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Genetic and Immunological Dissection of Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

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The only true wisdom is
in knowing you know nothing.

Socrates

To my family ... with love

ABSTRACT

Multiple Sclerosis (MS) is the most common chronic inflammatory disorders of the central nervous system (CNS) caused by a combination of environmental factors and multiple genes. Animal models offer possibilities to keep environmental factors and selected genes constant and very large ”families” may be created. Therefore, an animal model known as experimental autoimmune encephalomyelitis (EAE), which shows functional and pathological similarities to human MS is here utilized to characterize underlying genes and pathogenic mechanisms. We established congenic strains, PVG.LEW-*Eae30-31*, DA.PVG-*Eae20-22*, DA.BN-*Eae4* & LEW.BN-*Eae4* and DA.PVG-*Eae19*, encompassing previously identified quantitative trait loci (QTL) on rat chromosomes (RNO) 1, 4, 9 and 15 respectively. The DA and LEW strains are EAE susceptible both in the myelin oligodendrocyte glycoprotein (MOG) - and spinal cord (SC) -induced models while the PVG and BN strains are EAE-resistant.

With a combination of the tenth (G_{10}) generation of an advanced intercross line (AIL) (DAxPVG.AV1) and congenic breeding (DAc15ACI), *Eae19* on RNO15 was narrowed down to confidence interval of 13Mb. This region harbors 32 predicted and confirmed genes with some of them with an immunological role. Among the QTLs overlapping with *Eae19*, adjuvant-induced arthritis QTL 4 (*Aia4*) might be the most interesting one since EAE regulatory effects were demonstrated in rat strains congenic for arthritis-regulating QTL.
We identified two closely located QTLs on RNO4, *Eae20* and *Eae21*, in addition to a third epistatic QTL to *Eae20*, *Eae22*. The three QTLs were mapped on a region spanning 16.8Mb co-localized with several QTLs regulating experimental autoimmune diseases such as *Oia2*, *Pia1* and *Cia13*. We demonstrated an influence of *Eae20* on incidence and severity of EAE while *Eae21* was found to regulate EAE once a certain threshold of the disease is exceeded.
By breeding interval-specific congenic lines we were able to narrow down the susceptibility region of *Eae4* to ~1 cM conferring a milder clinical EAE course and lower expression and protein secretion of TNF, IFNγ and IL-2 in ConA-stimulated splenocytes compared to the susceptible background strains. We here positionally cloned Eae4 to be *Vav1* and correlated the disease-predisposing variant to higher pro-inflammatory cytokine regulation and low proportion of $Foxp3^{+}CD4^{+}$ regulatory T cells (Tregs). Association studies in seven human cohorts demonstrated association to one disease-predisposing CA haplotype (OR=1.18) that provides higher VAV1 expression and two protecting haplotypes (CG; OR=0.86 and TG, OR=0.90) associated to lower VAV1 expression. A higher expression of VAV1 was even noticed in peripheral blood (PBMCs) and cerebrospinal fluid (CSF) of MS patients compared to controls and correlated with TNF and IFN-γ expression.
Linkage analysis in G_{10} and G_{12} of AIL resolved the region on RNO1 in two narrow QTLs, *Eae30* and *Eae31*. *Eae30* showed linkage to MOG-EAE, anti-MOG antibodies and levels of IL-6. *Eae31* was linked to both EAE and PIA, anti-MOG antibodies and levels of TNF and IL-6. Association studies in four Nordic cohorts correlated a disease-predisposing variants of *RGMA* from *Eae30* to MS (OR=1.33) to higher expression of IFN-γ in CSF of MS patients. In *Eae31*, one positively and one negatively associated haplotype were detected in *IL-21R* (rs8060368-rs961914 CT, OR=0.87 and CC, OR= 1.16). The CT haplotype of *IL21R* also correlated to lower IFN-γ expression in PBMCs of MS patients.

In conclusion, thanks to a combination of high-resolution mapping in AIL we were able to fine-map several EAE-regulating QTLs. Applying the strategy of shared QTLs between autoimmune diseases and performing association studies in MS cohorts, we were able to point to three MS-associated genes.

Keywords: EAE, MS, QTL, linkage studies, association studies, *Vav1*, *RGMA*, *IL21R*.

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Submitted

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***RGMA* and *IL21R* associate with experimental inflammation and multiple sclerosis**
Submitted

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LIST OF ABREVIATIONS

Ab	antibody
ACI	AxC 9935 Irish
AIL	Advaced intercross line
BN	Brown Norway
CFA	comlpete Freund's adjuvant
cM	centimorgan
CNS	central nervous system
ConA	Concanavalin A
CSF	cerebrospinal fluid
DA	dark agouti
DZ	dizygotic
EAE	experimental autoimmune encephalomyelitis
HLA	human leukocyte antigen
IDDM	insulin dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
LD	Linkage Disequilibrium
Lod	logarithms of odds
LFA-1	The leukocyte function-associated antigen-1
Mb	megabase
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein (aa 1-125)
MZ	monozygotis
mRNA	messenger RNA
MS	multiple sclerosis
PCR	polymerase chain reaction
PLP	proteolipid protein
PPMS	primary progressive MS
PRMS	progressive relapsing MS
PVG	Piebald Virol Glaxo
QTL	quantitative trait loci
RRMS	relapsing remitting MS
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SPMS	secondary progressive MS
TCR	T-cell Receptor
TNF	tumor necrosis factor
VLA-4	Very late activation antigen 4

1. Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease causing demyelination in the central nervous system (CNS). As a result, the axons are blocked from conducting action potentials by saltatory conduction. The prevalence of MS is 0.1% in the Northern European population (Confavreux 2000) and 0.1-0.2% in Sweden with 5/100 000 new cases each year (Koch-Henriksen 1995).

Disease onset occurs during young adulthood and as in many diseases with a suspected autoimmune pathogenesis, women are affected more frequently than men with a female to male ratio of 2:1 (Whitacre 1999). Based on clinical and historical observations, the clinical course of multiple sclerosis is divided into four categories: Relapsing/Remitting MS (RRMS), Primary Progressive MS (PPMS), Progressive Relapsing MS (PRMS) and Secondary Progressive MS (SPMS) (Lublin and Reingold 1996) (figure 1).

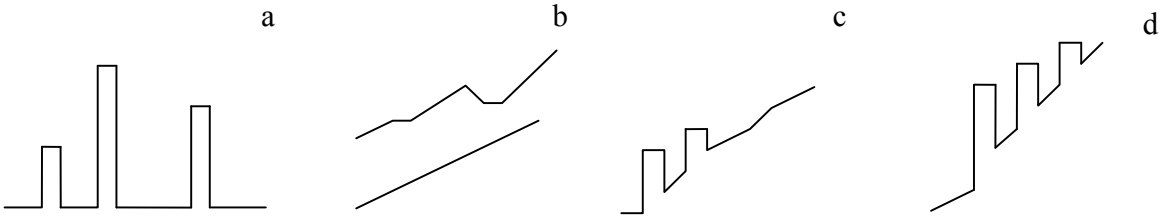

Figure 1: *The four courses of multiple sclerosis:* ***a)*** *Relapsing/ remitting (RRMS),* ***b)*** *Primary Progressive (PPMS),* ***c)*** *Secondary Progressive (SPMS) and* ***d)*** *Progressive Relapsing (PRMS). In b) two graphs are shown reflecting two different forms of the disease.*

The majority of the patients with symptoms are first diagnosed with the Relapsing/Remitting form that accounts for approximately 90% of MS cases and where the age of onset is often in the early twenties. This form is characterized by periods of relapses, or exacerbations, that last for days to weeks, during which new symptoms may appear and old ones worsen. This is followed by periods of slow remission where the patients fully or partially recover from the deficits acquired during relapses.

Approximately 50% of patients with RRMS convert to the *Secondary Progressive* form within 10 years from disease onset, and 90% after 30 years of onset. This form of MS is characterized by a steady progression of clinical neurological damage and tends to be associated with lower levels of inflammatory lesions than RRMS but the total burden of disease continues to progress, probable due to accumulation of axonal loss.

The *primary progressive form of MS* is characterised by a gradual progression of the disease from its onset that is typical in the late thirties, with no superimposed relapses and remissions at all. This form affects 10-15% of all people with MS with males and females equally represented, conversely to RRMS.

The fourth form of MS, *progressive relapsing MS* (PRMS), affects around 5% of individuals with MS and some neurologists believe that it is a variant of PPMS. It is characterised by a steady progression of clinical neurological damage with superimposed relapses and remissions.

1.1 What happens in the CNS during MS

Neurons in the white matter tissue of the CNS are the most liable to attacks in MS. In the normal situation, the axons of the neurons are coated with a sheath of insulating fatty protein, the myelin, which aids in transmitting nerve impulses along the axon to other neurons. Production and repair of the myelin sheaths in the CNS is the task of a sort of glial cells called oligodendrocytes.

During periods of multiple sclerosis activity an inflammation process is started during which myelin is stripped from the axons in a process called demyelination. After damage of myelin sheaths, transmission of nerve impulses is slowed or even stopped. The inflammation can proceed and damage the demyelinated, and hence unprotected, axon. During bouts in MS oligodendrocytes are also damaged and a decrease in their number is often noticed in MS lesions.

As disease progresses axons are also destroyed, though not necessarily only as a result of inflammation. During the secondary progressive phase of the disease inflammation is not as apparent and the axons continue to die. One theory for axonal death is the absence of oligodendrocytes that usually provide them with growth factors such as Insulin-like Growth Factor-1 (IGF-1) (Gutierrez-Ospina 2002) (Russell 1999) and Brain Derived Neutrophic Factor (BDNF) that has been linked to MS and other degenerative diseases of the CNS such as Parkinson's disease (Gold 2003).

1.2 Diagnostic criteria

Considering that many symptoms of MS are similar to symptoms of other neurological diseases and that no laboratory tests, symptoms or findings can separately determine if a person has MS, one criterion for the diagnosis of MS is to rule out all other explanations for the attacks or the symptoms (Natowicz and Bejjani 1994). Other criteria for the diagnosis of MS are based on evidence of two demyelinating attacks in the CNS lasting for at least 24 hours and separated in time by at least one month and in space in the form of inflammation and/or damage in different areas of the CNS.

Magnetic resonance imaging (MRI) scans are widely used in clinical investigations to evaluate the disease status by defining the degree and location of lesions within CNS. One drawback of MRI scans is that they correlate rather poorly with disability, probably because many MS lesions are silent with no detectable symptoms. A laboratory method widely used is analysis of immunoglobulin G (IgG) in cerebrospinal fluid (CSF) from spinal taps. While characteristic oligoclonal bands of IgG are not observed in sera. They are present in the CSF in more than

90% of the Swedish MS patients compared to healthy individuals in whom IgG bands are normally not present (Link 1977).

A revised diagnostic criterion for MS was recommended in April 2001 (McDonald 2001). In addition to the original requirements, McDonald's criteria provide specific guidelines using findings in MRI and CSF analyses to provide evidence and confirm diagnosis faster in the case of patients without second attacks and patients with steady progression of disabilities without distinct attacks. The McDonald's criteria have been lately updated to a newer version to simplify and speed diagnosis of MS (Polman 2005).

1.3 Multiple sclerosis and autoimmunity

The exact causes of MS remain to be defined. There are several theories but the overall process is so poorly understood that none has yet delivered the final cut.

That MS is an autoimmune disease is one of the leading theories. Autoimmune diseases (AIDs) are a group of chronic conditions often associated with severe morbidity and in some cases even mortality, characterized by destruction of the body's own tissues and organs. AIDs are the consequence of developing an immune response against self-antigens and result in organ damage (reviewed in (Albert and Inman 1999)).

The specific etiology of AIDs is still unknown but they are thought to arise as a result of a complex combination of genetic, environmental and stochastic factors (reviewed in (Vyse and Todd 1996), Rose 1998). Seven of the most common AIDs are rheumatoid arthritis (RA), Grave's disease (GD), familial psoriasis (PS), insulin-dependent diabetes mellitus (IDDM) also known as Type I diabetes (T1D), multiple sclerosis (MS), systemic lupus erythematosus (SLE) and Crohn's disease (CD) (Becker et al. 1998). Altogether AIDs affect 3-5% of the Western population with females represented in 75% of autoimmune patients (Jacobson 1997) (Rose 1997) (Davidson and Diamond 2001). The uniting factor combining all autoimmune diseases is the presence of autoimmune responses based on cumulative genetic risks combined with environmental factors (reviewed in (Vyse and Todd 1996), Rose 1998).

One hypothesis for the pathogenesis of MS is "molecular mimicry" through which there is a break of self tolerance where T cells activated by foreign antigens cross-react with self-antigens (Christensen 1999) (Fairweather 2001) (Martin 1999) (Oldstone 1998) (Ohashi 2002; Ohashi and DeFranco 2002) (Gronski 2004a) (Gronski 2004b). In essence, if epitopes located on a pathogen resemble epitopes in the body's own tissue then the immune system might also mistakenly attack its own body, giving rise to autoimmune diseases. In normal cases with a healthy immunological system, T-cells are components occurring widely. When structural similarities between microbial structures and CNS auto-antigens exist, molecular mimicry comes into action; activating these T-cells and making them attack the CNS. Innate anti-microbial immune responses creating inflammatory milieus in the brain might facilitate the activation of auto-reactive T cells (Wekerle 1984) (Wekerle 2006). Several viral and bacterial agents have been associated with MS, among them being Human Herpes Virus- 6 (HHV-6)

(Tejada-Simon 2003), Epstein Barr virus (EBV) (Lang 2002), Herpes Simplex Virus-1 (HSV-1) (Cortese 2001) and Measles Virus (MV) (Pette 1993).

Further evidence for a role of autoimmunity in MS is the increase in the number of CSF-derived B cells producing antibodies for MOG, PLP and MBP (Link 1990). Effector T cells specific for myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) were also raised in the peripheral blood and CSF of MS patients compared to healthy controls (Olsson 1990; Sun 1991a) (Sun 1991b; Sun 2001). The elevated responses of myelin-specific T and B cells, especially those reactive to MOG, in MS patients compared to controls support a role of autoimmunity in MS (Wallstrom 1998). MOG has for instance been associated with disintegrating myelin and was shown to induce demyelination both in vitro and in vivo (Vass 1992).

Additional pieces of evidence are the organ specificity in MS and the association with the HLA locus (reviewed in (Hemmer 2002)). The fact that MS shares many features with the animal model experimental autoimmune encephalomyelitis (EAE) with a noticed clustering of MS and EAE QTL, add to the evidence of autoimmunity playing a role in MS (reviewed in (Hemmer et al. 2002)).

1.4 Geographical distribution of MS

Multiple sclerosis is not evenly distributed and a decreasing risk is noticed when moving from the North and South poles towards the equator (Kurtzke 1977). Population studies divided the world map into low-, medium- and high-prevalence regions. Low risk areas have a prevalence of less than 5 per 100 000 individuals, medium risk areas of 5-29 per 100 000 and the prevalence in high risk areas is more than 30 per 100 000 individuals (Kurtzke 1991).

