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GENETICKÉ A KLINICKÉ ASPEKTY SYNDROMU NEKLIDNÝCH NOHOU

GENETIC AND CLINICAL ASPECTS OF THE RESTLESS LEGS SYNDROME

Disertační práce

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CONTENTS

Summary

Introduction: The Restless Legs Syndrome (RLS) is a frequent neurological disorder with a prevalence ranging from 5 – 10%. RLS is characterized by an urge to move the lower extremities during the night, thus RLS causes sleep disturbance. It presents as both idiopathic and secondary form. Idiopathic RLS is associated with common genetic variants in *MEIS1, BTBD9, PTPRD* and *MAP2K5/SCOR1*. Recently, multiple sclerosis (MS) was identified as a common cause for secondary RLS, the prevalence of RLS in patients with MS ranges from 13.3 to 37.5%.

The aim of our study was to analyse the clinical and genetic aspects of this disorder, especially in patients with multiple sclerosis.

In the clinical part, we evaluated the prevalence of RLS among Czech patients with MS and we compared the extent of brain damage between patients with and without RLS using magnetic resonance imaging (MRI). In the genetic part, we further analysed the impact of known genetic variants (*MEIS1, BTBD9, MAP2K5/SCOR1, PTPRD)* for RLS in other European populations and in patients with MS.

Methods: Clinical part: Each patient with MS underwent a semi-structured interview. A patient was considered to be affected by RLS if he/she met all four standard criteria at life-long interval. Lesion load (LL – T2), brain atrophy – T1 and brain parenchymal fraction (BPF) were assessed in some patients.

Genetic part included two genetic association studies. In the first study, we investigated these variants in 649 RLS patients and 1230 controls from the Czech Republic, Austria and Finland. Ten SNPs (single nucleotide polymorphisms) within the three genomic regions (*MEIS1, BTBD9* a *MAP2K5/SCOR1)* were selected. In the second study, 203 MS patients with RLS were compared to 438 MS patients without RLS. In total 12 SNPs within the four genomic regions (*MEIS1, BTBD9* a *MAP2K5/SCOR1*, *PTPRD*) were genotyped.

Results: Clinical part: A total of 765 subjects (553 females, mean age 36.54, ±SD 9.5) with MS were included in the study. The diagnosis of RLS was confirmed in 245 subjects (32.1%, 95% CI 28.7 – 35.4%) with MS. Patients suffering from both MS and RLS were significantly older (38.6 vs. 35.6 years), had longer durations of MS symptoms (11.0 vs. 8.2 years) and had higher EDSS score (2.9 vs. 2.3).

Quantitative MRI data were obtained in 385 patients without RLS and 215 patients with RLS. We found no difference between the two groups in the whole brain LL, brain atrophy and BPF, despite the fact that we were able to replicate the correlation of these data with clinical parameters of MS.

Genetic part: We replicated associations for all loci in the combined samples set (*MEIS1*, P = 1.26×10^{-5}, odds ratio (OR) = 1.47, *BTBD9*, P = 4.11×10^{-5}, OR = 1.58 and *MAP2K5/SCOR1*, P = 0.04764, OR = 1.27).

No significant association with *MEIS 1*, *BTBD9* and *PTPRD* was found in patients with MS despite sufficient statistical power for the first two loci. There was a trend for association with *MAP2K5/SCOR1* – the best model for the risk allele was the recessive model (p nominal = 0.0029, p corrected for four loci and allelic + recessive model = 0.023, odds ratio = 1.60 – 95% CI 1.17 – 2.18).

Conclusion: RLS is a common comorbidity of multiple sclerosis and MS should be considered among causes of secondary RLS forms. RLS is more prevalent in advanced stages of MS, but does not correlate with MRI markers of brain damage.

Our study confirmed that variants in these three loci (*MEIS1*, *BTBD9*, and *MAP2K5/SCOR1)* confer consistent disease risks in patients of European descent. On the contrary, RLS in MS patients shares only few genetic determinants with the idiopathic form, the gene variant *SCOR1* can partially contribute the phenotype (max. 50%).

SOUHRN

Úvod: Syndrom neklidných nohou (RLS – Restless Legs Syndrome) je časté neurologické onemocnění s prevalencí 5 – 10% v evropské populaci. Je charakterizované nutkáním pohybovat končetinami a v rozvinuté formě interferuje se spánkem. RLS je komplexní dědičné onemocnění, idiopatické formy jsou asociovány s variantami genů *MEIS1*, *BTBD9*, *PTPRD* a *MAP2K5/SCOR1*. Recentní studie uvádějí roztroušenou sklerózu jako novou příčinu sekundární formy RLS s prevalencí 19 – 37,5%.

Cílem naší práce bylo vyšetřit některé klinické a genetické aspekty tohoto onemocnění, hl. u pacientů s roztroušenou sklerózou (RS). V klinické části jsme vyšetřovali prevalenci RLS u českých pacientů s RS a porovnávali jsme rozsah postižení mozku na magnetické rezonanci (MR) u pacientů s RLS a bez RLS. V genetické části jsme zjišťovali, zda známé genetické varianty (*MEIS1*, *BTBD9*, *PTPRD* a *MAP2K5/SCOR1)* zvyšují riziko rozvoje RLS také u jiných evropských populací a u pacientů s RS.

Metodika: V klinické části (epidemiologické studii) byli pacienti s RS dotazováni na symptomy RLS; každý pacient absolvoval strukturovaný rozhovor cílený na přítomnost základních diagnostických kritérií, rodinnou anamnézu, komorbidity a terapii. U některých pacientů (radiologická studie) byla provedena MR mozku se zaměřením na objem T2 hyperintenzních ložisek (lesion load – LL), mozkovou atrofii a brain parenchymal fraction (BPF).

Genetická část zahrnuje 2 genetické asociační studie: 1. jsme vyšetřovali přítomnost genetických variant u 649 pacientů s idiopatickým RLS a 1230 kontrol ze 3 evropských populací (ČR, Rakousko, Finsko). 10 SNPs (single nucleotide polymorphisms) bylo vybráno na 3 genech (*MEIS1*, *BTBD9* a *MAP2K5/SCOR1).* Ve 2. studii jsme porovnávali přítomnost genetických variant u pacientů RS a RLS oproti pacientům s RS, ale bez symptomů RLS. Celkem 12 SNPs bylo vybráno na 4 genech (*MEIS1*, *BTBD9* a *MAP2K5/SCOR1*, *PTPRD*).

Výsledky: Do epidemiologické studie bylo zahrnuto celkem 765 pacientů s RS (553 žen, průměrný věk 36.54, ±SD 9.5). Diagnóza RLS byla potvrzena u 245 pacientů (32.1%, 95% CI 28.7 – 35.4%). V porovnání s pacienty bez RLS byli pacienti s RLS byli signifikantně starší (38.6 vs. 35.6 let), měli delší trvání roztroušené sklerózy (11.0 vs. 8.2 let) a měli vyšší EDSS skóre (2.9 vs. 2.3).

Kvantitativní data z MR (LL, BPF a mozková atrofie) byla porovnána u 385 pacientů bez RLS a u 215 pacientů s RLS, nebyl nalezen signifikantní rozdíl mezi pacienty s RLS a bez tohoto onemocnění, ačkoli jsme prokázali korelaci mezi uvedenými parametry a tíží RS.

V genetické části jsme replikovali asociaci všech lokusů v kombinovaném vzorku 3 populací (*MEIS1,* P = 1.26×10^{-5}, odds ratio (OR) = 1.47, *BTBD9,* P = 4.11×10^{-5}, OR = 1.58, *MAP2K5/SCOR1,* P = 0.04764, OR = 1.27). Ve studii s RS byl nalezen trend pro asociaci u *SCOR1*, nejlepším modelem pro tuto variantu byl recesivní model (p nom = 0.0029, p korigované pro model a 4 geny = 0.023, genotypické OR = 1.60 – 95% CI 1.17 – 2.18). Nebyla prokázána asociace s variantami *MEIS1* a *BTBD9* i přes dostatečnou statistickou sílu.

Závěr: Syndrom neklidných nohou je častou komorbiditou roztroušené sklerózy (prevalence 32%), může nepříznivě ovlivňovat kvalitu spánku a RS by měla být zahrnuta mezi sekundární formy RLS. RLS je častější v rozvinuté formě RS, ale nekoreluje s mírou postižení mozku na magnetické rezonanci.

Genetická studie s idiopatickým RLS potvrdila význam variant v intronických a intergenových oblastech *MEIS1, BTBD9* a *MAP2K5/SCOR1* v české, rakouské a finské populaci. Naopak RLS u pacientů s RS sdílí tedy jen malou část rizikových genetických faktorů s formami idiopatickými, varianta *SCOR1* přispívá k fenotypu z maximálně 50%.

INTRODUCTION

Restless legs syndrome (RLS) is a common neurological disorder. The disease is characterized by an imperative urge to move the legs associated with unpleasant sensations in the lower limbs. Symptoms typically occur at rest in the evening and at night and RLS is often associated with periodic limb movement in sleep (PLMS), thus RLS can lead to sleep disturbance and impaired quality of life in its developed form. Sleep disturbance typically involves initiating and maintaining sleep (1).

RLS is one of the commonest neurological sensorimotor disorders at least in Western countries, the prevalence in the European population ranges from 5% to 10%. However, it remains largely underdiagnosed and undertreated (2).

International Classification of Sleep Disorders – ICSD2 classes RLS as sleep related movement disorder. The term "restless legs syndrome" was first used by Karl Ekbom in 1945 (3).

Symptoms, clinical description and course, diagnosis:

RLS is typically characterised by an urge to move the limbs, accompanied by uncomfortable and unpleasant sensations in the legs.

The diagnosis of RLS is clinical and is based on the patient`s description. Subjective symptoms were first described by Thomas Willis in the 17th century and an extensive description of the disease was made by Ekbom in 1945, thus the condition is also referred to as Willis-Ekbom disease. The diagnostic criteria for RLS were established in 1995 by the IRLSSG (International RLS Study Group) and modified in 2003 (1). Accordingly, four essential criteria are required to establish the diagnosis of RLS.

1) an urge to move the legs, usually accompanied or caused by uncomfortable and unpleasant sensations in the legs

2) an urge to move or unpleasant sensations begin or worsen during period of rest or inactivity such as lying or sitting

3) an urge to move or unpleasant sensations are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues

4) an urge to move or unpleasant sensations are worse in the evening or at night than during the day or only occur in the evening or at night (when symptoms are very severe, the worsening at night may not be noticeable but must have been previously present).

A family history of RLS, a positive response to dopaminergic treatment, and an association with periodic limb movements in sleep (PLMS) are additional clinical features that may provide support for the diagnosis in some atypical clinical presentations (4-6).

RLS suffers present with a wide range of sensory and motor symptoms. The sensory symptoms include different unpleasant sensations (dysesthesia, paresthesia) such as tingling, burning, pricking, itching, leg cramps etc. and even pain in some cases. The legs are mostly affected, the disorder may also involve the arms and other body parts (7). There is often bilateral involvement but symptoms can be asymmetrical. The motor restlessness is another clinical feature, patients suffer from an urge to move, some people are unaware of a sensory component. Eighty percent of patients develop periodic limb movement in sleep (PLMS) (4, 8).

RLS usually begins or worsens during the period of inactivity (watching television, driving a car, during lectures etc.). Symptoms are relieved by activity such as flexing, stretching or walking. In most patients the relief is complete, but patients with severe RLS report only partial improvement. RLS has a typical circadian pattern with the maximum of symptoms in the evening and during the night resulting in insomnia (6, 9). Most patients report difficulty falling asleep and night awakenings. Studies with polysomnography showed prolonged sleep latency, reduced sleep efficiency and total sleep time (5, 10, 11). Several patients also complain about excessive daytime fatigue and sleepiness. Clinical course is variable from one patient to another, in one patient during the time and within the family. RLS can begin at any age and childhood cases have been reported. However, the majority of patients seen in clinical practice are middle aged or older. Patients with early onset (before age 45) tend to have slower progression milder, symptoms and strong family history, those with late onset show a more rapid progression (12).

RLS diagnosis is based on clinical description, four diagnostic criteria can be easily confirmed by history. An interview with a trained physician is necessary for the correct diagnosis, if only questionnaires with RLS criteria are given this results in

approximately 10 – 25% false positive cases due to "RLS mimics" (13). Patients with RLS mimics meet all essential criteria but do not actually have RLS; important mimics include akathasia, positional discomfort, cramps and anxiety disorder. RLS also needs to be differentiated from other conditions that can also coexist with RLS (neuropathy, multiple sclerosis, etc.). The diagnosis in the childhood, when a clear verbal description cannot be given, can be supported by a positive family history or polysomnography (PLM index) (14).

The performance of routine laboratory tests such as renal function, thyroid parameters, red blood cell count, iron status and physical examination are considered standard in RLS and are used to diagnose secondary RLS. Iron status involves measures of iron, serum ferritin and transferring saturation (14). Polysomnography is not required in all RLS patients, but useful in patients with other sleep related disorders. Polysomnography can be used as a supportive diagnostic tool and as an objective means of assessing treatment response and disease severity in RLS. Actigraphy represents an alternative less expensive method for PLMS measurement (15).

RLS is a heterogeneous disease, whose severity and frequency vary from one patient to another. Therefore, although RLS prevalence is rather high, only 3.4% need a drug therapy (16). International RLS scale was developed as a scoring system for RLS symptom severity by the International RLS Study Group. Dopaminergic agents (L-DOPA and dopamine agonist) are the first line RLS therapy (17), the major complication of this therapy represents augmentation. Augmentation is characterized by increasing intensity of symptoms, earlier onset of symptoms in the day, reduced time at rest before the symptoms start and spread of symptoms to other body parts. (18)

Morbidity of RLS and quality of life

RLS affects sleep, mood, health and quality of life. 20% of patients with RLS (3% of population) experience clinically and medically significant RLS symptoms – which occur at least twice a week and are reported as moderately or severely distressing. In these patients, RLS causes insomnia, patients sleep only 4–5 hours and chronic sleep loss leads to deficits in daily functions particularly in terms of wakefulness during work and social activities (2, 16).

Several large epidemiological studies have shown significantly decreased quality of life (QoL) in RLS patients when compared to the general population. The Medical Outcomes Study 36-Item Short Form Health Survey (SF-36), the physical and mental summary scores have been widely used to asses health status and quality of life. In SF-

36, 8 dimensions of health-related quality of life are evaluated: physical functioning, physical limitations on normal activities (role-physical), bodily pain, general health perceptions, energy and vitality, social functioning, emotional limitations on normal role activities (role emotional) and mental health. RLS patients had all of the SF-36 scale scores significantly lower than the general population (2, 16, 19) see Figure 1. Despite the negative impact on QoL and effective treatment, RLS remains an under-recognized and trivialized disorder.

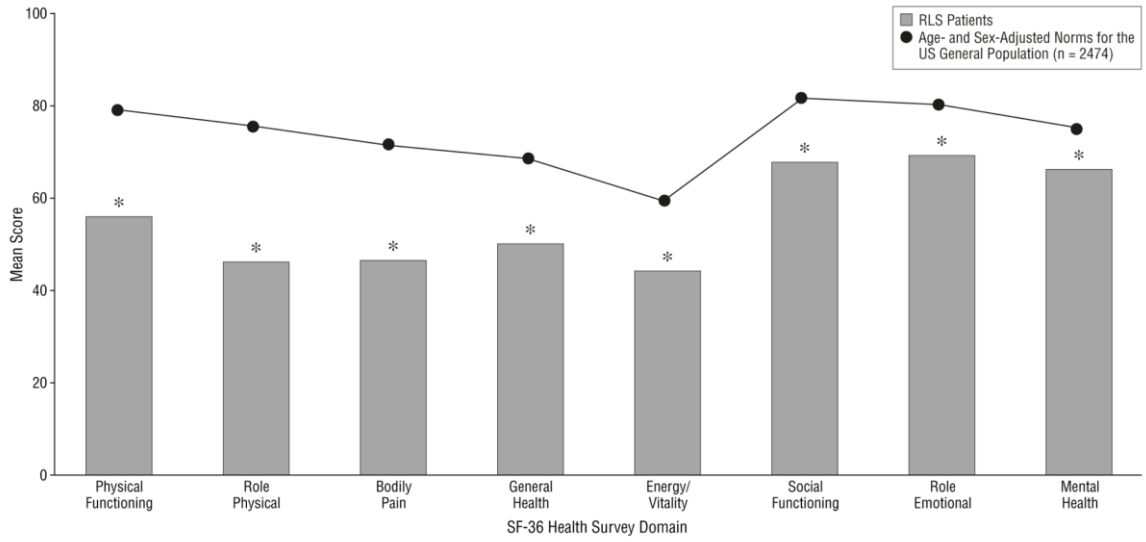


Figure 1: Comparison of mean Short Form 36 Health Survey (SF-36) scores of patients with restless legs syndrome with age- and sex-adjusted US population norms. Asterisks indicate that the scores of the RLS sufferer group were significantly below the norms for all 8 dimensions (2)

The sleep loss in untreated RLS patients produces cognitive deficits involving pre-frontal cognitive functions when compared to normal subjects. Similar changes have been described in patients with acute sleep deprivation, although RLS leads rather to chronic sleep deprivation (20).

Affective disorders, including depression and anxiety are also very common in RLS patients. Recent studies have demonstrated higher prevalence of major depressive disorder and panic disorder in RLS. Depression affects up to 50% patients; those who suffer RLS have two to four fold higher risk of developing depression (21).

Large epidemiological studies offer consistent and robust evidence for an association between RLS and cardiovascular disease. The underlying mechanism is complex. RLS may increase the risk for cardiovascular disorder and related conditions via action of the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis. Periodic limb movements in patients with RLS are associated with arousals, as

manifest in the electroencephalogram, and all are followed by elevations in nocturnal blood pressure and puls rate. Increased nocturnal cortisol level and HPA axis dysregulation may be responsible for the relation of RLS to diabetes and impaired glucose tolerance (22, 23).

Periodic limb movements in sleep (PLMS)

Periodic limb movements in sleep (PLMs) represent a very frequent objective finding in RLS and contribute to sleep disruption (24). Up to 80 per cent patients with RLS experience PLMS (11, 25), they also may have periodic limb movements while awake (PLMW) (2).

PLMS are best described as rhythmic extensions of the big toe and dorsiflexion of the ankle, PLMS are scored only if they are part of four or more consecutive movements lasting from 0.5 to 5 seconds with the inter-movement interval of 4 to 90 seconds. A PLMS index (number of PLMS per hour) greater than 5 is considered pathological (26). The number of PLMS varies from night to night. Roughly one third of PLMS are associated with arousals, as manifest in the electroencephalogram, leading to insomnia and increased cardiovascular risk in RLS patients.

Quantification of PLMS is performed with polysomnography (Figure 2) and actigraphy (15), it has become accepted as an objective tool to evaluate the severity and treatment outcome in RLS patients (17).

PLMS also occur in a wide range of disorders related to dopamine abnormalities such as narcolepsy, rapid eye movement behaviour (RBD) or Parkinson disease; they are often presented in sleep apnea syndrome, spinal cord lesions and are not specific to RLS. The PLMS are common in elderly patients without any sleep complaints. Some patients with otherwise unexplained insomnia or hypersomnia exhibited PLMS; this condition is defined as periodic limb movement disorder (PLMD). The clinical significance of PLMS continues to be controversially discussed (27).

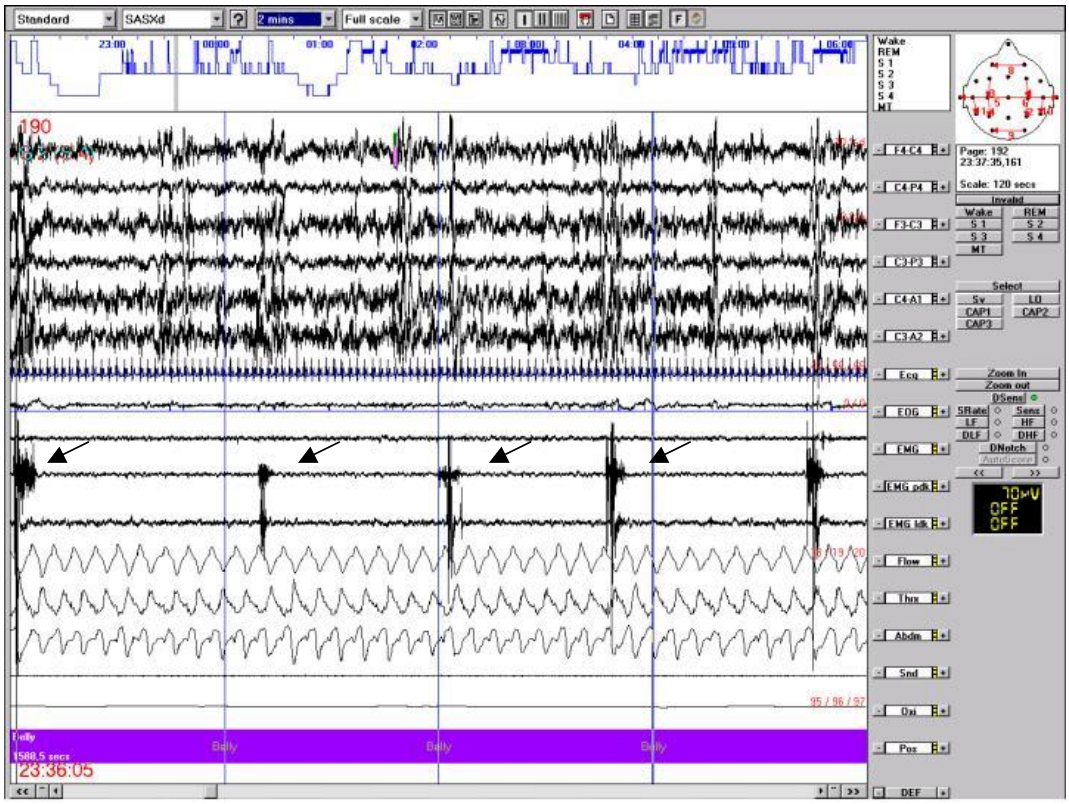


Figure 2: Polysomnogram of patients with RLS/PLMS. Duration of the hypnogram is 2 minutes. From the top to bottom: hypnogram (blue), EEG (six black leads), ECG (purple), EOG (two black leads), EMG (two black leads) with periodic leg movements (arrows indicate a periodicity of 20 – 30s).

Epidemiology

Prevalence rates of RLS, at least in Europe, identify this disorder as one of the most common neurological movement disorders. However, estimated prevalence in general populations does not overlap across studies even when the IRLSSG criteria are strictly applied. The subjective nature of the complaints, the fluctuating and variable course of symptoms, different populations and various methodological tools used such as questionnaires, telephone interviews, or direct face-to-face interviews may cause these discrepancies. Few studies in Western countries and Northern America have involved large population-based samples of subjects screened using the IRLSSG criteria. The RLS prevalence rate ranges from 7.2 to 11.5% in the general population (2, 16, 28).

A minority of sufferers (around 3% of the population) experience daily or severe symptoms (2, 16). RLS is twice as common in women as in men (29). Parity or postmenopausal intake of oestrogen are to be considered as major factors in explaining this sex difference (29).

RLS occurs in 3% of individuals from the Mediterranean or Middle Eastern region and in 1% of Asian population (30, 31), indicating that different genetic or environmental factors may play a role in the prevalence of this syndrome.

Prevalence of RLS also increases with age. Increasing co-morbidity in the very old, however, may interfere with the accurate identification of RLS.

Studies in children using diagnostic criteria for children established the prevalence rate in children to be 5.9% (32).

RLS classification

RLS can be divided into primary and secondary forms. Primary or idiopathic forms appear without apparent causes, they are not related to any medical conditions and include sporadic and inherited forms. Secondary forms occur in acquired forms associated with a variety of disorders. Iron deficiency, end-stage renal disease and pregnancy are thus well established secondary causes of RLS (5).

Pathophysiology of RLS

The pathophysiology of RLS is complex and remains unknown. RLS is predominantly a disorder of the central nervous system; dopamine and iron seem to play a fundamental role.

The dopamine hypothesis derives from the dramatic improvement of RLS with dopaminergic therapy. The hypofunctioning of A11 dopaminergic diencephalon spinal pathways seems to be implicated in RLS ethiopathogenesis (33). The impaired iron homeostatis is another important pathophysiological issue, most patients have normal ferritin serum levels, but reduced levels in the cerebrospinal fluid (34). There is a substantial evidence for a genetic contribution to RLS, more then 60% of cases are familial.

The different approaches used to define the pathophysiology of RLS are as follows:

1) studies attempting to localize the areas of abnormal central nervous system (CNS) function (e.g. basal ganglia or spinal cord)
2) studies of neurotransmitters
3) studies of iron
4) studies on the possible role of the peripheral nervous system in generating sensory symptoms in RLS

5) genetic studies

CNS structures and areas which are involved in RLS pathophysiology

There is a conflicting evidence for the cortical involvement in RLS pathophysiology. Electrophysiologic studies with transcranial magnetic stimulation have demonstrated an increased cortical excitability and decreased subcortical inhibiton, but normal motor threshold and conduction velocity suggest that the motor pathways are intact (35). The absence of corticospinal prepotentials on back-averaging, normal electroencephalogram, and the absence of high-amplitude cortical potentials in somatosensory evoked response argue against these movements being of cortical origin (36). An altered cortical excitability may be the result of subcortical inputs, probably at the level of the basal ganglia. Therefore, dopaminergic modulation of intracortical excitability might play a key role in these dynamics (37). Some MRI studies have revealed the presence of morphologic changes in the somatosensory cortex, motor cortex and thalamic gray matter. Significant regional decreases of gray matter volume were found in the primary somatosensory cortex (38). However, this study conflicts with two others in which no cortical changes were found (39). A functional MRI study revealed abnormal bilateral cerebellar and thalamic activation during the manifestation of sensory symptoms, with additional red nucleus and reticular formation activity during PLMS (40).

PLMS is likely to occur in patients with spinal cord lesions (41); several case reports described the onset of RLS in association with the spinal cord lesions (radiculopathy, traumatic lesion, etc.) (5). Sufficient evidence exists for hyperexcitability in motor and sensory spinal cord structures (42, 43). It is unclear wether the hyperexcitability arises in the spinal cord itself or in the supraspinal areas (44). Patients with PLMS have significantly increased spinal cord excitability, as indicated by lower threshold and greater spatial spread of the spinal flexor reflex, which is more prominent during sleep (42). Dopamine and opioids, two of the most effective therapies modulate spinal cord functions. L-DOPA depress flexors and nociceptive reflexes in the spinal cord (45). Opioids also play an important role in the control of sensory inputs to the spinal cord (see below).

RLS symptoms seem to be the result of abnormal sensorimotor integration at the spinal cord level and abnormal central somatosensory processing. The hypofunctioning of the A 11 dopaminergic diencephalospinal pathway seems to be implicated in the RLS pathophysiology (33). A11 neurons are located close to sleep

related nuclei in hypothalamus and receive diffuse projections from the suprachiasmatic nucleus, which largely controls circadian rhythms (37). A11 exists as the only source of dopaminergic innervation to the spinal cord (44, 46). This pathway is projecting from the hypothalamic area A 11 to D3 receptors located in the dorsal horns and intermediolateralis spinal nuclei and it is crucial for sensorimotor integration and pain control at the spinal cord level (47). There is extensive evidence for the existence of D1, D2 and D3 receptors in the spinal cord, the study with D3receptor – knock-out mices (D3KO) suggest that D3 receptors are involved in the spinal cord excitability (48). This fact is in accordance with clinical studies in which RLS is best relieved by D3 receptor – preferring agonists. *SCOR1* gene (see below) is selectively expressed in dorsal horns of the developing spinal cord, this fact may represent an interesting view on RLS pathophysiology.

Studies of neurotransmitters

Dopamine

The strongest evidence for a dopaminergic role in RLS is an excellent response to dopaminergic medication (49) and worsening with dopamine release blockers. Brain imaging studies of the dopaminergic system in the basal ganglia have not revealed consistent abnormalities. PET studies have shown little but significant reduction of mean caudate and putamen D2-receptor binding (50). Two SPECT studies did not find any difference in presynaptic DAT and striatal D2 receptor binding between RLS patients and controls suggesting normal pre-synaptic nigrostriatal terminal functions in RLS (51, 52), whereas one study (the only performed in the evening) reported a small but statistically significant difference, less binding to D2-receptors in RLS patients (53).

