Unravelling narcolepsy : from pathophysiology to measuring treatment effects

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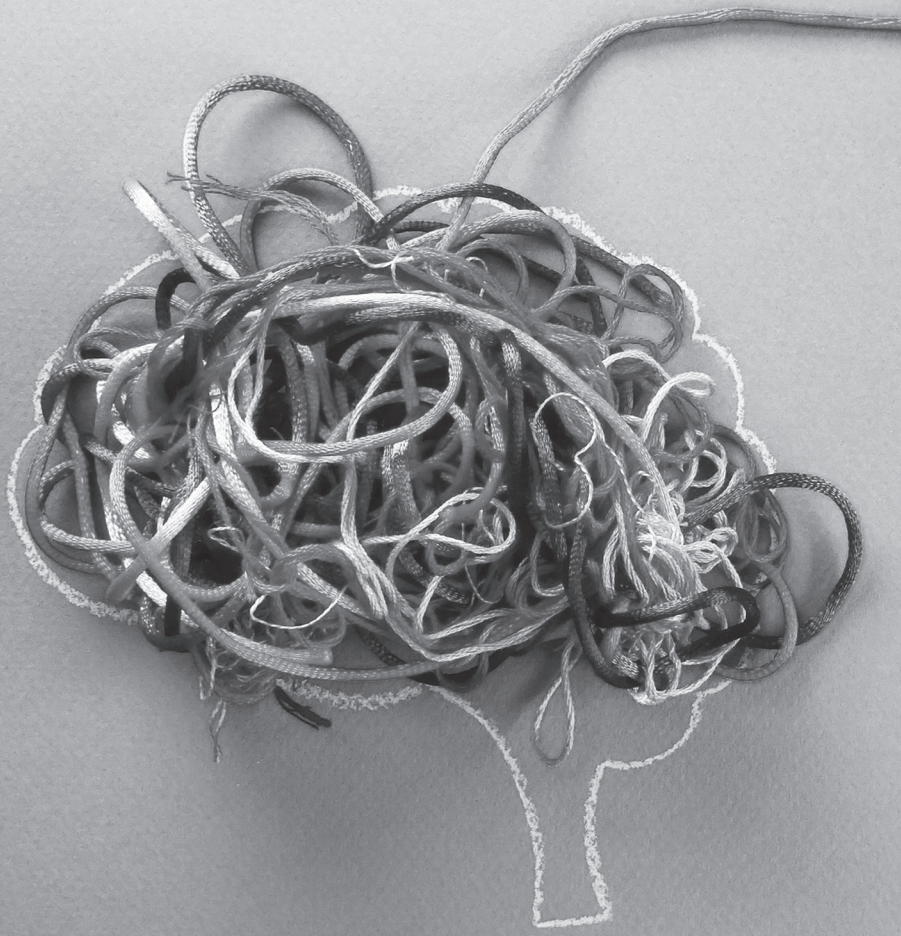

CHAPTER 2

HLA DOSAGE EFFECT IN NARCOLEPSY WITH CATAPLEXY

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ABSTRACT

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.[1,2] According to the current classification of sleep disorders, narcolepsy can be divided into narcolepsy with and without cataplexy.[3]

Narcolepsy with cataplexy is caused by a hypocretin-1 (orexin-A) deficiency,[4] which is thought to be the consequence of a selective loss of hypocretin producing neurons in the hypothalamus.[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*.[7] A functional *HLA-DQ* molecule originates from the binding of an α chain (DQA1) with a β chain (DQB1) (Figure 2.1). Not all *DQα* and *DQβ* 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*,[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 *DQβ* chains *DQB1*05:02*, *DQB1*06:04* and *DQB1*06:09*.[12] The *DQα* and *DQβ* 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.[13] For non-familial cases and those with typical cataplexy the association may even exceed the 98%.[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.[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[16] and celiac disease.[17,18] If a gene in close linkage with