While Caucasians are among the most prone to MS, resistance to MS has been attributed to some ethnic groups even in medium and high risk areas. These genetically and culturally distinct groups include black Africans (Dean 1967), Amerindians (Kurtzke 1979), Lapps in Norway (Gronning and Mellgren 1985), Hungarian gypsies (Kalman 1991), Orientals and American Blacks (Kurtzke et al. 1979). The reason for why Amerindians and Black Americans with African origin developed MS is thought to be due to a mixture of genes with Caucasians and/or as a result of change of environment (reviewed in (Ebers and Sadovnick 1994b)).

1.5 Environmental contribution

The non-uniform pattern of distribution of MS is thought to result from environmental factors such as temperate climate and diet such as fats that result in higher MS prevalence. Migration studies of people moving from high risk areas to low risk areas and vice-versa give an idea about environmental factors (Kurtzke 2000). When comparing two groups of the same ethnic origin, one of which had changed environment during adolescence into a new region with different prevalence than the original one, the frequency of MS was noticed to change into the new area's risk pattern (Gale and Martyn 1995). The age at which migration occurred was also

shown in the same studies to be of importance for developing MS. Further evidence for the role of the environment is an observation of a geographic distribution for MS with a decreasing rate from North to South in the Northern hemisphere, from South to North in the Southern hemisphere and West to East in the United States. Geographical distribution was also found in Australia, New Zealand, France, Japan and some populations of Northern European origin (reviewed in (Ebers and Sadovnick 1994b)) (Kurtzke 1975).

1.6 Treatments

In spite of extensive research around the world both to understand the reasons behind MS and for developing new therapeutic agents, there are still no medicines to cure MS. The most commonly prescribed disease-modifying drugs are Interferon beta (IFN-β) and Glatiramer acetate.

IFN-β treatments were the first drugs to be approved already in 1993 and found to alter the course of MS. Three interferon-based treatments are available in the market nowadays: Avonex® and Rebif® that are IFN-β1a medicines and betaferon® that is IFN-β1b based treatment (Jacobs 2000) ("TheIFNBMultipleSclerosisStudyGroup" 1993) ("The IFNB MS and The UBC MS/MRI Analysis Group" 1995) (Paty and Li 1993). While effectiveness of IFN-β treatment is at best when given to patients in the early stages of the disease more evaluating data are awaited regarding long-term treatments (Comi 2001) (Jacobs et al. 2000). The long-term clinical effect of these treatments on RRMS is uncertain although IFN-β1a have been used for treating relapses of MS and slowing progression of disability when given to mildly disabled patients with RRMS ("Interferon beta-1A for relapsing multiple sclerosis" 1996) (Munschauer and Stuart 1997). The IFN-based treatments are thought to be involved in a Th1/Th2 shifting, altering of cytokine secretion, reducing IFN-γ-induced antigen-presenting capacity of glial and B-cells and reduced infiltration of T-lymphocytes into the CNS (Jiang 1995) (Reder 1996; Stuve 1996).

Mitoxantrone, a drug primarily used to treat various malignant disorders, has lately been used in MS treatment. It was demonstrated to reduce progression of disability and clinical exacerbations (Edan 1997; Hartung 2002). A drug used for treatment in patients with secondary progressive MS is Alemtuzumab, which is a humanized anti-leucocyte (CD52) monoclonal antibody that decreases cerebral inflammation (Coles 1999a; Coles 1999b).

A recent addition to the list of treatments of MS is Natalizumab (Tysabri®) that has been used for treatments of relapsing MS (Virley 2005) (Johnson 2007). Tysabri is a humanized monoclonal antibody that binds to alpha-4-beta-1 integrins, such as the very late activation antigen 4 (VLA-4) and the leukocyte function-associated antigen-1 (LFA-1) (Virley 2005) (Simmons 2005). Upon attachment to integrins, interaction with endothelial adhesion molecules such as ICAM-1 and VCAM-1 is interrupted to diminish recruitment of cells through the blood brain barrier. Treatments with Tysabri pointed to decreased disability and number of relapses as well as reduced number of new upcoming lesions on MRI (Johnson 2007). In spite of the noted progress by Tysabri and being considered as the most effective disease-modifying treatment of relapsing-remitting MS (Ravnborg 2007), it is prescribed with

restriction and strict monitoring of patients in hospitals as a consequence of patients developing multifocal leukoencephalopathy (PML), an opportunistic infection of the brain with the polyoma virus JC (Engelhardt and Briskin 2005) (Johnson 2007).

2. Diseases with genetic causes

2.1 Genetic diseases in man

Human genetic diseases can be classified into at least three different categories: chromosomal, monogenic and polygenic. In chromosomal genetic diseases mutations whole chromosomes or chromosome segments are involved, i.e. trisomy-21 where three copies of chromosome 21 are present leading to Downs' syndrome. Most of the monogenic disorders that are caused by single mutated genes are rare and can often be initially identified by their characteristic Mendelian pattern of inheritance (autosomal dominant or recessive, X-linked dominant or recessive, or Y-linked).

A genetic contribution to a disease is usually measured by the grade of familial aggregation or relative risk, denoted λ, with a higher λ defining a higher degree of familial aggregation. This value describes the difference in the prevalence for relatives of an affected individual to get the disease compared to the rest of the population. The λ is defined as the ratio between a certain relative and the risk of the general population to develop a disease However, λ does not only reflect the degree of inheritance affecting the outcome of the disease, but also the environmental factors shared between relatives.

Many common chronic diseases with adult onset show familial aggregation that usually do not follow the Mendelian family patterns but appear to be caused by an unknown number of multiple genes, usually interacting with various environmental factors. Examples of such conditions are hypertension, diabetes, coronary heart diseases, Alzheimer's disease, multiple sclerosis and many others. Individuals affected by complex polygenic diseases often have an early age of onset and have severe clinical manifestations.

Defining genetic contribution to a disease has also been measured by comparing the concordance rate of a genetic factor between monozygotic twins, that are genetically identical, and dizygotic twins that only share around half of their genetic composition. Consequently, a higher concordance in two affected monozygotic twins than dizygotic twins is therefore an indication for a genetic contribution on the disease.

So far, the impact of modern genetics on defining genes underlying genetic diseases has been most noticeable in monogenic disorders. On the other hand, unravelling the genetic mechanisms of the majority of complex diseases has not been as clear and easy as in the case of the monogenic subtypes.

Genomes, whether it is the human genome or of any other art, are in general very large and contain many thousands of genes. Finding the particular gene responsible for human diseases is therefore a complicated task as finding a needle in a haystack.

Traditionally, a principal method commonly used for determination of disease-causing genes is linkage analysis, a method used to find the approximate location of genes relative to genetic markers with known positions.

2.2 Complex diseases

Complex disorders are defined as diseases with an unclear aetiology or those with a pattern of inheritance that is inconsistent with Mendelian diseases or do not show perfect cosegregation with any single locus. Alternatively, the reasons behind these diseases are often confounded in a combination of numerous genetic and environmental risk factors, each of them with a small contribution to the overall heritability. Multiple sclerosis has been defined as a complex disease with many factors influencing both disease onset and development (Oksenberg 2001). Genetic studies of complex disorders are encountered with complicating factors leading in some cases to inaccurate end results. The complex character of such disorders, as the case for MS, may be due to many features (Oksenberg et al. 2001) (Lander and Schork 1994):

- *Etiological heterogeneity* with identical genes resulting in different phenotypes
- *Genetic heterogeneity* showed when a phenotype is caused by more than one genotype either due to *locus heterogeneity* (multiple disease genes) or *allelic heterogeneity* (multiple mutations within a gene).
- *Epistasis* or gene-gene interactions occur when the risk of an allele isexpressed only in combination with another genetic factor. If there are large epistatic effects between susceptibility genes their relative effect may be dependent on the overall genetic background and therefore inherently less predictable.
- *Unknown genetic parameters* such as single or multiple genes, dominant versus recessive mode of inheritance and incomplete penetrance which is more complicated in polygenic diseases.
- *Post-genomic modifications* in form of genetic rearrangement, somatic mutations and post transcriptional regulatory mechanisms such as mRNA splicing and editing.
- *Environmental factors* of a non-heritable character. In the case of gene-environment interactions, individuals with identical genotypes may have different genetic risks when exposed to different environments (Guo 2000).
- *Epigenetic factors* that refer to all modifications to genes other than changes in the DNA sequence, including methylation and histone acetylation, that change the appearance and structure of DNA, altering how a gene can interact with important transcribing molecules in the cell's nucleus.

A further complicating factor in a complex polygenic disease is that each gene may have a very small effect on its own. The causative loci may then be difficult to identify by conventional linkage and association techniques without unrealistically large sample sizes and the use of many markers, even in phenotypes with high heritability. Even if a large sample size is provided, many of the studied populations are heterogeneous and admixture in these populations can lead to false association of markers with the disease (Rannala 2001).

An additional complexity that might create a heterogeneous group of patients is the imprecise definition of clinical symptoms leading to a weaker diagnosis. This might inflate the estimated heritability of the investigated phenotype. At the same time, a polygenic disease might appear discrete at the time when it is manifested only when the underlying phenotypic variables exceed a biological threshold. In a complex phenotype such as MS, the combination of genetic

factors influencing a polygenic phenotype and the magnitude of the environmental portion involved add to the difficulties of identifying susceptibility genes.

2.3. MS as a complex genetic disease

Even if most cases of MS are sporadic, epidemiological and family studies strongly support a role of genetic factors in the pathogenesis of MS (Sadovnick 1993a; Sadovnick 1996) (Sadovnick 2006) (Rose, 1998). Twin and adoptee studies have been the classical methods for distinguishing between environmental and genetic factors in multifactorial diseases such as MS. Twin studies from different populations indicate a higher risk for monozygotic (MZ) twin for a MS patient for developing MS than a dizygotic (DZ) twin. Concordance for MZ twins is 25.9% compared to a 2.3% concordance for dizygotic pairs (Ebers, 1986). In non twin siblings concordance is 1.9% while the general population displays a prevalence of 0.1% (Ebers and Sadovnick 1994a) (Ebers 1986).

The high concordance in twins compared to the general population suggests a genetic influence on MS susceptibility. Conversely, the difference of concordance between MZ and DZ twins attributes a polygenic character to MS. The hypothesis of MS being a polygenic disease is supported by numerous studies of familial aggregation when the relative risk was measured. The relative risk for: MZ twins was λ= 100-190 (Sadovnick 1993b) (Mumford 1994), full siblings λ= 13 (Robertson 1996a), half siblings λ= 7 (Sadovnick et al. 1996), first degree relatives λ= 7, second degree relatives λ= 3.5, children of a single affected parent λ= 5.5 (Robertson 1996b), conjugal parents λ= 60 (Robertson 1997) and adoptees λ= 1 (Ebers 1995). When comparing the number of alleles shared among affected siblings, the relative risk (λ_s) of loci outside the MHC region was determined to be a less than 1.2 based on the assumption of complete homogeneity in MS (Sawcer 2005).

2.3.1 The Major Histocompatibility Complex and MS

Once a role for a familial aggregation of MS was established, the hunt for specific genes behind MS began. Later on, this task was found not so easy to accomplish due to the polygenic character of the disease. Linkage and association studies were conducted on different sides of the world for this purpose in families in which more than one member was affected with MS. In fact, the major histocompatibility complex (MHC) human leukocyte antigen (HLA) complex was shown in 1972 to be involved in MS (Jersild 1973a) (Jersild and Fog 1972) (Jersild 1973b) and later shown to map mainly to the class II region (Hillert and Olerup 1993; Ebers and Sadovnick 1994a) (Lincoln 2005).

The MHC molecules, both class I and II, are highly polymorphic cell surface glycoproteins. Their primary role in the immune system is displaying and presenting short antigenic peptides to $CD4^+$ and $CD8^+$ T cells. The relative risk provided by HLA varies between 50 to 60% (Olerup and Hillert 1991) which indicates a significant role of non-MHC genes underlying MS susceptibility. The HLA genes are highly polymorphic, resulting in diverse genotypic combinations or haplotypes.

Disease predisposition in Caucasians of North-European descent has been associated with the DRB1*15 (DRB1*1501,DRB*0101,DQA1*0102,DQB1*0602) haplotype of the HLA class II locus(Olerup and Hillert 1991) (Hillert and Olerup 1993) (Rasmussen 2001a) (Chao 2008). The DR15 allele was previously known as HLA-DR2 or HLA-DR15/DQ6. The DRB1*15 is the most common haplotype of DR15 seen in white Europeans. It is present at a high frequency in Northern European populations with 60% frequency in MS patients and 30% in healthy controls. The Sardinian population was also associated to the same HLA haplotype albeit it was shown to be less prevalent with 2.5% in MS patients and 1.5% in healthy controls (Marrosu 1997) (Marrosu 2001). Additional haplotypes of HLA class II, the DR3 (DRB1*03) and DR4 (DRB1*04), were also associated in the same population (Marrosu et al. 1997) (Marrosu et al. 2001).

In a large association study three haplotypes of DRB1 were associated to MS. The DRB1*15 and DRB1*17 were positively associated to MS while the DRB1*14 conferred protection to MS (Dyment 2005). HLA-DR15 allele was associated with early onset of MS (Masterman 2000) (Hensiek 2002) with females being more prone to disease susceptibility and HLA-DR17 to RR and SP courses of MS.

Association to MHC class I has been tested in numerous studies with various results, some of them finding an association (Fogdell-Hahn 2000; de Jong 2002) (Rubio 2002; Harbo 2004; Rubio 2007) (Yeo 2007) and some others not(Lincoln et al. 2005). An allele of MHC class I, the HLA-A*0201, was also associated to MS protection and found that it might have a modifying effect on the HLA class II by contributing to its associated genetic susceptibility to MS (Harbo et al. 2004) (Fogdell-Hahn et al. 2000).

2.3.2 Finding non-MHC genes for MS by linkage studies

Linkage mapping uses information from recombination within families to identify markers co-segregating with a disease phenotype and presumably genetically linked with a disease locus. Several studies have been conducted in a number of groups with the hope of finding MS genes with the first studies dating back to 1996 and 1997 (Kuokkanen 1997; Sawcer 1997) (Ebers 1996; Haines 1996; Sawcer 1996).

Apart from the MHC, none of the first linkage scans showed significant linkage but some loci had a maximum logarithm of odds score (LOD score) higher than 0.5 in repetitive scans: 1p36-33, 2p23-21, 3p14-p13, 3q22-q24, 4q31-qter, 5p14-p12, 5p14-p22, 5q12-q13, 6p21 (the MHC-region), 6p27, 7q11-q22, 7q21-q22, 18p11 and 19q13 (Oksenberg et al. 2001).

In a recently performed large genome-wide linkage study no linkage with a genome-wide significance was observed in any non-MHC gene (Sawcer et al. 2005) but three chromosomal regions were suggestively linked, namely 5q33, 17q23 and 19p13.

Attempts of finding MS related QTLs in human cohorts have been repeatedly encountering power difficulties leading to no genome-wide significance in any study till our days (Modin

2003) (Vitale 2002). For this, foci have been directed to association studies that show higher potential in mapping MS genes.