Opioids

The therapeutic effects of opioids were already noted by Ekbom and were examined in several clinical trials. Neurophysiological studies suggest that the pain system may be abnormal, patients with RLS exhibit profound static mechanical hyperalgesia to pin-prick stimuli, but no dynamic mechanical hyperalgesia (allodynia). The hyperalgesia is reduced by long term dopaminergic treatment (43). Dopamine and opioids modulate the spinal cord functions, endogenous opioids may act upon dopamine systems to improve the symptoms of RLS (54).

Studies of iron

The connection between RLS and system or CNS iron deficiency has long been recognized (34). Studies in idiopathic cases found normal serum iron values, but significantly reduced values in the cerebrospinal fluid compared to healthy controls. This indicates brain iron insufficiency and decreased availability of iron in the CNS in RLS patients (55). Magnetic resonance (MR) studies revealed decreased iron stores in substantia nigra and putamen in RLS patients; the decrease was most evident in patients with a severe form and early onset (12, 56), this was also confirmed in other studies using ultrasound methods. Overall, these studies suggest that general changes rather than local changes may account for clinical manifestations of RLS. There are several interactions between iron and dopamine (34). Iron is a cofactor for tyrosine-hydroxylase, which is the rate-limiting step in the production of dopamine. Iron is a component of the dopamine type-2 (D2) receptor.

Iron levels decrease at night (30 – 50%) leading to the dopamine decrease in the evening hours and to the typical circadian pattern of the disorder.

Low serum ferritin levels are the best indicator of low iron store. Intravenous iron treatment showed successful, but transient improvement of RLS symptoms (57).

The peripheral nervous system in generating sensory symptoms in RLS

There is some evidence suggesting an association between RLS and peripheral neuropathy, but studies on this topic still remain controversial. Nerve conduction abnormalities and small-fibre neuropathy were found in a subset of RLS patients, especially in those with an older age of onset and negative familial history (58, 59). Other studies have failed to show this association; RLS was only 5.2% of 144 patients presenting with polyneuropathy, a prevalence not higher than that found in the general population (60).

Genetics of RLS:

Genetic factors participating in the RLS aetiopathogenesis has of late been repeatedly corroborated by several kinds of observations. About 40 – 60 % of idiopathic RLS patients report a positive family history; monozygotic twins are concordant for RLS in

80% (61). RLS is a highly familial phenotype with heredity estimates of about 50% (11), familial cases have a more slowly progressive course, the symptoms within a single RLS family can be variable. Based on the description of several large RLS families it is assumed that the disease follows an autosomal-dominant mode of inheritance. This was also confirmed by a segregation analysis in first degree family members. Findings in German and Czech populations suggest that heritability is higher in patients, who were younger than 30 at onset of symptoms (62).

Linkage studies have revealed 8 loci so far, but no causally related gene variant has been identified yet. Recessive model of inheritance was identified on chromosome 12 q in the family of French-Canadian origin. Further studies in families of Italian, American and German origin revealed loci on chromosome 14q (RLS 2), 9p (RLS3), 20p (RLS4) and 2q (RLS5) with the dominant mode of inheritance. Other two loci were found in one family on chromosome 4q a 17q, a suggestive locus is also on chromosome 19 (63).

So far, all of these linkage analyses of RLS families have met limited success and have not led to the identification of the disease-causing mutation. This fact provides indirect evidence for the complexity of RLS.

Apart from the linkage loci, which represent genetic variants of stronger effect, but are usually rare, association cases control studies are able to detect variants of smaller effect, which are more common in patients suffering from RLS. Association studies compare the frequencies of alleles in case and control populations. A higher frequency of the allele tested in cases is taken as evidence that the allele or genotype is associated with an increased risk for the disease. A genome-wide association study (GWAS) with German and Canadian RLS idiopathic cases revealed association with three gene variants in *MEIS1* on chromosome 2, *BTBD9* on chromosome 6 and in region between *MAP2K5* and *SCOR1* on chromosome 15q (64). A replication study from the USA confirmed the association of *MEIS1* and *BTBD9*, however the *MAP2K5/SCOR1* locus showed only a trend for association (65). Another GWAS conducted in US and Iceland population showed association of *BTBD 9* variants with periodic limb movements in sleep (PLMS) (66). The fourth loci, *PTPRD* on chromosome 9 was identified in the European and Canadian population (67). An association was identified with intronic variants, which suggests a functional role in the expression or alternative splicing of the gene. Carriers of one risk allele had a 50% increased risk for developing RLS. A closer inspection of the known function of the genes is surprising because some of them are developmental factors and did change the pathophysiological concept of RLS. The most recent GWAS including European

samples revealed new association loci, the first on chromosome 2 is an intergenic variant outside of *MEIS1* region and the second on chromosome 16 containing the 5'-end of *TOX3* (68).

MEIS1 (myeloid ecotropic viral integration site homeobox 1) is a member from a highly conserved family of TALE homeobox genes. *MEIS1* plays a role in proximodistal limb formation during embryonic development, it is also part of a HOX transcriptional regulatory network that specifies spinal motor neuron pool identity and connectivity (69). The specific function in postembryonic tissue remains to be established, *MEIS1* is expressed in the adult mouse brain in cerebellar granule cells, the forebrain and, notably, in dopaminergic neurons of substantia nigra. *MEIS1* has been found to be overexpressed in acute myeloid leukemia (64).

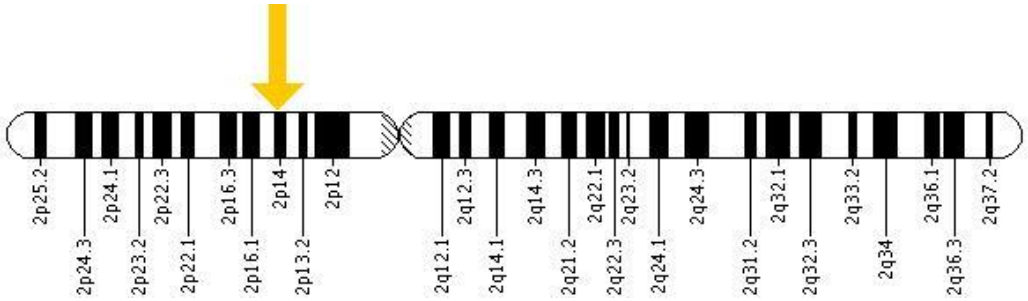


Figure 3: MEIS 1gene

The second region with significant association was found on chromosome 6p in intron of the *BTBD9* gene. *BTBD9* (BTB/POZ domain-containing protein 9) gene appears to be expressed in the periphery and in the central nervous system. The function of BTB (POZ) proteins includes transcription repression, cytoskeleton regulation, gating of ion channels and ubiquitin-dependent protein degradation (64). The specific function is not known due to universal occurrence. Interestingly, the association of *BTBD9* was found in subjects who had PLMS without RLS but not in subjects with RLS without PLMS. Furthermore, an analysis of parameters involving iron metabolism revealed that the risk allele was also associated with a 13% decrease of the serum ferritin levels (66).

The third region on 15p chromosome contains *MAP2K5*, a member of the mitogen activated protein kinase family, and the adjacent *SCOR1* gene (64). MAPK pathways are activated by a signaling cascade that mediate the transduction of extracellular signals to cytoplasmic nuclear effectors; this pathway is important in neuroprotection of dopaminergic neurons. MAP kinase cascade is critical at early stages of muscle cell differentiation. *SCOR1* acts as a corepressor of *LBX1*, this homeobox gene is critical in the development of sensory pathways in the dorsal horn of the spinal cord (70).

PTPRD (protein tyrosine phosphatase receptor type delta) belongs to the family of type IIa receptor-like protein tyrosine phosphatase; the involvement of *PTPRD* in RLS is unknown. Studies in *PTPRD* knockout mice have shown that these proteins function in axon guidance and termination of mammalian motoneurons during embryonic development (67, 71).

TOX3 is a member of the high mobility box group family of non-histone chromatin proteins which interacts with CREB and CBP and plays a critical role in mediating calcium-dependent transcription in neurons (68).

The identification of genetic variants has been a large step forward in unraveling the genetics of RLS. We still do not know whether RLS has components of a developmental disorder and whether the genes identified play a role in early embryonic days or have a completely different function in the elderly. None of these genes is related to dopamine or dopamine receptors, the genetic background of dopaminergic response remains to be solved.

It is now necessary to investigate relations between genetic and environmental factors such as iron deficiency, pregnancy, renal failure, etc. Secondary RLS cases may present genetically susceptible individuals with a clearly defined provoking factor (63). The only study, which demonstrated the influence of genetic factors in secondary RLS, was performed in patients with an end stage renal disease. Schormair et al. investigated the known genetic variants (*MEIS1*, *BTBD9*, *MAP2K5/SCOR1*, *PTPRD*) in a case-control association study of uremic patients from Germany and Greece. RLS in patients with an end stage renal disease was associated with *MEIS1* and *BTBD9* in the German sample, whereas, in the Greek sample, there was a trend for association for *BTBD9* and *MAP2K5/SCOR1* (72).

Secondary RLS:

Secondary RLS are related to other medical or neurological conditions. Well-documented associations include renal failure, iron deficiency and pregnancy. In fact, at least 20 disorders have been reported in the literature. Some of these are likely chance occurrences owing to the past underestimated prevalence of RLS. The diagnostic criteria for primary and secondary forms are identical (4-6). Although not yet formally studied, the secondary forms of RLS probably share the same clinical features as idiopathic RLS, suggesting a similar underlying pathophysiological basis, including the genetic factors (63).

RLS is associated with several neurological disorders such as spinal cord lesions (myelopathy, traumatic lesions, spinal anesthesia, etc.), genetic ataxias (SCA 1 – 3), Parkinson's disease, essential tremor and probably with neuropathy. RLS can be triggered by medication (e.g. SSRI) (6).

The prevalence of RLS in uremic patients ranges from 20% to 57% (73), RLS symptoms are usually very severe and RLS is associated with increased mortality. Kidney transplantation leads to dramatic improvement of RLS. Anemia, neuropathy and genetic factors (see above) may cause this secondary RLS form.

Pregnant women have 2–3 higher risk of developing RLS than the general population, the prevalence in pregnancy is estimated at 10–27%. Symptoms usually appear or get worse in the last trimester and RLS is resolved after the delivery in vast majority. Three factors are speculated in aethiology: 1) impaired metabolism of iron and folate, 2) hormonal changes (progesterone increases the excitability of the nervous system) and 3) psychosomatic factors (74).

All conditions which lead to system iron deficiency may cause RLS. RLS is common in anemic patients and regular blood donation may lead to iron deficiency (75).

Restless legs syndrome in patients with multiple sclerosis

The recent studies showed a higher prevalence of RLS in patients with multiple sclerosis, which ranges from 19% to 37.5%. Three major epidemiological investigations have been published so far. Auger et al. performed the first issue on this topic in the French-Canadian population, finding a very high prevalence in both patients and controls (37.5% vs. 16%), only a self-administered questionnaire without a personal interview was used (76). This methodology may overestimate the prevalence of RLS due to false positive cases; other two studies used a face to face interview. The second study, published by Spanish authors, showed different results – a similar prevalence rate of RLS in MS patients and in healthy subjects (13.3 % vs. 9.3%), but they did not use clear exclusion criteria for patients and subjects and the sample of patients was small (77). The largest study, published by an Italian group, showed a prevalence of 19% in MS and 4.2% in control subjects (78). They chose the frequency of symptoms occurrence at least twice a week as a threshold for the diagnosis of RLS. They did not include patients who experienced the symptoms with a frequency of occurrence lower than twice per week (further 7.3%, total RLS prevalence 26.3%). Figure 4 shows the strong dependency of RLS prevalence rate, in

both controls and cases groups, on the frequency of RLS symptom occurrence chosen as a threshold for the diagnosis of RLS. Different methodology, frequency criteria and population might explain discrepancies in absolute values in the estimation of prevalence rates among these studies.

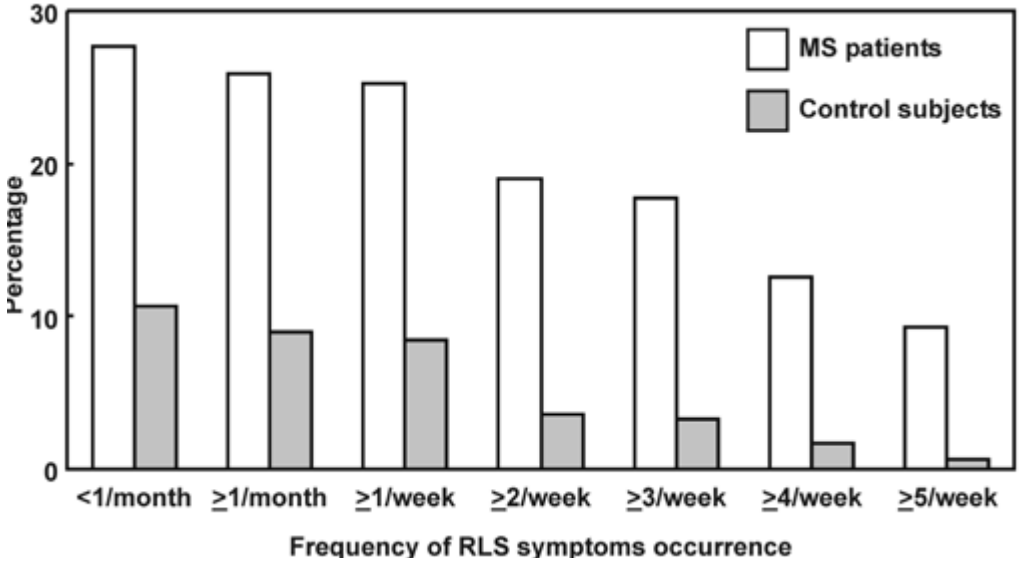


Figure 4: Likelihood of prevalence rate of restless legs syndrome (RLS), in both control subjects and patients with RLS, based on the frequency of RLS symptom occurrence chosen as a threshold for the diagnosis of RLS (78).

Among patients with MS, RLS is associated with older age, longer MS duration and more severe neurological disability specifically involving the pyramidal and the sensitive EDSS (Expanded disability status scale) functional systems (Figure 5). Patients with MS and RLS reported poorer sleep quality and higher intake of drugs (hypnotics, baclofen and antidepressants). RLS was more prevalent in the primary-progressive form when compared with relapse-remitting form (Figure 6). Thus RLS was associated with a higher MS disability and with the most severe MS course. The RLS clinical onset in the majority of MS patients followed MS onset, at least a small portion of patients (4%) may be idiopathic (RLS preceded MS). The severity of RLS symptoms was higher in patients with MS than in control subjects (78).

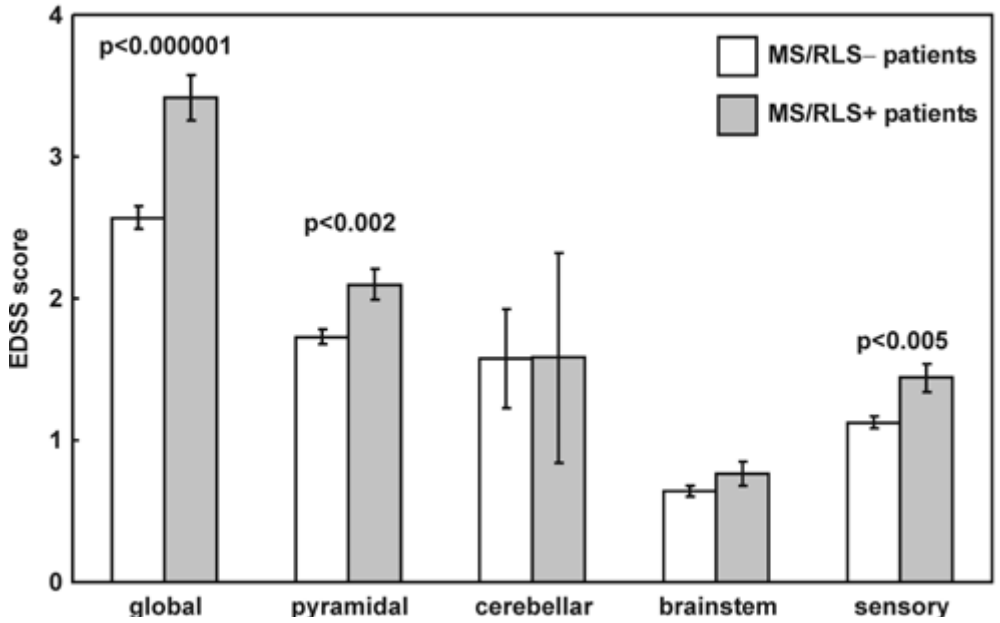


Figure 5: Comparison of Expanded Disability Status Scale (EDSS) scores found in MS/RLS+ and MS/RLS– patients. MS refers to multiple sclerosis; RLS to restless legs syndrome *mean ± SD (78)

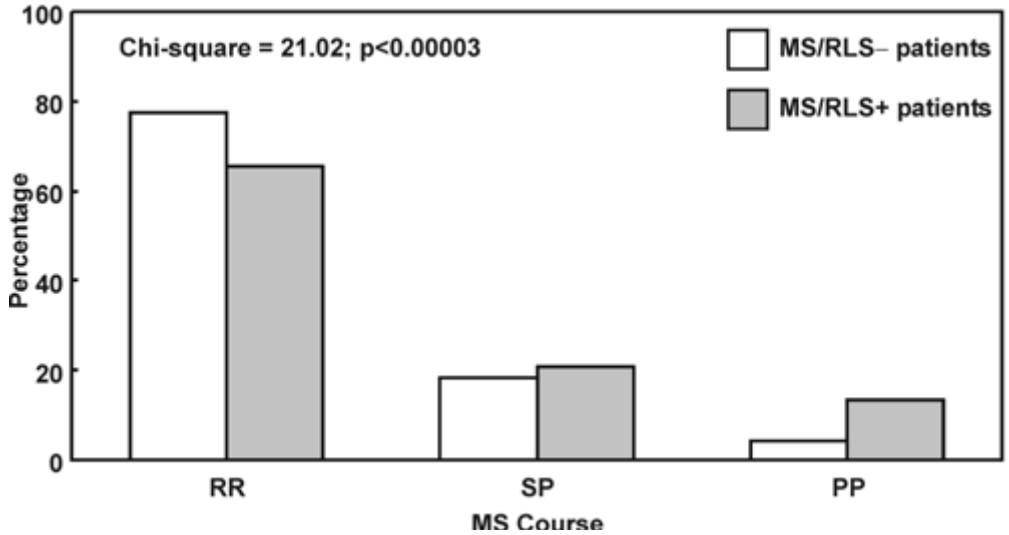


Figure 6: Distribution of the frequency of the 3 clinical courses of multiple sclerosis (MS) in patients with and without RLS. RR refers to relapsing remitting; SP to secondary progressive; PP to primary progressive (78).

The pathophysiology of this association remains to be investigated. Research on secondary forms may help in understanding which central nervous structure is responsible for RLS.

Manconi et al. compared the extent of brain and cervical cord damage in MS patients with and without RLS using conventional and diffusion tensor magnetic resonance imaging (MRI). Global and regional dual-echo lesion load (LL), number of cervical cord lesions, mean diffusivity (MD) and fractional anisotropy (FA) histograms of the brain and cervical cord were assessed. No difference between the two groups was found in the whole brain, cerebellar and brainstem lesion load, MD and number of cervical lesions. Cervical cord average FA was significantly reduced in MS patients with RLS compared to those without, pointing out that the cervical cord damage represents a significant risk factor for RLS in MS patients (79).

Conventional MR technique provides a crucial step for the diagnosis in MS. However, the gold diagnostic and prognostic standard, T2-weighted MRI has shown only a limited association with the disability progression due to the limited ability to characterize and quantify the heterogenous MS pathology (i.e. demyelination, axonal loss and gliosis)(80). The correlations between the conventional T2 lesion load and clinical outcome are strong in patients seen at the first presentation with clinically isolated syndromes suggestive of MS, but much weaker in established MS (81). Thus, more specific MR parameters and techniques (MR spectroscopy, functional MRI) have been developed to monitor disease progression and treatment efficacy. MRI volume parameters include brain atrophy and brain parenchymal fraction (82). Serial MRI studies have demonstrated that brain volume loss occurs at a rate around 0.5 – 0.1% per year in MS patients compared with a rate of about 0.1 – 0.3% per year in age matched healthy subjects (80). Brain atrophy begins early in the disease course and both global brain and selective grey matter measures of volume loss have been closely associated with the disease progression. Brain parenchymal fraction represents another approach to measure brain atrophy, it is defined as the ratio of brain parenchymal volume to the total cranial volume (83).

AIM OF OUR STUDY

The aim of our study was to further investigate the pathophysiology of primary and secondary forms of restless legs syndrome focusing on clinical and genetic aspects of this disorder, mainly in patients with multiple sclerosis. Our study is divided in a genetic and clinical part.

Clinical study:

The aim of the epidemiological and radiological study was to evaluate the prevalence of RLS among Czech patients with multiple sclerosis, to further analyze risk factors for developing RLS in patients with MS and to compare the extent of brain damage between MS patients with and without RLS using magnetic resonance imaging (MRI).

Hypothesis: MS is a new secondary RLS form, RLS is a common finding also among Czech patients with MS, the presence of RLS correlates with the clinical progression of MS and with the extent of brain damage on brain MRI.

Genetic study:

1) The aim of the study "Replication in three populations" in idiopathic RLS was to evaluate whether common genetic variants (*MEIS1*, *BTBD9* and *MAP2K5/SCOR1*) are also relevant among other Europeans (Czech, Austrian, and Finnish) and what is the difference of their impact between sporadic and familial cases.

Hypothesis: Common genetic variants also increase the risk for the idiopathic RLS form in other populations.

2) The aim of the study "Genetics of secondary RLS form in patients with multiple sclerosis" was to investigate whether the common genetic variants (*MEIS1*, *BTBD9*, *MAP2K5/SCOR1* and *PTPRD*) have also an impact on RLS in patients with multiple sclerosis.

Hypothesis: Secondary and primary RLS share at least some common genetic factors.

PATIENTS AND METHODS

Clinical study

Epidemiological and radiological study – prevalence of RLS in patients with multiple sclerosis and brain magnetic resonance imaging study in patients with RLS and MS

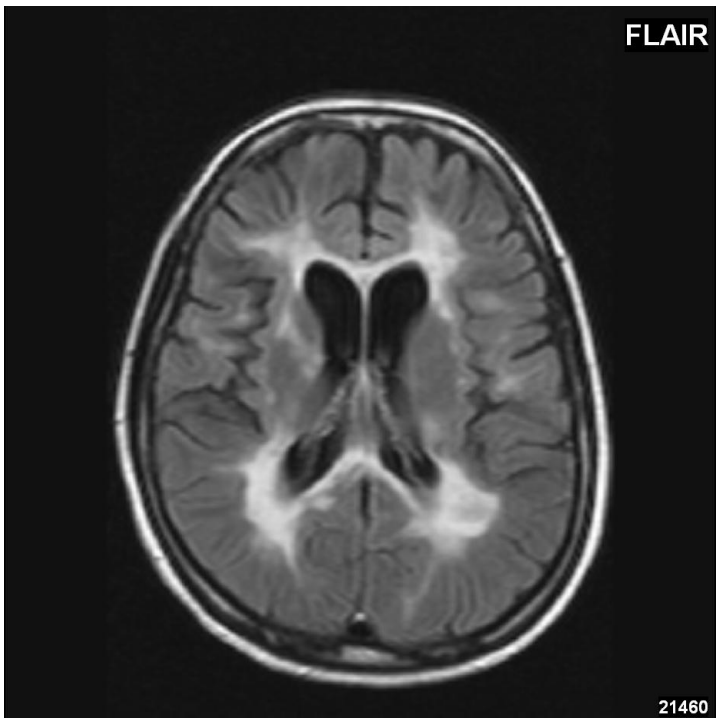
Patients

From April to December 2009, we recruited all patients with multiple sclerosis from the preselected population (patients with quantitative MRI data) in the MS Centre, Department of Neurology of First Faculty of Medicine, Prague. MS had been diagnosed according to McDonald criteria (84). Exclusion criteria for the study were dopaminergic and antidopaminergic drugs, renal failure, pregnancy, sideropenic anaemia, another disease known to be related to RLS, recent MS diagnosis (less than 6 months before the time of the interview) and recent clinical MS relapse (within 3 months of the interview). No specific limitations were used regarding chronic MS treatments with disease-modifying drugs. On the basis of its clinical course, MS was classified as primary progressive, secondary progressive or relapsing remitting. Each MS patient underwent a semi-structured interview and brain magnetic resonance imaging (MRI). An interview was conducted by a physician skilled in RLS diagnostics. A patient was considered to be affected by RLS if all four standard criteria had ever been met in his/her lifetime (2).

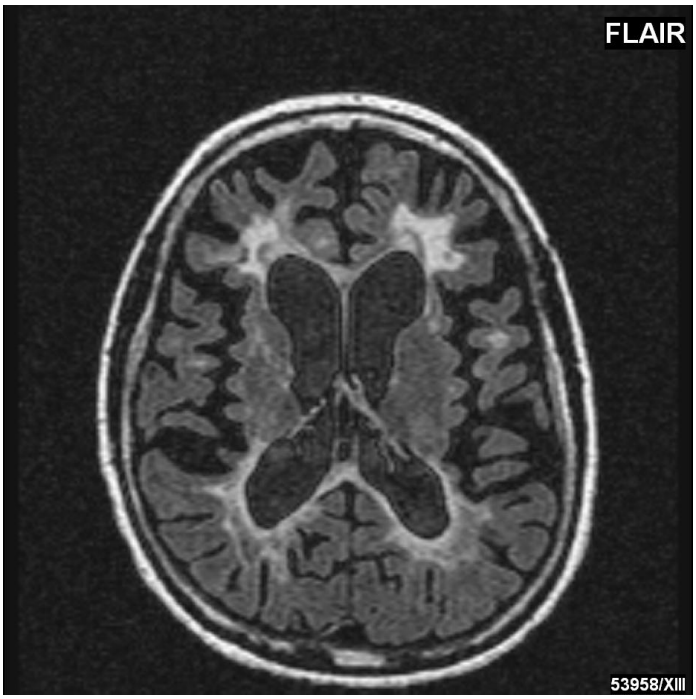
Magnetic resonance imaging (MRI) data for each patient were obtained during the year before the interview, MR analysis was performed by an experienced observer, who was unaware of the identity of scans.

All MRI scans were performed with Philips Gyroscan NT 1.5 T (Philips Medical Systems, Best, the Netherlands; software update in 2001, hardware update in 2004). Axial brain images were acquired using fast fluid-attenuated inversion recovery (FLAIR) and T1-weighted three-dimensional fast field echo images, we used our in-house developed software (ScanView 1.0.7). Image analysis was performed as described elsewhere (85). Three volumetric parameters (absolute values and changes against baseline) were measured: brain atrophy (Picture 1 – 3), brain parenchymal fraction (BPF) and T2 lesion load (T2LL) (Picture 4). T1-weighted images were used

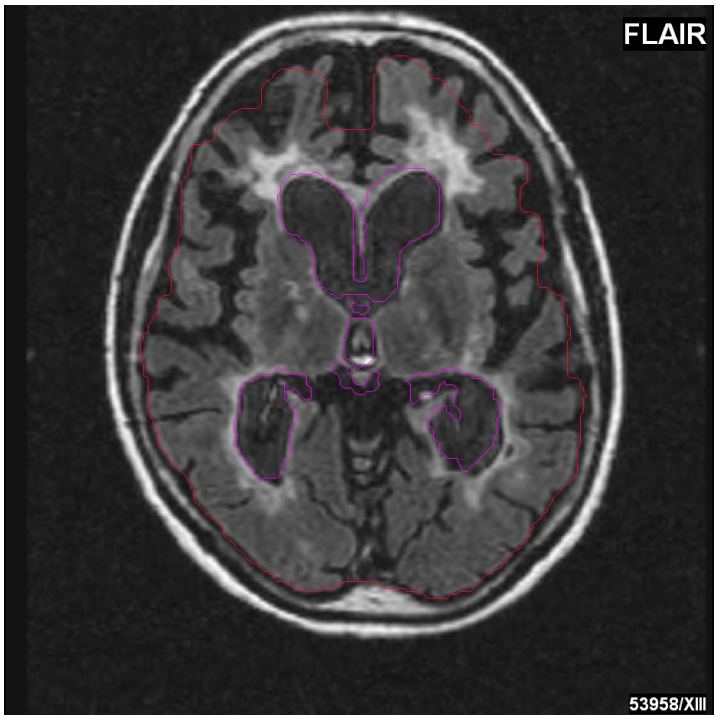
to assess brain atrophy, and brain tissue was outlined semi-automatically. BPF was calculated as the ratio of the brain tissue volume to the total volume contained within the brain surface contour (83). T2 lesion load was identified on FlAIR scans.



