06:03 were significantly less prevalent (OR 0.53 and 0.21, respectively). These alleles may be seen to be protective in developing narcolepsy when present in combination with DQB1*06:02, in particular DQB1*06:03.
HLA dosage effect in narcolepsy with cataplexy

Analysis of HLA-DQA1*01:02 homozygosity

The frequency of HLA-DQA1*01:02 located in trans with HLA-DQB1*06:02-DQA1*01:02 was compared between narcolepsy patients and controls (Table 2.3). In heterozygous DQB1*06:02 patients homozygosity for DQA1*01:02, was significantly increased compared to heterozygous controls (OR 2.37). Analysis of the frequency of the specific HLA-DQB1 alleles in trans with HLA-DQB1*06:02 in HLA-DQA1*01:02 homozygous revealed no differences between patients and controls (Table 2.4).
The aim of this study was to obtain more insight into the direct role of the HLA-DQ dimer DQ0602 in the aetiology of narcolepsy. In order to further explore the role of the DQ0602 dimer we studied the HLA-DQ alleles located in trans with HLA-DQB1*06:02-DQA1*01:02, with special attention to DQA1*01:02 homozygosity, in Dutch narcoleptic subjects compared with control subjects.

In concordance with previous studies, homozygosity for DQB1*06:02-DQA1*01:02 was more frequently seen in our patients than in controls.\textsuperscript{14,22} Homozygosity for DQA1*01:02-DQB1*06:02 leads to the maximum number of expressed predisposing dimers supporting the existence of a dosage effect in the aetiology.

The higher prevalence of homozygosity for DQA1*01:02 in DQB1*06:02 heterozygous narcolepsy patients compared to controls is in line with a direct role of the DQ0602 dimer in the aetiology of narcolepsy. This can be explained in the following way; a functional HLA-DQ molecule is formed by binding of a DQα with a DQβ chain. Every person carries two DQα chains and two DQβ chains. Theoretically, all these chains form functional HLA-DQ molecules.

### Table 2.3 Increased homozygosity for HLA-DQA1*01:02 in DQB1*06:02 heterozygous narcolepsy patients and controls

<table>
<thead>
<tr>
<th>DQA1 cis / trans</th>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
<th>ps</th>
</tr>
</thead>
<tbody>
<tr>
<td>01:02/01:02</td>
<td>36 (17.1)</td>
<td>95 (8.0)</td>
<td>2.366</td>
<td>1.565–3.578</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Presence of trans-HLA-DQA1*01:02 in patients and controls carrying HLA-DQB1*06:02-DQA1*01:02 on the cis haplotype. Ps = p value corrected for standard sample size.\textsuperscript{28} One of the DQA1*01:02 trans was derived an unusual haplotype with DRB1*15:01, DQB1*06:03 (in a Caucasian patient).

### Table 2.4 Analysis of HLA-DQB1 alleles in trans with HLA-DQB1*06:02 in HLA-DQA1*01:02 homozygous narcolepsy patients and controls

<table>
<thead>
<tr>
<th>DQB1</th>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
<th>pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>05:02</td>
<td>11 (31)</td>
<td>20 (21)</td>
<td>1.661</td>
<td>0.710–3.887</td>
<td>0.259</td>
<td>0.698</td>
</tr>
<tr>
<td>06:03</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>8.070</td>
<td>0.321–202.751</td>
<td>0.275</td>
<td>0.723</td>
</tr>
<tr>
<td>06:04</td>
<td>21 (58)</td>
<td>64 (67)</td>
<td>0.677</td>
<td>0.310–1.477</td>
<td>0.413</td>
<td>0.881</td>
</tr>
<tr>
<td>06:09</td>
<td>3 (8)</td>
<td>11 (12)</td>
<td>0.768</td>
<td>0.217–2.711</td>
<td>0.757</td>
<td>0.997</td>
</tr>
</tbody>
</table>

HLA*DQB1 alleles present on the other chromosome in narcolepsy patients (n=36) and controls (n=95) heterozygous for HLA-DQB1*06:02 and homozygous for HLA-DQA1*01:02. Pc = p value corrected for multiple testing.\textsuperscript{25}

### DISCUSSION

The aim of this study was to obtain more insight into the direct role of the HLA-DQ dimer DQ0602 in the aetiology of narcolepsy. In order to further explore the role of the DQ0602 dimer we studied the HLA-DQ alleles located in trans with HLA-DQB1*06:02-DQA1*01:02, with special attention to DQA1*01:02 homozygosity, in Dutch narcoleptic subjects compared with control subjects.

In concordance with previous studies, homozygosity for DQB1*06:02-DQA1*01:02 was more frequently seen in our patients than in controls.\textsuperscript{14,22} Homozygosity for DQA1*01:02-DQB1*06:02 leads to the maximum number of expressed predisposing dimers supporting the existence of a dosage effect in the aetiology.