2.3.3 Association studies and MS

Association mapping methods examine marker alleles in affected and non-affected individuals to detect differences of allele frequencies between the two groups (Rannala 2001). Since the discovered association of the Human Leukocyte Antigens (HLA) in 1972, no consistent results were found until very recently. Candidate gene studies were mostly focused on genes known for their relevance as myelin components or immune-mediating targets, such as the Alzheimer-associated *APOEε4*, a gene involved in the transport of lipids necessary for membrane repair, that was also linked to MS (Masterman 2002b).

Experiments of the cytotoxic T-lymphocyte antigen 4 (*CTLA4*) on experimental autoimmune encephalomyelitis (EAE) established a protective role with suppressed clinical manifestations and inhibition of production of pro-inflammatory cytokines of Th1 type (Khoury 1995) (Khoury 1996). At the time that *CTLA4* was associated to MS susceptibility in some studies with higher frequencies in MS patients than controls (Harbo 1999) (Maurer 2002; Kantarci 2003) (Malferrari 2005) this association lacked a confirmation in some others (Rasmussen 2001b) (Masterman 2002a).

The protein Kinase C alpha (*PRKCA*) gene involved in signal transduction of T cell signalling is one additional gene associated to MS in a British MS population (Barton 2004) (reviewed in (Olsson 2006)) and a Canadian and Finish population (Saarela 2006) but with different polymorphisms. This could point to population-specific variants of *PRKCA*. Additional studies are therefore required for confirming this association to *PRKCA*.

A recent publication from our group reported an association of the MHC class II transactivator (*MHC2TA*) to three MS, RA and myocardial infarction (Swanberg 2005). Further studies on this gene came up with both a confirmative role in MS (Martinez 2007) and some with no confirmation (Akkad 2006a) (Akkad 2006b). A main reason for why this association was not confirmed in other studies is the lack of power in those studies. For this, additional studies with larger cohorts are required to validate this finding.

Currently, few hot candidates are being widely tested based on the strong association in MS from various studies mainly from the first genome wide study testing for risk alleles for MS (Hafler 2007). Two of these non-HLA MS genes have been confirmed in a couple of studies, namely *IL7R* and *IL2RA* ("Refining genetic associations in multiple sclerosis" 2008) (Hafler et al. 2007) (Lundmark 2007) (Weber 2008). Furthermore, evidence for additional MS genes is emerging and includes *CLEC16A*, *RPL5*, *EVI5*, *CD226*, and *CD58* (Rubio 2008) (Hafler et al. 2007) ("Refining genetic associations in multiple sclerosis" 2008) (Burton 2007). With the developing techniques such as genome-wide association studies (GWAS), hopes are raised on achieving better success in unraveling genetic mechanisms and finding additional MS associated genes.

3. Animal models

Understanding complex human diseases has been facilitated by using animal models mimicking the human disease with regard to clinical course and the pattern of histopathological lesions. Inbred mouse and rat strains with a broad variance in susceptible to induction of a disease following immunization with different antigens have been valuable in identifying genetic risk factors for disease. Phenotypic and genetic monitoring of these animals can enable the identification of susceptibility loci and disease genes.

The use of inbred animal models offer advantages over human research mainly by offering a controlled variability of genetic background, in contrast to the existing heterogeneity in humans. Furthermore environmental conditions can be kept constant. The possibility of breeding very large number of offsprings from a single couple of animals results in an increasing power and higher genetic resolution. A more practical benefit from animal models is the ability of performing genetic manipulations, i.e. knock in and knock out experiments.

3.1 Experimental autoimmune Encephalomyelitis

Due to the polygenic character and complexity of MS, studies of animal models were considered to offer greater advantages compared to humans (Risch 1993). An animal model known as experimental autoimmune encephalomyelitis (EAE) which shows functional and pathological similarities to human MS was established in the early 1930s in monkeys injected with brain extracts (Rivers and Schwentker 1935).

In table 1 shared common features between EAE in rats and MS that makes EAE a good model for MS to be used in the hunt of genes are summarized (Steinman 1999).Animal models of EAE are usually divided into three categories, depending on the induction mode of the disease: the passively transferred EAE, the spontaneous model of EAE and the actively immunized EAE, the model of use in our group and for all experiments of this thesis.

In *passive transfer* of EAE, myelin-specific T-cell clones isolated from actively immunized rats affected with EAE are transferred to naïve hosts resulting in EAE. The encephalitogenic effect is then conferred by $CD4^+$ T cells recognizing epitopes on MHC class II and secreting cytokines of Th1 type such as IL-17, IFN-γ and TNF (Liblau 1995) (Stromnes 2008). No production of Th2 cytokines such as IL-4 and IL-5 is noticed from these cells (Bettelli and Kuchroo 2005). The most common models of transferred EAE involve encephalitogenic $CD4^+$ T cells, with only few models transferred by $CD8^+$ T cells, including MBP-specific $CD8^+$ T cell cloned from MBP deficient shiverer mice and MOG-reactive $CD8^+$ T BL mice (Huseby 2001) (Sun et al. 2001).

To date, no spontaneous EAE models have been discovered. The actual spontaneity of the claimed spontaneous models has been widely questioned. Unlike the NOD mouse model that develops spontaneous autoimmune diabetes, there are no spontaneous models for EAE. Instead, different models of genetically modified mouse strains showed similar symptoms as EAE. Goverman et al. developed in 1993 a transgenic mouse model for MBP-specific T-cell

receptors (TCRs) that develop a spontaneous EAE-like disease in the cases where clean conditions were lacking in the animal facilities (Goverman 1993). The severity of symptoms of the spontaneous EAE was proportional to the hygienic levels in the environment with a full protection of the model in clean circumstances.

Lately, two groups developed in parallel transgenic mice with MOG-specific TCRs and 30% of their B cell subsets binding to a conformational MOG epitope (Krishnamoorthy 2006) (Bettelli 2006) (Probert 1995). These B cells also secrete auto-antibodies with the same specificity to MOG. As a result from the cooperation between the MOG-recognizing B- and T-cells, the transgenic mice develop a Devic's-like disease with optico-spinal symptoms. High infiltration of $CD4^+$ and $CD8^+$ T cells in the parenchyma of the CNS in addition to other inflammatory demyelinating characteristic of the human CNS disorders were also noted in TCR transgenic model (Probert et al. 1995). With the emerging evidence of MS pathogenesis being a result of autoimmune B and T cells contributing to the autoimmunity of MS, it is believed that the transgenic models will show a potential in understanding the interplay between these two cell subsets.

Table 1. Comparison between multiple sclerosis and EAE. (Adapted and modified from Steinman L., 1999)

	MS	EAE
Clinical phenotype		
Relapses and remission	Present	Present
Paralysis	Present	Present
Ataxia	Present	Present
Visual impairment	Present	Present
Genetics		
MHC-linked susceptibility	Yes	Yes
Females more susceptible	Yes	Yes
Pathology in lesions		
T cells reactive to myelin	Present	Present
Antibodies to myelin	Present	Present
Demyelination	Present	Present
TNFα, IFNγ	Present	Present

3.2 Active induction of EAE

The advantages of EAE lie in the rapid induction of the disease in susceptible animals and the ease in following the disease course visually. Usually the experimental animals are immunized with whole spinal cord homogenate or myelin antigens such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP). Freund's adjuvant is used in immunization as an enhancer of immune responses to the antigens.

Approximately 9-15 days post immunization clinical signs start appearing. Classical EAE models are characterized by ascending paralysis starting by tail weakness, followed by paresis in the hind legs and then the front legs. Paralysis is usually preceded by one or two days of weight loss. Also observed are histopathological alterations of the CNS and infiltrations of mononuclear cells such as T cells, macrophages and plasma cells (Raine 1997).

Discussions have been raised about the accuracy of using EAE as a model for MS since this model requires immunization with an antigen to induce disease. The immunization could be seen as a parallel virus infection that might induce MS through molecular mimicry, especially since exacerbation of MS was noticed after viral infections such as Epstein-Barr virus (EBV) and Human Herpes virus (HHV) (Challoner 1995; Soldan 1997) (Sola 2002) (Ascherio and Munch 2000) (Sundstrom 2004). Additional arguments for EAE being a good model for MS are the clinical and histopathological similarities between them and the overlap between many human and rat quantitative trait loci (QTL) regions (Serrano-Fernandez 2004).

Different rat-EAE models have been used and three of them are employed in our group.
The *MBP-EAE* is induced by active immunization with myelin MBP and complete Freund's adjuvant (CFA). It is characterized by a predominantly monophasic clinical course autoimmune response, mostly mediated by proinflammatory MBP-specific $CD4^+$ T cells, which is strengthened by studies of adoptive transfer with such autoreactive T cells (Holda and Swanborg 1982) (Bouwer and Hinrichs 1994). Demyelination is not a common feature of MBP-EAE.

The *SC-EAE* model is induced through injections with spinal cord (SC) homogenate either in complete or incomplete Freund's adjuvant (IFA). This model usually results in a chronic relapsing EAE model with an MS-like demyelination and presence of MOG-specific antibodies in the serum (Lorentzen 1995). These model shares important T cell response mechanisms with the Th_1 cell-mediated EAE models that include all mouse- EAE models as well as MBP- and spinal cord-induced EAE in LEW rats. These mechanisms include association with T cell responses against MBP (Lorentzen et al. 1995) and the proinflammatory cytokine profiles in the CNS such as TNF and IFN-γ (Issazadeh 1996) (Issazadeh 1995b).
The spinal cord model of EAE has also been associated with anti-MOG responses in serum (Lorentzen et al. 1995). Persistant production of proinflammatory cytokines such as TNF, IFN-γ, IL-1β, IL-12 and absence of downregulatory cytokines such as IL-4, IL-10 and TGF-beta (Issazadeh 1995a) (Issazadeh et al. 1996) (Issazadeh et al. 1995b). Widespread inflammation and demyelination is a common feature of this model.

MOG-EAE. Induction with myelin oligodendrocyte glycoprotein results in an MS-like disease both in the chronic relapsing/remitting disease course and a B cell mediated response (Linington 1988; Genain 1995). MOG-EAE also shows T cell infiltration and focal widespread inflammation and demyelination in the CNS similar to MS (Storch 1998) (Weissert 1998). Demyelination is associated with IgG deposition together with macrophages (Lucchinetti 2000) (Stefferl 1996) (Stefferl 2000) (Stefferl 1999). MOG is considered to be the only myelin component eliciting pathogenic T- and B-cell autoimmune responses (Linington 1993) (Genain and Hauser 1996).

3.3 The choice of experimental animals for EAE.

Numerous factors are to be taken into consideration when choosing to work with animal models such as EAE, including the choice of the animal species and the antigen to be used for immunization.

EAE has been developed in many species such as monkeys, dogs, guinea pigs, sheep, mice and rats (Bigazzi 1995). However, rodent models of EAE have been preferably used due to many reasons, among them being the good genetic and immunologic resources and the striking similarity between rodent and human immune responses.

Even if mice are cheaper to work with and take less space in the animal facilities, and even if more genomic information is available about them such as microsatellite markers and genome sequence, the group has chosen to work with the rat EAE. One raison d'être is the stability of the rat model which tends to be higher than the mouse model. Another motivation is the more MS-like displayed phenotype in the rat model of MOG-EAE compared to the mice models. In addition, the rat model has a lower requirement of adjuvant and hence a milder immunization protocol than for mice that often need repeated immunizations.

3.4 MHC influence on rat-EAE

The major histocompatibility complex is an important susceptibility locus underlying MS both in humans and in experimental models such as rat EAE. Experiments in different LEW congenics that share the same non-MHC background genes but with different MHC genes were performed to investigate an eventual role of MHC genes in EAE susceptibility (Weissert et al. 1998). In this experiment a difference in the clinical phenotype was noticed between the various congenic strains, with LEW.N being the most susceptible congenic followed by LEW.AV1, LEW.1W and LEW.L, classified in decreasing disease severity. The number of anti-MOG antibody secreting B cells also paralleled the differences in the clinical phenotype with the highest levels in LEW.N and decreasing in the same order as above. If instead MBP is used as the EAE-inducing antigen, the MHC restriction pattern is reversed (Muhallab 2005). These findings in the experiments imply a role of MHC in rat-EAE (Weissert et al. 1998).

3.5 Non-MHC genes influencing EAE in rats

In order to verify the influence of non-MHC genes on EAE, clinical differences and histopathological studies were performed in DA, PVG.AV1, LEW.AV1 and ACI rat strains that share the same MHC gene combination but with variable non-MHC genetic background (Weissert et al. 1998). Non-MHC genes were found to be protective in PVG.AV1 and ACI, and permissive in DA and LEW, with only the permissive strains showing a strong MOG-specific B-cell response (Weissert et al. 1998) (Ben-Nun 1982).

Having demonstrated a role of non-MHC genes influencing EAE in the rat, the next task would be to identify these genes or the loci harboring these genes. Even if the first genome scan in rodents was performed on mice in 1995 (Sundvall 1995), it was not until four years later that the first three whole genome scans identifying a number of loci in rats were performed (Dahlman 1998; Dahlman 1999a) (Dahlman 1999b) (Roth 1999). A locus on rat chromosome 9 was identified in a genome-wide scan of an (DAxBN)F2 cross of a spinal cord- induced EAE (Dahlman et al. 1999a). In addition, a suggestive linkage to an EAE phenotype was assigned to seven new regions on rat chromosomes 1, 2, 5, 7, 8, 12 and 15. Another locus on chromosome 18 was identified in MOG induced EAE in a (DAxACI)F_2 intercross together with three suggestive loci on chromosomes 10, 12 and 13 (Dahlman et al. 1999b). The region on chromosome 10 was later confirmed in a DAxACI congenic strain (Jagodic 2001) and narrowed-down thanks to an advanced intercross line (AIL) consisting of a (DAxPVG.1AV1)G_7 intercross (Jagodic 2004). A scan in a myelin induced model of a (LEWxBN)F2 cross identified the two loci on chromosomes 4 and 10 (Roth et al. 1999).

Some of these loci have been confirmed, some others are still not, while additional ones have been discovered along the way. In a spinal cord model of a (DAxE3)F2 intercross, 11 loci were identified on chromosomes 1, 4, 5, 6, 11, 14, 18, 19 and 20 (Bergsteinsdottir 2000). A (LEWxPVG)F2 cross showed linkage to EAE phenotype on a new locus on chromosome 8 and two other suggestive loci on chromosomes 1 and 17 (Becanovic 2003b). To date, a number of significant loci have been linked to the rat chromosomes 1, 2, 4, 8, 9, 10, 12, 13, 15, 17, 18 and 19 with some other suggestive loci.

Since the first whole genome scans we have been able to fine map a number of regions in our lab, thanks to the AIL and congenic strategies applied in different strain combinations. Among the confirmed and fine-mapped region are the regions on rat chromosome 4 (RNO 4) (Becanovic 2003a; Jagodic et al. 2004) (Marta, submitted), RNO 10 (Jagodic et al. 2001), RNO 8 (Nohra, unpublished data of this thesis), RNO 12 (Becanovic 2006), RNO 9 (paper III) and RNO 15 (Sheng 2005). Even if the large whole genome scans have provided us with solid data about susceptibility loci for rat EAE, these regions are generally too large to be able to decode the exact genes defining EAE. Additional mapping techniques are therefore required in order to reach our goal of deciphering the enigma of EAE genes.