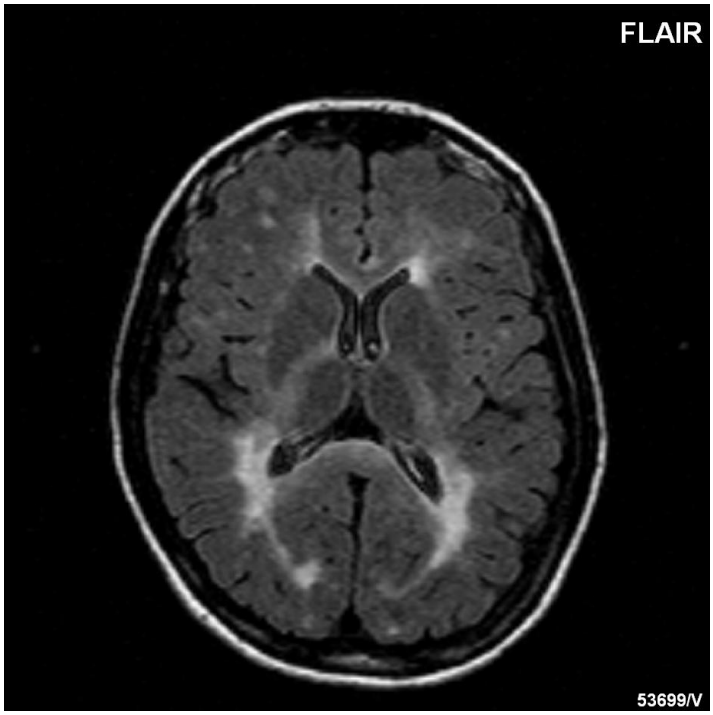
Picture 1: Brain atrophy in 31-year-old patient with MS (FLAIR)



Picture 2: Brain atrophy 8 years later in the same patient (FLAIR)



Picture 3: Brain atrophy – technique of measurement



Picture 4: Lesion load (FLAIR)

Statistical analysis:

The data were analyzed using the software package Statistica 8 (StatSoft, Inc. STATISTICA for Windows, Tulsa, OK: 2300 East 14th Street, Tulsa, OK 74104, http://www.statsoft.com). Results are presented as mean ± one standard deviation; nonparametric descriptive statistics and an inter-group comparative test (Mann-Whitney) were used to analyze EDSS scores. T-tests were employed for all other parameters.

Genetic study

1) Replication in three populations:

Patients and Controls

The diagnosis of all RLS cases was made according to the diagnostic criteria of the International RLS Study Group by personal examination by a neurologist in the respective study center. Positive family history was defined as at least one first-degree family member being affected by RLS (reported by the proband) in all three populations. The control samples originate from the general population and were not screened for presence of RLS.

Czech subjects – The patients were recruited in the center for Disorders of Sleep and Wakefulness, Department of Neurology of First Faculty of Medicine and the General Teaching Hospital, Prague. In total, 290 patients were included (107 males, mean age 55.7 ± 15.3 years (± SD), mean age at onset of RLS 38.3 ± 18.1 years). Positive family history was reported by 110 patients, in 175 cases it was negative and in five the data were not available. Altogether 450 sex matched controls were selected randomly from the Czech blood and bone marrow donors registry (166 males, mean age 45.3 ± 9.9). Since the maximum age for the controls was 63 years, 38 male and 51 female cases in the age group from 64 to 91 years could not be age matched.

Austrian subjects – 269 (104 males) patients were recruited in 2 centers: at the Department of Neurology, Medical University of Vienna and the Department of Neurology, University Clinic Innsbruck, (mean age 59.0 ± 14.3, mean age at onset of RLS 37.14 ± 19.5). Positive family history was reported by 107 patients, in 108 cases it was negative and in 54 the data were not available. The patients were matched by sex to 611 controls from the German KORA project, whose procedures were described elsewhere (86) (236 males, mean age 59.9 ± 11.35). KORA controls were already used

in the previous GWA study, which showed only negligible effect of population stratification (64).

Finnish subjects – 90 (24 males) patients were recruited in the Sleep Research Center in Turku (mean age 46.5 ± 18.1, mean age at onset of RLS 19.4 ± 13.4). Positive family history was reported by 81 patients and 9 patients had a negative family history. A random sample from the general Finnish population, comprising 169 sex matched individuals (45 males) was used as control. Data on age of controls were not available. Studies were performed according to the declaration of Helsinki and approved by the Ethical Committee of the respective study centers. Written informed consent was obtained from all RLS patients.

Genotyping

Ten SNPs within the three genomic regions were selected according to the results of previous GWA scans (64, 66). Samples were genotyped on two Sequenom platforms in Munich and Helsinki (Sequenom MassArray system, Sequenom Inc, San Diego, CA, USA) with a genotype discordance rate of 1.3% in 158 comparisons, when analyzing repeatedly genotyped internal control samples. Automated genotype calling was done with SpectroTYPER 3.4 software and genotype clustering was visually checked by an experienced scientist. Assays were designed using AssayDesign 3.1.2.2 with iPLEX Gold chemistry default parameters. SNP quality control criteria leading to exclusion from analysis were a call rate < 90%, MAF < 1% and P < 0.001 for deviations from HWE in controls.

Statistical analysis

Genotype data were analyzed using standard association tests (allelic, genotypic, dominant and recessive models) including Cochran-Armitage test for trend, Cochran-Mantel-Haenszel test for estimation of ORs in the stratified sample (including Breslow-Day test for homogeneity) and haplotype tests, as implemented in the PLINK statistical package v1.0.v (87). The sample was stratified only according to the country of origin. Logistic regression implementing the Cochran-Armitage test for trend (using genotypes as ordinal values rather than categorical) in the combined sample using age, sex and country of origin as covariates was performed by generalized linear modeling routines incorporated in R package v.2.6.0 (http://www.r-project.org/). Bonferroni correction for multiple testing of ten markers was employed. All P-values given are

one-sided, with the direction of the alternative hypothesis given by the original report (66). Power calculations were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (87). For input parameter we used a RLS prevalence of 8%, an alpha-level of 0.05% and ORs and allele frequencies according to results from the GWA experiment (64). Association tests were conducted in three different settings: 1. All patients (i.e. familial and sporadic) combined versus all controls, 2. familial cases versus controls, and 3. sporadic cases versus controls.

2) Genetics of secondary RLS form in patients with multiple sclerosis

Patients

Participants in the epidemiological study (see above) were asked to take part also in the genetic association study. We also recruited more patients with clear cut secondary RLS to increase statistical power and did not use all the RLS negative patients so as not to exceed the 2:1 ratio between controls and cases. As a reference population, blood donors were used – the same sample as described in the previous study (p. 39). The genetic association study included 642 subjects; 203 MS patients (45 men, 158 women, mean age 40.7 years, SD ±10.7) with RLS were compared to 438 MS patients (122 men, 316 women, mean age 35.8 years, SD ±9.3) without RLS and to a reference population of 450 blood donors (166 males, 284 females, mean age 45.3 ±9.9).

We excluded patients who had experienced RLS prior to the first symptoms of MS and patients with a positive family history of RLS to minimize the admixture of idiopathic cases.

Association tests were conducted in different settings: 1) patients with MS with RLS combined versus patients with MS without RLS 2) patients with MS with and without RLS versus population controls (blood donors not screened for RLS) and Czech sample of idiopathic RLS (see above).

The Local Ethics Committee approved the study and written informed consent was obtained from all subjects.

Twelve single nucleotide polymorphisms (SNPs) within the four genomic regions were selected according to the results of previous GWA scans (64, 67). Samples were genotyped on Sequenom platform (Sequenom MassArray system, Sequenom Inc, San Diego, California, USA). Automated genotype calling was done with SpectroTYPER 3.4 software and genotype clustering was visually checked by an experienced scientist.

Assays were designed using AssayDesign 3.1.2.2 with iPLEX Gold chemistry default parameters. SNP quality control criteria leading to exclusion from analysis were a call rate, 90%, minor allele frequencies (MAF), 1% and p=0.001 for deviations from Hardy-Weinberg equilibrium (HWE) in controls.

Statistical analysis:

Genotype data were analyzed using standard association tests (allelic, genotypic, dominant and recessive models) including the Cochran-Armitage test for trend and test for empirical significance as these are implemented in the PLINK statistical package v1.0.11. (87).

Bonferroni correction for multiple testing of 4 regions (all genotyped SNPs within each region are in close linkage disequilibrium, except for chromosome 9) and 2 different models (allelic and the best model) were employed. All p values given are two-sided.

Power calculations were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (88). For input parameter we used an RLS prevalence of 8%, an alpha level of 5%, and ORs and the allele frequencies according to the results of the GWA experiment (64, 67).

RESULTS

Clinical study

Epidemiological and radiological study – prevalence of RLS in patients with multiple sclerosis and brain magnetic resonance imaging study in patients with RLS and MS

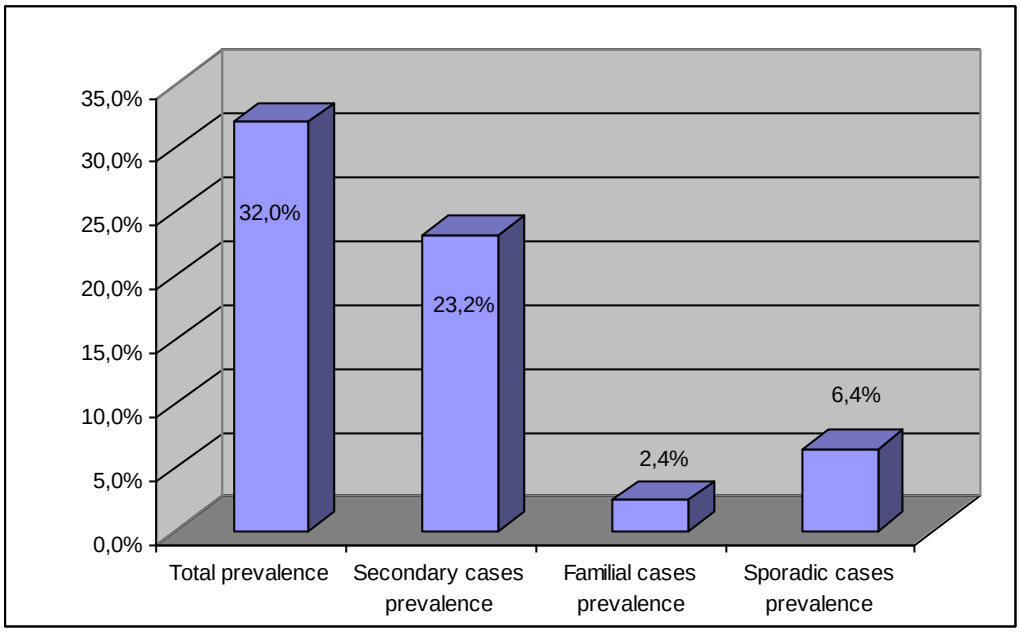
In total, we enrolled 765 MS patients (553 females and 212 males). The mean age was 36.5 ±9.5 years with average disease duration of 9.1 ±7.36 years. The median EDSS score was 2.0 (quartiles 1.5 and 3.5).

Out of all the examined patients, 76% had relapse-remitting MS, 14.4% had clinically isolated syndrome, 5.6% were in secondary progression and 0.9% had a primary progressive form of MS.

The diagnosis of RLS was confirmed in 245 subjects (32%, 95% CI 28.7–35.4%) with MS, mean age at onset of RLS symptoms was 29.1 ±10.4 years. In 49 patients (6.4%), RLS symptoms preceded the MS onset and 19 patients (2.4%) had a positive family history and RLS symptoms preceding the MS onset, and therefore were subsequently excluded from all genetic studies (Graph 1). In 177 patients (23.2%) RLS followed the MS onset, 520 patients (68%) never experienced RLS. The average delay between the onset of MS and that of RLS was 2.5 ±8.7 years.

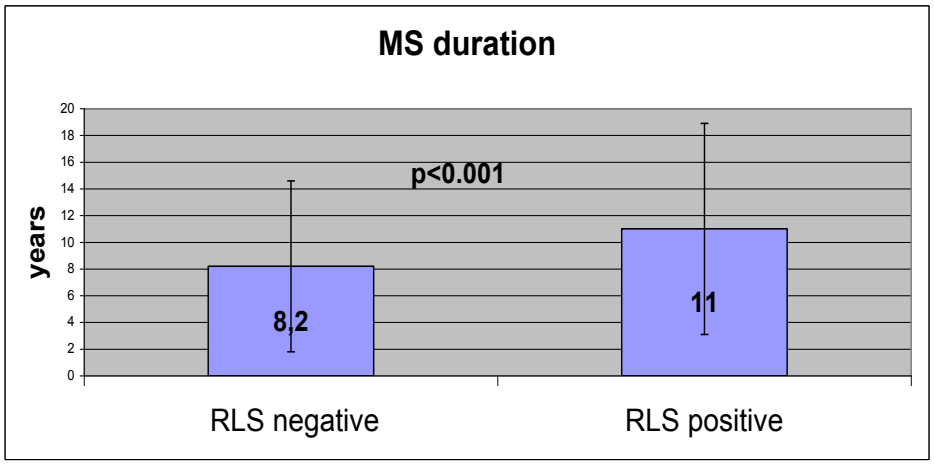
For individual subtypes of MS, using the same strict criteria (exclusion of patients with positive family history of RLS and onset of RLS symptoms before MS) we observed the following RLS prevalence: In relapse-remitting MS 39.6% (95% CI 35.36% to 43.84%), in clinically isolated syndrome 21.7% (95% CI 12.83% to 30.57%) and in secondary progressive MS 61.5% (95% CI 46.23% to 76.77%) (Graph 2).

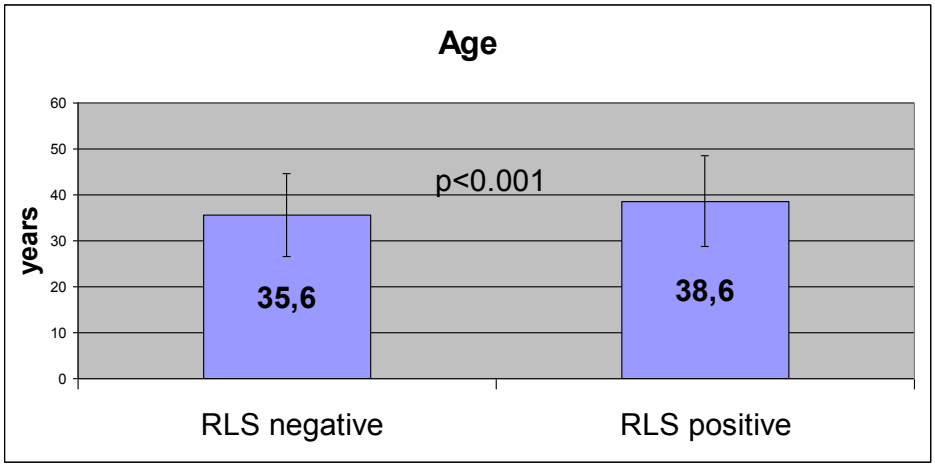
Compared to patients without RLS, patients suffering from both MS and RLS were significantly older (38.6 vs. 35.6 years, $p<0.001$, Students t-test), had longer durations of MS symptoms (11.0 vs. 8.2 years, $p<0.001$, Student's t-test) and had higher EDSS scores (2.9 vs. 2.3, $p<0.001$, Mann-Whitney test) (Graph 3–5). There were significantly more affected women in the RLS affected group (78% vs. 69%, $p= 0.0072$, χ^2).

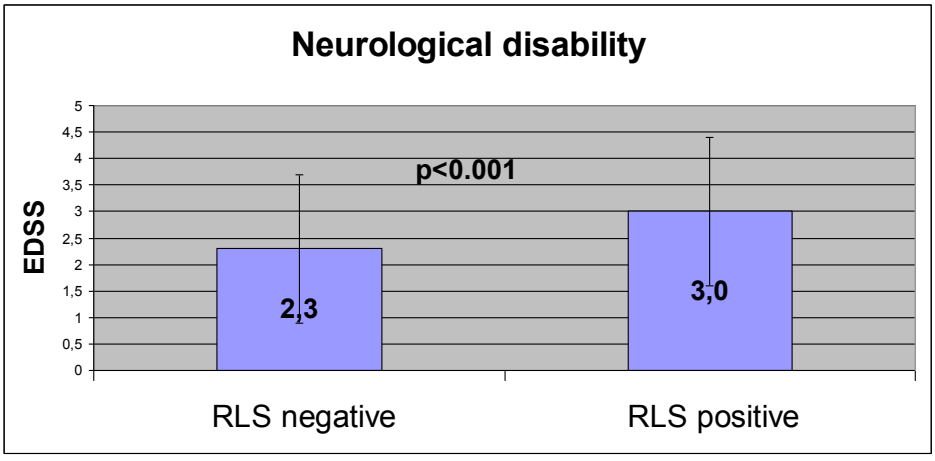


Graph 1: Prevalence RLS in MS patients

Graph 2: Prevalence of RLS in different clinical courses of MS, CIS – cilinical isolated syndrome, RR – relapse-remitting, SP – secondary progressive







Graphs 3–5: Risk factors for developing RLS in MS patients

Quantitative MRI data were obtained in 385 patients without RLS (mean age 38.3 ± 10.2 years, mean MS duration 12.0 ± 6.3 years, median EDSS score 2.6 ± 1.5) and 215

patients with RLS (mean age 41.4 ± 10.4 years, mean MS duration 14.5 ± 7.6 years, mean EDSS 3.2 ± 1.4).

We found no difference between the two groups in the whole brain lesion load, brain atrophy and brain parenchymal fraction (Table 1), despite the fact that we were able to replicate the correlation of these data with clinical parameters of MS (Table 2).

Table 1: Quantitative brain MRI data from MS-patients) without RLS (MS/RLS-) and with RLS (MS/RLS+)

	MS/RLS-	**MS/RLS+**	**t-value**	**p-value**
Age	38.318	41.426	-3.67371	0.00026
Lesion load – T2	6.555	6.579	-0.03019	0.975928
Brain parenchymal fraction	84.495	84.5	-0.02666	0.978737
Atrophy – T1 %	98.44	98.436	0.02306	0.981608

Table 2: Correlation of MRI data with clinical parameters of MS

	Lesion load – T2	**Brain parenchymal fraction**	**Brain atrophy**
Age	0.2786	-0.3131	0.1991
	p=.000	**p=.000**	**P=.007**
Age at RLS onset	0.1083	-0.1815	0.0758
	p=.144	**p=.014**	**P=.308**
MS duration	0.406	-0.3517	0.0851
	p=.000	**p=.000**	**P=.252**
EDSS	0.4331	-0.4308	0.0141
	p=.000	**p=.000**	**P=.850**

Genetic study

1) Replication in three populations:

All SNPs (Table 4) tested were in HWE ($p > 0.01$) in both patients and controls. Under assumption of genetic homogeneity, the combined sample had good power to detect association using previously published parameters (64) (98% for *MEIS1* and *BTBD9*, 89% for *MAP2K5/SCOR1*). In the Czech sample alone the power was 82.5% for *MEIS1* and *BTBD9*, 71.8% for *MAP2K5/SCOR1*; in the Austrian sample, the powers were 84.8% and 74.8%, respectively; and in the Finish sample separately 38.7% and 30.4%.

Allele frequencies in the Czech and KORA control samples were not significantly different (lowest P in χ^2 test = 0.2045 for rs4236060). Significant allele frequency differences were observed between the Finnish and the combined Czech and KORA control samples within *BTBD9* ($P < 7.67 \times 10^{-6}$ for all SNP markers within *BTBD9*). A similar, nominally significant, difference in allele frequencies in *BTBD9* markers was also observed between Finnish cases and combined Czech and Austrian cases (in χ^2 test lowest P = 0.01063 for rs9296249), but we did not observe a significant difference between allele frequencies of Czech and Austrian RLS patients (lowest P in χ^2 test was 0.4608 for rs2300478). Logistic regression showed no significant interaction with country for any SNP tested, and Breslow-Day test showed homogeneous ORs in all samples (Table 5).

Significant association after correction for multiple testing at significance level alpha = 5 % was found in at least one SNP for all tested loci in the combined samples (Table 3), and in the Czech and Austrian samples separately. Analyzing the Finnish sample, we confirmed only the association to *BTBD9*. The association to rs2300478 in *MEIS1* was only nominally significant and *MAP2K5/SCOR1* showed no association (Table 4).

In the combined sample we observed a strong association with the haplotype formed by markers rs6710341 and rs12469063, both located within *MEIS1*. Carriers of the "AG" haplotype had ORs for developing RLS of 1.98 ($P = 9.1 \times 10^{-10}$). Results for this haplotype were similar when testing the Czech ($P = 3.2\ 10^{-7}$, OR = 2.38), Austrian ($P = 8.3 \times 10^{-5}$, OR = 1.82), and Finnish samples ($P = 2.0 \times 10^{-4}$, OR = 2.46) separately. No other common polymorphic phased haplotypes (MHF > 1%) yielded significant results. An allele dosage model best described the association for *MEIS1* and *BTBD9*

(Armitage trend test). In contrast, a recessive model for the risk allele fitted best for the *MAP2K5/SCOR1* locus (Table 6).

Analyzing only familial cases (n = 217) and all controls, all three loci were significantly associated. Using sporadic cases only (n = 283), we would confirm the association to *BTBD9* but not to *MEIS1* and *MAP2K5/SCOR1*. We omitted patients of Finnish origin from this sub-analysis due to very low proportion of sporadic cases and different allele frequencies in these samples. The Breslow-Day test did not show significant heterogeneity between sporadic and familial cases.

Table 3: Genotyped SNPs and Results of Association in Combined Samples

The Genetic positions in bp and gene alignments are derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (89) r^2 – linkage disequilibrium relative to preceding marker, data were computed using genotypes observed in both cases and controls using Haploview 4.0 from HapMap project (http://www.hapmap.org, release 21a) (90) OR – Odds ratio for the risk allele (Cochran-Mantel-Haenszel test) with 95% confidence intervals, P nom – Logistic regression implementing Armitage trend test with country of origin, sex and age as covariates, P corr – adjusted P values for multiple testing, MAF – minor allele frequencies observed in combined Czech and Austrian sample, in sporadic and familial cases, Best model corresponds to model, under which lowest P values were observed (TREND – Armitage trend test, REC – recessive model), P corr Fam. – comparison of allele frequencies between familial cases and all controls, P corr Spor – comparison of allele frequencies between sporadic cases and all controls. † risk allele is the major allele.

Chr	**Gene**	**SNP ID**	**Genome**	**r^2**	**OR (95% Conf. Int)**	**P nom**	**P corr**	**MAF Fam.**	**MAF Spor.**	**Best model**	**P corr Fam.**	**P corr Spor.**
2p	*MEIS1*	rs6710341	66611926		0.84 (0.64-1.11)	0.30646	1	0.1270	0.1288	TREND	1	1
2p	*MEIS1*	rs12469063	66617812	0.413	1.43 (1.16-1.78)	4.15E-06	4.15E-05	0.3522	0.2727	TREND	2.24E-05	0.3245
2p	*MEIS1*	rs2300478	66634956	0.969	1.47 (1.18-1.82)	1.26E-06	1.26E-05	0.3575	0.2860	TREND	3.10E-05	0.1520
6p	*BTBD9*	rs9296249	38473818		1.59 (1.26-2.01) †	0.00011	0.00107	0.1694	0.1553	TREND	0.0544	0.0012
6p	*BTBD9*	rs3923809	38548947	0.512	1.58 (1.28-1.96) †	4.11E-06	4.11E-05	0.2204	0.2330	TREND	0.0018	0.0022
6p	*BTBD9*	rs4236060	38578315	0.829	1.49 (1.19-1.86) †	1.93E-05	0.00019	0.1882	0.2110	TREND	0.0008	0.0049
15q	MAP2K5	rs11635424	65824631		1.26 (1.02-1.55) †	0.00602	0.06023	0.2446	0.2992	REC	0.0203	1
15q	MAP2K5	rs3784709	65859328	0.935	1.24 (1.01-1.52) †	0.00530	0.05301	0.2392	0.2917	REC	0.0393	1
15q	MAP2K5	rs1026732	65882138	0.966	1.27 (1.03-1.56) †	0.00428	0.04278	0.2339	0.2936	REC	0.0116	1
15q	MAP2K5/ LBXCOR1	rs6494696	65890259	0.999	1.27 (1.03-1.56) †	0.00476	0.04764	0.2339	0.2936	REC	0.0108	1

Table 4: Analysis in individual populations

MAF – minor allele frequencies in each subsample in patients and healthy individuals, N – numbers of successfully genotyped individuals bypassing quality control criteria, Best P corr – P values corrected for multiple testing according to the full association model in Table 1, OR - Odds ratio and corresponding 95% confidence intervals.

	Czech Republic				**Austria**				**Finland**			
SNP ID	**MAF Cases N=276**	**MAF Controls N=412**	**Best P corr**	**OR (95% Conf. Int)**	**MAF Cases N=222**	**MAF Controls N=570**	**Best P corr**	**OR (95% Conf. Int)**	**MAF Cases N=88**	**MAF Controls N=246**	**Best P corr**	**OR (95% Conf. Int)**
rs6710341	0.1309	0.1456	1	1.13 (1.55-0.83)	0.1306	0.1412	1	0.91 (0.66-1.26)	0.1207	0.1585	1	0.73 (0.43-1.22)
rs12469063	0.2971	0.2172	0.0492	1.52 (1.19-1.95)	0.3108	0.2426	0.0064	1.41 (1.11-1.79)	0.3161	0.2439	0.6093	1.43 (0.98-2.10)
rs2300478	0.3025	0.2209	0.0285	1.53 (1.20-1.96)	0.3243	0.2487	0.0017	1.45 (1.14-1.84)	0.3276	0.2459	0.3676	1.49 (1.02-2.18)
rs9296249	0.1649	0.2306	0.0252	1.52 (1.15-2.00)	0.1644	0.2378	0.0116	1.59 (1.19-2.11)	0.2414	0.3516	0.1081	1.70 (1.15-2.53)
rs3923809	0.2301	0.2998	0.0374	1.43 (1.12-1.84)	0.223	0.3133	0.0049	1.59 (1.23-2.05)	0.2651	0.4119	0.0124	1.94 (1.32-2.87)
rs4236060	0.2047	0.2662	0.1903	1.41 (1.09-1.83)	0.1968	0.2891	0.0028	1.66 (1.27-2.17)	0.2674	0.3921	0.0497	1.77 (1.20-2.60)
rs11635424	0.2772	0.3350	0.0135	1.31 (1.04-1.66)	0.2793	0.3229	0.1014	1.23 (0.97-1.57)	0.3046	0.2866	1	1.09 (0.75-1.59)
rs3784709	0.2754	0.3289	0.0124	1.29 (1.02-1.63)	0.2725	0.3185	0.0522	1.25 (0.98-1.59)	0.3046	0.2744	1	1.16 (0.79-1.69)
rs1026732	0.2717	0.3350	0.0050	1.35 (1.07-1.71)	0.2748	0.322	0.0519	1.25 (0.98-1.60)	0.3046	0.2764	1	1.15 (0.79-1.67)
rs6494696	0.2717	0.3350	0.0050	1.35 (1.07-1.71)	0.2748	0.3229	0.0416	1.26 (0.99-1.60)	0.3046	0.2764	1	1.15 (0.79-1.67)

Table 5: Results of Cochran-Mantel-Haenszel test in all 3 populations

TEST – TOTAL represents all sources of variance, ASSOC association without assuming heterogeneity, HOMOG test for homogeneity, 1 Czech samples, 2 Austrian samples and 3 Finish samples. **CHISQ** – the actual value of chi-square statistic. **DF** – number of degrees of freedom, **P** – nominal P values. **OR** – Odds ratio.