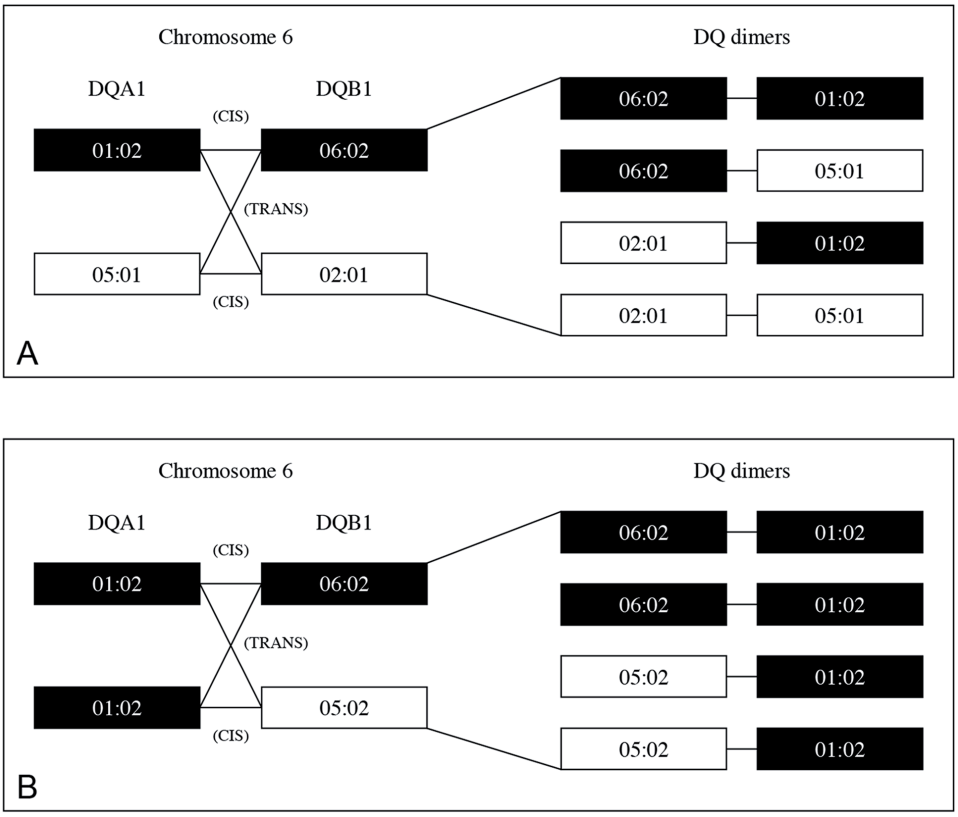

Figure 2.1 Dimerisation of *DQα* and a *DQβ* chains to a functional *HLA-DQ* molecule.
A functional *HLA-DQ* molecule is formed by binding of a *DQα* (*DQA1*) and a *DQβ* (*DQB1*) chain. Every person carries two *DQα* chains and two *DQβ* chains. All these chains form functional *HLA-DQ* molecules by (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.

*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,[19,20] whereas heterozygosity of *DQB1*06:02* with *DQB1*06:01*,

*DQB1*06:03*, *DQB1*05:01* or *DQA1*01* (non *DQA1*01:02*) turned out to decrease the risk.[11,20,21] Furthermore, a two to four times increased risk of developing narcolepsy is reported in Caucasians homozygous for *DQB1*06:02*.[14,22]

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.[3] 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.[23] 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.[24] 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.[25] Odds ratios and corresponding 95% confidence intervals (CI) were calculated according to the Woolf Haldane test.[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.[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*.

Table 2.1 Increased homozygosity for *HLA-DQB1*06:02-DQA1*01:02* in narcolepsy patients

Patients n (%)	Controls n (%)	OR	95% CI	p	ps
37 (14.9)	91 (7.2)	2.290	1.524–3.440	<0.001	<0.001

Narcolepsy patients (n=248) and controls (n=1272) were compared with respect to the incidence of *DQB1*06:02-DQA1*01:02* homozygosity. Ps = p value corrected for standard sample size.[28]

Table 2.2 Analysis of *HLA-DQB1* alleles in trans with *HLA-DQB1*06:02*

DQB1 trans	Patients n (%)	Controls n (%)	OR	95% CI	p	ps
02	34 (16.1)	315 (26.7)	0.534	0.363–0.786	0.001	0.016
03:01/03:04	65 (30.8)	216 (18.3)	1.994	1.439–2.764	<0.001	0.001
03:02/03:05	28 (13.3)	137 (11.6)	1.180	0.765–1.819	0.488	0.500
03:03	9 (4.3)	67 (5.7)	0.775	0.386–1.553	0.511	0.500
04	11 (5.2)	37 (3.1)	1.751	0.889–3.447	0.149	0.500
05:01	19 (9.0)	187 (15.8)	0.537	0.329–0.878	0.008	0.128
05:02	11 (5.2)	20 (1.7)	3.250	1.554–6.794	0.004	0.060
05:03	6 (2.8)	36 (3.0)	0.993	0.425–2.317	0.986	0.500
06:01	1 (0.5)	5 (0.4)	1.524	0.249–9.327	0.607	0.500
06:03	3 (1.4)	86 (7.3)	0.213	0.072–0.625	<0.001	0.006
06:04	21 (10.0)	64 (5.4)	1.955	1.172–3.262	0.018	0.259
06:09	3 (1.4)	11 (0.9)	1.709	0.512–5.704	0.457	0.500

*HLA*DQB1* alleles present on the other chromosome in narcolepsy patients (n=211) and controls (n=1181) heterozygous for *HLA-DQB1*06:02*. Ps = p value corrected for multiple testing[25] and for standard sample size.[28]

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).

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

DQA1 cis / trans	Patients n (%)	Controls n (%)	OR	95% CI	p	ps
01:02/01:02	36 (17.1)	95 (8.0)	2.366	1.565–3.578	<0.001	<0.001

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.[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

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

*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.[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.[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 DQ*α* 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).[11] 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.[14] 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*[11,20,21] 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*[11,20,21] 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.[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,[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 *DQα* and *DQβ* chains of the predisposing *DQ0602* dimer.[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.[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.

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