4. Aims of the study

The purpose of the studies of this thesis was to define genetic influences of non-MHC regions on rat chromosomes 1, 4, 9 and 15 (*Eae30-31*, *Eae20-21, Eae4* and *Eae19* respectively).

The specific aims were to:

- Define and fine-map previously defined quantitative trait loci (QTLs) on rat chromosomes 1, 4, 9 and 15 using AIL and congenic mapping.
- Identify candidate genes within these regions.
- Dissect pathogenic mechanisms underlying the narrowed QTLs.
- Test if genes/pathways mapped in the animal model also predispose for human disease.

5. Methodological considerations- Mapping disease genes

5.1 Genetic variations

With almost 99.9% of the genomic composition being identical between humans, the remaining 0.1% still consists of millions of base pairs varying among different individuals (Kruglyak and Nickerson 2001). Except for monozygotic twins, there are no completely genetically identical human beings. This is due to genetic variations that result from spontaneous changes in the DNA sequence. A mutation in a somatic cell is usually new and not transmitted to the progeny. On the other hand, mutations in germ cells are transmitted to the offsprings.
The genetic variations are of varying types and sizes, ranging from single nucleotides to larger segments. Most of these mutations are non-functional and do not lead to any particular change in any phenotype or gene expression. In contrast, some of them lead to a noticeable phenotypic appearance or an alteration in gene expression, with some of them being more disadvantageous and thus, raising the risk of disease. How long a variation persists in a population is depending on a number of factors. In optimal situations, a highly penetrant mutation leading to a non-fit phenotype for a population's survival, i.e. sterility or a fatal disease, usually disappears rapidly and is not inherited through many generations. Highly penetrant mutations are those that almost always lead to a changed phenotype or cause the rise of a disorder once present in an individual. In non-optimal circumstances, a variation with low penetrance or a late age of onset of a disease caused by a certain mutation forces this mutation to survive longer in a population and transmitted to more generations.
The frequency of a mutation in a population is determined by the size of a population in addition to some other factors such as migration, genetic drift and linkage disequilibrium with other variations under selective pressure. Once a mutation persists in a population with a frequency of 0.01 or higher, it is called a polymorphism. Researchers have taken advantage of variations in the genome by using them as genetic markers during genetic mapping.

5.2. Genetic markers

Genetic mapping used for tracing inheritance patterns of genes and DNA segments is accomplished by using genetic markers. For a marker to be useful and informative, it has to be polymorphic and localized at identifiable position, locus, in the genome. In human analysis a heterozygosity test is measured in order to define the polymorphism and hence, the informativity of a marker. The heterozygosity is a measure of how many individuals in the population are heterozygous it can be calculated for a marker with n number of:

$$H = 1 - (p_1^2 + p_2^2 + p_3^2 + ... p_n^2)$$

with "H" stands for heterozygosity, "*p*" for the frequency of respective allele and "n" for the number of alleles for the tested marker.
The most commonly used markers in genetic analysis are single nucleotide polymorphisms (SNPs) and microsatellites but even insertion/deletions and copy number variations (CNV) have been employed.

5.2.1 Single Nucleotide Polymorphisms

As indicated by the name, single nucleotide polymorphisms (SNPs) are differences in single nucleotides. They are the most prevalent types of polymorphisms with a minor allele frequency of at least 1%. In the addition to their stability, the high density of SNPs in the genome (about 1 SNP every 500 bp-1kb) is one of the benefits that one can take advantage of since they allow detection of QTLs of lesser effect (Moore and Nagle 2000). The functional importance of SNPs, i.e. by altering amino acid sequences and in some cases resulting in a different protein, in addition to their abundance have been plausible for studying them extensively in medical genetics.
An obstacle in using SNP markers for genotyping has been the extensive costs of genotyping. Since SNPs are variations between two bases, their limited heterozygosity was a plausible reason for not employing them in linkage analysis to as large extend as microsatellites. Instead, a great value of SNPs has been shown in association studies and studies of linkage disequilibrium. Lately, the development of SNP arrays raised the possibility of using SNPs in linkage analysis (Kennedy 2003) (Oliphant 2002). SNP arrays enabled a cost-effective, accurate and high throughput genotyping with thousands of SNPs analysed with limited efforts, compensating for the low amount of information gained from particular SNPs (Kennedy et al. 2003) (Oliphant et al. 2002). While SNPs have been widely used in humans, their use in rats is still not very common.

5.2.2 Insertion and deletions

Limited efforts have been put into studying insertions and deletions (also called indels) even though they are the second most common type of variations. The size of indels could vary from one bp to larger segments of inserted or deleted DNA sequences that might affect a specific phenotype or a disease in a similar way as SNPs by interfering with gene expression and subsequently, altering protein translations. More studies are required before we are able to map all indels and define their prevalence in the genome. It is believed that only a small amount of the indels have been reported so far and more to be discovered in the close future (Newman 2005) (Szamalek 2006) (Tuzun 2005). However, it is estimated that almost 16-25% of the genome consists of indels (Mills 2006).

5.2.3 Repeats

A considerable fraction of the genome consists of repetitive sequences of varying repeat sizes and they are classified into three groups:
Megasatellites are repeats of longer sequences up to several Kb long.
Minisatellites are slightly smaller tandem repeats of about 10-100bp. They are widely dispersed in the genome but mostly clustered at the telomeres of the chromosomes.
*Microsatellite*s, also known as Short Tandem Repeats, are short stretches of di-, tri- or tetra-nucleotide repeats found in most genomes (figure 2). They account for almost 3% of the genome (Lander 2001). They have a mutation rate of 10^{-2} to 10^{-4} per locus and generation and

are not as stable or frequent as SNPs (Ellegren 2000) (Lai and Sun 2003) (Ellegren 2004). Still, they are the genetic markers of choice used for linkage mapping, due their high polymorphism in organisms.

Figure 2: *A microsatellite of seven di-nucleotide repeats (CA). Black lines are segments of flanking DNA*

The popularity of microsatellite markers lies in their heterozygozity and the ease in their amplification by traditional polymerase chain reaction (PCR). PCR products are then analysed by separation either in a gel or using automated capillary electrophoresis to determine the difference in the size of the PCR products which reflects the difference in the number of repeats. Microsatellites have been widely used for mapping in both humans and rats. In linkage studies of the rat regions of this thesis, we used exclusively microsatellite markers.

5.2.4 Copy-Number variations

Recently, a new sort of markers called Copy-Number Variations (CNV) has been discovered (Sebat 2004) (Iafrate 2004). They are structural modifications in the DNA sequences longer than 1 kb and sometimes ranging up to 3 Mb (Redon 2006) (Sharp 2005) (reviewed in (Feuk 2006)). CNVs arise from gains or loss of large DNA sequences (Sharp et al. 2005) (Tuzun et al. 2005). They are considered as a joint name for indels, duplications and complex multi-site variants. If a CNV has a frequency of more than 1%, it is then called Copy-Number Polymorphism (CNP). CNVs co-localize with genes and hereby disrupt the gene expression or alter the gene dosage. Recently, numerous diseases in both humans and rats have been associated to CNVs in genes including HIV-1/AIDS (Gonzalez 2005), glomerulonephritis in rats and humans (Aitman 2006), systemic autoimmunity (Fanciulli 2007), systemic lupus erythematosus (Yang 2007) and rheumatoid arthritis (McKinney 2008). Recent reports from the Database of Genomic Variants (http://projects.tcag.ca/variation/) show that more than 7332 genes are overlapped by CNVs (http://projects.tcag.ca/variation/). This raises the chance of CNVs being implicated in diseases in the near future.

5.3 Mapping of quantitative trait loci and susceptibility genes.

Continuous traits that are given a quantitative value are referred to as quantitative traits. A QTL is the genomic region that is strongly associated with a trait. Finding genetic regions contributing to a disease is a three step procedure that involves: QTL detection, QTL mapping and QTL fine mapping (Darvasi and Pisante-Shalom 2002).

Traits are traditionally defined by genetic research in terms of variances, both environmental and genetic. The genetic contribution is usually due to a combination of many genes working either in an additive, dominant or epistatic pattern of action. For this reason, mapping of QTLs

that consists of both localizing QTLs and estimating their effect on a trait is not an easy task. The larger effect a QTL has on a trait, the easier it is to map it.

Since quantitative traits may be a result of the expression of numerous genes working in concert, a QTL test must include enough markers dispersed evenly for full genome coverage. In order to achieve the aim of QTL analysis, which is to characterize the genetic aspects underlying complex traits, it is of great interest to ensure large populations with reliable phenotypic data in addition to informative markers. Molecular markers associated with a certain QTL are identified by first scoring a reasonably large number of a segregating population for a quantitative trait and genotyping every member of this population. Mapping of disease predisposing gene and loci is performed either by linkage studies or association studies.

5.3.1 The principle of linkage analysis

When a phenotype and a gene at a specific locus are inherited together, they are said to be linked. Genetic linkage is the tendency of genetic segments, i.e. genes or markers, to be inherited together due to their physical proximity on a chromosome. Through linkage analysis, polymorphic genetic markers of a known genomic position, i.e. microsatellites or single nucleotide polymorphisms (SNPs), are used to trace their pattern of inheritance from the parents and link them with a disease.

The ability of determining the parental origin of the markers allows us to show whether a recombination has taken place in the germ cells during meiosis (Figure 3).

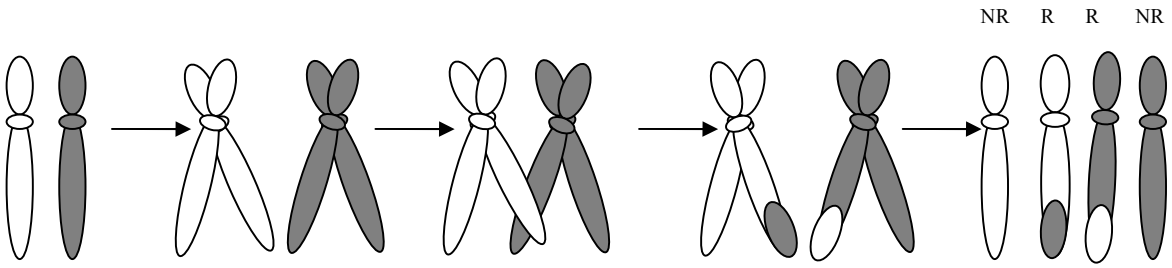

Figure 3: *Crossing-over and homologous recombination of sister chromatids during meiosis. Chromosome material is exchanged between the sister chromatids, giving rise to new gene combinations. (NR: non-recombinant; R: recombinant)*

During meiosis, the precursor cells of the sperms and eggs must multiply and at the same time reduce the number of chromosomes to one full set. During the early stages of cell division in meiosis, the two homologous parental chromosomal pairs may exchange segments through recombination, or crossing over (figure 3). Recombination is of great importance from the evolutionary point of view because it creates diversity and makes individuals more fit to adjust to new environments. Recombination takes place between any two locations on the

chromosome and the amount of crossing-over is a function of the distance these locations. A larger distance leads to a higher recombination rate. When crossing-over has occurred the resulting gametes are then called recombinants. In contrast, when two markers are closely positioned, they will recombine only rarely. In cases where markers do not recombine with the disease gene due to their close proximity they are said to be linked. Disease genes are mapped by measuring recombination between different markers spread throughout the genome.

Through linkage analysis we are able to compare the segregation of particular markers and the disease phenotype compared to random segregation. The observed number of recombined gametes produced by a parent is called the recombination fraction, denoted by θ (theta), where unlinked genes show 50% recombination. In other words, theta measures the extent of genetic linkage. A recombination fraction of 0.5 means no linkage at all but as θ approaches 0 the linkage becomes tighter. The recombination fraction is thus high for loci far apart and low for adjacent loci.

Linkage is calculated by the score of the logarithm of the odds (the LOD score) method that was developed by Newton E. Morton. The LOD score is calculated by consideration of two hypotheses: the null hypothesis (H_0) that corresponds to free recombinations between the gene and the marker ($\theta = 0.5$) and the H_1 hypothesis that represents linkage ($0 \leq \theta \leq 0.5$). The likelihood ratio is expressed as the $\log_{10}$ ratio between the likelihood that the investigated loci are linked rather than unlinked and calculated according to the following equation.

$$\text{LOD} = \log_{10} \frac{\text{Likelihood of linkage } (H_1)}{\text{Likelihood of free recombination } (H_0)}$$

Linkage studies have been successful in mapping loci of high penetrance both in Mendelian and complex diseases, but proven less powerful for detecting variants with low penetrance (Risch et al. 1993) (Risch and Merikangas 1996) (Altmuller 2001). It has also shown some weakness in identifying disease genes and pinpointing to their particular effect in disease outcome. This is due to lack of power resulting from either too small family materials or the low number of markers employed through genome wide linkage studies in contrast to the contribution of the particular gene (Hirschhorn and Daly 2005). However, the difficulties encountered in linkage mapping in human in the animal models have been more successful with good examples presented by work from our group

5.3.2 Parametric and non-parametric linkage analysis

The standard method for LOD score calculations utilizes *parametric linkage analysis*. In human research, this method requires information about inheritance pattern, penetrance and allele frequencies for all used markers. Two ways of calculating parametric linkage: a *two-point linkage* and a *multipoint linkage*. In a *two-point linkage* allele data is compared with phenotypic data at one marker locus at the same time as *multipoint linkage* compares the phenotype data to allelic data from several markers from different loci.

Non-parametric linkage analysis is a model-free method utilized in the cases where data about inheritance pattern, i.e. in complex diseases, penetrance and/or allele frequencies is absent. This method does not require healthy siblings and searches for shared alleles only among affected cases. This method has a lower power compared to the *parametric linkage analysis* and requires larger material of patients.

5.3.3 Association studies

Association study is a method used for testing of allele segregation with a hereditary disease. It is based on the hypothesis that individuals that share a common ancestor for a common mutation will most likely also share a common allele at an adjacent locus to the disease gene. Closely positioned markers can be co-inherited as a haplotype that, once segregating with a disease, becomes valuable in evaluating an ancestral mutation. Haplotype analysis shows the same increase of power for association studies in a similar matter as multipoint linkage (Rannala and Reeve 2001) (Rannala and Slatkin 1998) (Rannala and Slatkin 2000). In contrast to linkage studies where inheritance patterns are investigated conclusively in families, association studies can be achieved both in families and in a set of cases and healthy controls of no particular familial relatedness.

In family-based association studies the co-occurrence of alleles from a healthy parent to an affected child is tested. Family-based studies are generally protected from the effects of ethnic admixture owing to their use of internal controls. While the investigated families in linkage studies preferably are larger and from several generations, in an association study a transmission disequilibrium test (TDT) can be achieved in trio families consisting of the two parents and one affected child (Spielman and Ewens 1996) (Spielman 1993). There are two ways for testing association in trio families, the TDT test and haplotype relative risk (HRR). In a TDT test, distortion from the transmission frequency is tested. Transmission of alleles from unaffected heterozygous parents to affected children is then compared to non-transmitted alleles. In an HRR, a control group of haplotypes not inherited by a sick child is compared with the haplotypes inherited by this child. The non-inherited chromosomes will then function as controls to be compared with the allele frequencies of the affected children. Including incomplete families could bias the association studies performed through TDT and make them less powerful than case-control association studies (Schulze and McMahon 2002).