The higher prevalence of homozygosity for DQA1*01:02 in DQB1*06:02 heterozygous narcolepsy patients compared to controls is in line with a direct role of the DQ0602 dimer in the aetiology of narcolepsy. This can be explained in the following way; a functional HLA-DQ molecule is formed by binding of a DQα with a DQβ chain. Every person carries two DQα chains and two DQβ chains. Theoretically, all these chains form functional HLA-DQ molecules.
by dimerisation. In principle, the DQα and DQβ chains of both chromosomes (cis and trans) can dimerise, theoretically leading to four different HLA-DQ molecules in heterozygous individuals. However, DQα and DQβ chains differ in affinity for each other and only a portion of the theoretically possible dimers will actually be formed. The affinity of DQB1*06:02 and DQA1*01:02 for each other is high. In case of homozygosity for DQA1*01:02-DQB1*06:02, all expressed DQ molecules are DQA1*01:02-DQB1*06:02. In individuals heterozygous for both the DQα and the DQβ chain, a maximum of four different DQ molecules can be expressed, of which only one is the predisposing one. When a person is homozygous for DQA1*01:02 and heterozygous for DQB1*06:02, only two different DQ molecules can be formed, which subsequently results in a higher number of predisposing dimers compared to fully heterozygous individuals. In contrast to the present study, a recent Chinese study did not report a higher prevalence of homozygosity for DQA1*01:02 in DQB1*06:02 heterozygous narcolepsy patients (OR=1.15, not significant). Nevertheless, the prevalence of homozygous DQA1*01:02 heterozygous DQB1*06:02 patients was reported to fall between the prevalence of DQA1*01:02-DQB1*06:02 heterozygous patients and that of DQA1*01:02-DQB1*06:02 homozygous patients, suggesting both amount and ratio of DQA1*01:02/DQB1*06:02 may be important. Remarkably, Tafti et al. recently reported a protective effect of DQB1*06:09, which is in linkage disequilibrium with DQA1*01:02. This finding is in contradiction with our hypothesis, as a predisposing effect would be expected. Although the numbers are small, neither a predisposing effect nor a protective effect of this allele was found in the present study.

In fully heterozygous patients the known predisposing and protective effects of several alleles in trans cannot be explained by this mechanism. In these patients, different hypotheses have been described. One of those is the DQA1-DQB1 allelic competition model, in which the trans DQβ molecule competes with DQB1*06:02 for dimerization with the DQA1*01:02 molecule. If cross dimerisation is possible, the number of disease predisposing HLA-DQ0602 dimers on the cell surface may decrease, resulting in a lowered risk of developing narcolepsy. The currently identified and previously reported decreased prevalence of DQB1*06:03 and DQB1*06:01 is in line with this allelic competition model, as both these alleles are not in linkage disequilibrium with DQA1*01:02, but are able to dimerise with DQA1*01:02, and therefore compete with DQB1*06:02. However, the currently and previously reported predisposing effect of DQB1*03:01 and currently reported protective effect of DQB1*02, cannot be explained by one of the above described hypotheses. The mechanism behind the predisposing effect of DQB1*03:01 can possibly
be found in a theory similar to the DQA1-DQB1 allelic competition model based on cross dimerisation. Such a mechanism is also suggested to play an important role in the development of type 1 diabetes.\textsuperscript{29} DQB1*03:01 is in linkage disequilibrium with DQA1*03. DQB1*03:01 is not able to cross dimerise with DQA1*01:02 in vitro,\textsuperscript{12} but DQB1*06:02 is able to do so with DQA1*03. Subsequently, all DQA1*01:02 chains will exclusively dimerise with DQB1*06:02, leading to a maximal expression of the predisposing dimer DQ0602, explaining the increased susceptibility for the disease. Nevertheless, the observed protective effect of DQB1*02 in trans with DQB1*06:02 in our population does not fit into one of the described hypotheses; DQB1*02 is in linkage-disequilibrium with DQA1*02 and DQA1*05, but these alleles are not able to form stable heterodimers with the DQ\textalpha{} and DQ\textbeta{} chains of the predisposing DQ0602 dimer.\textsuperscript{12} Subsequently, this would not influence the availability of the predisposing dimer.

Since it is not possible to explain all protective and predisposing effects of the described alleles by one or multiple of the mechanisms described in de previous section, the explanation may lie in another direction. A possible mechanism explaining the protective or predisposing effect of a certain heterodimer, could be found in a different affinity for the epitopes relevant for the development of narcolepsy.\textsuperscript{30} Heterodimers may compete to bind a particular epitope and when a protective heterodimer has a higher affinity for the epitope, this will negatively affect binding of this peptide to the predisposing heterodimer, resulting in a reduced risk of developing narcolepsy. The opposite might be true for a predisposing heterodimer.

The current genetic study provides additional evidence for a direct role of the HLA-DQ0602 molecule in the aetiology of the disease. The direct role of the HLA-DQB1*06:02-DQA1*01:02 dimer fits perfectly in the autoimmune hypothesis in narcolepsy. The function of HLA-class II molecules like the DQ0602 dimer is to present peptides derived from foreign proteins to the immune system in order to elicit a T cell mediated immune response. Sometimes, T cells reactive with foreign peptides may cross react with self-structures leading to destruction of autologous cells and autoimmunity.

In conclusion, the present study shows a significantly higher prevalence of homozygosity for DQA1*01:02 in HLA-DQB1*06:02 heterozygous patients compared to controls and clearly supports a direct role of the DQB1*06:02-DQA1*01:02 dimer molecule in the development of disease.
REFERENCES