CHR	SNP ID	TEST	CHISQ	DF	P	OR
2	rs6710341	TOTAL	1.9040	3	0.5926	NA
2	rs6710341	ASSOC	1.5190	1	0.2178	NA
2	rs6710341	HOMOG	0.3851	2	0.8248	NA
2	rs6710341	1	0.5741	1	0.4486	0.8858
2	rs6710341	2	0.2722	1	0.6018	0.9180
2	rs6710341	3	1.0580	1	0.3038	0.7537
2	rs12469063	TOTAL	21.5900	3	0.0001	NA
2	rs12469063	ASSOC	21.3600	1	0.0000	NA
2	rs12469063	HOMOG	0.2341	2	0.8895	NA
2	rs12469063	1	11.2100	1	0.0008	1.5230
2	rs12469063	2	7.7250	1	0.0054	1.4090
2	rs12469063	3	2.6610	1	0.1028	1.3990
2	rs2300478	TOTAL	24.1900	3	0.0000	NA
2	rs2300478	ASSOC	24.0900	1	0.0000	NA
2	rs2300478	HOMOG	0.1051	2	0.9488	NA
2	rs2300478	1	11.5800	1	0.0007	1.5300
2	rs2300478	2	9.2940	1	0.0023	1.4510
2	rs2300478	3	3.3170	1	0.0686	1.4510
6	rs9296249	TOTAL	26.3400	3	0.0000	NA
6	rs9296249	ASSOC	25.8700	1	0.0000	NA
6	rs9296249	HOMOG	0.4634	2	0.7932	NA
6	rs9296249	1	8.6440	1	0.0033	0.6604
6	rs9296249	2	9.8720	1	0.0017	0.6335
6	rs9296249	3	7.8210	1	0.0052	0.5564
6	rs3923809	TOTAL	32.1700	3	0.0000	NA
6	rs3923809	ASSOC	30.0900	1	0.0000	NA
6	rs3923809	HOMOG	2.0840	2	0.3528	NA
6	rs3923809	1	8.0210	1	0.0046	0.6992
6	rs3923809	2	12.4900	1	0.0004	0.6309

6	rs3923809	3	11.6600	1	0.0006	0.4925
6	rs4236060	TOTAL	27.7400	3	0.0000	NA
6	rs4236060	ASSOC	26.6300	1	0.0000	NA
6	rs4236060	HOMOG	1.1150	2	0.5726	NA
6	rs4236060	1	6.6790	1	0.0098	0.7106
6	rs4236060	2	13.6700	1	0.0002	0.6047
6	rs4236060	3	7.3950	1	0.0065	0.5700
15	rs11635424	TOTAL	8.3850	3	0.0387	NA
15	rs11635424	ASSOC	5.2620	1	0.0218	NA
15	rs11635424	HOMOG	3.1240	2	0.2098	NA
15	rs11635424	1	5.1000	1	0.0239	0.7622
15	rs11635424	2	2.7930	1	0.0947	0.8141
15	rs11635424	3	0.4924	1	0.4829	1.1540
15	rs3784709	TOTAL	8.3920	3	0.0386	NA
15	rs3784709	ASSOC	4.7050	1	0.0301	NA
15	rs3784709	HOMOG	3.6870	2	0.1582	NA
15	rs3784709	1	4.4100	1	0.0357	0.7763
15	rs3784709	2	3.1370	1	0.0766	0.8031
15	rs3784709	3	0.8455	1	0.3578	1.2080
15	rs1026732	TOTAL	10.4000	3	0.0154	NA
15	rs1026732	ASSOC	5.8410	1	0.0157	NA
15	rs1026732	HOMOG	4.5610	2	0.1022	NA
15	rs1026732	1	6.1260	1	0.0133	0.7417
15	rs1026732	2	3.2910	1	0.0697	0.7993
15	rs1026732	3	0.9856	1	0.3208	1.2260
15	rs6494696	TOTAL	10.5200	3	0.0146	NA
15	rs6494696	ASSOC	5.9440	1	0.0148	NA
15	rs6494696	HOMOG	4.5780	2	0.1014	NA
15	rs6494696	1	6.1260	1	0.0133	0.7417
15	rs6494696	2	3.4100	1	0.0648	0.7961
15	rs6494696	3	0.9856	1	0.3208	1.2260

Table 6: All tested model based statistics in the combined sample

TEST TREND – Armitage trend test, REC – recessive model, DOM – dominant model, ALLELIC– allelic test (double numbers, individual alleles treated separately), GENO – genotypic test comparing both homozygotes and heterozygotes individually, with 2 degrees of freedom, AFF – distribution for the given test in affected patients, UNAFF – distribution in controls, CHISQ **–** the actual value of chi-square statistic. DF – number of degrees of freedom, P – nominal P values.

CHR	SNP	TEST	AFF	UNAFF	CHISQ	DF	P
2	rs6710341	GENO	4/64/207	11/98/303	NA	NA	NA
2	rs6710341	TREND	72/478	120/704	0.5835	1	0.4449
2	rs6710341	ALLELIC	72/478	120/704	0.5947	1	0.4406
2	rs6710341	DOM	68/207	109/303	NA	NA	NA
2	rs6710341	REC	4/271	11/401	NA	NA	NA
2	rs12469063	GENO	22/120/134	18/143/251	11.1000	2	0.0031
2	rs12469063	TREND	164/388	179/645	11.5100	1	0.0007
2	rs12469063	ALLELIC	164/388	179/645	11.2700	1	0.0008
2	rs12469063	DOM	142/134	161/251	10.2600	1	0.0014
2	rs12469063	REC	22/254	18/394	3.9160	1	0.0478
2	rs2300478	GENO	22/123/131	18/146/248	12.7000	2	0.0024
2	rs2300478	TREND	167/385	182/642	12.4000	1	0.0005
2	rs2300478	ALLELIC	167/385	182/642	11.6400	1	0.0006
2	rs2300478	DOM	145/131	164/248	10.8300	1	0.0010
2	rs2300478	REC	22/254	18/394	3.9160	1	0.0478
6	rs9296249	GENO	8/75/193	22/146/244	8.7540	2	0.0126
6	rs9296249	TREND	91/461	190/634	8.6860	1	0.0032
6	rs9296249	ALLELIC	91/461	190/634	8.7870	1	0.0030
6	rs9296249	DOM	83/193	168/244	8.1720	1	0.0043
6	rs9296249	REC	8/268	22/390	2.3620	1	0.1243
6	rs3923809	GENO	16/95/165	44/159/209	7.7900	2	0.0204
6	rs3923809	TREND	127/425	247/577	7.5980	1	0.0058
6	rs3923809	ALLELIC	127/425	247/577	8.1100	1	0.0044
6	rs3923809	DOM	111/165	203/209	5.4620	1	0.0194
6	rs3923809	REC	16/260	44/368	4.9500	1	0.0261

6	rs4236060	GENO	13/87/176	30/153/217	6.6260	2	0.0364
6	rs4236060	TREND	113/439	213/587	6.5640	1	0.0104
6	rs4236060	ALLELIC	113/439	213/587	6.7600	1	0.0093
6	rs4236060	DOM	100/176	183/217	6.0790	1	0.0137
6	rs4236060	REC	13/263	30/370	2.1340	1	0.1441
15	rs11635424	GENO	28/97/151	48/180/184	6.7650	2	0.0340
15	rs11635424	TREND	153/399	276/548	4.8430	1	0.0278
15	rs11635424	ALLELIC	153/399	276/548	5.1430	1	0.0233
15	rs11635424	DOM	125/151	228/184	6.6820	1	0.0097
15	rs11635424	REC	28/248	48/364	0.3813	1	0.5369
15	rs3784709	GENO	28/96/152	46/179/187	6.4100	2	0.0406
15	rs3784709	TREND	152/400	271/553	4.1900	1	0.0407
15	rs3784709	ALLELIC	152/400	271/553	4.4470	1	0.0350
15	rs3784709	DOM	124/152	225/187	6.2010	1	0.0128
15	rs3784709	REC	28/248	46/366	0.1792	1	0.6721
15	rs1026732	GENO	28/94/154	48/180/184	8.3620	2	0.0153
15	rs1026732	TREND	150/402	276/548	5.7840	1	0.0162
15	rs1026732	ALLELIC	150/402	276/548	6.1800	1	0.0129
15	rs1026732	DOM	122/154	228/184	8.2020	1	0.0042
15	rs1026732	REC	28/248	48/364	0.3813	1	0.5369
15	rs6494696	GENO	28/94/154	48/180/184	8.3620	2	0.0153
15	rs6494696	TREND	150/402	276/548	5.7840	1	0.0162
15	rs6494696	ALLELIC	150/402	276/548	6.1800	1	0.0129
15	rs6494696	DOM	122/154	228/184	8.2020	1	0.0042
15	rs6494696	REC	28/248	48/364	0.3813	1	0.5369

2) Genetics of secondary RLS form in patients with multiple sclerosis

A. Testing of MS patients positive for RLS versus MS patients negative for RLS

All SNPs tested were in HWE (p>0.01) in both patients and controls. One of the tested SNP failed to by-pass genotyping criteria (rs4236060 at *BTBD9*). After excluding patients with probably idiopathic RLS, the power for *MEIS1* and *BTBD* remained sufficient – 89.6 and 85.2 respectively. For *PTPRD*, the power was below 50%, for *MAP2K5/SCOR1* it was below 70%.

No significant association with *MEIS 1*, *BTBD9* and *PTPRD* was found in 203 patients with multiple sclerosis. There was a trend for association with *MAP2K5/SCOR1* – the best model for the risk allele was the recessive model (p nominal = 0.0029, p permutated after correction = 0.0248, p nominal corrected for 4 loci and 2 models, i.e. 8 tests = 0.029, odds ratio = 1.60 – 95% CI 1.17 – 2.18). Thus, the one sided p value with the direction of the alternative hypothesis given by the original report is p corrected 0.019. Results for all tested loci are summarized in Table 7, 8.

Finally, we did the association analysis only in relapse-remitting MS form in order to distinguish the MS subtypes. We included 192 MS patients with RLS and 373 MS patients without RLS in the analysis after excluding patients who had a family history and RLS symptoms before MS onset.

The results show the same trend for association as when using patients with all MS forms, but due to the lower sample size the significance is lower and does not bypass correction for multiple testing (Table 7).

B. Testing of MS patients versus population controls and idiopathic RLS patients

When testing MS patients negative for RLS versus population controls and idiopathic RLS, one SNP was not available in previously genotyped population controls (rs11788684 from *PTPRD*). Otherwise, no significant differences were found within the remaining tested SNP. Maximal observed χ^2 statistics was 1.9 at rs4626664 on chromosome 9, all other values were below 1.0. The sample of 438 MS patients without RLS symptoms and 450 population controls had 80% power to detect association with

MS (assuming prevalence of 0.001) with OR over 1.58 (*MAP2K5/SCOR1*) (Table 10 and 13).

When testing RLS MS patients positive for RLS vs. idiopathic RLS patients, the allele frequencies were very similar for *MAP2K5/SCOR1* and *PTPRD* markers - maximal observed χ^2 statistics was 0,4. However, idiopathic RLS patients in variants in *MEIS 1* and *BTBD9* genes present with different allele frequencies, but this contrast is only nominally significant (Table 9 and 12).

The last performed comparison was of MS patients positive for RLS versus population controls shows similar results and in the same directions, as when comparing to MS patients negative for RLS. However the statistical significance is lower, because the blood donor were not screened for presence of RLS and have different sex and age distribution. (Table 11 and 14)

Table 7 Results of genetic association study

Genome – The Genetic positions in bp derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (89) **, OR best model –** Odds-ratio according to best model in original locus description (Allelic for TREND, Allele negativity for REC) including 95% confidence interval. **MAF MS+RLS+ -** minor allele frequency in MS patients with RLS symptoms, **MAF MS+RLS- -** minor allele frequency in MS patients without RLS symptoms, **MAF controls –** minor allele frequency in unscreened population sample of blood donors. **Best model –** Best model corresponds to the model under which the lowest P values were observed (TREND – Armitage trend test, REC – recessive model) in the original and replication publications. (1) **P-nom model –** raw nominal p-values observed under the best model, **P nom allelic** – comparison of allele frequencies between MS+ RLS+ and MS+RLS- patients. **P nom Model RR-MS –** raw nominal p-values observed under the best model using relapse-remitting MS patients. All p-values shown are 2-sided. † risk allele is the major allele.

Chr	Gene	SNP ID	Genome	OR best model (95% Conf. Int)	MAF MS+ RLS+	MAF MS+ RLS-	MAF controls	Best model	P nom Model	P nom Allelic	P nom Model RR-MS
2p	*MEIS1*	Rs6710341	66611926	1.19 (0.86 - 1.64)	0.1533	0.1323	0.1407	TREND	0.4552	0.2954	0.3861
2p	*MEIS1*	rs12469063	66617812	1.12 (0.86 - 1.45)	0.2588	0.2384	0.2194	TREND	0.3887	0.4128	0.4660
2p	*MEIS1*	Rs2300478	66634956	1.13 (0.87 - 1.47)	0.2622	0.2396	0.2229	TREND	0.3767	0.3668	0.4000
6p	*BTBD9*	Rs9296249	38473818	1.14 (0.86 - 1.5) †	0.2102	0.2326	0.2361	TREND	0.1519	0.3541	0.3084
6p	*BTBD9*	Rs3923809	38548947	1.03 (0.8 - 1.32) †	0.2978	0.3037	0.3060	TREND	0.5883	0.8235	0.7501
9p	*PTPRD*	rs11788684	8846420	1.01 (0.73 - 1.4)	0.1422	0.1407	NA	TREND	0.5714	0.9400	0.3782
9p	*PTPRD*	Rs4626664	9261737	1.15 (0.84 - 1.58) †	0.1467	0.1655	0.1409	TREND	0.4507	0.3762	0.8330
15q	MAP2K5	rs11635424	65824631	1.53 (1.12 - 2.08) †	0.2788	0.3341	0.3349	REC	0.0070	0.0402	0.0355
15q	MAP2K5	Rs3784709	65859328	1.60 (1.17 - 2.18) †	0.2765	0.3345	0.3291	REC	0.0029	0.0316	0.0167
15q	MAP2K5	Rs1026732	65882138	1.54 (1.13 - 2.10) †	0.2753	0.3314	0.3349	REC	0.0059	0.0367	0.0272
15q	MAP2K5/ *SCOR1*	Rs6494696	65890259	1.56 (1.15 - 2.13) †	0.2765	0.3329	0.3356	REC	0.0045	0.0361	0.0253

Table 8: Results of model based association in MS patients RLS + vs. RLS –

TEST TREND – Armitage trend test, REC – recessive model, DOM – dominant model, ALLELIC– allelic test (double numbers, individual alleles treated separately), GENO – genotypic test comparing both homozygotes and heterozygotes individually, with 2 degrees of freedom, **AFF** – distribution for the given test in MS+RLS+ patients, **UNAFF** – distribution in MS+RLS-**, CHISQ** – the actual value of chi-square statistic. **DF** – number of degrees of freedom, **P** – nominal P values.

CHR	SNP	TEST	AFF	UNAFF	CHISQ	DF	P
2	rs6710341	GENO	9/51/165	15/84/332	1.101	2	0.5767
2	rs6710341	TREND	69/381	114/748	0.9581	1	0.3277
2	rs6710341	ALLELIC	69/381	114/748	1.095	1	0.2954
2	rs6710341	DOM	60/165	99/332	1.1	1	0.2942
2	rs6710341	REC	9/216	15/416	0.1133	1	0.7364
2	rs12469063	GENO	16/85/125	23/159/248	0.9082	2	0.635
2	rs12469063	TREND	117/335	205/655	0.6737	1	0.4118
2	rs12469063	ALLELIC	117/335	205/655	0.6709	1	0.4128
2	rs12469063	DOM	101/125	182/248	0.3377	1	0.5612
2	rs12469063	REC	16/210	23/407	0.7937	1	0.373
2	rs2300478	GENO	17/84/124	23/161/248	1.348	2	0.5097
2	rs2300478	TREND	118/332	207/657	0.8159	1	0.3664
2	rs2300478	ALLELIC	118/332	207/657	0.8146	1	0.3668
2	rs2300478	DOM	101/124	184/248	0.3176	1	0.573
2	rs2300478	REC	17/208	23/409	1.288	1	0.2563
2	rs11683508	GENO	6/70/149	15/128/286	0.4001	2	0.8187
2	rs11683508	TREND	82/368	158/700	0.007393	1	0.9315
2	rs11683508	ALLELIC	82/368	158/700	0.007316	1	0.9318
2	rs11683508	DOM	76/149	143/286	0.01309	1	0.9089
2	rs11683508	REC	6/219	15/414	0.327	1	0.5674
6	rs9296249	GENO	10/75/141	32/137/263	2.223	2	0.3291
6	rs9296249	TREND	95/357	201/663	0.7982	1	0.3716
6	rs9296249	ALLELIC	95/357	201/663	0.8588	1	0.3541
6	rs9296249	DOM	85/141	169/263	0.1427	1	0.7056
6	rs9296249	REC	10/216	32/400	2.209	1	0.1372
6	rs3923809	GENO	19/96/110	46/168/214	1.248	2	0.5358
6	rs3923809	TREND	134/316	260/596	0.04781	1	0.8269
6	rs3923809	ALLELIC	134/316	260/596	0.04974	1	0.8235
6	rs3923809	DOM	115/110	214/214	0.07283	1	0.7873
6	rs3923809	REC	19/206	46/382	0.8728	1	0.3502

9	rs11788684	GENO	6/52/167	7/107/316	1.005	2	0.605
9	rs11788684	TREND	64/386	121/739	0.005666	1	0.94
9	rs11788684	ALLELIC	64/386	121/739	0.005662	1	0.94
9	rs11788684	DOM	58/167	114/316	0.04108	1	0.8394
9	rs11788684	REC	6/219	7/423	0.8193	1	0.3654
9	rs4626664	GENO	5/56/164	11/120/298	0.8369	2	0.6581
9	rs4626664	TREND	66/384	142/716	0.7878	1	0.3748
9	rs4626664	ALLELIC	66/384	142/716	0.783	1	0.3762
9	rs4626664	DOM	61/164	131/298	0.8348	1	0.3609
9	rs4626664	REC	5/220	11/418	0.07228	1	0.788
15	rs11635424	GENO	20/86/120	39/210/182	7.565	2	0.02277
15	rs11635424	TREND	126/326	288/574	4.402	1	0.0359
15	rs11635424	ALLELIC	126/326	288/574	4.209	1	0.04021
15	rs11635424	DOM	106/120	249/182	7.053	1	0.007913
15	rs11635424	REC	20/206	39/392	0.007195	1	0.9324
15	rs3784709	GENO	20/85/121	38/213/181	8.863	2	0.0119
15	rs3784709	TREND	125/327	289/575	4.865	1	0.02741
15	rs3784709	ALLELIC	125/327	289/575	4.621	1	0.03159
15	rs3784709	DOM	105/121	251/181	8.098	1	0.004431
15	rs3784709	REC	20/206	38/394	0.000524	1	0.9817
15	rs1026732	GENO	20/85/122	39/209/185	7.81	2	0.02014
15	rs1026732	TREND	125/329	287/579	4.532	1	0.03326
15	rs1026732	ALLELIC	125/329	287/579	4.363	1	0.03673
15	rs1026732	DOM	105/122	248/185	7.269	1	0.007017
15	rs1026732	REC	20/207	39/394	0.007054	1	0.9331
15	rs6494696	GENO	20/85/121	39/211/184	8.011	2	0.01821
15	rs6494696	TREND	125/327	289/579	4.583	1	0.03229
15	rs6494696	ALLELIC	125/327	289/579	4.392	1	0.03611
15	rs6494696	DOM	105/121	250/184	7.424	1	0.006435
15	rs6494696	REC	20/206	39/395	0.003407	1	0.9535

Table 9: Comparison of secondary RLS in MS vs. idiopathic RLS patients, allelic association

BP – The Genetic positions in bp derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (89), **MAF MS+RLS+ -** minor allele frequency in MS patients with RLS symptoms, **MAF RLS- -** minor allele frequency in idiopathic patients, Czech sample, **CHISQ** – the actual value of chi-square statistic. **P** – nominal P values, **OR –** Odds-ratio according to allelic test.

CHR	SNP	BP	MAF MS+RLS+	MAF RLS	CHISQ	P	OR
2	Rs6710341	66611926	0.1553	0.1295	2.189	0.139	1.236
2	Rs12469063	66617812	0.2594	0.3061	4.144	0.04178	0.7939
2	Rs2300478	66634957	0.2628	0.3152	5.126	0.02357	0.7746
6	Rs9296249	38473819	0.2	0.1754	1.585	0.2081	1.175
6	Rs3923809	38548948	0.2799	0.2329	4.612	0.03174	1.28
9	Rs4626664	9251737	0.1565	0.1689	0.443	0.5057	0.9125
15	Rs11635424	65824632	0.284	0.281	0.01777	0.8939	1.015
15	Rs3784709	65859329	0.2772	0.2776	0.00028	0.9867	0.9981
15	Rs1026732	65882139	0.2787	0.2771	0.005017	0.9435	1.008
15	Rs6494696	65890260	0.2789	0.2767	0.009224	0.9235	1.011

Table 10: Comparison of MS patients without RLS vs. population controls, allelic association

BP – The Genetic positions in bp derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (89), **MAF MS+RLS- -** minor allele frequency in MS patients without RLS symptoms, **MAF Controls- -** minor allele frequency in population based controls (blood donors), **CHISQ** – the actual value of chi-square statistic. **P** – nominal P values**.**

CHR	SNP	BP	MAF MS+RLS-	MAF Controls	CHISQ	P
2	rs6710341	66611926	0.1331	0.147	0.6801	0.4096
2	rs12469063	66617812	0.2392	0.2194	0.9403	0.3322
2	rs2300478	66634957	0.2404	0.2229	0.7379	0.3903
6	rs9296249	38473819	0.2368	0.2361	0.0013	0.9717
6	rs3923809	38548948	0.3104	0.306	0.0381	0.8452
9	rs4626664	9251737	0.1651	0.1409	1.9150	0.1664
15	rs11635424	65824632	0.3381	0.3349	0.0202	0.8870
15	rs3784709	65859329	0.3385	0.3291	0.1697	0.6804
15	rs1026732	65882139	0.3353	0.3349	0.0004	0.9843
15	rs6494696	65890260	0.3369	0.3356	0.0030	0.9562

Table 11: Comparison of MS patients with RLS vs. population controls, allelic association

BP – The Genetic positions in bp derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006) (89) **, MAF MS+RLS+ –** minor allele frequency in MS patients with RLS symptoms, **MAF Controls- –** minor allele frequency in population based controls (blood donors), **CHISQ** – the actual value of chi-square statistic, **P** – nominal P values, **OR –** Odds-ratio according to allelic test

CHR	SNP	BP	MS+RLS+	Controls	CHISQ	P	OR
2	rs6710341	66611926	0.1553	0.147	0.1883	0.6644	1.0670
2	rs12469063	66617812	0.2594	0.2194	3.103	0.0781	1.2460
2	rs2300478	66634957	0.2628	0.2229	3.065	0.0800	1.2430
6	rs9296249	38473819	0.2	0.2361	2.652	0.1034	0.8088
6	rs3923809	38548948	0.2799	0.306	1.147	0.2841	0.8814
9	rs4626664	9251737	0.1565	0.1409	0.6777	0.4104	1.1310
15	rs11635424	65824632	0.284	0.3349	4.203	0.0404	0.7879
15	rs3784709	65859329	0.2772	0.3291	4.423	0.0355	0.7819
15	rs1026732	65882139	0.2787	0.3349	5.165	0.0230	0.7675
15	rs6494696	65890260	0.2789	0.3356	5.241	0.0221	0.7656

Table 12: Comparison of secondary RLS in MS vs. idiopathic RLS patients, genotypic (model based) association

TEST TREND – Armitage trend test, REC – recessive model, DOM – dominant model, ALLELIC – allelic test (double numbers, individual alleles treated separately), GENO – genotypic test comparing both homozygotes and heterozygotes individually, with 2 degrees of freedom, **MS+RLS+ –** test distribution in MS patients with RLS symptoms, **RLS- –** test distribution in idiopathic patients, Czech sample –**, CHISQ** – the actual value of chi-square statistic. **DF** – number of degrees of freedom, **P** – nominal P values.

CHR	SNP	TEST	MS+RLS+	RLS	CHISQ	P
2	rs6710341	GENO	13/65/215	12/128/447	4.1500	0.1255
2	rs6710341	TREND	91/495	152/1022	2.0300	0.1542
2	rs6710341	ALLELIC	91/495	152/1022	2.1890	0.1390
2	rs6710341	DOM	78/215	140/447	0.8053	0.3695
2	rs6710341	REC	13/280	12/575	4.0530	0.0441
2	rs12469063	GENO	20/112/161	56/248/284	4.1090	0.1282
2	rs12469063	TREND	152/434	360/816	4.1080	0.0427
2	rs12469063	ALLELIC	152/434	360/816	4.1440	0.0418
2	rs12469063	DOM	132/161	304/284	3.4590	0.0629
2	rs12469063	REC	20/273	56/532	1.8060	0.1790
2	rs2300478	GENO	21/112/160	59/252/276	5.1060	0.0779
2	rs2300478	TREND	154/432	370/804	5.0710	0.0243
2	rs2300478	ALLELIC	154/432	370/804	5.1260	0.0236
2	rs2300478	DOM	133/160	311/276	4.5030	0.0338
2	rs2300478	REC	21/272	59/528	1.9670	0.1608
6	rs9296249	GENO	14/90/191	18/171/401	2.0230	0.3638
6	rs9296249	TREND	118/472	207/973	1.5590	0.2117
6	rs9296249	ALLELIC	118/472	207/973	1.5850	0.2081
6	rs9296249	DOM	104/191	189/401	0.9209	0.3372
6	rs9296249	REC	14/281	18/572	1.6210	0.2029
6	rs3923809	GENO	24/116/153	31/210/343	4.7420	0.0934
6	rs3923809	TREND	164/422	272/896	4.5900	0.0322
6	rs3923809	ALLELIC	164/422	272/896	4.6120	0.0317
6	rs3923809	DOM	140/153	241/343	3.3700	0.0664

6	rs3923809	REC	24/269	31/553	2.7590	0.0967
9	rs4626664	GENO	9/74/211	22/155/412	0.4450	0.8005
9	rs4626664	TREND	92/496	199/979	0.4188	0.5175
9	rs4626664	ALLELIC	92/496	199/979	0.4430	0.5057
9	rs4626664	DOM	83/211	177/412	0.3126	0.5761
9	rs4626664	REC	9/285	22/567	0.2629	0.6081
15	rs11635424	GENO	25/117/152	57/217/315	0.8609	0.6502
15	rs11635424	TREND	167/421	331/847	0.0167	0.8973
15	rs11635424	ALLELIC	167/421	331/847	0.0178	0.8939
15	rs11635424	DOM	142/152	274/315	0.2493	0.6176
15	rs11635424	REC	25/269	57/532	0.3209	0.5711
15	rs3784709	GENO	25/113/156	59/209/321	1.0160	0.6018
15	rs3784709	TREND	163/425	327/851	0.0003	0.9872
15	rs3784709	ALLELIC	163/425	327/851	0.0003	0.9867
15	rs3784709	DOM	138/156	268/321	0.1632	0.6862
15	rs3784709	REC	25/269	59/530	0.5219	0.4700
15	rs1026732	GENO	25/115/156	58/211/321	1.0210	0.6003
15	rs1026732	TREND	165/427	327/853	0.0046	0.9457
15	rs1026732	ALLELIC	165/427	327/853	0.0050	0.9435
15	rs1026732	DOM	140/156	269/321	0.2303	0.6313
15	rs1026732	REC	25/271	58/532	0.4451	0.5047
15	rs6494696	GENO	25/114/155	58/210/321	1.0130	0.6027
15	rs6494696	TREND	164/424	326/852	0.0085	0.9265
15	rs6494696	ALLELIC	164/424	326/852	0.0092	0.9235
15	rs6494696	DOM	139/155	268/321	0.2495	0.6174
15	rs6494696	REC	25/269	58/531	0.4158	0.5190

Table 13: Comparison of MS patients without RLS vs. population controls, genotypic (model based) association

TEST TREND – Armitage trend test, REC – recessive model, DOM – dominant model, ALLELIC – allelic test (double numbers, individual alleles treated separately), GENO – genotypic test comparing both homozygotes and heterozygotes individually, with 2 degrees of freedom, **MS+RLS-** – test distribution in MS patients without RLS symptoms, Controls- – test distribution in population controls (blood donors), **CHISQ** – the actual value of chi-square statistic. **DF**– number of degrees of freedom, **P** – nominal P values.