In case-control studies, a frequency of alleles at a marker co-segregating disease allele is compared between a group of patients affected with the investigated disease (referred to as cases) and a group of healthy individuals (referred to as controls). The cases and controls should not be related for this purpose but it is important to use ethnically homogenous cohorts to avoid false positive associations due to population stratification. In the association studies of this thesis (both paper III and IV) we use the case-control strategy.

Two ways can be used in a case-control study: the odds ratio (OR) and the relative risk (RR), even called risk ratio.

The OR is a method employed to compare the occurrence of a disease allele between cases and controls. An OR of 1 indicates that both cases and controls are at equal risk of developing a disease. An OR greater than 1 in a case-control study implies that the cases have higher risk for developing disease with OR < 1 meaning that cases show a lower risk. The OR is calculated according to the following equation:

$$OR= (C.a. / C.b) / (N.a / N.b)$$

Where "C" represents cases; "N" controls, "a" represents the investigated allele a and "b" stands for allele b.
The RR is a more direct measure to compare the probability in the two groups, cases and controls and calculated according to the following equation: $RR = P_{cases} / P_{controls}$ where P_{cases} is the probability in the cases and $P_{controls}$ is the probability in controls.

5.3.4 Linkage disequilibrium

Once an association between a disease phenotype and an allele is established, a functional contribution of the associated allele can be investigated for a better understanding of the disease. However, in most cases, an associated allele may not be causative but in linkage disequilibrium (LD), or in allelic association, with the causative allele. The power of an association study is therefore increasing with the degree of LD. LD is defined as the non-random association of alleles at two or more loci, either on the same chromosome or on different ones. When two alleles are inherited together more or less frequently than expected from the allele frequency in the total population, they are then in allelic association or in LD. Closely positioned markers in LD can be inherited as an entire set forming a haplotype of ancestral co-inherited alleles. LD is influenced by a number of factors including the rate of mutations, natural selection and random drift, the recombination fraction, non-random mating and migration. With the increasing number of generations the extent of LD is expected to decrease as a result of accumulating recombinations that disrupt haplotype blocks (Rannala and Slatkin 1998). Over time, recombinations will result in a reduction in the ancestral haplotype blocks, and consequently, facilitating the restriction of disease-associated regions through the determination of minimal shared haplotype (Rannala and Slatkin 1998). Conversely, for the 1% of SNPs lying outside an LD block (Frazer 2007), a disease causing variant might not be detected unless they are directly genotyped.

Accumulation of factors affecting LD to diverse extents in different populations leads to a vast variety of LD patterns throughout the genome in these populations. It has been suggested that the genome is divided into high recombination regions, referred to as "recombination hotspots" and regions of high LD and low recombination frequency (Gabriel 2002). Genomic blocks of high LD show less variations leading to a limited number of haplotypes. These have been denoted "haplotype blocks" or "recombination coldspots". An advantage of the LD blocks is that an association can be detected with a marker in proximity of the causative allele, or in some cases even up to several Kb apart, if they are in high LD and hence, limiting the number of markers needed. The occurrence of LD blocks in the genome has been beneficial by

reducing the required number of SNPs to be genotyped by 90-95%, without interfering with the final outcome to large extent (Plenge and Rioux 2006).

In fact, an international project was initiated in 2002 with the goal of characterizing LD blocks along the human genome. This project, named the "HapMap project", resulted in a database (www.hapmap.org) widely used as a tool for mapping LD blocks ("The International HapMap Project" 2003). HapMap offered the possibility of tagging SNPs (tagSNPs) within a haplotype block and hence, limiting the number of SNPs to be used in an association study without interfering with the power of the study. By genotyping a SNP in an LD block, sufficient genotype information is captured for all the correlating markers within this block. Concerns about the HapMap being constructed based on a limited number of datasets leading to an overestimation of the degree of LD, ethnic admixture and allelic heterogeneity are among factors hazardous to tagSNPs that may result in false results (Terwilliger and Hiekkalinna 2006).

There are two methods to calculate the significance of LD, either through the χ^2 (chi square) test (Koillinen 2003) or by calculating the allelic excess (Hastbacka 1992). The χ^2 test, or Fisher exact test, tests cases and controls in a two-by-two contingency table. The allelic excess (P_{excess}) of an associated marker is calculated through this equation:

$$P_{excess} = (P_{affected} - P_{control}) / (1 - P_{control})$$

Where $P_{affected}$ is the frequency of an allele in chromosomes carrying the disease mutation and $P_{control}$ is the frequency of the same marker allele in normal chromosomes. When the frequency of a marker allele is equal in cases and controls, this means there is no LD exists and P_{excess} is then 0.

The "effect size" of LD is measured in several ways with the two most common methods being by calculating the r^2 and the D' (Devlin and Risch 1995).

The r^2 method takes into consideration the correlation between two alleles at two loci (Lewontin 1964) and is calculated through the following equation:

$$r^2 = D^2 / [f(A) \times f(a) \times f(B) \times f(b)]$$
$$D = f(AB) - [f(A) \times f(B)]$$

where D represents the difference between the observed and expected frequencies of haplotypes; f stands for frequency of respective haplotype (AB) or allele (A, B, a or b). A complete LD is present when $r^2 = 1$. The r^2 value is preferably used rather than the D' value since it is not affected by small sample sizes and intermediate values of r^2 are not as interpreted. A generally acquired cut-off is $r^2 > 0.8$

D' is a normalization value employed to facilitate the interpretation of D and is described as:

$$D' = D / D_{max}$$

where D_{max} is the maximum value of D taking into account the alleles at two different loci. A full LD is present when $D' = 1$. A drawback of the D' value is the overestimation often seen as a result from small sample sizes and rare allele frequencies and the difficulties in interpreting intermediate value of $D' < 1$.

5.3.5 Genome-wide association studies

The first attempt of whole genome approach, genome-wide association studies (GWAS), started already in 1980 by Botstein and colleagues when they were about to construct a linkage map for the human genome by using molecular markers (Botstein 1980). Earlier experiments of genetic mappings were for Mendelian disorders but soon enough, Lander and Botstein found out that most of the diseases display a more complex inheritance pattern, a fact that would require different views in adjusting this task. One of these suggestions was the LD mapping. This application came first into use in 1989 when a gene for cystic fibrosis was mapped by fine-mapping genes discovered in family linkage study. The first time that LD structures were utilized in a whole genome scan was in 1994 by mapping a gene for benign recurrent intrahepatic cholestasis (Houwen 1994).

Today, GWAS are made much faster. Recent advances in genotyping techniques enable rapid genotyping of enormous numbers of samples and markers to lower costs than what was possible a decade ago. The availability of databases with information about millions of tagSNPs and the mapping of LD patterns has enabled GWAS. With a *no-priori* hypothesis about genes effect and their contribution to a disease, a GWAS offers a possibility of detecting disease-causative variants. Compared to linkage analysis where location of rare variants with strong effects have been achieved, the power of GWAS lies in detecting common variants with moderate or high contribution on disease risk (Risch and Merikangas 1996).

In 1996 Lander postulated that common complex diseases are caused mainly by common variants with moderate effects that are quite frequent in the population, calculated both in healthy and affected individuals (Lander 1996). This hypothesis is called the “common disease- common variant” (CDCV) and is still used today. According to the CDCV, a powerful association study seems to be an adequate method in the search for such risk variants. Indeed, numerous susceptibility genes have been mapped through GWAS focusing on complex diseases, with or without an autoimmune characteristic, including systemic lupus erythematosus (Graham 2008) (Harley 2008) (Hom 2008), T1D (Zeggini 2008), MS (Burton et al. 2007) (Hafler et al. 2007), RA (Plenge 2007)and a number of autoimmune diseases ("Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls" 2007) (Burton et al. 2007).

Unfortunately, the high-throughput approach of GWAS is not performed without inferring biases and errors through every step from the procedure. GWAS generate enormous amounts of data, for this, great care has to be taken for a correct study design, accurate sample and data collection and statistical analysis. Moreover, the number of samples from cases and controls has to be sufficiently high, with at least thousands of samples to be used in order to distinguish true signals of moderate size from the noise of spurious signals (Sawcer 2008) (Wang 2005a).

Also, replication of positive findings in additional cohorts is essential for elimination of false positive results.

5.3.6. Candidate gene approaches

In candidate gene studies a gene is selected from a mapped QTL, based on its previously known biological function in similar diseases to the investigated one or for its contribution in similar functional pathways. This strategy is even called a hypothesis-based search. In-depth studies of candidate genes usually start by mapping for genetic variations within this gene, i.e. tagSNPs. Subsequently, investigation of additional SNPs within this gene might lead to a putative association or functional contribution to the disease outcome. For instance, association to a SNP can hint on an altering in a binding site of a transcription factor for the gene with an effect on regulation of expression of the gene. In other cases, it might link to a missense mutation, altering the translational state of the gene and resulting in a different protein.

Occasionally, sequencing (alternatively re-sequencing) of the gene in a selected number of patients and controls is carried out when suspicions about previously unidentified functional variants are present. This is usually accomplished either by sequencing the entire gene, or by focusing on regulatory regions in form of promoters or splicing sites or even only exons or conserved regions among species.

If the biggest advantage of GWAS lies in it being hypothesis-free not requiring any prior information about diseases pathogenesis and gene functions, the candidate gene approach on the hand offers a denser mapping. Allowing a focus on fewer genes, a closer and more in-depth mapping of these genes can be performed with fewer requirements of multiple testing corrections to lower costs.

5.3.7 Mutations and their functionality

Mapping genetic factors of complex disorders is carried out with the goal of understanding the pathological mechanisms of the diseases. Conversely, translating any mapped association into deeper functional disease causation is a task easier said than done. Polymorphisms altering splicing sites of a gene leading to an amino acid substitution or change in a protein structure is one example of complicating factor. In other cases, alteration in regulatory sequences may lead to distorted levels and sites of gene expression, interfere with binding sites for transcription factors or other regulatory elements or even affect epigenetic factors such as DNA methylation sites.

However, the constant improvement of experimental methods will significantly aid in achieving this goal. In addition, the development of good bioinformatics have become a priceless tool assisting scientists in the interpretation of data and combining data from numerous databases and numerous methods from association studies to expression and analysis.

5.4. Investigated experimental populations

Traditionally, three types of populations have been used for identification of QTLs in experimental animals, each of which has its advantages and disadvantages. These populations are the recombinant inbred (RI) lines, the backcross and F2 population.

The RI lines are the most suitable alternatives for the third stage of QTL analysis, the fine mapping of QTLs (discussed a bove in the section of linkage mapping) Their advantage is the ability to perform larger experiments at several locations repetitively over a longer period of time, a fact that allows us to profit from the extensive recombinations that result from continuous breeding. A drawback is that this strategy is time and resource consuming, especially since no specific information about gene action can be estimated through this procedure. Our lab used the first advanced intercross line (AIL) for QTL fine mapping in the rat model of EAE (Jagodic et al. 2004). This thesis contains three papers where we mapped QTLs using this strategy (papers I, II and IV).

While QTLs with low effect are easily detected in F_2 populations, backcross populations lack this property and they are used for QTLs with large impact on traits. Using F_2 crosses many EAE regulating QTLs have been identified in our group in different rat strains (Dahlman et al. 1999a) (Dahlman et al. 1999b) (Becanovic et al. 2003b). A disadvantage of F_2 populations is the limited number of recombinations, a fact that limits narrowing down of QTL regions.

5.4.1 High Resolution mapping in an advanced intercross line (AIL)

An AIL was developed with the aim of increasing the resolution of QTL mapping by increasing the number of accumulated recombinations in experimental animals rather than increasing the population size. An AIL is obtained by random breeding for several generations of two inbred strains with different clinical predispositions to EAE (figure 4) (Darvasi and Soller 1995).

Compared to F_2 and backcross populations, an AIL provides three- to fivefold decrease in the confidence interval of a QTL map location between the different generations without any increase of the population size (Darvasi and Soller 1995). A 20 cM confidence interval in F_2 generation could be theoretically narrowed down to 3.5 cM in the G_{10} generation. The only requirements are to avoid brother-sister mating and that the size of the breeding population is not reduced to fewer than 100 individuals per generation.

A fair coverage of markers is important for a powerful analysis when assessing data for QTL mapping in AIL. The number of markers used for an F2 population is not sufficient to create enough power for in AIL, especially with the increasing number of generations. A distance of 10-25 cM between markers is acceptable when analyzing data from an F_2 cross or a backcross population while in an AIL, the optimal distance between markers is 2-5 cM for an G_{10} generation.

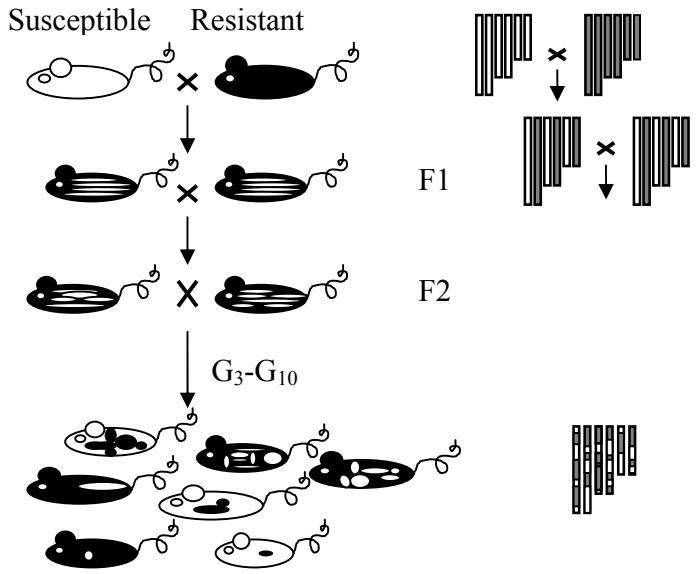

Figure 4. *A schematic representation of AIL breeding and comparison of recombination frequency between F_1 and G_{10} generations*

5.4.2 Fine mapping of QTLs in congenics

Once markers have been detected that are associated with QTLs through use of an AIL, the logical next step is to confirm these QTL obtained by statistical analyses to narrow down the size of the confidence interval of the QTL and investigate the pathogenic mechanisms. The obvious method would be to only advance those lines which contain those alleles with a positive effect on the quantitative trait by developing a congenic model for the QTL and select for the interval of interest.

The first step in the breeding of a congenic strain starts in the same way as for AIL where 2 inbred rat strains with different clinical predispositions to EAE are crossed, with one of the strains being more susceptible than the other. The progenies are then backcrossed for numerous generations into one of the parental strains (recipient) to eliminate alleles from the other parental strain (donor) (figure 5). After approximately 10 generations of backcrossing the progeny will contain less than 0.1% of the donor's alleles outside the congenic interval. These animals are then intercrossed and homozygotes for the recombinant haplotype are selected to establish an interval-specific congenic strain ready for EAE experiments.

A conventional congenization procedure requires approximately 10 backcross generations, a process that takes three to four years. A faster system is the "speed congenic" strategy that saves half of the time (Wakeland 1997). This approach is based on the same basis as the classical model but here the progeny are screened over the entire genome, and not only over the QTL region, with markers dispersed at 20 cM. The same level of contamination obtained after 10 generation with the classical congenization model is reached after five "speed congenic" generations (Wakeland et al. 1997).

The choice of the donor and recipient strains might be a delicate task. The best choice would be to have reciprocal congenics where the donor and the recipient can be alternated between the susceptible and resistant parental rat strains. A limiting factor for applying this strategy is economy. For this reason, when one has to choose, the most favourable selection would be to have the susceptible rat strain as the recipient rather than the resistant one since in many cases the clinical phenotype is easier to detect in the more vulnerable background.

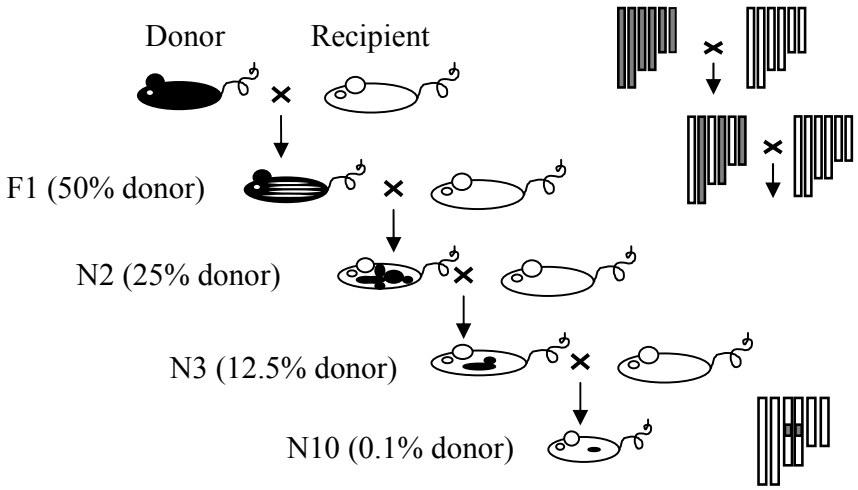

Figure 5. *A schematic representation of the congenization procedure*

In this thesis we use congenic strains between DA as a background strain and the PVG donor strain (papers I and II). In paper III we employed congenic strains with the susceptible dark agouti (DA) or LEW.1AV1 rat strain as a background (recipient) strain and the brown Norwegian (BN) resistant strain. For the forth paper where we mapped *RGMA* and *IL21R*, the congenic strain utilized was bred with PVG as the resistant background strain and the LEW.1AV1 donor strain.

5.4.3 What do we choose- AIL or Congenics?

The starting point of QTL identification in experimental animals is usually an F2 cross. Once a QTL region has been identified a congenic model can be constructed to further narrow down the region. The combination of both AIL and congenic models provides a good ground for investigating the genetic mechanisms influencing EAE from different points of view, since each one of these two models has its own advantages and disadvantages. The benefit of the AIL model lies in the reserved influence from other genes due to epistatic interactions that may easily be lost during a congenization of a QTL. On the other hand, a great drawback of this model is that it only gives a statistical approximation of a QTL and a region of interest cannot be narrowed down enough. This goal is accomplished in the congenic model since genes can be isolated and evaluated in this model. The weakness of the congenic model, the break of epistasis from other QTLs, is compensated for by the AIL model. Other limitations of a

congenic model are that this model is tedious and that it has a limited number of recombinations. In addition, a small effect of a QTL might be undetectable in this model and there is always an uncertainty of transferring the correct fragment to the congenic.

5.5 Experimental design

The investigated phenotypes in experimental animals should be reflected by genetic predisposition to the disease and should be easily measured with high reproducibility and objectivity. All rats used in our experiments were kept in a specific pathogen- free environment with controlled day/night rhythm, temperature and humidity. External stress such as noise was avoided due to its pronounced influence on the appearance of clinical signs of EAE (Correa, 1998).

5.5.1 How do we choose the experimental rats?

Age, sex and weight of experimental animals have been shown to have an impact on the outcome of the experiments. Young animals with immature immune systems differ in their response to EAE induction and old animals might be harder to induce EAE in due to the increase of weight. The relationship between weight and dose of antigen is particularly obvious in mild immunization protocols while a high antigen dose protocol is independent of the animal's weight (Dahlman et al. 1999b) (Wallstrom 1997). A sex effect has been shown in some congenic strains developed in our lab with females being more susceptible to EAE than males (unpublished data) and in sex-segregated linkage analysis where sex-specific QTLs have been found (Bergsteinsdottir et al. 2000; Butterfield 2000). The sex effect might be reflected by the more dramatic increase of weight in male animals compared to female rats. Having these factors in mind, considerations have been taken when choosing groups of experimental animals. We aimed for as homologous groups as possible, being both age and sexually matched, with the age of rats varying between 8 and 14 weeks.

5.5.2 Disease induction protocols

Animals were injected in the tail base with a single subcutaneous injection of an antigen emulsion containing either recombinant MOG protein (1-125 aa) in incomplete Freund's adjuvant (IFA) or homogenized spinal cord (SC) from DA rats in complete Freund's adjuvant (CFA). The adjuvant is used as an enhancer for the immunological responses to antigens (Gupta 1993). Incomplete Freund's adjuvant (IFA) that consists of mineral oil protects MOG from rapid degradation and prolongs its exposure to the immune system (Warren 1986). CFA includes, in addition to the oil, heat-deactivated *Mycobacterium tuberculosis* (Freund 1947) that boosts the proinflammatory immune responses. The MOG/IFA model is a relapsing/remitting form of EAE while the SC/CFA immunization protocol leads to a relapsing/remitting form of EAE that usually develops into a secondary progressive form after manifestation of the first bout of symptoms. The use of two immunization protocols with different clinical, histopathological and immunological characteristics is profitable since MS is

thought to have multiple aetiologies and the disease predisposing genomic regions in EAE might act differently depending on the induction protocol.

The dose of antigens is adjusted through pilot experiments with rats of both the susceptible and the resistant strains. The concentration is fine-tuned in a way that rats of the susceptible strain develop the clinical disease without dying while rats of the resistant strain remain unaffected. In general, there is no strain that is completely resistant and increasing the antigen dose enough can even induce EAE even in the resistant rats. There is hence a threshold that differs between the two categories of rats and defines one of them as being more susceptible than the other.

5.5.3 Phenotyping

Following immunization experimental animals were scored for clinical signs for 30-40 days. The rats usually start developing clinical signs of EAE on day 9-15 preceded by 1-2 days of weight loss. The severity of the disease is scored using a six-point non-linear scale (0-5) with 0 assigned to unaffected animals and 5 for dead animals. Symptoms start with tail weakness (score 1) followed by hint leg paralysis (score 2) and front leg paralysis (score 3). Full paresis is scored as 4 on the scoring scale. The phenotypes used during linkage analysis of the AIL set of animals were:

- Weight loss: between day 8 after immunization and the initial weight before immunization.
- Day of onset: the day where the first clinical signs of EAE are observed.
- Maximum score: the highest clinical score noted in the immunized rats.
- Sum score: cumulative values of all scores during the whole experiment.
- Incidence: a binary trait stating if the rats were affected by EAE or not.
- Duration: the number of days with EAE.

Serum levels of anti-MOG immunoglubulins (Ig) were measured from MOG-immunized rats sampled from day of disease onset. Differences in Ig levels reflect variation between humoral autoimmune responses between rat strains and the relation between T1 and T2 immune responses (Gracie and Bradley 1996). Responses of Th1 character are figured in IgG2b and IgG2c levels, while induction of IgG1 production is a result of Th2 responses (Gracie and Bradley 1996).

Activation of splenocytes and whole blood with potent T-cell mitogens and proliferation assays of lymphocytes give a good idea about production and expression of pro-inflammatory cytokines such as IL-2, TNF and IFN-γ in addition of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β. These were screened in dissected splenocytes from DA.BN-*Eae4* congenics and stimulated with concanavalin-A (conA). Whole blood sampled from the same animals and was stimulated with LPS and serum screened for the same cytokines as above.

Histopathological investigations determine the degree of inflammation and demyelination in the CNS. In the congenic model described in paper III, rats were sacrificed on day 40 and perfused with 4% paraformaldehyde. Brains and spinal cords were sectioned and stained with Hematoxylin-eosin and Luxol fast blue. Cellular infiltration and demyelination were scored

according to a semi-quantitative scale as described (Storch et al. 1998). No difference was noted between the different groups of rats (controls and congenics) since sampling occurred at the final stages of the experiments at the time of relapses.

6.6 Statistical analysis

Linkage analysis in the AIL material was performed using R/qtl (Broman 2003). An advantage of the R/qtl program is that it allows for permutation testing that defines the threshold of significant LOD score levels. In paper III, differences in cytokine levels and in the various clinical phenotypes except of mortality and incidence were tested using Mann-Whitney's U-test and the GraphPad Prism version 3.0 (San Diego, CA). Mortality and incidence phenotypes were analysed in the same program and Fisher's exact test.

Discussions about the threshold of significance when performing linkage studies have been numerous. In 1995 Lander and Kruglyak defined through guidelines for interpreting linkage results in complex traits that still hold till our days. In these guidelines, a LOD score of 3.3 of higher is often considered significant on a genome-wide level (Kruglyak and Lander 1995b) (Kruglyak and Lander 1995a) (Lander and Kruglyak 1995). A LOD value of 3.3 is equivalent to a p-value of 4.9×10^{-5}, or in other words, a 5% probability of association by chance in a genome-wide scan.

In monogenic disorders significant linkage of a locus to a disease is assigned when the LOD score is ≥ 3 which is equivalent to a probability of 1000:1 in favour of linkage. A LOD value of -2 excludes linkage between a certain marker and the investigated disease while a LOD value between -2 and 3 is considered inconclusive. The LOD score is a widely used technique not only in human research but also in plant and animal linkage analyses. In the linkage studies of this thesis performed in AIL significance threshold has been defined in two ways; either through a permutation analysis in AIL-G_7 or by calculating the residual threshold (Churchill and Doerge 1994).

Due to phenotypic variations observed between families from the breeding couples of AIL, determination of significance levels through permutation analysis is not preferable. Therefore a residual threshold approach, where each animal's value for a phenotype was subtracted from the mean value of this particular phenotype for the family of origin, was applied in AIL-G_{10}.

5.7 Quantification of mRNA using Real-time RT-PCR

Studies of differences in gene expression are additional steps for understanding disease regulations. Changes in gene expression can be tested using diverse techniques such as RNAse protection assay, *in situ* hybridization and Northern blotting. Even if they are very accurate and successful, these methods require large amounts of RNA, a need that is not always easily accomplished due to a limited sample size. A method developed to compensate for a previously mentioned pitfall of RNA requirement is the TaqMan RT-PCR (Heid 1996). This PCR-based method is more sensitive than Northern blotting and with higher resolution than *in*

situ hybridization, allows quantitative analysis of mRNA expression and screening of numerous transcripts from small amounts of RNA.

RNA is first extracted from tissues or cells and used as templates for construction of complementary DNA (cDNA) by reverse transcriptase enzyme. Amplification of cDNA by TaqMan is conducted by the use of two primers and a fluorogenic probe that anneals specifically to one strand of the DNA between the primers. The use of probe is an approach used to guarantee the precise amplification of the aimed target sequence. Another alternative is the SYBR® green method that is used for the same purpose as TaqMan. This alternative method is mainly used as a cheaper choice than the classical TaqMan application since no probes are required.

Primers for both TaqMan and SYBR green were designed in a way so that at least one of the primers from each pair was located on exon-exon borders to avoid amplification of genomic DNA. In the case of TaqMan, the probe was designed in the sequence between the two flanking markers used for mRNA amplification. To assure a complete removal of any contaminating genomic DNA, RNA samples are usually treated with DNase prior to cDNA amplification. In this thesis, the targets TNF, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IL-23 were amplified from splenocytes following stimulation with conA. Standardization for the amount of starting material was performed using GAPDH or 18S. Amplification of standard curves for the targets was carried out using cDNA from conA-stimulated lymph node cells.

5.8 Measuring protein levels with enzyme-linked immunosorbent assay (ELISA)

In addition to expression of cytokines, protein levels of the above mentioned cytokines were analysed using enzyme-linked immunosorbent assay (ELISA). The cytokine levels of TNF, IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-13 production were measured in the supernatants of conA stimulated splenocytes.

5.9 Proliferation assay

One of the most familiar and widely used method for testing cell viability and growth as a result of activation by the used mitogen is the proliferation assay. Antigen-specific T cell proliferation is quantified by measurement of tritiated thymidine ([^{3}H]-thymidine) incorporation into newly synthesized DNA during cell division. The amount of incorporation is usually measured by a β-counter.

6. Results and interpretations

6.1 A new EAE-regulating locus, *Eae19*

Previous studies in (DAxACI)F_2 of a MOG-EAE model and (DAxBN)F_2 of a SC-EAE model showed linkage to a region on rat chromosome 15 (Dahlman et al. 1999b) (Dahlman et al. 1999a). In order to investigate the importance of this QTL in MOG-EAE, we applied a speed congenic technique to construct a congenic strain with EAE-susceptible Dark Agouti (DA) rat strain and the MHC-similar but EAE-resistant ACI strain. In this, a 25cM fragment spanning from D15Rat6 microsatellite marker till D15Rat 71 was transferred from the ACI donor strain to the DA background strain. After six generations of backcrossing and one intercross generation, experimental animals from the DA.ACI-*D15Rat6-D15Rat71* (N7F1) were subjected to a MOG-EAE experiment. In addition to the full length congenic, we also used four recombinant congenic strains, C15R1 that span from D15Rat6 till D15Rat13, C15R3a strain (D15Rat6-D15Rat48), C15R3B (D15Rat6-D15Rat48; D15Rat126- D15Rat71), the C15R4 strain (D15Rat23-D15Rat71) and the parental DA strain that was used as a susceptible control strain. A result from six independent but normalized experiments showed a difference in the displayed clinical phenotype between DA.ACI-*D15Rat6-D15Rat71*, C15R3b and C15R4 that demonstrated a lower maximal and cumulative EAE score compared to the control DA strain and the C15R1and C15R3a recombinant strains. The lack of polymorphic markers between DA and ACI does not allow us to define the exact borders of the congenic but we think that the fragment transferred from the donor strain could be between 2.5Mb to a maximum of 16.7 Mb large.

In a parallel we performed linkage analysis in the MOG-EAE model of the seventh generation of an advanced intercross line (AIL) between the DA strain and the EAE-resistant PVG.AV1 strain that share the same MHC background as DA. A profit gained from the AIL strategy is the reduction of confidence interval with the increasing number of generations due the accumulated number of recombinations. A significant linkage was found in AIL with the LOD value of 4.7 for the maximal EAE score. We were also able to further narrow down the confidence interval to 13Mb defined with a 1 LOD drop that overlaps with C15R3b and C15R4 at the peak marker D15Rat71.