CHR	SNP	TEST	MS+RLS-	Controls	CHISQ	P
2	rs6710341	GENO	14/83/320	11/105/316	2.6950	0.2598
2	rs6710341	TREND	111/723	127/737	0.6289	0.4278
2	rs6710341	ALLELIC	111/723	127/737	0.6801	0.4096
2	rs6710341	DOM	97/320	116/316	1.4550	0.2277
2	rs6710341	REC	14/403	11/421	0.4883	0.4847
2	rs12469063	GENO	22/155/239	19/152/262	0.9647	0.6173
2	rs12469063	TREND	199/633	190/676	0.9632	0.3264
2	rs12469063	ALLELIC	199/633	190/676	0.9403	0.3322
2	rs12469063	DOM	177/239	171/262	0.8193	0.3654
2	rs12469063	REC	22/394	19/414	0.3743	0.5407
2	rs2300478	GENO	22/157/239	19/155/259	0.7714	0.6800
2	rs2300478	TREND	201/635	193/673	0.7610	0.3830
2	rs2300478	ALLELIC	201/635	193/673	0.7379	0.3903
2	rs2300478	DOM	179/239	174/259	0.6098	0.4349
2	rs2300478	REC	22/396	19/414	0.3552	0.5512
6	rs9296249	GENO	31/136/251	24/156/252	2.0330	0.3619
6	rs9296249	TREND	198/638	204/660	0.0012	0.9724
6	rs9296249	ALLELIC	198/638	204/660	0.0013	0.9717
6	rs9296249	DOM	167/251	180/252	0.2585	0.6112
6	rs9296249	REC	31/387	24/408	1.2150	0.2703
6	rs3923809	GENO	46/165/203	48/169/216	0.0676	0.9668
6	rs3923809	TREND	257/571	265/601	0.0355	0.8506
6	rs3923809	ALLELIC	257/571	265/601	0.0381	0.8452

6	rs3923809	DOM	211/203	217/216	0.0613	0.8045
6	rs3923809	REC	46/368	48/385	0.0001	0.9905
9	rs4626664	GENO	11/115/289	10/102/321	2.1240	0.3458
9	rs4626664	TREND	137/693	122/744	1.8940	0.1687
9	rs4626664	ALLELIC	137/693	122/744	1.9150	0.1664
9	rs4626664	DOM	126/289	112/321	2.1210	0.1453
9	rs4626664	REC	11/404	10/423	0.1021	0.7493
15	rs11635424	GENO	38/206/173	50/190/193	3.0760	0.2148
15	rs11635424	TREND	282/552	290/576	0.0211	0.8845
15	rs11635424	ALLELIC	282/552	290/576	0.0202	0.8870
15	rs11635424	DOM	244/173	240/193	0.8251	0.3637
15	rs11635424	REC	38/379	50/383	1.3570	0.2441
15	rs3784709	GENO	37/209/172	48/189/196	3.7310	0.1549
15	rs3784709	TREND	283/553	285/581	0.1789	0.6723
15	rs3784709	ALLELIC	283/553	285/581	0.1697	0.6804
15	rs3784709	DOM	246/172	237/196	1.4690	0.2255
15	rs3784709	REC	37/381	48/385	1.1800	0.2773
15	rs1026732	GENO	38/205/176	50/190/193	2.7600	0.2516
15	rs1026732	TREND	281/557	290/576	0.0004	0.9840
15	rs1026732	ALLELIC	281/557	290/576	0.0004	0.9843
15	rs1026732	DOM	243/176	240/193	0.5719	0.4495
15	rs1026732	REC	38/381	50/383	1.4120	0.2347
15	rs6494696	GENO	38/207/175	50/190/192	2.9830	0.2250
15	rs6494696	TREND	283/557	290/574	0.0032	0.9552
15	rs6494696	ALLELIC	283/557	290/574	0.0030	0.9562
15	rs6494696	DOM	245/175	240/192	0.6701	0.4130
15	rs6494696	REC	38/382	50/382	1.4680	0.2257

Table 14: Comparison of MS patients with RLS vs. population controls, genotypic (model based) association

TEST TREND – Armitage trend test, REC – recessive model, DOM – dominant model, ALLELIC – allelic test (double numbers, individual alleles treated separately), GENO – genotypic test comparing both homozygotes and heterozygotes individually, with 2 degrees of freedom, **MS+RLS+** – test distribution in MS patients with RLS symptoms, Controls**- –** test distribution in population controls (blood donors), **CHISQ** – the actual value of chi-square statistic. **DF** – number of degrees of freedom, **P** – nominal P values.

CHR	SNP	TEST	MS+RLS+	Controls	CHISQ	P
2	rs6710341	GENO	13/65/215	11/105/316	2.2210	0.3293
2	rs6710341	TREND	91/495	127/737	0.1740	0.6766
2	rs6710341	ALLELIC	91/495	127/737	0.1883	0.6644
2	rs6710341	DOM	78/215	116/316	0.0047	0.9451
2	rs6710341	REC	13/280	11/421	1.9500	0.1626
2	rs12469063	GENO	20/112/161	19/152/262	3.3290	0.1893
2	rs12469063	TREND	152/434	190/676	3.1340	0.0767
2	rs12469063	ALLELIC	152/434	190/676	3.1030	0.0781
2	rs12469063	DOM	132/161	171/262	2.2210	0.1361
2	rs12469063	REC	20/273	19/414	2.0430	0.1529
2	rs2300478	GENO	21/112/160	19/155/259	3.5510	0.1694
2	rs2300478	TREND	154/432	193/673	3.0990	0.0783
2	rs2300478	ALLELIC	154/432	193/673	3.0650	0.0800
2	rs2300478	DOM	133/160	174/259	1.9420	0.1635
2	rs2300478	REC	21/272	19/414	2.5930	0.1074
6	rs9296249	GENO	14/90/191	24/156/252	3.0290	0.2199
6	rs9296249	TREND	118/472	204/660	2.6030	0.1067
6	rs9296249	ALLELIC	118/472	204/660	2.6520	0.1034
6	rs9296249	DOM	104/191	180/252	3.0280	0.0818
6	rs9296249	REC	14/281	24/408	0.2321	0.6300
6	rs3923809	GENO	24/116/153	48/169/216	1.6770	0.4323
6	rs3923809	TREND	164/422	265/601	1.0850	0.2975
6	rs3923809	ALLELIC	164/422	265/601	1.1470	0.2841
6	rs3923809	DOM	140/153	217/216	0.3809	0.5371

6	rs3923809	REC	24/269	48/385	1.6390	0.2005
9	rs4626664	GENO	9/74/211	10/102/321	0.7008	0.7044
9	rs4626664	TREND	92/496	122/744	0.6544	0.4186
9	rs4626664	ALLELIC	92/496	122/744	0.6777	0.4104
9	rs4626664	DOM	83/211	112/321	0.4991	0.4799
9	rs4626664	REC	9/285	10/423	0.3888	0.5329
15	rs11635424	GENO	25/117/152	50/190/193	4.1390	0.1262
15	rs11635424	TREND	167/421	290/576	4.1200	0.0424
15	rs11635424	ALLELIC	167/421	290/576	4.2030	0.0404
15	rs11635424	DOM	142/152	240/193	3.5680	0.0589
15	rs11635424	REC	25/269	50/383	1.7540	0.1854
15	rs3784709	GENO	25/113/156	48/189/196	4.5060	0.1051
15	rs3784709	TREND	163/425	285/581	4.3120	0.0378
15	rs3784709	ALLELIC	163/425	285/581	4.4230	0.0355
15	rs3784709	DOM	138/156	237/196	4.2610	0.0390
15	rs3784709	REC	25/269	48/385	1.2920	0.2556
15	rs1026732	GENO	25/115/156	50/190/193	5.1340	0.0768
15	rs1026732	TREND	165/427	290/576	5.0360	0.0248
15	rs1026732	ALLELIC	165/427	290/576	5.1650	0.0230
15	rs1026732	DOM	140/156	240/193	4.6570	0.0309
15	rs1026732	REC	25/271	50/383	1.8320	0.1759
15	rs6494696	GENO	25/114/155	50/190/192	5.2360	0.0729
15	rs6494696	TREND	164/424	290/574	5.1090	0.0238
15	rs6494696	ALLELIC	164/424	290/574	5.2410	0.0221
15	rs6494696	DOM	139/155	240/192	4.8030	0.0284
15	rs6494696	REC	25/269	50/382	1.7810	0.1821

DISCUSSION

In our work, we wanted to further investigate clinical and genetic aspects of primary and secondary RLS.

The aim of the clinical part was to verify the high prevalence of RLS among Czech patients with multiple sclerosis, to identify the risk factors for RLS and correlation of magnetic resonance imaging parameters with RLS in patients with MS.

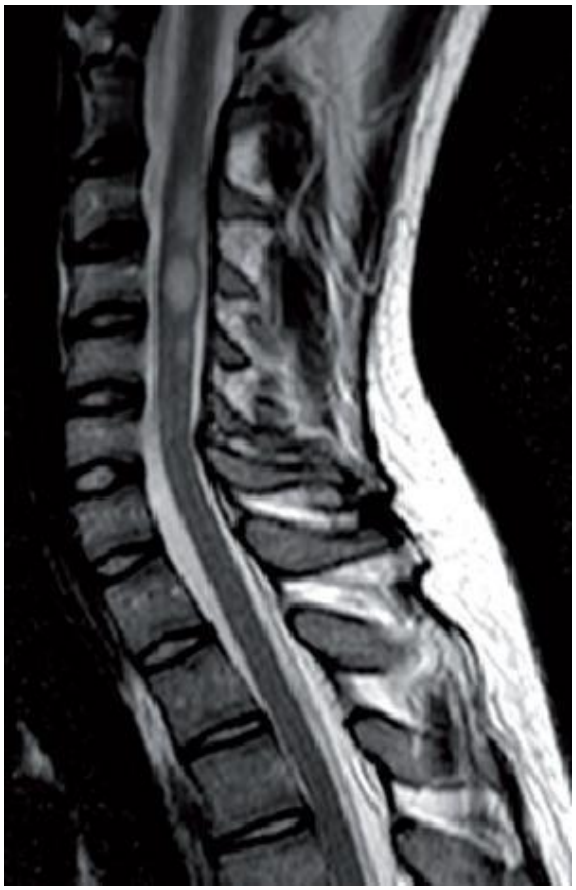
In the clinical part, we confirmed the previous findings that the prevalence of RLS is high in patients with MS.

An earlier study investigating the association between RLS and MS in the French-Canadian population showed a difference in prevalence between patients and controls of 37.5% vs. 16% (76). A later study published by an Italian group showed a prevalence of 19% in MS and 4.2% in control subjects (78). They did not include patients who experienced the symptoms with a frequency of occurrence lower than twice per week (a further 7.3%, total RLS prevalence 26.3%). Another study published by Spanish authors showed different results – a similar prevalence rate of RLS in MS patients and in healthy subjects (13.3% vs. 9.3%) (77). A different methodology and different frequency criteria might explain the discrepancies in absolute values in estimation of prevalence rates among these studies. In our study, we did not use any frequency threshold for the diagnosis of RLS: a patient was considered to be affected if he/she had ever met all criteria in their lifetime. The total prevalence was 32%, and in 68 (8.8%) subjects the RLS symptoms preceded the MS onset and 19 patients (2.4% of total) from this group reported a positive family history. Thus our estimate of the prevalence of RLS is very similar to those observed in the larger studies.

In patients with MS, among others, the following risk factors for RLS were found: older age, longer MS duration and higher neurological disability; therefore, the patients with RLS seem to be in a more advanced stage of MS as was previously suggested (78).

We conclude that RLS is significantly associated with MS and can lead to sleep disturbance in MS patients. In clinical praxis, we encouraged the routine screening of patients for insomnia and symptoms of RLS. However, patients with MS often report sensitive symptoms (dysaesthesia and paraesthesia, spasticity) and it is important to clearly differentiate between RLS and neurological symptoms not associated with RLS. We therefore stress that all the essential criteria should be met and patients should be personally interviewed to avoid false-positive diagnosis.

The radiological study has also confirmed the previous investigations of Manconi et al. that the presence of RLS symptoms does not correlate with MRI markers of brain damage in MS despite the fact that RLS is more prevalent in advanced stages of MS. We used MRI volume parameters which better correlate with the MS clinical progression. The results may be caused by the low sensitivity of our MRI analysis approach, however Manconi et al. assessed more specific scans with mean diffusion (MD) and fractional anisotropy (FA) analysis and found no association between RLS and a particular brain MRI lesion pattern (79). The study revealed significantly reduced cervical cord average FA in MS patients with RLS compared to those without. Cervical cord damage may play a role in the pathophysiology of the association between RLS and MS. The cord damage may interrupt descending or ascending pathways and this could lead to a higher spinal motor excitability in RLS patients, this is supported by several clinical and neurophysiological studies (91). The possible target may be the A 11 dopaminergic diencephalon pathways projecting from the A 11 hypothalamic area to D3 receptors located in the dorsal and intermediolateralis spinal nuclei (33).



Picture 5: Spinal cord lesions in MS patients

Our second study, "Replication in three populations" showed an association of common genetic variants in *MEIS1*, *BTBD9* and *MAP2K5/SCOR1* with RLS in a combined sample of Czech, Austrian, and Finnish RLS cases. Similar findings were observed in the US population (65). In accordance to the original report, the strongest effect was observed with the haplotype "AG" formed by markers rs6710341 and rs12469063 located in the 9th intron of *MEIS1*, providing ORs of about 2.0 for this haplotype. However, the OR may be underestimated, because the controls samples were not screened to exclude RLS and therefore may contain approximately 10% of individuals actually affected by RLS. The best models observed for individual loci are in good agreement with previous findings in German and Canadian populations (64). The significance of these loci to RLS can therefore be regarded as well established.

The sub-analysis in Czech and Austrian populations show the same trends for association as the combined sample, but in the Finnish sample, only association with *BTBD9* was confirmed and there was a trend for association to *MEIS1*. Moreover, the allele frequencies and proportions of familial cases in the Finnish sample were different from the other two, but the smaller size of this sample limits further implications.

In our sample set we have not observed significant differences between familial and sporadic cases concerning the *BTBD9* locus. The 95% confidence intervals of OR also overlapped between familial and sporadic cases for both *MEIS1* (1.357 – 2.1 in familial and 1.019 – 1.534 in sporadic cases vs. all controls for rs12469063) and *MAP2K5/SCOR1* (1.164 – 1.841 in familial and 0.951 – 1.408 in sporadic cases for rs6494696). There is a trend that *MEIS1* and *MAP2K5/SCOR1* possibly play a more important role in familial RLS, but due to limited number of patients, we were not able to prove significant heterogeneity. Generally the risk alleles in these loci are common and exert only small to moderate effects. They do not explain the familial clustering of RLS. Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations and is also most independent of familial clustering. We conclude that the observed genetic determinants are risk factors for RLS in multiple populations. Further studies including genotyping with genome-wide SNP Arrays might give us a more comprehensive picture and answer the question as to whether further genetics factors besides the known RLS factors are involved.

The last part of the genetic study "Genetics of secondary RLS form in patients with MS" attempted to reveal whether the genetic variants known to increase the risk in idiopathic RLS cases (*MEIS1, BTBD9, MAP2K5/SCOR1, PTPRD*) also contribute to the secondary RLS in patients with multiple sclerosis. So far only one genetic association study with secondary RLS cases has been published (72).

Our study, despite its sufficient statistical power, showed no association to variants in *MEIS1* and *BTBD9* with secondary RLS in MS patients. There was a trend for the association with *MAP2K5/SCOR1,* the best model was the recessive one. This model and the direction of the association are in accordance with the previous genome-wide scans and replication studies in idiopathic cases (64, 67).

To exclude the possible genetic influence of an MS diagnosis we conducted the second association study comparing patients suffering with MS without RLS to the unscreened population and found no association for all tested variants, taking into account the above described statistical power.

The *MAP2K5/SCOR 1* gene variant showed significant evidence for the association in the genome-wide scans in idiopathic cases. *MAP2K5* is important for the early stages of muscle differentiation and is important in the neuroprotection of dopaminergic neurons. *SCOR 1* acts as a transcriptional co-repressor of LBX1 (64, 92). This homeobox is critical in the development of sensory pathways in the dorsal horn of the spinal cord (70). Its role and function in RLS as well as in patients with RLS/MS, however, is not known.

Spinal cord is also a common lesion target for autoimmune inflammation in MS. Patients with MS may be due to this *SCOR1* variant more susceptible to development of RLS, in presence of another provoking factor (i.e. lesion in spinal cord). The most probable structure can be dopaminergic pathways from the hypothalamic area A 11 to dorsal horns in the spinal cord (33). This second-hit theory is supported by findings of the older age, longer MS duration and higher disability of MS patients with RLS compared to the patients without RLS. However, other genes of smaller effects may contribute to this RLS phenotype.

Further studies with more accurate spinal cord MRI and genetic association with other secondary RLS cases, such as in pregnancy, are necessary to disclose the pathogenesis of both secondary and primary RLS.

FINAL CONCLUSION

Clinical part:

A. The prevalence of RLS in patients with MS is high (32% in the Czech population), MS should be considered amongst secondary RLS forms. RLS is associated with more severe disability and clinical course in MS patients.

We should investigate MS patients for RLS symptoms, if they report sleep difficulties, because the effective treatment is available.

B. The extent of brain damage using MRI does not correlate with the presence of RLS in MS patients. Therefore, further studies with the spinal cord MRI are necessary to disclose the etiopathogenesis

Genetic part

A. Our study shows that variants in three loci confer consistent disease risks in patients of European descent. Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations and is also most independent of familial clustering.

B. The idiopathic RLS forms do not share all the major genetic features with secondary RLS forms in patients with MS. However, we were able to confirm the mild impact of the SCOR1 gene variants on a higher prevalence of RLS in MS patients.

LIST OF TABLES

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PhD Candidate's publications list: IF in total 45.6

A. Publications in extenso, which form the basis of the PhD thesis

1. Kemlink D, Polo O, Frauscher B, Gschliesser V, Högl B, Poewe W, Vodicka P, Vavrova J, Sonka K, Nevsimalova S, Schormair B, Lichtner P, Silander K, Peltonen L, Gieger C, Wichmann HE, Zimprich A, Roeske D, Müller-Myhsok B, Meitinger T, Winkelmann J. Replication of Restless Legs Syndrome Loci in Three European Populations. J Med Genet. 2009 Mar 10. 46(5):315–8 IF = 5.5 **Attachment B**

2. Vávrová J, Kemlink D, Sonka K, Havrdová E, Horáková D, Pardini B, Müller-Myhsok B, Winkelmann J. Restless legs syndrome in Czech patients with multiple sclerosis: An epidemiological and genetic study. Sleep Med. 2012 May 18. [Epub ahead of print] IF = 3.4 – **Attachment A**

3. Winkelmann J, Czamara D, Schormair B, Knauf F, Schulte EC, Trenkwalder C, Dauvilliers Y, Polo O, Högl B, Berger K, Fuhs A, Gross N, Stiasny-Kolster K, Oertel W, Bachmann CG, Paulus W, Xiong L, Montplaisir J, Rouleau GA, Fietze I, Vávrová J, Kemlink D, Sonka K, Nevsimalova S, Lin SC, Wszolek Z, Vilariño-Güell C, Farrer MJ, Gschliesser V, Frauscher B, Falkenstetter T, Poewe W, Allen RP, Earley CJ, Ondo WG, Le WD, Spieler D, Kaffe M, Zimprich A, Kettunen J, Perola M, Silander K, Cournu-Rebeix I, Francavilla M, Fontenille C, Fontaine B, Vodicka P, Prokisch H, Lichtner P, Peppard P, Faraco J, Mignot E, Gieger C, Illig T, Wichmann HE, Müller-Myhsok B, Meitinger T. Genome-wide association study identifies novel restless legs syndrome susceptibility loci on 2p14 and 16q12.1. PLoS Genet. 2011 Jul;7(7):e1002171. Epub 2011 Jul 14. IF = 9.5 **Attachment D**

4. Schormair B & Kemlink D, Roeske D, Eckstein G, Xiong L, Lichtner P, Ripke S, Trenkwalder C, Zimprich A, Stiasny-Kolster K, Oertel W, Bachmann CG, Paulus W, Högl B, Frauscher B, Gschliesser V, Poewe W, Peglau I, Vodicka P, Vávrová J, Sonka K, Nevsimalova S, Montplaisir J, Turecki G, Rouleau G, Gieger C, Illig T, Wichmann HE, Holsboer F, Müller-Myhsok B, Meitinger T, Winkelmann J.*PTPRD* (protein tyrosine phosphatase receptor type delta) is associated with restless legs syndrome.Nat Genet. 2008 Aug;40(8):946–8. IF 25.5. **Attachment C**

B. Publications in extenso which are not related to the thesis topic with IF

Volná J, Kemlink D, Kalousová M, Vávrová J, Majerová V, Mestek O, Svarcová J, Sonka K, Zima T. Biochemical oxidative stress-related markers in patients with obstructive sleep apnea. Med Sci Monit. 2011 Sep;17(9):CR491–7. IF = 1.7.

C. Publications in extenso which are not related to the thesis topic without IF

Sonka K, Fialová L, Volná J, Jiroutek P, Vávrová J, Kemlink D, Pretl M, Kalousová M. Advanced oxidation protein products in obstructive sleep apnea. Prague Med Rep. 2008;109(2–3):159–65.

ATTACHMENT A

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Original Article

Restless legs syndrome in Czech patients with multiple sclerosis: An epidemiological and genetic study

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ABSTRACT

Background: Restless legs syndrome (RLS) is a frequent neurological disorder which is presented in idiopathic and secondary form. Idiopathic RLS is associated with common genetic variants in four chromosomal regions. Recently, multiple sclerosis (MS) was identified as a common cause for secondary RLS. The aim of our study was to evaluate the prevalence of RLS among Czech patients with MS and to further analyze the impact of known genetic risk factors for RLS in patients with MS.
Methods: Each patient underwent a semi-structured interview. A patient was considered to be affected by RLS if all four standard criteria had ever been met in their lifetime. The sample was genotyped using 12 single nucleotide polymorphisms within the four genomic regions, which were selected according to the results of previous genome-wide association studies.
Results: A total of 765 subjects with MS were included in the study and the diagnosis of RLS was confirmed in 245 subjects (32.1%, 95% CI 28.7–35.4%). The genetic association study included 642 subjects; 203 MS patients with RLS were compared to 438 MS patients without RLS. No significant association with *MEIS 1*, *BTBD9*, and *PTPRD* gene variants was found despite sufficient statistical power for the first two loci. There was a trend for association with the *MAP2K5/SCOR1* gene – the best model for the risk allele was the recessive one (p nominal = 0.0029, p corrected for four loci and two models = 0.023, odds ratio = 1.60).
Conclusion: We confirmed that RLS prevalence was high in patients with multiple sclerosis, but this form did not share all genetic risk variants with idiopathic RLS.

1. Introduction

Restless legs syndrome (RLS) is a common movement disorder characterized by an urge to move the legs associated with an unpleasant sensation in the lower limbs, typically occurring when patients are at rest in the evening and at night. Symptoms are partially or totally relieved by movement. RLS can lead to the disturbance of sleep and impaired quality of life [1]. The diagnosis is based on the patient's clinical description and the presence of typical symptoms in diagnostic criteria [1]. The diagnosis is further supported by the presence of periodic limb movements in sleep (PLMS) and positive response to dopaminergic treatment [2].

RLS is a complex genetic disorder with a prevalence of 5–10% in the European population and with a female preponderance [2].

Genome-wide association studies (GWAs) identified intronic and intergenic RLS-associated single nucleotide polymorphisms (SNPs) within four genomic regions: the *MEIS1*, *BTBD9*, *PTPRD*, and *MAP2K5/SCOR1* genes on chromosome 2, 6, 9, and 15, respectively [3–6]. These findings were replicated in multiple independent studies from different European and North American populations [7].

Whilst most cases may be idiopathic, RLS may also occur in acquired forms. Iron deficiency, end-stage renal disease, and pregnancy are well established secondary causes of RLS [8–10]. Three recent studies reported a higher prevalence of RLS in patients with multiple sclerosis (MS) compared to healthy subjects [11–14]. The pathogenic mechanism of RLS in patients with MS is unclear. It is

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not known whether the same genetic risk variants that lead to increased susceptibility to idiopathic RLS may also have an impact on secondary RLS, such as that in MS.

Thus, the aim of our study was (i) to evaluate the prevalence of RLS among Czech patients with MS and (ii) to further analyze the impact of known genetic risk factors for idiopathic RLS in patients suffering from both MS and RLS.

2. Study population and methods

2.1. Epidemiological study

From April to December 2009 we recruited all patients from the preselected population (patients with quantitative MRI data) in the MS Centre, Department of Neurology of First Faculty of Medicine, Prague. MS had been diagnosed according to McDonald criteria [15]. Exclusion criteria for the study were dopaminergic and antidopaminergic drugs, renal failure, pregnancy, sideropenic anaemia, recent MS diagnosis (less than six months before the time of the interview) and recent clinical MS relapse (within three months of the interview). Each MS patient underwent a semi-structured interview conducted by a physician skilled in RLS diagnostics. A patient was considered to be affected by RLS if all four standard criteria had ever been met in their lifetime [1].

2.2. Genetic association study

Participants in the epidemiological sample were asked to also take part in the genetic association study. We also recruited more patients with clear-cut secondary RLS to increase statistical power and did not use all the RLS negative patients so as not to exceed the 2:1 ratio between controls and cases. As a reference population, blood donors were used and then epidemiological procedures and genotyping are described elsewhere [7].

We excluded patients who had experienced RLS prior to the first symptoms of MS and patients with a positive family history of RLS to minimize the admixture of idiopathic cases.

Association tests were conducted in two different settings:

(1) MS patients with RLS vs. MS patients without RLS.
(2) MS patients without RLS vs. population controls (blood donors not screened for RLS) to exclude the possible genetic influence of RLS genetic risk factors on MS.

The Local Ethics Committee approved the study and written informed consent was obtained from all subjects.

Twelve single nucleotide polymorphisms (SNPs) within the four genomic regions were selected according to the results of previous GWAS [5,6]. Samples were genotyped on the Sequenom platform (Sequenom MassArray system, Sequenom Inc., San Diego, California, USA). Automated genotype calling was done with SpectroTYPER 3.4 software and genotype clustering was visually checked by an experienced scientist. Assays were designed using AssayDesign 3.1.2.2 with iPLEX Gold chemistry default parameters. SNP quality control criteria leading to exclusion from analysis were a call rate, 90%, minor allele frequencies (MAF), 1% and $p = 0.001$ for deviations from Hardy–Weinberg equilibrium (HWE) in controls.

3. Statistical methods

3.1. Epidemiological study

The data were analyzed using the software package Statistica 8 (StatSoft, Inc. STATISTICA for Windows Tulsa, OK: 2300 East 14th Street, Tulsa, OK 74104, http://www.statsoft.com). Results are presented as mean ± one standard deviation, nonparametric descriptive statistics, and inter-group comparative test (Mann–Whitney) were used to analyze EDSS scores. *T*-tests were employed for all other parameters.

3.2. Genetic association study

Genotype data were analyzed using standard association tests (allelic, genotypic, dominant, and recessive models), including the Cochran–Armitage test for trend and test for empirical as these are implemented in the PLINK statistical package v1.0.11 [16].

Bonferroni correction for multiple testing of four regions (all genotyped SNPs within each region are in close linkage disequilibrium, except for chromosome 9) and two different models (allelic and the best model) was employed. All p values given are two sided.

Power calculations were performed using the Genetic Power Calculator (pngu.mgh.harvard.edu/~purcell/gpc/) [17]. For input parameter we used an RLS prevalence of 8%, an alpha level of 5%, and ORs and the allele frequencies according to the results of the GWAS [5,6].

4. Results

4.1. Epidemiological study

In total, we enrolled 765 MS patients (553 females and 212 males). The mean age was 36.5 ± 9.5 years, with an average disease duration of 9.1 ± 7.36 years. The median EDSS score was 2.0 (quartiles 1.5 and 3.5).

Out of all the examined patients, 76% had relapse-remitting MS, 14.4% had clinically isolated syndrome, 5.6% were in secondary progression, and 0.9% had a primary progressive form of MS.

The diagnosis of RLS was confirmed in 245 subjects (32%, 95% CI 28.7–35.4%) with MS, mean age at onset of RLS symptoms was 29.1 ± 10.4 years. In 49 patients (6.4%), RLS symptoms preceded the MS onset and 19 patients (2.4%) had a positive family history and RLS symptoms preceding the MS onset and, therefore, were subsequently excluded from all genetic studies. In 177 patients (23.2%) RLS followed the MS onset; 520 patients (68%) never experienced RLS. The average delay between the onset of MS and that of RLS was 2.5 ± 8.7 years.

For individual subtypes of MS, using the same strict criteria (exclusion of patients with positive family history of RLS and onset of RLS symptoms before MS) we observed the following RLS prevalence: 39.6% in relapse-remitting MS (95% CI 35.36–43.84%), 21.7% in clinically isolated syndrome (95% CI 12.83–30.57%), and 61.5% in secondary progressive MS (95% CI 46.23–76.77%).