We were hence able to identify a new locus, named *Eae19*, on rat chromosome 15 that harbours 32 confirmed or predicted genes. Among interesting genes are immune-responsive gene 1 and neuronal ceroid lipofuscione gene 5. Of course we can at this stage not rule out genes outside the here defined confidence interval. The observation that *Eae19* region also overlaps with a QTL region for adjuvant-induced arthritis QTL 4 (*Aia4*) is interesting since it is highly likely that the two diseases share some genetically regulating mechanisms, a fact that has been shown in arthritis-regulating congenics subjected to EAE experiments (Becker 1998; Kawahito 1998) (Becanovic et al. 2003a). The potential importance of *Eae19* is shown in the implications of shared genetic regulation mechanisms with cancer and cardiovascular diseases. This is shown by the overlap of serum cholesterol QTL1 (Kato 2000), blood pressure QTL cluster 12 (Stoll 2000) and gastric cancer susceptibility QTL 1 (Ushijima 2000). However, such overlaps must be interpreted with caution, since QTLs identified in conventional F2

crosses usually are large, and significant overlaps are expected by chance due to the large number of QTLs identified.
No evidence of significant linkage was presented in the human syntenic region on chromosome 13 (13q22.1-q31.2). This may be due to the fact that human studies have lacked power or because the rat and human region might not share the same regulating genes but rather the same disease regulating pathways. Another reason might be that the region harbours genes regulating mechanisms unique to the rat.

6.2 Rat 4q42-43 region, a multiple QTL region

Previous linkage studies showed an effect on a particularly interesting region on rat chromosome 4, a 16.8Mb region on 4q42-43, harbouring a striking accumulation of QTLs linked to EAE, arthritis, and diabetes (Dahlman et al. 1998; Martin et al. 1999). The interest in this region increased specially since the syntenic human region on 12p13.31-13.2 has been implicated in both multiple sclerosis and rheumatoid arthritis (Xu 2001) (Cornelis 1998).

An interesting finding is that the normally EAE resistant PVG allele that should confer less disease severity to a congenic strain was found to exacerbate encephalomyelitis in the C4R3 congenic at the same time that it was proved to provide protection against arthritis (Becanovic, 2003b).

We performed a QTL analysis on the 16.8Mb region overlapping with C4R3 in a set of 794 animals of the tenth generation of AIL. In this we identified linkage to two closely located loci, *Eae20* and *Eae21* that are situated 3.1Mb apart. The effect of *Eae20* was illustrated in its influence on all investigated clinical phenotypes of EAE. *Eae21* only influenced the severity of EAE with no influence on incidence and onset of EAE. This suggests that *Eae21* acts as a modifier locus controlling the disease phenotype once a threshold level has been reached.

We investigated the possibility of interacting QTLs in a two-dimensional scan with a two-QTL model analysis. This revealed a third locus, E*ae22*, situated 10Mb on the telomeric side of *Eae20* and epistatic with it. Epistatic interactions, explained as a situation in which a genotype at one locus affects the phenotypic expression of a genotype at another locus, add another level of complication on the genetic dissection of complex diseases. We also discovered that the disease promoting DA allele on *Eae20* was, alone, sufficient for disease regulation at this locus. On the other hand, the PVG allele on *Eae20* conferred an even more severe disease when present together with a DA allele on *Eae22*. This might explain the observed disease exacerbation in *Eae20*. This epistatic interaction suggests that a similar interaction might be present in other organisms than the rat's model of EAE, a fact that should be considered when analyzing in the syntenic human region on 12p13.31-13.2.

The paradoxical finding of exacerbation of disease in the 10 Mb C4R3 region with the down-regulation in EAE clinical phenotypes in the smaller congenic strain C4R11 of 1.44Mb could be explained by different scenarios. One hypothesis would be an interaction between *Eae20* and *Eae21* resulting in different outcomes depending on the combination of a PVG allele at one locus and a DA allele at the other one. This interaction could not be confirmed, probably

due to the limited number of animals in the G10 AIL that have different allele combinations. Another theory suggests the presence of several QTL within *Eae20*, *Eae20a* and *Eae20b*, each one with different effect on EAE. This speculation proposes the presence of *Eae20a* on the centromeric region of *Eae20* not overlapping with C4R11 that exacerbates EAE and *Eae20b* overlapping with C4R11 that demonstrates a protection of the EAE phenotype.

A look at the genes harboured in *Eae20*, located in the 1.44 Mb C4R11 congenic strain, showed that it encloses a limited number of immunologically important genes. Among these genes is a cluster of C-type lectin superfamily members (*Clecsf*) (Flornes 2004), consisting of a family of stimulatory and inhibitory receptors expressed on APCs that are important for leukocyte activation (Flornes et al. 2004).

Candidate genes in *Eae21* include the TNF receptor superfamily TNFR1, CD27 and lymphotoxin-β receptor. Experiments in knockout mice showed an effect of TNFR1 on EAE severity but not on disease induction (Eugster 1999). These data reflect the noticed results of the linkage analysis we performed where Eae21 did not show any effect on the incidence. (A more detailed list about the genes harboured in these regions is present in paper II). The TNFR superfamily is of particular interest since its known role as a multiple effector might be another explanation for the exacerbation in C4R3. TNF receptors showed a disease-predisposing effect in EAE induction (Kassiotis and Kollias 2001) and at the same time a positive role displayed in a beneficial removal of pathogenic T cells (Probert 2000).

6.3 *Vav1*, a genetic regulator of proinflammatory cytokines, MS, RA and EAE severity

We previously identified a locus on rat chromosome 9, *Eae4*, in (DAxBN)F_2 rats immunized with whole spinal cord (WSC) homogenate in CFA (Dahlman et al. 1999a). *Eae4* displayed regulation of severity and chronicity of SC-EAE and of the number of IFN-γ mRNA-expressing cells in CNS. A correlation between EAE severity and IFN-γ and TNF has been previously shown as well as the potential role of these cytokines in encephalitogeneity of T cells (Issazadeh et al. 1996). The genes underlying *Eae4* might reveal a common mechanism shared by QTLs regulating other inflammatory conditions such as *eae5* in mouse encephalomyelitis, *Ciaa3* and *Cia15* in arthritis (Furuya 2000; Griffiths 2000), IgE responses (Mas 2000), *Aiid3* that regulates gold salt-triggered glomerulonephritis (Mas 2004) and $CD45RC^{high}/CD45RC^{low}$ ratio in T cell subsets (Subra 2001) (Butterfield 1998) (Xystrakis 2004).

We here fine-mapped *Eae4* using DA.BN-*Eae4* and LEW.BN-*Eae4* (henceforth denoted DA.BN and LEW.BN) two congenic strains containing the *Eae4* region from the EAE-resistant BN strain transferred to the EAE-susceptible DA and LEW backgrounds respectively, in addition to a panel of interval-specific recombinant congenic strains.

Fine-mapping of the *Eae4* region was performed by a combination of analysis of the clinical EAE phenotype, investigation of immunological responses to *in vitro* stimulation of spleen cells with ConA and numbers of $Foxp3^+CD4^+$ regulatory T cells. ConA is a potent T cell

mitogen known to activate expression of proinflammatory cytokines, thus mimicking an important EAE subphenotype (Wang 2005b).

For clinical assessment of EAE phenotypes, we induced a chronic disease in DA rats together with the DA.BN through immunization with emulsions of either MOG or WSC-homogenate in IFA and CFA, respectively. For the LEW rats and the LEW.BN congenics, a monophasic EAE model was induced by immunizing with MBP in IFA. The *Eae4* region transferred from the resistant BN strain to the susceptible parental DA strain conferred less disease severity in the DA.BN-*Eae4* and LEW.BN congenics compared to susceptible parental strains, DA and LEW respectively. In DA and DA.BN, a better recovery from the initial bout of EAE independently of the antigen used during immunization was observed, with significantly lower cumulative clinical score in both models of the chronic EAE, the MOG and WSC-induced, ($p<0.03$ and 0.05, respectively). In two of the four experiments that were performed independently, a significant effect on mortality was demonstrated in addition decrease of the cumulative and maximum score in the congenic strain during the second bout. The MBP model induced in LEW and LEW.BN also presented a significantly lower incidence ($p<0.05$) and milder disease ($p<0.008$) conferred by the BN allele.

An influence of inflammatory cytokines and regulatory T cells (Tregs) on EAE has been previously established (Korner 1997) (McGeachy and Anderton 2005) (McGeachy 2005) (Fontenot 2003). For this, we tested the impact of *Eae4* on these two phenotypes. We measured TNF both on the expression level with TaqMan and on the protein level with ELISA in naïve splenocytes stimulated with Con A and their supernatants, respectively. TNF secretion in naïve splenocytes stimulated with Con A was significantly lower in DA.BN ($p<0.008$) and LEW.BN ($p<0.01$) compared to the parental DA and LEW strains, respectively. Assessing the impact of Eae4 on the proportion of Tregs was conducted by analyzing the percentage of CD4+Foxp3+ cells. The BN allele allele at *Eae4* conferred higher proportions of Tregs in comparison to the DA ($p<0.008$) and LEW ($p<0.009$) alleles respectively.

Starting from the full length congenic strains we created a panel of overlapping interval-specific congenic lines to narrow down the *Eae4* region. The smallest recombinants of both DA.BN, hereby referred to as R25, and the one of LEW.BN, displayed both a significantly milder EAE. These minor recombinants narrowed down the *Eae4* interval to approximately 2cM

Although the smallest recombinants of *Eae4* displayed influence on severity and duration of EAE, we could not use this parameter for positional cloning since this would have required larger amounts of animals to be screened. We employed the sub-clinical phenotypes of TNF and Tregs to narrow down this QTL. TNF expression was used as a subphenotype due to its association with EAE and MS (Issazadeh et al. 1995a) (Issazadeh et al. 1996) (Issazadeh et al. 1995b). Both the TNF and Treg sub-phenotypes co-segregated with the clinical disease, with lower TNF expression and higher Treg proportion in the congenic animals. The correlation of these phenotypes to a lower disease severity enabled us to narrow down the *Eae4* region further more to a roughly 1 cM.
The combination of both clinical assessment of data and the utilization of subphenotypes provided a solid ground for positional cloning of genes underlying the EAE phenotype. We

believe that a milder disease is a result from a combination of up-regulation of Tregs and lower TNF expression, and that all these phenotypes might be regulated by the same gene/genes within *Eae4* between D9Wox24 and D9Got8 markers.

When the impact of the *Eae4* region on TNF regulation was tested in different subsets of cells sorted from splenocytes and stimulated with Con A, we observed a lower TNF production in T cells, in particular the $CD4^+$ subsets, of the congenic strain compared to the same cell subsets of the DA strain. No influence of TNF regulation was indicated neither in the monocyte/macrophages nor the NK/NK-T cells. To verify that the effects shown were not secondary to macrophage activation we tested TNF expression in bone marrow-derived and peritoneal macrophages after stimulation with LPS. Even though LPS stimulation upregulated TNF and NO production, no difference was noticed between the congenic and DA strains. This implicates major effect in lymphocytes that is further supported by:

The fact that the prime target of ConA, used during splenocyte stimulation, is the T cells, since it is known to bind to the TCR:CD3 complex (Kanellopoulos 1985). Another indication is the coordinate regulation of IL-2 and IFN-γ that are the primary products of lymphocytes. A third indirect proof is the effect of a major regulator of EAE, the MHC complex, as a restriction element for T cells, especially since its effect goes well along with the influence on T cells (Weissert et al. 1998).

Since the correlation with subphenotypes could provide additional insights into the underlying mechanisms of gene regulation of EAE, we expanded our functional studies of the *Eae4* effect. We noticed a regulation of IFN-γ and interleukin-2 (IL-2) with no effect exerted on Th2 cytokines (figure 6 A and B). Cell proliferation was also affected by *Eae4*, a response that may be reflected by the effect on IL-2 (Figure 6 C).

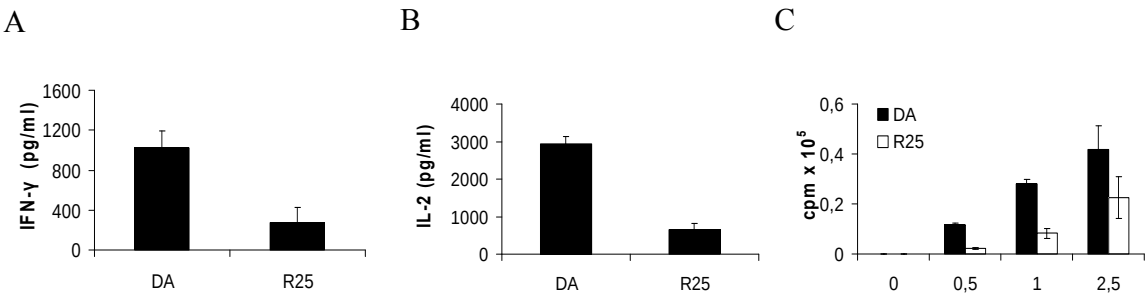

Figure 6: *Mean IFN-gamma and IL-2 production measured in supernatants of naïve splenocytes stimulated with ConA (A and B, respectively) and mean proliferative response (C), measured in splenoctyes stimulated with ConA in parental DA and R25 recombinants. BN alleles within the R25 strain that contains the narrowest Eae4 region conferred lower IFN-gamma, IL-2 and proliferative response in splenocytes ($p<0.05$). The error bars represent SD.*

It is worth to mention is that the noticed effect of *Eae4* on the expression of proinflammatory cytokines and proliferation is not crucial in the induction of the disease. It rather showed a disease promoting effect after the first bout. ‘

A list of genes harboured in the *Eae4* region was not easy to obtain since the rat genome sequence of this region displays some inconsistencies. We therefore retrieved a list from the homologous region at 53.8-54.9Mb on mouse chromosome 17 (the homologous region is available at http://www.ensembl.org, MMU17: 57.3-57.45 Mb). We developed a minor congenic of 120 Kb of LEW.BN encompassing the five genes tumor necrosis factor ligand superfamily member 14 (*Tnfsf14*), complement 3 precursor (*C3*), G protein coupled receptor 108 (*Gpr108*), Cdc42-interacting protein 4 (*Cip4*/*Trip10*) and Vav1 oncogene (*Vav1*). Although clinical influence of this smaller congenic was not tested due to the resistant BN background, the correlation to the sub-clinical phenotypes of TNF and Tregs was validated.

The five genes in this interval did not differ in their expression level. We therefore aimed on sequencing the coding regions of these genes in DA and BN, and subsequently the identified SNPs in additional six rat strains to search for shared haplotypes between strains of similar susceptibility, alternatively resistance, to various inflammatory diseases. A combination of previously known data about QTLs and susceptibility to different diseases in this region and the SNP mapping revealed one SNP in the first exon of *Vav1* segregating between strains sharing the same susceptibility to inflammatory diseases. This SNP resulting in a substitution of Arginin to Tryptophan (R63W) in the conserved Calponin domain of Vav1 was the only one fulfilling these criteria ($p<0.005$). While an Arginin on aa position 63 in exon one leads to higher TNF and lower Tregs levels, the substitution at this position to Tryptophan present in the DA.BN congenic resulted in lower levels of TNF and higher Treg proportions.