Compared to patients without RLS, patients suffering from both MS and RLS were significantly older (38.6 vs. 35.6 years, $p < 0.001$, Students *t*-test), had longer durations of MS symptoms (11.0 vs. 8.2 years, $p < 0.001$, Students *t*-test), and had higher EDSS scores (2.9 vs. 2.3, $p < 0.001$, Mann–Whitney test). There were significantly more affected women in the RLS affected group (78% vs. 69%, $p = 0.0072$, χ^2).

4.2. Genetic association study

The genetic association study included 642 subjects; 203 MS patients (45 men, 158 women, mean age 40.7 years, SD ± 10.7) with RLS were compared to 438 MS patients (122 men, 316 women, mean age 35.8 years, SD ± 9.3) without RLS and to a reference population of 450 blood donors (166 males, 284 females, mean age 45.3 ± 9.9) whose epidemiological procedures and genotyping are described elsewhere [7].

Table 1
Results of genetic association study.

Chr	Gene	SNP ID	Genome	OR best model (95% CI)	MAF MS+RLS+	MAF MS+RLS−	MAF controls	Best model	*P* nom model	*P* nom Allelic	*P* nom Model RR-MS
2p	*MEIS1*	rs6710341	66611926	1.19 (0.86–1.64)	0.1533	0.1323	0.1407	TREND	0.4552	0.2954	0.3861
2p	*MEIS1*	rs12469063	66617812	1.12 (0.86–1.45)	0.2588	0.2384	0.2194	TREND	0.3887	0.4128	0.4660
2p	*MEIS1*	rs2300478	66634956	1.13 (0.87–1.47)	0.2622	0.2396	0.2229	TREND	0.3767	0.3668	0.4000
6p	*BTBD9*	rs9296249	38473818	1.14 (0.86–1.5)[a]	0.2102	0.2326	0.2361	TREND	0.1519	0.3541	0.3084
6p	*BTBD9*	rs3923809	38548947	1.03 (0.8–1.32)[a]	0.2978	0.3037	0.3060	TREND	0.5883	0.8235	0.7501
9p	*PTPRD*	rs11788684	8846420	1.01 (0.73–1.4)	0.1422	0.1407	NA	TREND	0.5714	0.9400	0.3782
9p	*PTPRD*	rs4626664	9261737	1.15 (0.84–1.58)[a]	0.1467	0.1655	0.1409	TREND	0.4507	0.3762	0.8330
15q	*MAP2K5*	rs11635424	65824631	1.53 (1.12–2.08)[a]	0.2788	0.3341	0.3349	REC	0.0070	0.0402	0.0355
15q	*MAP2K5*	rs3784709	65859328	1.60 (1.17–2.18)[a]	0.2765	0.3345	0.3291	REC	0.0029	0.0316	0.0167
15q	*MAP2K5*	rs1026732	65882138	1.54 (1.13–2.10)[a]	0.2753	0.3314	0.3349	REC	0.0059	0.0367	0.0272
15q	*MAP2K5/SCOR1*	rs6494696	65890259	1.56 (1.15–2.13)[a]	0.2765	0.3329	0.3356	REC	0.0045	0.0361	0.0253

Genome – The genetic positions in bp derived from UCSC genome browser (http://genome.ucsc.edu, assembly March 2006) [21]; *OR best model* – Odds-ratio according to best model in original locus description (Allelic for TREND, Allele negativity for REC) including 95% confidence interval. *MAF MS+RLS+* – minor allele frequency in MS patients with RLS symptoms; *MAF MS+RLS−* – minor allele frequency in MS patients without RLS symptoms; *MAF controls* – minor allele frequency in unscreened population sample of blood donors. *Best model* – Best model corresponds to the model under which the lowest *P* values were observed (TREND – Armitage trend test, REC – recessive model) in the original and replication publications [5–7]. *P-nom model* – raw nominal *p*-values observed under the best model; *P nom allelic* – comparison of allele frequencies between MS+RLS+ and MS+RLS− patients. *P nom Model RR-MS* – raw nominal *p*-values observed under the best model using relapse-remitting MS patients. All *p*-values shown are 2-sided.

[a] Risk allele is the major allele.

4.2.1. Testing of MS patients positive for RLS vs. MS patients negative for RLS

All SNPs tested were in HWE ($p > 0.01$) in both patients and controls. One of the tested SNPs failed to pass genotyping criteria (rs4236060 at *BTBD9*). After excluding patients with probable idiopathic RLS, the power for the *MEIS1* and *BTBD* genes remained sufficient – 89.6 and 85.2, respectively. For the *PTPRD* locus, the power was below 50%, for the *MAP2K5/SCOR1*locus it was below 70%.

No significant association with the *MEIS 1*, *BTBD9*, and *PTPRD* genes was found in 203 patients with MS. There was a trend for association with the *MAP2K5/SCOR1* gene – the best model for the risk allele was the recessive model (p nominal = 0.0029, p permutated after correction = 0.0248, p nominal corrected for four loci and two models, i.e., eight tests = 0.029, odds ratio = 1.60–95% CI 1.17–2.18). Thus, the one sided p value with the direction of the alternative hypothesis given by the original report is p corrected 0.019. Results for all tested loci are summarized in Table 1.

Finally, we did the association analysis only in relapse – remitting MS form in order to distinguish the MS subtypes. We included 192 MS patients with RLS and 373 MS patients without RLS in the analysis after excluding patients who had a family history and RLS symptoms before MS onset.

The results show the same trend for association as when using patients with all MS forms, but due to the lower sample size the significance is lower and does not pass correction for multiple testing.

4.2.2. Testing of MS patients negative for RLS vs. population controls

One SNP was not available in previously genotyped population controls (rs11788684 from *PTPRD locus*). No significant differences were found within the tested SNPs. The maximal observed χ^2 statistics were 1.9 at rs4626664 on chromosome 9, all other values were below 1.0. The sample of 438 MS patients without RLS symptoms and 450 population controls had 80% power to detect association with MS (assuming prevalence of 0.001) with OR over 1.58 (*MAP2K5/SCOR1* locus).

5. Discussion

We confirmed the previous findings that the prevalence of RLS is high in patients with multiple sclerosis.

An earlier study investigating the association between RLS and MS in the French–Canadian population showed a difference in prevalence between patients and controls of 37.5% vs. 16% [12]. A later study published by an Italian group showed a prevalence of 19% in MS and 4.2% in control subjects [11]. They did not include patients who experienced the symptoms with a frequency of occurrence lower than twice per week (a further 7.3%, total RLS prevalence 26.3%). Another study published by Spanish authors showed different results – a similar prevalence rate of RLS in MS patients and in healthy subjects (13.3% vs. 9.3%) [14]. A different methodology and different frequency criteria might explain discrepancies in absolute values in estimation of prevalence rates among these studies. In our study, we did not use any frequency threshold for the diagnosis of RLS: a patient was considered to be affected if he/she had ever met all criteria in their lifetime. The total prevalence was 32%, and in 68 subjects (8.8%) the RLS symptoms preceded the MS onset and 19 patients (2.4% of total) from this group reported a positive family history. Thus our estimate of the prevalence of RLS is very similar to those observed in the larger studies.

In patients with MS, among others, the following risk factors for RLS were found: older age, longer MS duration, and higher neurological disability; therefore, the patients with RLS seem to be in a more advanced stage of MS as was previously suggested [11,13].

Therefore, we conclude that RLS is significantly associated with MS and can lead to sleep disturbance in MS patients. In clinical praxis we encouraged the routine screening of patients for insomnia and symptoms of RLS. However, patients with MS often report sensitive symptoms (dysaesthesia and paraesthesia spasticity) and it is important to clearly differentiate between RLS and neurological symptoms not associated with RLS. We therefore stress that all the essential criteria should be met and patients should be personally interviewed to avoid false-positive diagnosis.

The pathophysiology of this association is still unknown. Our study attempted to reveal whether the genetic variants known to increase the risk in idiopathic RLS cases also contribute to the secondary RLS in patients with MS.

Our study, despite its sufficient statistical power, showed no association to variants in the *MEIS1* and *BTBD9* genes with secondary RLS in MS patients. There was a trend for the association with the *MAP2K5/SCOR1* gene, and the best model was the recessive one. This model and the direction of the association are in accordance

with previous genome wide association studies and replication studies in idiopathic cases [5,18].

To exclude the possible influence of RLS genetic risk factors on MS we conducted the second association study comparing patients suffering from MS without RLS to the unscreened population and found no association for all tested variants, taking into account the above described statistical power. Association of RLS risk factors to MS would, if presented, cause the false negative results when comparing MS patients with RLS vs. MS patients without RLS.

The *MAP2K5/SCOR 1* gene variant showed significant evidence for the association in the GWAs in idiopathic cases. The *MAP2K5* locus is important for the early stages of muscle differentiation and is important in the neuroprotection of dopaminergic neurons [19]. The *SCOR 1* locus acts as a transcriptional co-repressor of LBX1. This homeobox is critical in the development of sensory pathways in the dorsal horn of the spinal cord [20]. Its role and function in RLS, as well as in patients with RLS/MS, however, is not known.

Further studies including genotyping with genome-wide SNP Arrays might give us a more comprehensive picture and answer the question as to whether further genetics factors besides the known RLS factors are involved.

6. Conclusion

We concluded that RLS is significantly associated with multiple sclerosis, especially in patients with more severe disability. MS should be considered amongst the secondary RLS forms. The idiopathic RLS forms do not share all of their major genetic features with secondary RLS forms in patients with MS. However, we were able to confirm the mild impact of the *MAP2K5/SCOR1* gene variant on a higher prevalence of RLS in MS patients.

Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: doi:10.1016/j.sleep.2012.03.012.

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ATTACHMENT B

Original article

Replication of restless legs syndrome loci in three European populations

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ABSTRACT

Background: Restless legs syndrome (RLS) is associated with common variants in three intronic and intergenic regions in *MEIS1, BTBD9,* and *MAP2K5/LBXCOR1* on chromosomes 2p, 6p and 15q.

Methods: Our study investigated these variants in 649 RLS patients and 1230 controls from the Czech Republic (290 cases and 450 controls), Austria (269 cases and 611 controls) and Finland (90 cases and 169 controls). Ten single nucleotide polymorphisms (SNPs) within the three genomic regions were selected according to the results of previous genome-wide scans. Samples were genotyped using Sequenom platforms.

Results: We replicated associations for all loci in the combined samples set (rs2300478 in *MEIS1*, $p = 1.26 \times 10^{-5}$, odds ratio (OR) = 1.47, rs3923809 in *BTBD9*, $p = 4.11 \times 10^{-5}$, OR = 1.58 and rs6494696 in *MAP2K5/LBXCOR1*, p = 0.04764, OR = 1.27). Analysing only familial cases against all controls, all three loci were significantly associated. Using sporadic cases only, we could confirm the association only with *BTBD9*.

Conclusion: Our study shows that variants in these three loci confer consistent disease risks in patients of European descent. Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations and is also most independent of familial clustering.

Restless legs syndrome (RLS) is characterised by an urge to move the legs associated with unpleasant sensations in the lower limbs, typically occurring at rest in the evening or at night.[1] Since the maximum number of symptoms appear at bedtime, RLS can lead to disturbances of sleep resulting in a decreased quality of life.[1] The diagnosis is further supported by the presence of periodic limb movements in sleep (PLMS) and positive response to dopaminergic treatment.[1]

A recent genome-wide association study (GWA) with German and Canadian RLS cases identified intronic or intergenic variants within three genomic regions: *MEIS1* (myeloid ecotropic viral integration site homeobox 1) on chromosome 2p, *BTBD9* (BTB/POZ domain containing protein 9) on chromosome 6p, and a third region on chromosome 15q containing *MAP2K5* (mitogen activated protein kinase kinase 5) and *LBXCOR1* (ladybird homeobox co-repressor 1).[2] A similar study conducted in Icelandic and US cases showed an association of *BTBD9* to PLMs.[3]

MEIS1 belongs to the family of TALE homeobox genes involved in limb development, the determination of the megakaryocytes and central nervous system (CNS) structures, such as the retina, cerebellar granule cells, hindbrain and spinal motor neuron pools.[4–6] So far, very little is known about *BTBD9*. It consists of a BTB/POZ domain, a BACK domain and a coagulation factor domain. Known functions of similar proteins containing these domains include ubiquitin dependent protein degradation.[7] The variants located in the third genetic region are in strong linkage disequilibrium with two surrounding genes: *MAP2K5*, which is critical at early stages of muscle cell differentiation,[8] and *LBXCOR1*, which is a transcriptional co-repressor of LBX1 and is highly expressed in spinal dorsal horn and midbrain–hindbrain border.[9] The involvement of these genes in the aetiopathogenesis of RLS is still unknown.

The aim of our study was to investigate whether these variants are also relevant among other European (Czech, Austrian, and Finnish) RLS cases and what is the difference of their impact between sporadic and familial cases.

PATIENTS AND METHODS

Patients and controls

The diagnosis of all RLS cases was made according to diagnostic criteria of the International RLS Study Group[1] by personal examination by a neurologist in the respective study centre. The positive family history was defined as at least one first degree family member being affected by RLS (reported by the proband) in all three populations. The control samples originate from the general population and were not screened for presence of RLS.

Czech subjects

The patients were recruited in the Centre for Disorders of Sleep and Wakefulness, Department of Neurology of First Faculty of Medicine and the General Teaching Hospital, Prague. In total, 290 patients were included (107 males, mean (SD) age 55.7 (15.3) years, mean age at onset of RLS 38.3 (18.1) years). Positive family history was reported by 110 patients, in 175 cases it was negative, and in five the data were not available. Altogether 450 sex matched controls were selected randomly from the Czech blood and bone marrow donors registry (166 males, mean age 45.3 (9.9) years). Since the maximum age for the controls was 63 years, 38 male and 51 female cases in the age group from 64 to 91 years could not be age matched.

Austrian subjects

A total of 269 (104 males) patients were recruited in 2 centres: at the Department of Neurology, Medical University of Vienna, and the Department of Neurology, University Clinic Innsbruck, (mean age 59.0 (14.3) years, mean age at onset of RLS 37.14 (19.5) years). Positive family history was reported by 107 patients, in 108 cases it was negative, and in 54 the data were not available. The patients were matched by sex to 611 controls from the German KORA project, the procedures for which have been described elsewhere[10] (236 males, mean age 59.9 (11.35) years). KORA controls were already used in the previous GWA study, which showed only a negligible effect of population stratification.[2]

Finnish subjects

Ninety (24 males) patients were recruited in the Sleep Research Center in Turku (mean age 46.5 (18.1) years, mean age at onset of RLS 19.4 (13.4) years. Positive family history was reported by 81 patients and nine patients had a negative family history. A random sample from the general Finnish population, comprising 169 sex matched individuals (45 males), was used as control. Data on age of controls were not available. Studies were performed according to the declaration of Helsinki and approved by the ethical committees of the respective study centres. Written informed consent was obtained from all RLS patients.

Genotyping

Ten single nucleotide polymorphisms (SNPs) within the three genomic regions were selected according to the results of previous GWA scans.[2 3] Samples were genotyped on two Sequenom platforms in Munich and Helsinki (Sequenom MassArray system, Sequenom Inc, San Diego, California, USA) with a genotype discordance rate of 1.3% in 158 comparisons, when analysing repeatedly genotyped internal control samples. Automated genotype calling was done with SpectroTYPER 3.4 software and genotype clustering was visually checked by an experienced scientist. Assays were designed using AssayDesign 3.1.2.2 with iPLEX Gold chemistry default parameters. SNP quality control criteria leading to exclusion from analysis were a call rate <90%, minor allele frequencies (MAF) <1% and p<0.001 for deviations from Hardy-Weinberg equilibrium (HWE) in controls.

Statistical analysis

Genotype data were analysed using standard association tests (allelic, genotypic, dominant and recessive models) including Cochran–Armitage test for trend, Cochran–Mantel–Haenszel test for estimation of odds ratios (ORs) in the stratified sample (including Breslow–Day test for homogeneity), and haplotype tests, as implemented in the PLINK statistical package v1.0.[11] The sample was stratified only according to the country of origin. Logistic regression implementing the Cochran–Armitage test for trend (using genotypes as ordinal values rather than categorical) in the combined sample using age, sex and country of origin as covariates was performed by generalised linear modelling routines incorporated in R package v.2.6.0 (http://www.r-project.org/). Bonferroni correction for multiple testing of 10 markers was employed. All p values given are one sided, with the direction of the alternative hypothesis given by the original report.[3] Power calculations were performed using the Genetic Power Calculator (pngu.mgh.harvard.edu/~purcell/gpc/).[12] For input parameter we used an RLS prevalence of 8%, an α level of 5%, and ORs and allele frequencies according to results from the GWA experiment.[2] Association tests were conducted in three different settings: (1) all patients (that is, familial and sporadic) combined versus all controls; (2) familial cases versus controls; and (3) sporadic cases versus controls.

RESULTS

All SNPs tested were in HWE (p>0.01) in both patients and controls. Under the assumption of genetic homogeneity, the combined sample had good power to detect association using previously published parameters[2] (98% for *MEIS1* and *BTBD9*, 89% for *MAP2K5/LBXCOR1*). In the Czech sample alone the power was 82.5% for *MEIS1* and *BTBD9*, and 71.8% for *MAP2K5/LBXCOR1*, in the Austrian sample the powers were 84.8% and 74.8%, respectively, and in the Finnish sample separately 38.7% and 30.4%.

Allele frequencies in the Czech and KORA control samples were not significantly different (lowest p in χ^2 test = 0.2045 for rs4236060). Significant allele frequency differences were observed between the Finnish and the combined Czech and KORA control samples within *BTBD9* ($p<7.67\times10^{-6}$ for all SNP markers within *BTBD9*). A similar, nominally significant, difference in allele frequencies in *BTBD9* markers was also observed between Finnish cases and combined Czech and Austrian cases (in χ^2 test lowest p = 0.01063 for rs9296249), but we did not observe a significant difference between allele frequencies of Czech and Austrian RLS patients (lowest p in χ^2 test was 0.4608 for rs2300478). Logistic regression showed no significant interaction with country for any SNP tested, and the Breslow–Day test showed homogeneous ORs in all samples.

Significant association after correction for multiple testing at significance α level of 5% was found in at least one SNP for all tested loci in the combined samples (table 1), and in the Czech and Austrian samples separately. Analysing the Finnish sample, we confirmed only the association to *BTBD9*. The association to rs2300478 in *MEIS1* was only nominally significant and *MAP2K5/LBXCOR1* showed no association (table 2).

In the combined sample we observed a strong association with the haplotype formed by markers rs6710341 and rs12469063, both located within *MEIS1*. Carriers of the "AG" haplotype had ORs for developing RLS of 1.98 ($p=9.1\times10^{-10}$). Results for this haplotype were similar when testing the Czech ($p=3.2\ 10^{-7}$, OR = 2.38), Austrian ($p=8.3\times10^{-5}$, OR = 1.82), and Finnish samples ($p=2.0\times10^{-4}$, OR = 2.46) separately. No other common polymorphic phased haplotypes (MHF >1%) yielded significant results. An allele dosage model best described the association for *MEIS1* and *BTBD9* (Armitage trend test). In contrast, a recessive model for the risk allele fitted best for the *MAP2K5/LBXCOR1* locus.

Analysing only familial cases (n = 217) and all controls, all three loci were significantly associated. Using sporadic cases only (n = 283), we could confirm the association to *BTBD9* but not to *MEIS1* and *MAP2K5/LBXCOR1*. We omitted patients of Finnish origin from this sub-analysis due to very low proportion of sporadic cases and different allele frequencies in these samples. The Breslow–Day test did not show significant heterogeneity between sporadic and familial cases.

DISCUSSION

Our study showed an association of variants in *MEIS1*, *BTBD9* and *MAP2K5/LBXCOR1* with RLS in a combined sample of Czech, Austrian, and Finnish RLS cases. Similar findings were

Table 1 Genotyped single nucleotide polymorphisms (SNPs) and results of association in combined samples

Chr	Gene	SNP ID	Genome	r^2	OR (95% CI)	p nom	p corr	MAF fam	MAF spor	Best model	p corr fam	p corr spor
2p	MEIS1	rs6710341	66611926		0.84 (0.64 to 1.11)	0.30646	1	0.1270	0.1288	TREND	1	1
2p	MEIS1	rs12469063	66617812	0.413	1.43 (1.16 to 1.78)	4.15E-06	4.15E-05	0.3522	0.2727	TREND	2.24E-05	0.3245
2p	MEIS1	rs2300478	66634956	0.969	1.47 (1.18 to 1.82)	1.26E-06	1.26E-05	0.3575	0.2860	TREND	3.10E-05	0.1520
6p	BTBD9	rs9296249	38473818		1.59 (1.26 to 2.01)*	0.00011	0.00107	0.1694	0.1553	TREND	0.0544	0.0012
6p	BTBD9	rs3923809	38548947	0.512	1.58 (1.28 to 1.96)*	4.11E-06	4.11E-05	0.2204	0.2330	TREND	0.0018	0.0022
6p	BTBD9	rs4236060	38578315	0.829	1.49 (1.19 to 1.86)*	1.93E-05	0.00019	0.1882	0.2110	TREND	0.0008	0.0049
15q	MAP2K5	rs11635424	65824631		1.26 (1.02 to 1.55)*	0.00602	0.06023	0.2446	0.2992	REC	0.0203	1
15q	MAP2K5	rs3784709	65859328	0.935	1.24 (1.01 to 1.52)*	0.00530	0.05301	0.2392	0.2917	REC	0.0393	1
15q	MAP2K5	rs1026732	65882138	0.966	1.27 (1.03 to 1.56)*	0.00428	0.04278	0.2339	0.2936	REC	0.0116	1
15q	MAP2K5/LBXCOR1	rs6494696	65890259	0.999	1.27 (1.03 to 1.56)*	0.00476	0.04764	0.2339	0.2936	REC	0.0108	1

OR, odds ratio for the risk allele (Cochran–Mantel–Haenszel test) with 95% confidence intervals (CI); p nom, logistic regression implementing Armitage trend test with country of origin, sex and age as covariates; p corr, adjusted p values for multiple testing; MAF, minor allele frequencies observed in combined Czech and Austrian sample, in sporadic and familial cases; Best model corresponds to model under which lowest p values were observed (TREND, Armitage trend test; REC, recessive model); p corr fam, comparison of allele frequencies between familial cases and all controls; p corr spor, comparison of allele frequencies between sporadic cases and all controls; SNP, single nucleotide polymorphism.
The genetic positions in bp and gene alignments are derived from UCSC Genome browser (http://genome.ucsc.edu, assembly March 2006),[25] r^2– linkage disequilibrium relative to preceding marker; data were computed using genotypes observed in both cases and controls using Haploview 4.0 from HapMap project (http://www.hapmap.org, release 21a).[26]
*Risk allele is the major allele.

Table 2 Analysis in individual populations

	Czech Republic				Austria				Finland			
SNP ID	MAF cases n = 276	MAF controls n = 412	Best p corr	OR (95% CI)	MAF cases n = 222	MAF controls n = 570	Best p corr	OR (95% CI)	MAF cases n = 88	MAF controls n = 246	Best p corr	OR (95% CI)
rs6710341	0.1309	0.1456	1	1.13 (1.55 to 0.83)	0.1306	0.1412	1	0.91 (0.66 to 1.26)	0.1207	0.1585	1	0.73 (0.43 to 1.22)
rs12469063	0.2971	0.2172	0.0492	1.52 (1.19 to 1.95)	0.3108	0.2426	0.0064	1.41 (1.11 to 1.79)	0.3161	0.2439	0.6093	1.43 (0.98 to 2.10)
rs2300478	0.3025	0.2209	0.0285	1.53 (1.20 to 1.96)	0.3243	0.2487	0.0017	1.45 (1.14 to 1.84)	0.3276	0.2459	0.3676	1.49 (1.02 to 2.18)
rs9296249	0.1649	0.2306	0.0252	1.52 (1.15 to 2.00)	0.1644	0.2378	0.0116	1.59 (1.19 to 2.11)	0.2414	0.3516	0.1081	1.70 (1.15 to 2.53)
rs3923809	0.2301	0.2998	0.0374	1.43 (1.12 to 1.84)	0.223	0.3133	0.0049	1.59 (1.23 to 2.05)	0.2651	0.4119	0.0124	1.94 (1.32 to 2.87)
rs4236060	0.2047	0.2662	0.1903	1.41 (1.09 to 1.83)	0.1968	0.2891	0.0028	1.66 (1.27 to 2.17)	0.2674	0.3921	0.0497	1.77 (1.20 to 2.60)
rs11635424	0.2772	0.3350	0.0135	1.31 (1.04 to 1.66)	0.2793	0.3229	0.1014	1.23 (0.97 to 1.57)	0.3046	0.2866	1	1.09 (0.75 to 1.59)
rs3784709	0.2754	0.3289	0.0124	1.29 (1.02 to 1.63)	0.2725	0.3185	0.0522	1.25 (0.98 to 1.59)	0.3046	0.2744	1	1.16 (0.79 to 1.69)
rs1026732	0.2717	0.3350	0.0050	1.35 (1.07 to 1.71)	0.2748	0.322	0.0519	1.25 (0.98 to 1.60)	0.3046	0.2764	1	1.15 (0.79 to 1.67)
rs6494696	0.2717	0.3350	0.0050	1.35 (1.07 to 1.71)	0.2748	0.3229	0.0416	1.26 (0.99 to 1.60)	0.3046	0.2764	1	1.15 (0.79 to 1.67)

MAF, minor allele frequencies in each subsample in patients and healthy individuals; n, number of successfully genotyped individuals by passing quality control criteria; Best p corr, p values corrected for multiple testing according to the full association model in table 1; OR, odds ratio and corresponding 95% confidence intervals (CI); SNP, single nucleotide polymorphism.

observed in the US population.[13] In accordance with the original report, the strongest effect was observed with the haplotype "AG" formed by markers rs6710341 and rs12469063 located in the ninth intron of *MEIS1*, providing ORs of about 2.0 for this haplotype. However, the OR may be underestimated, because the controls samples were not screened to exclude RLS and therefore may contain approximately 10% of individuals actually affected by RLS. The best models observed for individual loci are in good agreement with previous findings in German and Canadian populations. The significance of these loci to RLS can therefore be regarded as well established.

The sub-analyses in Czech and Austrian populations show the same trends for association as the combined sample, but in the Finnish sample, only association with *BTBD9* was confirmed and there was a trend for association to *MEIS1*. Moreover, the allele frequencies and proportions of familial cases in the Finnish sample were different from the other two, but the smaller size of this sample limits further implications.

In our sample set we have not observed significant differences between familial and sporadic cases concerning the *BTBD9* locus. The 95% confidence intervals of OR also overlapped between familial and sporadic cases for both *MEIS1* (1.357 to 2.1 in familial and 1.019 to 1.534 in sporadic cases *vs* all controls for rs12469063) and *MAP2K5/LBXCOR1* (1.164 to 1.841 in familial and 0.951 to 1.408 in sporadic cases for rs6494696). There is a trend that *MEIS1* and *MAP2K5/LBXCOR1* possibly play a more important role in familial RLS, but due to the limited number of patients, we were not able to prove significant heterogeneity. Generally the risk alleles in these loci are common and exert only small to moderate effects. They do not explain the familial clustering of RLS.[2] Besides these association signals, six linkage regions for RLS on chromosomes 2q, 9p, 12q, 14q, 19p and 20p,[14–19] under a recessive or autosomal dominant model of inheritance, have been described. These variants must be of larger effects and less frequent, since only some have been successfully confirmed in independent populations or in single families.[20–24] Among the known loci, *BTBD9* seems to be the most consistent in its effect on RLS across populations, and is also most independent of familial clustering.

We conclude that the observed genetic determinants are risk factors for RLS in multiple populations.

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ATTACHMENT C

nature genetics

PTPRD (protein tyrosine phosphatase receptor type delta) is associated with restless legs syndrome

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We identified association of restless legs syndrome (RLS) with *PTPRD* at 9p23–24 in 2,458 affected individuals and 4,749 controls from Germany, Austria, Czechia and Canada. Two independent SNPs in the 5′ UTR of splice variants expressed predominantly in the central nervous system showed highly significant *P* values (rs4626664, $P_{nominal/\lambda\ corrected} = 5.91 \times 10^{-10}$, odds ratio (OR) = 1.44; rs1975197, $P_{nominal/\lambda\ corrected} = 5.81 \times 10^{-9}$, OR = 1.31). This work identifies *PTPRD* as the fourth genome-wide significant locus for RLS.

Restless legs syndrome (RLS) is a frequent neurological phenotype characterized by a diurnal occurrence of an urge to move, usually accompanied by uncomfortable sensations in the lower limbs. Symptoms manifest at rest and improve with walking. RLS can lead to severe sleep disturbances and impaired quality of life[1]. Dopaminergics provide effective treatment, but their use is limited because of side effects[1]. A genome-wide association study (GWAS) with German and Canadian RLS cases revealed association with variants in *MEIS1*, *BTBD9* and a locus comprising *MAP2K5* and *LBXCOR1*, with ORs above 2 (ref. 2). Another GWAS conducted with Icelandic and US RLS cases showed association of *BTBD9* variants with periodic limb movements in sleep (PLMS), an associated motor feature of RLS[3]. The association with *MEIS1* and *BTBD9* was also confirmed in an independent case-control study in the US population[4]. None of these genes is located in any of the previously described linkage regions for RLS (RLS1–RLS5)[5]. Analysis of these loci in our GWAS data[2] showed nominally significant signals in RLS3 on 9p23–24. Despite criticism of the statistical analysis concerning the original linkage finding[6] and variation in the precise definition of the disease-containing interval, this is the most robust RLS linkage region, having been identified in two US families and replicated in two German families[7–10]. We therefore carried out an association study with 3,270 SNPs from this 31-Mb region (9p, 0.5–31.5 Mb).

For the exploratory genome-wide scan (stage 1), we genotyped 628 RLS cases and 1,644 population-based controls from the KORA-S3/F3 survey using Affymetrix Mapping 500K array sets (401 cases and 1,644 controls)[2] and Affymetrix Genome-Wide Human SNP 5.0 arrays (227 cases). Application of stringent quality control criteria yielded 208,733 SNPs throughout the genome for analysis. Eigenvalue-based analysis and genomic control showed minimal population substructure ($\lambda = 1.07$). Of 3,270 SNPs analyzed in RLS3, 8 SNPs with a nominal *P* value corrected for $\lambda < 10^{-3}$ passed our criterion for replication (**Fig. 1**, **Supplementary Methods** and **Supplementary Table 1** online).

In the replication phase (stage 2), we genotyped these SNPs in German (1,271 cases, 1,901 controls), Czech (279 cases, 368 controls) and Canadian (285 cases, 842 controls) samples using multiplex mass spectrometry. Part of the German and Canadian samples had been used in the replication stage of our previous GWAS[2]. Details of demographic data, recruitment, diagnosis for subjects and genotyping of both stages are shown in **Supplementary Methods** and **Supplementary Table 2** online. Genomic control analysis resulted in inflation factors of 1.10 in the German, 1.23 in the Czech and 1.26 in the Canadian sample. Separate analysis of stage 2 samples showed significantly different minor allele frequencies (MAFs) across samples but comparable ORs with unidirectional allelic association (**Supplementary Table 3** online). Heterogeneity with respect to MAFs

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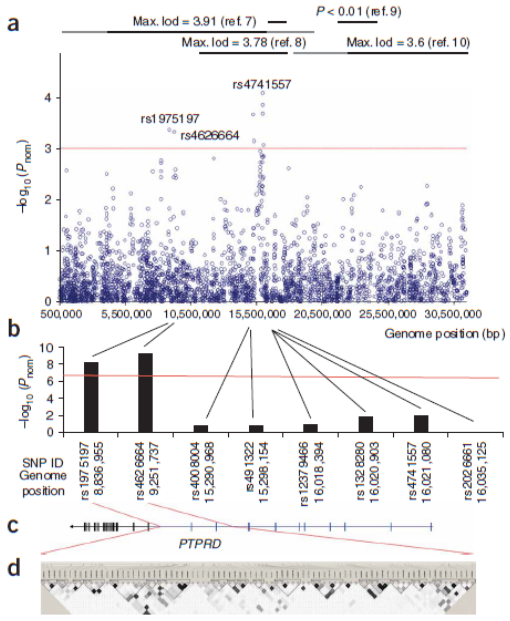

Figure 1 Association results for stages 1 and 2 over the chromosomal segment analyzed. (**a**) Results of stage 1 ($-\log_{10}$ of *P* nominal (P_{nom}) corrected for λ) for chromosome 9p, 0.5–31.5 Mb. The red line indicates the cut-off for selection of SNPs for replication. Position and extent of linkage signals[7–10] are shown as horizontal bars. Black bars represent the narrowest suggested region as defined by intrafamilial recombination events; gray bars extend to the maximum size. Maximum multi-point lod scores[7,8,10] and the *P* value from nonparametric linkage analysis[9] are denoted above the bars. Genomic positions refer to the UCSC Genome Browser Human March 2006 assembly (http://genome.ucsc.edu/). (**b**) Results of joint analysis of stages 1 and 2 for the eight SNPs within RLS3 selected for replication, given as $-\log_{10}$ of *P* nominal (P_{nom}) corrected for λ. Red line represents the cut-off for genome-wide significance after correction for multiple testing ($-\log_{10}(P_{nom}) = 6.62$, $P_{nom} < 2.4 \times 10^{-7}$). (**c**) Position of associated SNPs with genome-wide significance in *PTPRD*. Exons are depicted as bars, introns as lines. The noncoding 5′ UTR is highlighted in blue. Position of SNPs is indicated by red lines. (**d**) LD structure of region between rs1975197 and rs4626664. Gray shading indicates extent of LD (dark gray, high LD; light gray, low LD). Haploview 4.0 (http://www.broad.mit.edu/mpg/haploview/) and data from 1,639 KORA controls were used for visualization.

necessitated inclusion of country of origin as a covariate, allowing joint analysis of all stage 1 and 2 samples. This resulted in two SNPs with genome-wide significance after Bonferroni (B) correction for multiple testing: rs4626664, with $P_{nominal/\lambda\ corrected} = 5.91 \times 10^{-10}$, $P_{corrected(B)} = 0.00012$, OR = 1.44 and rs1975197, with $P_{nominal/\lambda\ corrected} = 5.81 \times 10^{-9}$, $P_{corrected(B)} = 0.0012$, OR = 1.31 (**Table 1** and **Supplementary Table 4** online). Both SNPs were also significant after Bonferroni correction in the German subsample and in the combined analysis of all stage 2 samples. In the Canadian subsample, both SNPs were nominally significant (rs4626664, $P_{nominal/\lambda\ corrected} = 0.018$; rs1975197, $P_{nominal/\lambda\ corrected} = 0.024$), whereas the Czech sample showed only a trend for association for the stronger signal (rs4626664, $P_{nominal/\lambda\ corrected} = 0.075$), most likely explainable by lack of power due to the smaller sample size (**Table 1** and **Supplementary Table 3**). Because cases and controls were not perfectly matched for age and sex, we used these factors as covariates in all analyses.

The association signals are located 0.41 Mb apart and map to introns 8 and 10 of *PTPRD*, within two separate linkage disequilibrium (LD) blocks. Logistic regression did not show any significant interaction between these SNPs ($P = 0.986$), as also evidenced by the lack of LD between them ($r^2 = 0$). They are separated by 17 haplotype boundaries, indicating a hot spot of recombination between them[11]. There is no significant interaction with risk alleles in *MEIS1* (rs4626664, $P = 0.463$; rs1975197, $P = 0.957$), *BTBD9* (rs4626664, $P = 0.487$; rs1975197, $P = 0.246$) and *LBXCOR1-MAP2K5* (rs4626664, $P = 0.510$; rs1975197, $P = 0.859$), and therefore no evidence for epistasis. Haplotype analysis showed no increase in significance compared to single SNP analysis. Power for the joint analysis was 77.4% and 99.4% to detect an allelic association with an OR of 1.3 and 1.4 with genome-wide significance level $\alpha = 0.05$ and a MAF of 0.17 (**Supplementary Methods**).

Sequence analysis revealed no mutations in 35 coding and 10 noncoding exons of *PTPRD* among nine affected individuals from

Table 1 Association results for rs1975197 and rs4626664

dbSNP ID	Genome position	Gene	Risk allele[a]	MAF stage 1 GER ca. (623) co. (1,639)	MAF stage 2 GER ca. (1,271) co. (1,900)	MAF stage 2 CZ ca. (279) co. (368)	MAF stage 2 CAN ca. (285) co. (842)	Stage 1 $P_{nom/\lambda\ corrected}$	Stage 2 $P_{corrected\ (B)}$ GER CZ CAN	Stage 2 combined analysis $P_{corrected\ (B)}$	Joint analysis stage 1 & 2 $P_{nom/\lambda\ corrected}$	Joint analysis stage 1 & 2 OR (95% CI)
rs1975197	Chr 9p: 8,836,955	*PTPRD*	T	0.216 0.164	0.196 0.157	0.158 0.136	0.203 0.156	4.42E-04	1.55E-03 1 1.81E-01	3.29E-05	5.81E-09	1.31 (1.20–1.44)
rs4626664	Chr 9p: 9,251,737	*PTPRD*	A	0.175 0.133	0.167 0.117	0.196 0.146	0.159 0.117	4.73E-04	6.88E-05 7.47E-01 2.44E-01	7.53E-07	5.91E-10	1.44 (1.31–1.59)

SNPs with genome-wide significant association located in *PTPRD*. Genome positions refer to the Human March 2006 (hg18) assembly. ca, cases; co, controls; numbers in parentheses denote successfully genotyped sample numbers; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; P_{nom}, nominal *P* value. P_{nom} values in stage 1 were calculated using logistic regression with age, sex and the first four components from the MDS analysis of the IBS matrix as covariates. P_{nom} values in the individual analysis of stage 2 were calculated using logistic regression with age and sex as covariates. In the combined stage 2 analysis and the joint analysis of stage 1 and 2, country of origin was included as an additional covariate. P_{nom} in all analyses were corrected for population stratification by dividing the corresponding χ^2 by the inflation factor λ ($P_{nom/\lambda\ corrected}$). $P_{corrected(B)}$, λ–corrected *P* value corrected for multiple testing according to Bonferroni correcting for 208,733 SNPs in stage 1 and the joint analysis of stage 1 and 2, and 10 SNPs in the stage 2 analyses.
[a]Unidirectional association in all samples.

an RLS3-linked family, three index cases from families with RLS in which linkage to RLS3 was not excluded and one control compared to the reference sequence (NM_002839). We also did not find any exon deletions or duplications using quantitative real-time PCR. Among eight nonsynonymous coding SNPs genotyped in replication samples, only rs10977171 and rs35929428 were polymorphic, and these did not show any association (**Supplementary Tables** 5 and 6 online). The familial relative risk figures estimated by the risk to siblings of an affected individual (λ_s) were all below 1.04 and explain only a minor portion of the original RLS3 linkage signal[7].

PTPRD belongs to the family of type IIa receptor-like protein tyrosine phosphatases. These molecules are characterized by an extracellular region containing cell adhesion motifs and an intracellular region containing two phosphatase domains[12]. Several *PTPRD* mRNA isoforms are expressed in a developmental and tissue-specific manner[13]. Both RLS-associated SNPs are located within the 5′ UTR, consisting of ten noncoding exons contained in two known long splice variants expressed predominantly in fetal and adult brain tissue[13,14]. The involvement of *PTPRD* in RLS is unknown. Studies in *Ptprd* and *Ptprs* knockout mice have shown that these proteins function in axon guidance and termination of mammalian motorneurons during embryonic development[12]. Investigations in neuroblastoma tumor tissue and cell lines have identified microdeletions and aberrant splicing patterns in the 5′ UTR of *PTPRD* that may influence mRNA stability and thereby gene expression[15].

The RLS-associated SNPs are common (MAF (CEU) $>$ 0.13) and show weak effects (rs4626664, OR = 1.44, 95% CI = 1.3–1.6; rs1975197, OR = 1.31, 95% CI = 1.2–1.4). We failed to detect rare alleles with strong effects within this gene that could explain the linkage signal. The association of two independent signals strengthens the evidence for *PTPRD* as a gene influencing risk of RLS.

PTPRD is the fourth locus associated with RLS at a significance level that withstands correction for multiple testing in a genome-wide analysis for common variants. The two newly identified association signals on chromosome 9p add another four to the previous six risk alleles from chromosomes 2p, 6p and 15q, making a total of ten possible risk alleles (referring to homozygous carriers). Analysis of the receiver operating characteristic curve shows limited usefulness for individual risk prediction, with the area under the curve estimated at 0.624. This is in line with heritability estimates of 0.6, pointing to genetic and nongenetic effects contributing to the risk of RLS[7]. Dependent on the number of risk alleles, there is an increased risk for RLS with an empirical OR larger than 9, as we found when analyzing 309 carriers with at least 7 risk alleles (**Supplementary Methods**). This makes RLS highly amenable to association studies using common variants.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design: B.M.-M., T.M., J.W. Recruitment and biobanking of RLS cases: C.T., A.Z., K.S.-K., W.O., C.G.B., W. Paulus, B.H., B.F., V.G., W. Poewe, I.P., T.M., J.W. Recruitment and biobanking of KORA controls: C.G., T.I., H.-E.W. Recruitment and biobanking of Canadian RLS cases and controls: L.X., J.M., G.T., G.R. Recruitment and biobanking of Czech RLS cases and controls: D.K., P.V., J.V., K.S., S.N. Affymetrix genotyping: B.S., G.E., P.L. Sequenom genotyping: B.S., D.K., P.L. Supervision of all markers typed: P.L., J.W. Statistical analysis: D.R., S.R., B.M.-M. Clustering of Affymetrix genotypes: D.R., S.R., B.M.-M. Manuscript writing: B.S., D.K., D.R., F.H., B.M.-M., T.M., J.W.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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ATTACHMENT D

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Genome-Wide Association Study Identifies Novel Restless Legs Syndrome Susceptibility Loci on 2p14 and 16q12.1

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Abstract

Restless legs syndrome (RLS) is a sensorimotor disorder with an age-dependent prevalence of up to 10% in the general population above 65 years of age. Affected individuals suffer from uncomfortable sensations and an urge to move in the lower limbs that occurs mainly in resting situations during the evening or at night. Moving the legs or walking leads to an improvement of symptoms. Concomitantly, patients report sleep disturbances with consequences such as reduced daytime functioning. We conducted a genome-wide association study (GWA) for RLS in 922 cases and 1,526 controls (using 301,406 SNPs) followed by a replication of 76 candidate SNPs in 3,935 cases and 5,754 controls, all of European ancestry. Herein, we identified six RLS susceptibility loci of genome-wide significance, two of them novel: an intergenic region on chromosome 2p14 (rs6747972, $P = 9.03 \times 10^{-11}$, OR = 1.23) and a locus on 16q12.1 (rs3104767, $P = 9.4 \times 10^{-19}$, OR = 1.35) in a linkage disequilibrium block of 140 kb containing the 5′-end of *TOX3* and the adjacent non-coding RNA *BC034767*.

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Introduction

Restless legs syndrome (RLS) is a common neurological disorder with a prevalence of up to 10 %, which increases with age [1]. Affected individuals suffer from an urge to move due to uncomfortable sensations in the lower limbs present in the evening or at night. The symptoms occur during rest and relaxation, with walking or moving the extremity leading to prompt relief. Consequently, initiation and maintenance of sleep become defective [1]. RLS has been associated with iron deficiency, and is pharmacologically responsive to dopaminergic substitution. Increased cardiovascular events, depression, and anxiety count among the known co-morbidities [1].

Genome-wide association studies (GWAs) identified genetic risk factors within *MEIS1*, *BTBD9*, *PTPRD*, and a locus encompassing *MAP2K5* and *SKOR1* [2–4]. To identify additional RLS susceptibility loci, we undertook an enlarged GWA in a German case-control population, followed by replication in independent case-control samples originating from Europe, the United States of America, and Canada. In doing so, we identified six RLS susceptibility loci with genome-wide significance in the joint analysis, two of them novel: an intergenic region on chromosome 2p14 and a locus on 16q12.1 in close proximity to *TOX3* and the adjacent non-coding RNA *BC034767*.

Results/Discussion

We enlarged our previously reported [2,4] GWA sample to 954 German RLS cases and 1,814 German population-based controls from the KORA-S3/F3 survey and genotyped them on Affymetrix 5.0 (cases) and 6.0 (controls) arrays. To correct for population stratification, as a first step, we performed a multidimensional scaling (MDS) analysis, leading to the exclusion of 18 controls as outliers. In a second step, we conducted a variance components analysis to identify any residual substructure in the remaining samples, resulting in an inflation factor λ of 1.025 (Figures S1 and S2). The first four axes of variation from the MDS analysis were included as covariates in the association analysis of the genome-wide stage and all P-values were corrected for the observed λ.

Prior to statistical analysis, genotyping data was subjected to extensive quality control. We excluded a total of 302 DNA samples due to a genotyping call rate <98 %. For individual SNP quality control, we adopted a stringent protocol in order to account for the complexity of an analysis combining 5.0 and 6.0 arrays. We excluded SNPs with a minor allele frequency (MAF) <5%, a callrate <98%, or a significant deviation from Hardy-Weinberg Equilibrium (HWE) in controls ($P<0.00001$). In addition, we dropped SNPs likely to be false-positive associations due to differential clustering between 5.0 and 6.0 arrays by adding a second set of cases of an unrelated phenotype and discarding SNPs showing association in this setup (see Materials and Methods). Finally, we tested 301,406 SNPs for association in 922 cases and 1,526 controls. Based on a threshold level of a nominal λ-corrected $P_{GWA}<10^{-4}$, a total of 47 SNPs distributed over 26 loci were selected for follow-up in the replication study (Figure 1, Table S1).

We genotyped these 47 SNPs together with 29 adjacent SNPs in strong linkage disequilibrium (LD, $r^2=0.5–0.9$) using the Sequenom iPLEX platform in seven case-control populations of European descent, comprising a total of 3,935 cases and 5,754 controls. Eleven SNPs with a call rate <95%, MAF<5%, and $P<0.00001$ for deviation from HWE in controls as well as 432 samples with a genotyping call rate <90% were excluded. A set of 47 SNPs, genotyped in 186 samples on both platforms (Affymetrix and Sequenom), was used to calculate an average concordance rate of 99.24 %.

The combined analysis of all replication samples confirmed the known four susceptibility loci and, in addition, identified two novel association signals on chromosomes 2p14 and 16q12.1 (Table 1). To address possible population stratification within the combined replication sample, we performed a fixed-effects meta-analysis. For four of the replication case-control populations, we included λ inflation factors which were available from a genomic controls experiment in a previous study in these populations [4]. These were used to correct the estimates for the standard error. Joint analysis of GWA and all replication samples showed genome-wide significance for these two novel loci as well as for the known RLS loci in *MEIS1*, *BTBD9*, *PTPRD*, and *MAP2K5/SKOR1* with a nominal λ -corrected $P_{JOINT}<5\times10^{-8}$ (Table 1). Depending on the variable power to detect the effects, the separate analyses of individual subsamples in the replication either confirmed the association after correction for multiple testing or yielded nominally significant results (Tables S2 and S3). The differing relevance of the risk loci in the individual samples is illustrated in

Author Summary

Restless legs syndrome (RLS) is one of the most common neurological disorders. Patients with RLS suffer from an urge to move the legs and unpleasant sensations located mostly deep in the calf. Symptoms mainly occur in resting situations in the evening or at night. As a consequence, initiation and maintenance of sleep become defective. Here, we performed a genome-wide association study to identify common genetic variants increasing the risk for disease. The genome-wide phase included 922 cases and 1,526 controls, and candidate SNPs were replicated in 3,935 cases and 5,754 controls, all of European ancestry. We identified two new RLS–associated loci: an intergenic region on chromosome 2p14 and a locus on 16q12.1 in a linkage disequilibrium block containing the 5′-end of *TOX3* and the adjacent non-coding RNA *BC034767*. *TOX3* has been implicated in the development of breast cancer. The physiologic role of TOX3 and BC034767 in the central nervous system and a possible involvement of these two genes in RLS pathogenesis remain to be established.

forest plots (Figure 2). There was no evidence of epistasis between any of the six risk loci ($P_{Bonferroni} > 0.45$).

The association signal on 2p14 (rs6747972: nominal λ-corrected $P_{JOINT} = 9.03 \times 10^{-11}$, odds ratio (OR) = 1.23) is located in an LD block of 120 kb within an intergenic region 1.3 Mb downstream of *MEIS1* (Figure 3). Assuming a long-range regulatory function of the SNP-containing region, *in silico* analysis for clusters of highly conserved non-coding elements using the ANCORA browser (http://ancora.genereg.net) identified *MEIS1* as well as *ETAA1* as potential target genes [5,6].

The second locus on chromosome 16q12.1 (rs3104767: nominal λ-corrected $P_{JOINT} = 9.4 \times 10^{-19}$, OR = 1.35) is located within an LD block of 140 kb (Figure 3), which contains the 5′UTR of *TOX3* (synonyms *TNRC9* and *CAGF9*) and the non-coding RNA *BC034767* (synonym *LOC643714*). *TOX3* is a member of the high mobility box group family of non-histone chromatin proteins which interacts with *CREB* and *CBP* and plays a critical role in mediating calcium-dependent transcription in neurons [7]. GWAs have identified susceptibility variants for breast cancer in the identical region [8]. The best-associated breast cancer SNP, rs3803662, is in low LD ($r^2 \sim 0.1$, HapMap CEU data) with rs3104767, but showed association to RLS (λ-corrected nominal $P_{GWA} = 7.29 \times 10^{-7}$). However, logistic regression analysis conditioned on rs3104767 demonstrated that this association is dependent on rs3104767 (rs3803662: $P_{GWA/conditioned} = 0.2883$).

BC034767 is represented in GenBank by two identical mRNA transcripts, BC034767 and BC029912. According to the gene model information of the UCSC and Ensembl genome browsers (http://genome.ucsc.edu and http://www.ensembl.org/index.html), these mRNAs are predicted to be non-coding. Additional *in silico* analysis using the Coding Potential Calculator (http://cpc.cbi.pku.edu.cn) supported this by attributing only a weak coding potential to this RNA, suggesting a regulatory function instead [9]. We also searched for rare alleles with strong effects and performed a mutation screening by sequencing all coding and non-coding

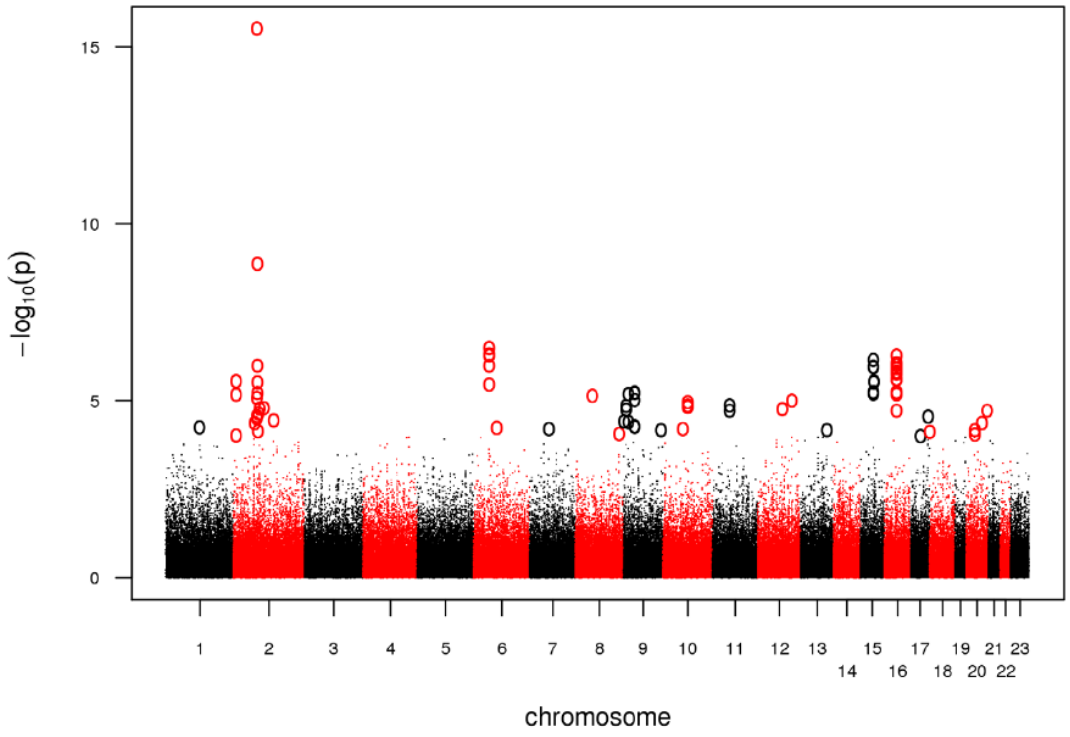

Figure 1. Manhattan plot of the GWA. Association results of the GWA stage. The x-axis represents genomic position along the 22 autosomes and the x-chromosome, the y-axis shows -log10(P) for each SNP assayed. SNPs with a nominal λ-corrected $P < 10^{-4}$ are highlighted as circles.
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exons of *TOX3* and *BC034767* in 188 German RLS cases (Table S4). In *TOX3*, a total of nine variants not listed in dbSNP (Build 130) were found, three of which are non-synonymous. Only one of these is also annotated in the 1000 Genomes project (November 2010 data release). Three additional new variants were located in putative exons 1 and 2 of *BC034767*. Analysis of the frequency of these variants as well as all known non-synonymous, frameshift, and splice-site coding SNPs in *TOX3* in a subset of one of the replication samples (726 cases and 735 controls from the GER1 sample) did not reveal any association to RLS. For a power of >80%, however, variants with an OR above 4.5 and a MAF ≥0.01 would be required. For even lower MAFs, ORs ≥10 would be necessary for sufficient power. Furthermore, the described CAG repeat within exon 7 of *TOX3* was not polymorphic as shown by fragment analysis in 100 population-based controls.

According to publicly available expression data (http://genome.ucsc.edu), in humans, *BC034767* is expressed in the testes only, while *TOX3* expression has been shown in the salivary glands, the trachea, and in the CNS. Detailed in-depth real time PCR profiling of *TOX3* showed high expression levels in the frontal and occipital cortex, the cerebellum, and the retina [10]. To assess a putative eQTL function of rs6747972 or rs3104767, we studied the SNP-genotype-dependent expression of *TOX3* and *BC034767* as well as of genes known to directly interact with *TOX3* (*CREB-1/CREBBP/CITED1*) and potential target genes of long-range regulatory elements at the locus on chromosome 2 (*MEIS1/ETAA1*) in RNA expression microarray data from peripheral blood in 323 general population controls [11]. No differential genotype-dependent expression variation was found.

To assess the potential for genetic risk prediction, we split our GWA sample in a training and a test set and determined classifiers for case-control status in the training set to predict case-control status in the test set. Training and test set were independent of each other – not only with respect to included individuals but also with respect to the genotyping procedure as we used genotypes generated on different genotyping platforms. As training set, we used those cases of the current GWA which had been genotyped on 500K arrays in a previous GWA and the corresponding control set [2], in total, 326 cases and 1,498 controls. The test set comprised 583 cases and 1,526 controls, genotyped on 5.0/6.0 arrays as part of the current study. Prior to the analysis, we removed the six known risk loci and performed LD-pruning to limit the analysis to SNPs not in LD with each other. In the end, a total of 76,532 SNPs were included in the pruned dataset. We conducted logistic regression with age and sex as covariates. Based on these association results, the sum score of SNPs showing the most significant effects (i.e. the number of risk alleles over all SNPs) weighted by the ln(OR) of these effects was chosen as predictor variable in the test set. We then varied the P-value threshold for SNPs included in the sum score. For a P-value <0.6, we observed a maximum area under the curve (AUC) of 63.9% and an explained genetic variance of 6.6% (Nagelkerke's R), values comparable to estimates obtained for other complex diseases such as breast cancer or diabetes (Table S5) [12–14]. Inclusion of the six known risk loci in this analysis resulted in a maximum AUC of 64.2% and an explained genetic variance of 6.8%.

Additionally, we performed risk prediction in the combined GWA and replication sample including only the six established RLS risk loci. For this purpose, we used the weighted risk allele score resulting in ORs of up to 8.6 (95% CI: 2.46–46.25) and an AUC of 65.1% (Figures S3 and S4).

By increasing the size of our discovery sample, we have identified two new RLS susceptibility loci. The top six loci show effect sizes between 1.22 and 1.77 and risk allele frequencies between 19 and 82%, and reveal genes in neuronal transcription pathways not previously suspected to be involved in the disorder.

Materials and Methods

Study population and phenotype assessment

Ethics statement. Written informed consent was obtained from each participant in the respective language. The study has

Table 1. Association results of GWA and joint analysis of GWA and replication.

Chr	Locus	LD block (Mb)	SNP	Position (bp)	Risk allele	Risk allele frequency cases/controls	P_{GWA}	$P_{REPLICATION}$	P_{JOINT}	Odds ratio (95% CI)
Known risk loci (1 SNP per locus)										
2	MEIS1	66.57–66.64	rs2300478	66634957	G	0.35/0.24	7.77×10^{-16}	4.39×10^{-35}	3.40×10^{-49}	1.68 (1.57–1.81)
6	BTBD9	37.82–38.79	rs9357271	38473851	T	0.82/0.76	6.74×10^{-7}	2.01×10^{-16}	7.75×10^{-22}	1.47 (1.35–1.47)
9	PTPRD	8.80–8.88	rs1975197	8836955	A	0.19/0.16	4.94×10^{-5}	1.07×10^{-6}	3.49×10^{-10}	1.29 (1.19–1.40)
15	MAP2K5/SKOR1	65.25–65.94	rs12593813	65823906	G	0.75/0.68	1.49×10^{-6}	1.54×10^{-17}	1.37×10^{-22}	1.41 (1.32–1.52)
New genome-wide significant loci ($P_{JOINT} < 5.2\times10^{-8}$)										
2	intergenic region	67.88–68.00	rs6747972	67923729	A	0.47/0.44	1.37×10^{-6}	3.73×10^{-6}	9.03×10^{-11}	1.23 (1.16–1.31)
			rs2116050	67926267	G	0.49/0.47	7.84×10^{-6}	4.85×10^{-6}	4.83×10^{-10}	1.22 (1.15–1.30)
16	TOX3/BC034767	51.07–51.21	rs3104767	51182239	G	0.65/0.58	7.38×10^{-7}	2.16×10^{-13}	9.40×10^{-19}	1.35 (1.27–1.43)
			rs3104788	51196004	T	0.65/0.58	1.19×10^{-6}	2.42×10^{-13}	1.63×10^{-18}	1.33 (1.25–1.43)

RLS-associated SNPs with genome-wide significance. P_{GWA}, λ-corrected nominal P-value of GWA stage. $P_{REPLICATION}$, nominal P-value obtained from meta-analysis of the replication stage samples. P_{JOINT}, nominal P-value of the joint meta-analysis of GWA and replication stage, λ-corrected in samples where λ-values were available. Nominal P-values in GWA were calculated using logistic regression with sex, age, and the first four components from the MDS analysis of the IBS matrix as covariates. For nominal $P_{REPLICATION}$ and P_{JOINT} -values, a fixed-effects inverse-variance meta-analysis was performed. Risk allele frequencies and odds ratios were calculated in the joint sample. LD blocks were defined by D′ using Haploview 4.2 based on HapMap CEU population data from HapMap release #27. CI, 95% confidence interval. Genome positions refer to the Human March 2006 (hg18) assembly.

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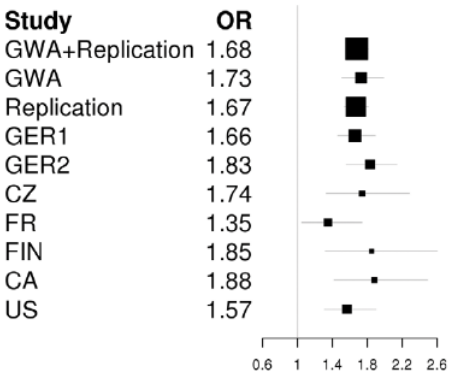
A



OR and CI for rs2300478

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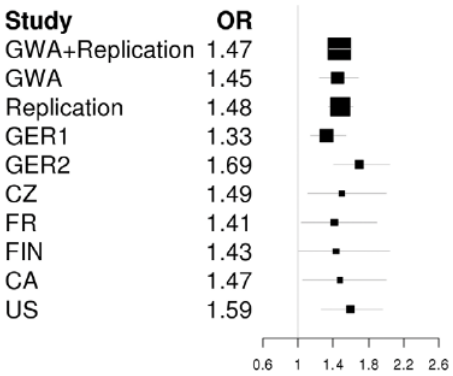

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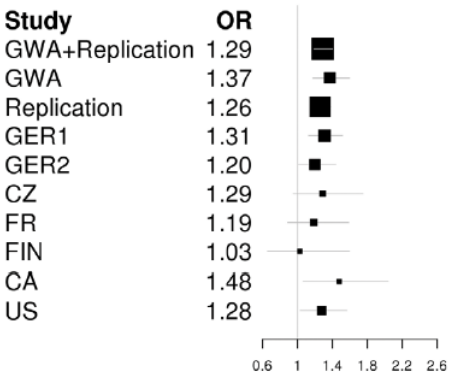

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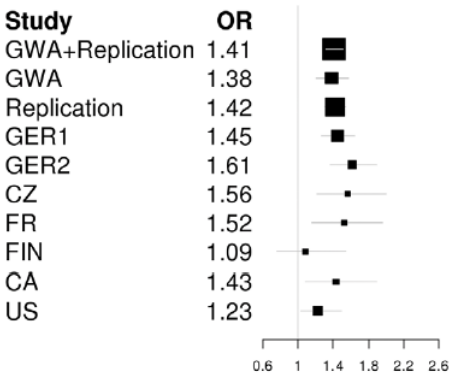

OR and CI for rs12593813

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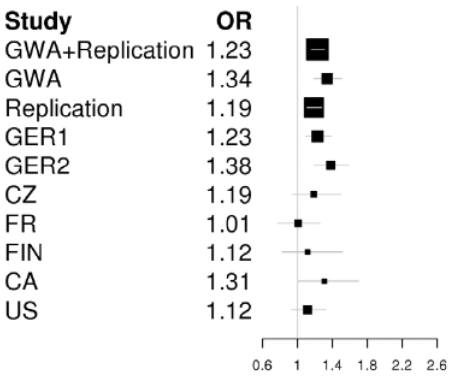

OR and CI for rs6747972

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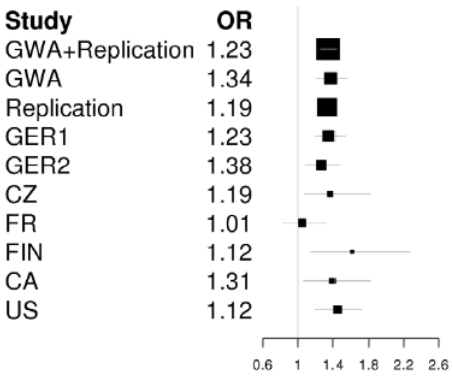

OR and CI for rs3104767

Figure 2. Forest plots of the RLS risk loci (1 SNP per locus). OR and corresponding confidence interval for the GWA sample, all individual replication samples, the combined replication sample as well as the combined GWA and replication sample are depicted. ORs are indicated by squares with the size of the square corresponding to the sample size for the individual populations. (A) rs2300478 in *MEIS1*; (B) rs9357271 in *BTBD9*; (C) rs1975197 in *PTPRD*; (D) rs12593813 in *MAP2K5/SKOR1*; (E) rs6747972 in intergenic region on chromosome 2; (F) rs3104767 in *TOX3/BC034767*.
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been approved by the institutional review boards of the contributing authors. The primary review board was located in Munich, Bayerische Ärztekammer and Technische Universität München.

RLS patients (GWA and replication phase). A total of 2,944 cases (GWA = 954, replication = 1,990) of European descent were recruited in two cycles via specialized outpatient clinics for RLS. German and Austrian cases for the GWA (GWA) and the replication sample (GER1) were recruited in Munich, Marburg, Kassel, Göttingen, Berlin (Germany, n in GWA = 830, n in GER1 = 1,028), Vienna, and Innsbruck (Austria, n in GWA = 124, n in GER1 = 288). The additional replication samples originated from Prag (Czech Republic (CZ), n = 351), Montpellier (France (FR), n = 182), and Turku (Finland (FIN), n = 141). In all patients, diagnosis was based upon the diagnostic criteria of the International RLS Study Group [1] as assessed in a personal interview conducted by an RLS expert. A positive family history was based on the report of at least one additional family member affected by RLS. We excluded patients with secondary RLS due to uremia, dialysis, or anemia due to iron deficiency. The presence of secondary RLS was determined by clinical interview, physical and neurological examination, blood chemistry, and nerve conduction studies whenever deemed clinically necessary.

In addition, 1,104 participants (GER2) of the "Course of RLS (COR-) Study", a prospective cohort study on the natural course of disease in members of the German RLS patient organizations, were included as an additional replication sample. After providing informed consent, study participants sent their blood for DNA extraction to the Institute of Human Genetics, Munich, Germany. A limited validation of the RLS diagnosis among the majority of members was achieved through a diagnostic questionnaire. Five percent had also received a standardized physical examination and interview in one of the specialized RLS centers in Germany prior to recruitment. To avoid doublets, we checked these subjects against those recruited through other German RLS centers and excluded samples with identical birth date and sex.

556 cases (US) were recruited in the United States at Departments of Neurology at Universities in Baltimore, Miami, Houston, and Palo Alto. Diagnosis of RLS was made as mentioned above.

285 cases (CA) were recruited and diagnosed as above in Montréal, Canada. All subjects were exclusively of French-Canadian ancestry as defined by having four grandparents of French-Canadian origin.

Detailed demographic data of all samples are provided in Table S6.

Control populations (GWA and replication phase). Controls for German and Austrian cases were of European descent and recruited from the KORA S3/F3 and S4 surveys, general population-based controls from southern Germany. KORA procedures and samples have been described [15]. For the GWA phase, we included 1,814 subjects from S3/F3, and, for the replication stage, 1,471 subjects from S4.

For replication of the GER2 sample, we used controls from the Dortmund Health Study (DHS), a population-based survey conducted in the city of Dortmund with the aim of determining the prevalence of chronic diseases and their risk factors in the general population. Sampling for the study was done randomly from the city's population register stratified by five-year age group and gender [16]. 597 subjects selected at random from the Czech blood and bone marrow donor registry served as Czech controls [17]. French controls included 768 parents of multiple sclerosis patients recruited from the French Group of Multiple Sclerosis Genetics Study (REFGENSEP) [18]. Finnish controls comprised 360 participants of the National FINRISK Study, a cross-sectional population survey on coronary risk factors collected every five years. The current study contains individuals recruited in 2002. Detailed description of the FINRISK cohorts can be found at www.nationalbiobanks.fi.

French-Canadian controls were 285 unrelated individuals recruited at the same hospital as the cases.

1,200 participants of the Wisconsin Sleep Cohort (WSC), an ongoing longitudinal study on the causes, consequences, and natural course of disease of sleep disorders, functioned as US controls [19].

None of the controls were phenotyped for RLS. All studies were approved by the institutional review boards in Germany, Austria, Czech Republic, France, Finland, the US, and Canada. Written informed consent was obtained from each participant. Detailed demographic data of all samples are provided in Table S6.

Genotyping

GWA. Genotyping was performed on Affymetrix Genome-Wide Human SNP Arrays 5.0 (cases) and 6.0 (controls) following the manufacturer's protocol. The case sample included 628 cases from previous GWAs [2,4] and 326 new cases. After genotype-calling using the BRLMM-P clustering algorithm [20], a total of 475,976 overlapping SNPs on both Affymetrix arrays were subjected to quality control. We added 655 cases of a different phenotype unrelated to RLS, genotyped on 5.0 arrays, to the analysis and excluded those SNPs which showed a significant difference of allele frequencies in cases (RLS and unrelated phenotype on 5.0) and controls (6.0) (n = 92). Thereby, we filtered out SNPs likely to be false-positive associations. We excluded SNPs with a minor allele frequency (MAF) <5% (n = 88,582), a callrate <98% (n = 65,906) or a significant deviation from Hardy-Weinberg Equilibrium (HWE) in controls (P<0.00001) (n = 20,060). Cluster plots of the GWA genotyping data for the best-associated SNPs in Table 1 are shown in Figure S5. Genotypes of these SNPs are available in Table S7.

Replication. We selected all SNPs with a λ-corrected $P_{nominal}<10^{-4}$ in the GWA for replication. These SNPs clustered in 26 loci (defined as the best associated SNP ±150 kb of flanking sequence). We genotyped a total of three SNPs in each of the 26 regions. These were either further associated neighbouring SNPs with a λ-corrected $P_{nominal}<10^{-3}$ or, in case of singleton SNPs, additional neighbouring SNPs from HapMap with the highest possible r^2 (at least >0.5) with the best-associated SNP. We also genotyped the best-associated SNPs identified in the previous GWAs [2,4].

Genotyping was performed on the MassARRAY system using MALDI-TOF mass spectrometry with the iPLEX Gold chemistry (Sequenom Inc, San Diego, CA, USA). Primers were designed using AssayDesign 3.1.2.2 with iPLEX Gold default parameters. Automated genotype calling was done with SpectroTYPER 3.4. Genotype clustering was visually checked by an experienced evaluator.

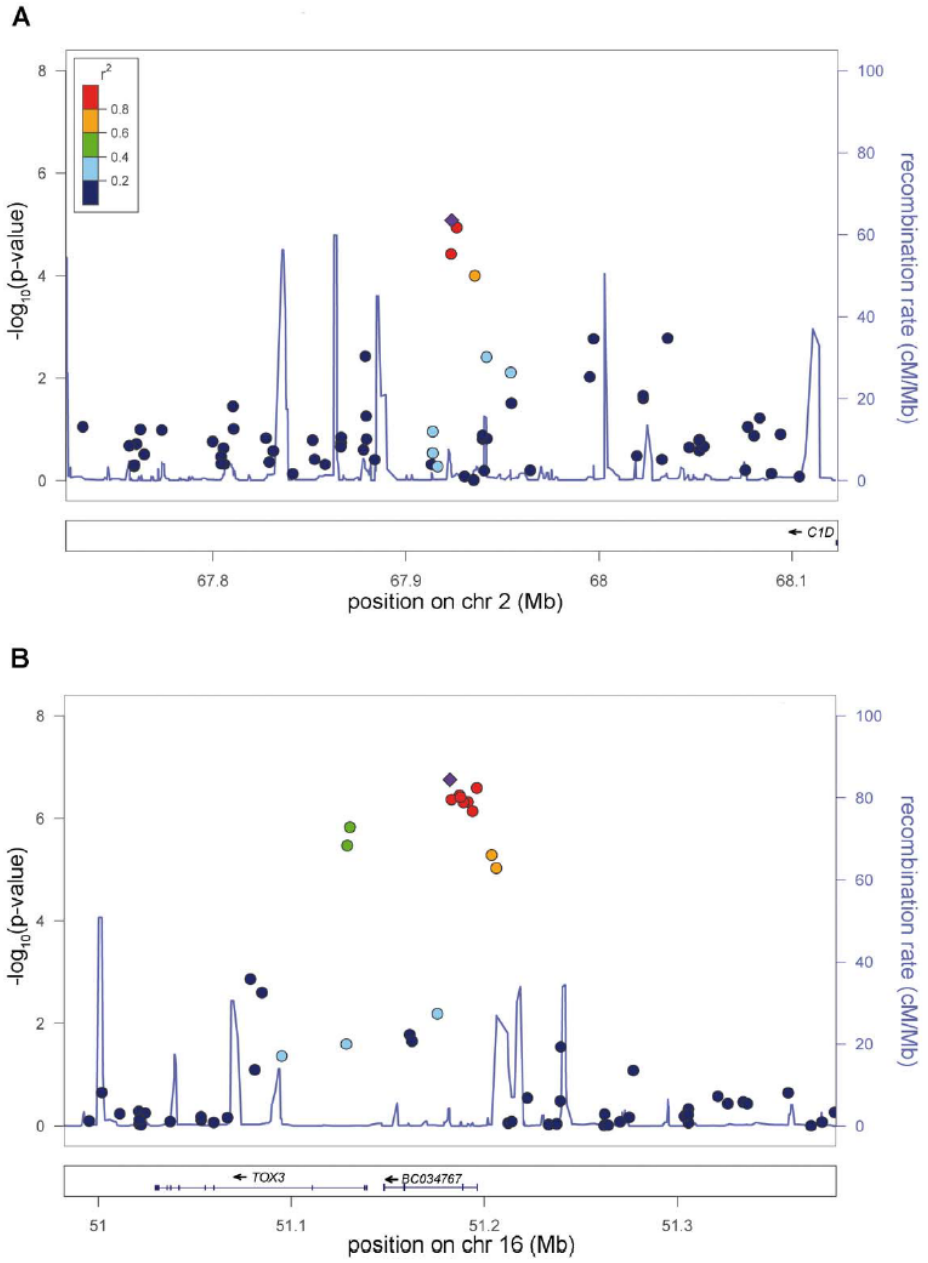

Figure 3. New genome-wide significant RLS loci. a) Risk locus on chromosome 2p14, showing the best-associated SNP rs6747972 and ±200 kb of surrounding sequence. b) Risk locus on chromosome 16p21, showing the best-associated SNP rs3104767 and ±200 kb of surrounding sequence. The left-hand x-axis shows the negative log10 of the nominal λ-corrected P-values of the GWA stage for all SNPs genotyped in the respective region. The right-hand x-axis shows the recombination frequency in cM/Mb. The y-axis shows the genomic position in Mb based on the hg18 assembly. The r^2-based LD between SNPs is colour-coded, ranging from red (r^2>0.8) to dark blue (r^2<0.2) and uses the best-associated SNP as reference. This SNP is depicted as a violet diamond. Recombination frequency and r^2 values are calculated from the HapMap II (release 22) CEU population. Plots were generated with LocusZoom 1.1 (http://csg.sph.umich.edu/locuszoom/).
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SNPs with a call rate<95%, MAF<5%, and P<0.00001 for deviations from HWE in controls were excluded. DNA samples with a call rate<90% were also excluded.

Population stratification analysis

GWA. To identify and correct for population stratification, we performed an MDS analysis as implemented in PLINK 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink, [21]) on the IBS matrix of our discovery sample. After excluding outliers by plotting the main axes of variation against each other, we performed logistic regression with age, sex, and the values of the MDS components as covariates. Using the Genomic Control approach [22], we obtained an inflation factor λ of 1.11.

Additionally, we performed a variance components analysis using the EMMAX software (http://genetics.cs.ucla.edu/emmax, [23]) and, again, calculated the inflation factor with Genomic Control, now resulting in a λ of 1.025. EMMAX uses a mixed linear model and does not only correct for population stratification but also for hidden relatedness. We, therefore, decided to base correction for population substructure on the EMMAX results.

Replication. Correction for population stratification was performed for the German, Czech, and the Canadian subsamples. The λ-values of 1.1032, 1.2286, and 1.2637 were derived from a previous Genomic Control experiment within the same samples using 176 intergenic or intronic SNPs [4]. Here, we had applied the expanded Genomic Control method GCF developed by Devlin and Roeder [24]. In the meta-analysis of all replication samples, the λ-corrected standard errors were included for the German, Czech, and Canadian samples. For the other replication samples from France, Finland, and the USA, no such data was available and, therefore, no correction factor was included in the analysis.

Statistical analysis

Statistical analysis was performed using PLINK 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink, [21]). In the GWA sample, we applied logistic regression with age, sex, and the first four axes of variation resulting from an MDS analysis as covariates.

P-values were λ-corrected with the λ of 1.025 from the EMMAX analysis. In the individual analysis of the single replication samples, we tested for association using logistic regression and correcting for gender and age as well as for population stratification where possible (see Population Stratification). Each replication sample was Bonferroni-corrected using the number of SNPs which passed quality control for the respective sample.

For the combined analysis of all replication samples, we performed a fixed-effects inverse-variance meta-analysis. Where available, we used λ-corrected standard errors in this analysis. Bonferroni-correction was performed for 74 SNPs, i.e. the number of SNPs which passed quality control in at least one replication sample.

For the joint analysis of the GWA and the replication samples, we also used a fixed-effects inverse-variance meta-analysis and again included λ-corrected values as far as possible. For the conditioned analysis, the SNP to be conditioned on was included as an additional covariate in the logistic regression analysis as implemented in PLINK.

Interaction analysis was performed using the –epistasis option in PLINK. Significance was determined via Bonferroni-correction (i.e. 0.05/28, as 28 SNP combinations were tested for interaction).

Power calculation

Power calculation was performed using the CaTS power calculator [25] using a prevalence set of 0.08 and an additive genetic model (Table S3). The significance level was set at 0.05/74 for replication stage analysis and at 0.05/301,406 for genome-wide significance in the joint analysis of GWA and replication. For the rare variants association study, the significance level was set at 0.05/12.

Mutation screening of *TOX3* and *BC034767*

All coding and non-coding exons including adjacent splice sites of *TOX3* (reference sequence NM_001146188) and *BC034767* (reference sequence IMAGE 5172237) were screened for mutations in 188 German RLS cases.

Mutation screening was performed with high resolution melting curve analysis using the LightScanner technology and standard protocols (IDAHO Technology Inc.). DNAs were analyzed in doublets. Samples with aberrant melting pattern were sequenced using BigDyeTerminator chemistry 3.1 (ABI) on an ABI 3730 sequencer. Sequence analysis was performed with the Staden package [26]. Primers were designed using ExonPrimer (http://ihg.gsf.de) or Primer3plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). All identified variants were then genotyped in 735 RLS cases and 735 controls of the general population (KORA cohort) on the MassARRAY system, as described above.

In addition, fragment analysis of exon 7 of *TOX3* was performed to screen for polymorphic CAG trinucleotide repeats. DNA of 100 controls (50 females, 50 males) was pooled and analyzed on an ABI 3730 sequencer with LIZ-500 (ABI) as a standard. Primers were designed using Primer3plus, the forward Primer contains FAM for detection. Analysis was performed using GeneMapper v3.5.

Expression analyses

Associations between *MEIS1/ETAA1* RNA expression and rs6747972 and between *TOX3/BC034767/CREB-1/CREBBP/CITED1* expression and rs3104767 were assessed using genome-wide SNP data (Affymetrix 6.0 chip) in conjunction with microarray data for human blood samples (n = 323 general population controls from the KORA cohort, Illumina Human WG6 v2 Expression BeadChip) [11]. A linear regression model conditioned on expression and controlling for age and sex was used to test for association.

Prediction of genetic risk

Based on the performance of P-value-threshold selected SNPs in a training and a test sample. As training sample, we used those GWA-cases which had also been genotyped for our previous study [2]. We also included the control samples from this study. As a first quality control step, we carried out an association analysis comparing the Affymetrix 500K genotypes of these GWA-cases to the Affymetrix 5.0 genotypes of the same cases. Significant P-values would indicate systematic differences in the genotyping between the different chips. For further analysis, we only used those 259,302 SNPs with P-values >0.10. We performed a second quality control step in which IDs with a callrate below 98% and SNPs with a callrate below 98%, a MAF lower than 5%, or a P-value for deviation from HWE<0.00001 were removed.

Further, we excluded the four already known risk loci as well as the two newly identified loci and performed LD-pruning to limit the analysis to SNPs not in LD with each other. This was performed using a window-size of 50 SNPs. In each step, this window was shifted 5 SNPs. We used a threshold of 2 for the VIF (variance inflation factor). 76,532 SNPs, 326 cases, and 1,498 controls were included in the final training dataset. We conducted logistic regression with age and sex as covariates. Based on these association results, the sum score of SNPs showing the most

significant effects (i.e. the number of risk alleles over all SNPs) weighted by the ln(OR) of these effects was chosen as predictor variable in the test set, comprising the remaining 583 cases of the GWA sample and 1,526 controls. None of these cases/controls were included in the training-sample, i.e. the test-sample constitutes a completely independent sample. Based on this sum score, we calculated the ROC curve and Nagelkerke's R to measure the explained variance.

Based on a weighted risk allele score. To evaluate the predictive value in our sample, we calculated a weighted sum score of risk alleles in the combined GWA and replication sample. To this end, we used one SNP from each RLS risk region and also included markers from the two newly identified regions on chromosome 16q12 and 2p14 (*MEIS1:* rs2300478, *2p14*: rs6747972, *BTBD9*: rs9296249, *PTPRD*: rs1975197, *MAP2K5:* rs11635424, *TOX3/BC034767*: rs3104767). At each SNP, the number of risk alleles was weighted with the corresponding ln(OR) for this SNP. The corresponding distribution of the score in cases and controls is illustrated in Figure S3. Employing this score for risk prediction resulted in an AUC of 0.651 (Figure S4).

Supporting Information

Figure S1 MDS analysis plot for GWA. Distribution of cases (red) and controls (black) along the two main axes of variation identified in the MDS analysis. The three visible clouds are due to a common 3.8 Mb inversion polymorphism on chromosome 8 (described in: Tian C, Plenge RM, Ransom M, Lee A, Villoslada P, et al. (2008) Analysis and Application of European Genetic Substructure Using 300 K SNP Information. PLoS Genet 4: e4. doi:10.1371/journal.pgen.0040004).
(TIFF)

Figure S2 QQ-plot of GWA results. QQ-plot showing the P-value distribution before (red) and after (blue) correction for population stratification using Genomic Control.
(TIFF)

Figure S3 Weighted risk allele score analysis. Histogram of the weighted risk allele scores for cases and controls. The corresponding OR and CI for each category against the median category is depicted in green. The left y-axis refers to the number of individuals (in %), the right-axis refers to the OR values.
(TIFF)

Figure S4 ROC curve for weighted risk score analysis. Receiver operating characteristic (ROC) curve for the weighted risk allele score approach of risk prediction. The area under the curve (AUC) is 65.1%.
(TIFF)

Figure S5 Cluster plots of GWA genotyping for the six risk loci. For the best-associated SNPs at each risk locus, clusterplots were generated for cases and controls. Intensities of the A and B allele (based on the Affymetrix annotation of the SNPs) are given on the x- and y-axes and the respective genotypes are indicated in blue, green, and orange.
(PDF)

Table S1 GWA results for SNPs with λ-corrected $P_{GWA}<10–4$ and additional SNPs selected for replication. A star (*) indicates SNPs which had been identified in previous RLS GWAs [2–4]. P-values of the GWA phase are given as λ-corrected nominal P-values. Two different methods for λ correction were applied, multi-dimensional-scaling (MDS)-analysis using PLINK and variance components (VC)-analysis using the EMMAX software with the P-values listed in the respective columns "MDS λ-corrected P_{GWA}" and "VC λ-corrected P_{GWA}". The selection of SNPs for replication was based on the MDS λ-corrected P-values. r^2-values based on Hapmap CEU data are given for those SNPs which were selected for replication based on their LD with the best-associated SNP in each region. Genomic position and gene annotation refer to the hg18 genome.
(DOC)

Table S2 Replication stage association results for individual replication samples. P-values are derived from logistic regression and correcting for gender and age as well as for population stratification where possible (see Materials and Methods). Each replication sample was Bonferroni-corrected using the number of SNPs which passed quality control for the respective sample. The OR refers to the minor allele. NA; SNP could not be analysed due to failing quality control in the respective sample.
(DOC)

Table S3 Power analysis for GWA, replication and joint analysis of GWA and replication. Power calculation was performed using the CaTS power calculator [25] using a prevalence set of 0.08 and an additive genetic model. The significance level α was set at 0.05/74 for replication stage analysis and at 0.05/301,406 for genome-wide significance in the joint analysis of GWA and replication.
(DOC)

Table S4 Results of TOX3 and BC034767 mutation screening. * "A" refers to the mutant allele, "B" to the reference allele. Position refers to hg18 genome annotation. Codon numbering refers to the reference sequence NM_001146188. Data of the 1000 genomes project was obtained from the November 2010 release via the 1000 genomes browser (http://browser.1000genomes.org/index.html).
(DOC)

Table S5 Prediction of genetic risk; training- and test-set approach. Inclusion threshold P-values were derived from a logistic regression with age and sex as covariates in the training sample. # SNPs indicates the number of SNPs passing the inclusion threshold. Based on these association results, the sum score of SNPs showing the most significant effects (i.e. the number of risk alleles over all SNPs) weighted by the ln(OR) of these effects was chosen as predictor variable in the test set. Based on this sum score, an AUC and Nagelkerke's R were calculated.
(DOC)

Table S6 Demographic data of GWA and replication samples. Mean age, mean age of onset and respective standard deviations and ranges are given in years. N: number of individuals; SD: standard deviation; AAO: age of onset. GWA: Genome-wide association study; CZ: Czechia; FR: France; FIN: Finland; CA: Canada; US: United States. - indicates that this information is not applicable for the respective sample.
(DOC)

Table S7 Genotype data of GWA samples. Genotypes of the GWA samples are given for the eight best-associated SNPs (see Table 1). SNP alleles are ACGT-coded. Phenotype information includes gender (1 = male, 2 = female) and disease status (1 = unaffected, 2 = affected).
(XLS)

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