Having mapped variations of *Vav1* in the experimental inflammatory model, we aimed in characterizing its contribution to human inflammation. We performed an initial screening in 1039 MS patients and 1206 controls with 47 SNPs in the synthenic human region on chromosome 19p13.3 covering *TRIP10*, *SH2D3A* and *VAV1*. We observed an association to numerous SNPs within *VAV1* and a CGC haplotype of three markers between blocks 6, 10 and 11 less frequent in MS patients. The haplotype markers were analyzed in additional MS patients and healthy controls from Sweden, Norway, Denmark, Finland, France and Canada summing up to 12 735 individuals. A joint meta-analysis of all these materials showed an association to three haplotypes: a common CA haplotype (frequency ~85%) with a higher frequency in MS patients than healthy controls (Odds Ratio (OR) =1.18, $p<2x10^{-5}$) and two haplotypes, CG and TG, less frequent in cases than controls (OR=0.86; $p<0.002$ and OR=0.90; $p<0.02$ respectively).

To estimate the probability of our data being true associations we calculated the false positive report probability (FPRP). The FPRP is a function of the prior probability of association and the power of the study. It is based on three factors determine its magnitude namely, the prior probability of a true association of the tested genetic variants with the disease (π), the observed *P* value and the statistical power to detect the OR of the alternative hypothesis at the given *P* value. It is calculated according to these formula: FPRP= 1/(1+ posterior odds for true association). The Posterior odds for true association = (prior odds x power)/significance

threshold. Assuming a prior π value between 0.01 and 0.002, the FPRP for our material was less 10 %, meaning that there is less than 10% risk that our found associations of *VAV1* are false positive.

Interestingly, the disease predisposing AC haplotype of *VAV1* also correlated to higher expression of VAV1 in PBMC of MS patients both in an additive and dominant manner ($p<0.05$ and $p<0.005$ respectively), while the disease protecting TG haplotype resulted in lower Vav1 expression in PBMC of MS patients in an additive way ($p<0.005$). Additionally, a higher mRNA expression of VAV1, TNF and IFN-γ was noticed both in BPMCs and CSF of MS patients ($p<0.0001$ for all phenotypes). Furthermore, we could show a correlation between levels of VAV1 and TNF ($r^2=0.61$; $p<0.0001$) and VAV1 and IFN-γ ($r^2=0.40$; $p<0.0001$).

Along with increasing evidence of shared genes between inflammatory diseases (Zhernakova 2009) and given the inflammatory regulation of Vav1 summarized in all previously known phenotypes, our interest in testing its association to other inflammatory diseases was raised. We therefore performed association in rheumatoid arthritis (RA) due to the overlap of experimental QTL of *Eae4* with a QTL of collagen induced arthritis and anti-collagen IgG levels (*Cia15* and *Ciaa3* respectively) (Furuya et al. 2000) (Griffiths et al. 2000). We presented in this context an association of *VAV1* in patients negative for antibodies to citrullinated peptide antigens (ACPA negative) RA patients ($p<0.008$) with no evidence in ACPA positive RA patients.

We were thus able to, thanks to QTL mapping in the animal model of EAE in rats, position *Vav1* and define its role in regulating proinflammatory cytokine productions and the human inflammatory diseases MS and RA.

6.4 *RGMA* and *IL-21R* associate to MS and EAE.

We previously mapped an approximately 80 Mb large QTL on rat chromosome one (RNO1) linked to LPS-induced TNF response in an F_2 cross between the EAE-resistant PVG.1AV1 and EAE-susceptible LEW.1AV1 strains (Xu 2002). For a confirmation we performed a linkage analysis in peripheral blood mononuclear cells (PBMCs) from rats of the 12^{th} generation (G_{12}) of an AIL (AIL-G_{12}) stimulated with LPS and screened for TNF and IL-6 production. The original QTL was considerably refined and resolved into two QTLs. The proximal 6 Mb large QTL affects IL-6 production while the distal 8.6 Mb large QTL was linked to IL-6 and overlaps with a 10Mb QTL affecting TNF. TNF and IL-6 showed linkage in the whole group and for sex as an interactive covariate. When different sexes were treated as subsets, an effect on IL-6 production was more pronounced in females.

We aspired to find any particular influence of this cytokine-regulating region on the outcome of inflammatory diseases. We therefore performed a linkage analysis in rats of the 10^{th} generation of AIL (AIL-G_{10}) subjected to MOG-EAE and in AIL-G_{12} subjected to pristane induced arthritis (PIA).

Analysis in AIL-G_{10} revealed two independent EAE-regulating QTLs, hereby named *Eae30* and *Eae31*, of 6 and 10 Mb respectively. *Eae30* showed linkage to all clinical phenotypes as well as production of anti-MOG IgG2 and overlapped with the proximal IL-6 QTL. The *Eae31* QTL was for all clinical phenotypes and to anti-MOG IgG1, IgG2b and total IgG titers and overlapped with the distal IL-6/TNF QTL. Analysis of allelic effect revealed that the disease susceptibility and increased anti-MOG IgG2b levels of *Eae30* were conferred by the EAE-susceptible DA alleles, in contrast to the overlapping IL-6 QTL where higher levels were conferred by the PVG allele.

In order to rule out any potential interaction along the region, a two-QTL model was carried out in AIL-G_{10}. This model provides information about interaction of both an additive and epistatic character between two loci by making an association between all pairs of markers and inter-marker positions along the investigated region of a chromosome. The analysis showed the presence of a third interactive QTL, *intQTL*, interacting with *Eae30* with the peak marker D1Rat131, located ~ 16 Mb telomeric to D1Rat270, the peak marker of *Eae30*. The epistatic *intQTL* mainly influences incidence and day of onset of EAE. A 60% incidence was noticed when a homozygous DA allele on *intQTL* was present in combination with a homozygote PVG allele on *Eae30*. A homozygous PVG allele or a heterozygous DA/PVG on *intQTL* in combination with a homozygous PVG on *Eae30* did not increase incidence of disease. No evidence for interaction was found between neither *Eae30* and *Eae31* nor *intQTL* and *Eae31*.
We constructed a fit model to confirm the linkage to *Eae30* and *Eae31* and the observed interactive *intQTL* using the following formula of variance in R/qtl software: y ~ *Eae30* + *intQTL* + *Eae31* + *Eae30*: *intQTL*. When dropping the effect of each QTL and the interaction at a time, an impact of the particular QTL is then determined. Excluding *Eae30* and *Eae31* in subsequent steps showed an impact of each of these two QTL on all clinical phenotypes independently. Dropping the contributing effect from *intQTL* alone and the interaction between *intQTL* and *Eae31* confirmed the data from the interaction analysis with *intQTL* influencing incidence and day of onset of EAE.

The parallel linkage study in the PIA model of AIL-G_{12} showed a 2.1 Mb QTL overlapping with *Eae31* and the QTLs of IL-6 and TNF, hereby called *Pia32*, and linked to disease incidence, onset and disease severity. This shared QTL is here forth called *Eae31*/*Pia32*.

To verify the linkage data of EAE, we induced MOG-EAE in CFA in a congenic strain that was bred based on data of the F_2 cross. The PVG.LEW-*D1Rat270-D1Rat68* (hereafter named PVG.LEW) congenic was developed by selectively breeding the EAE-susceptible LEW strain into a background of the EAE-resistant PVG strain. Prediction of how the congenic would behave is based on the AIL data. In accordance with the AIL data, the allelic combination at the different QTL within the PVG.LEW congenic strain should drive a more severe disease and higher levels of TNF and IL-6. Indeed, *in vitro* experiments with LPS stimulation on PBMCs from naïve PVG.LEW congenics and PVG demonstrated that PVG.LEW produced higher levels of TNF and IL-6 compared to the parental PVG.1AV1 strain. Reflecting the sex-effect from the linkage analysis in AIL; the IL-6 production was more pronounced in females. In all performed EAE experiments, the PVG.LEW congenics immunized with MOG-EAE in CFA displayed a higher incidence and mortality and a more severe disease course displayed by a higher maximum and cumulative EAE score.

With increasing evidence of shared genes between inflammatory diseases (Zhernakova et al. 2009) we hypothesized that the linked phenotypes on respective QTL were controlled by the same genetic variation. Combining data on respective QTL, the shared regulatory region between *Eae30*, the QTL for IgG2b and IL-6, was reduced to less than 2.4 Mb spanning 127.9-130.3 Mb on RNO1 (figure 7).

Similarly, the shared region between *Eae31*/*Pia32*, the QTLs for anti-MOG antibodies, TNF and IL-6 was reduced to a confidence interval smaller than 2.1 Mb. A glance at the genes harbored within these shared regions on respective positions showed that they are not gene dense (figure 7). Applying a candidate gene approach, our choice was on *RGMa* over the *Eae30* region due its contributive role in the nervous system and regulation of demyelination about CNS injury (Matsunaga and Chedotal 2004) (Matsunaga 2004) (Schwab 2005b). For the shared QTLs in *Eae31*/*Pia32*, previous association studies of *IL4R* in MS (Hackstein 2001) (Mirel 2004) (Suppiah 2005) (Quirico-Santos 2007) and knowledge about *IL21R* in EAE and inflammation (Liu 2008a), were reasons to choose them for further studies.

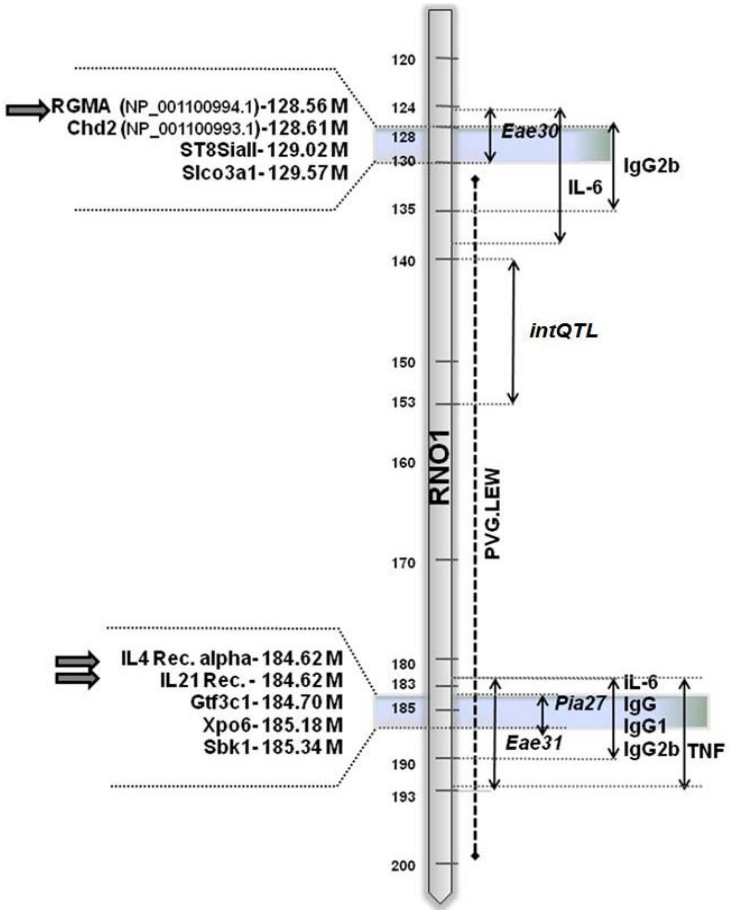

Figure 7: *A summarizing figure showing RNO1 with all QTL depicted along the chromosome. Shadowed regions symbolize the shared areas between the different QTLs at respective position. Thick dashed line shows the region covered by the PVG.LEW congenic. A list of the genes harbored within the shared QTLs is shown to the right. Arrows to the left point to the genes tested in the association studies.*

We therefore focused on *RGMA* from the synthenic human chromosome 15 in addition to *IL4R* and *IL21R* on human chromosome 16 in a meta-analysis of four Nordic cohorts of MS cases and controls, two of them from Sweden, SWE I and SWE II, one from Norway (NOR)and one from Denmark (DEN).

For *RGMA*, at least one marker was associated to MS in every material but the most remarkable finding was the difference in association between males and females. In view of the gender influence observed in the rat

experiments, we stratified the human material for gender. Differences in the allele frequencies were mainly evident in male and not female MS patients. Since gender information from all NOR control samples were not accessible, we performed the sex stratified joint meta-analysis including the SWE I, SWE II and DEN materials. The association between males and females showed a significant heterogeneity ($p<0.04$) for the rs34925346 marker. An overall significant association for a disease predisposing C allele of was found among males (OR 1.33 95% CI 1.04-1.69, $p<0.005$) but not among females. A difference in the frequency of this allele was noticed between male cases (13%) and controls (9.7%) with no difference in females (11%).

We also tested for any particular interaction between rs34925346 and sex by estimating the relative excess risk due to interaction (RERI) (Zou 2008). If no interaction from the sex is present, RERI is then expected to be 0. When analyzing all cohorts, except of NOR, the RERI value was 2.45 (95% CI 0.44-4.99) indicating a clear interaction of the gender to the association of this marker. Worth to mention here that we expect a higher significance from males in SWE I, reaching at least to the same level as for the case of SWE II, if the frequency of males in SWE I would have been as high as in SWE II.

For *IL4R*, we found an association to one haplotype and one single marker in SWE I. None of these were confirmed in the SWE II material. One of the markers associated in SWE I, rs1801275, has been previous tested in other association studies for MS (Mirel et al. 2004) (Suppiah et al. 2005) (Quirico-Santos et al. 2007). We therefore run a joint meta-analysis of this marker run in NOR and SWE II combining them to data from the previously run studies. There was no association for this *IL4R* marker, nor was there significant heterogeneity between the materials.

For *IL21R*, we analyzed the five markers of the previously associated haplotype of SWE I in the SWE II, NOR and DEN materials. In the joint meta-analysis of all these materials revealed an association to one protective and one predisposing rs8060368-rs961914 haplotype of *IL21R* to MS. The MS protective TC haplotype of these markers (OR 0.87 95%CI 0.80-0.94, $p<0.0005$) had a frequency of 27.6% in patients and 30.5% in controls. The MS predisposing CC haplotype (OR 1.16 95%CI 1.07-1.24, $p<0.0002$) was more frequent in MS patients (58.4%) compared to controls (54.9%).

In order to evaluate the significance of our findings we have estimated the probability that the association between MS and *Eae30* and *Eae31* is false by calculating the false positive report probability (FPRP) for the reported associations (Wacholder 2004). For RGMA, when the prior odds is ranging from 0.01 to 0.002, then our estimated FPRP is less than 0.06, which is less than the FPRP used for genome-wide significance in GWAS ("Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls" 2007). For *IL21R*, if the prior odds are ranging from 0.01 to 0.002 then FPRP is less than 0.04.

To investigate if variants of our candidate genes have any impact on regulation of cytokine levels we measured the expression of TNF and IL6 that were studied in the rat as well as the Th1 associated cytokine IFN-γ. We thus tested for a correlation between the genotype of *RGMA*, *IL4R* and *IL21R* and expression levels of these cytokines in CSF and PBMCs of MS patients and patients with other non-inflammatory neurological diseases (OND).

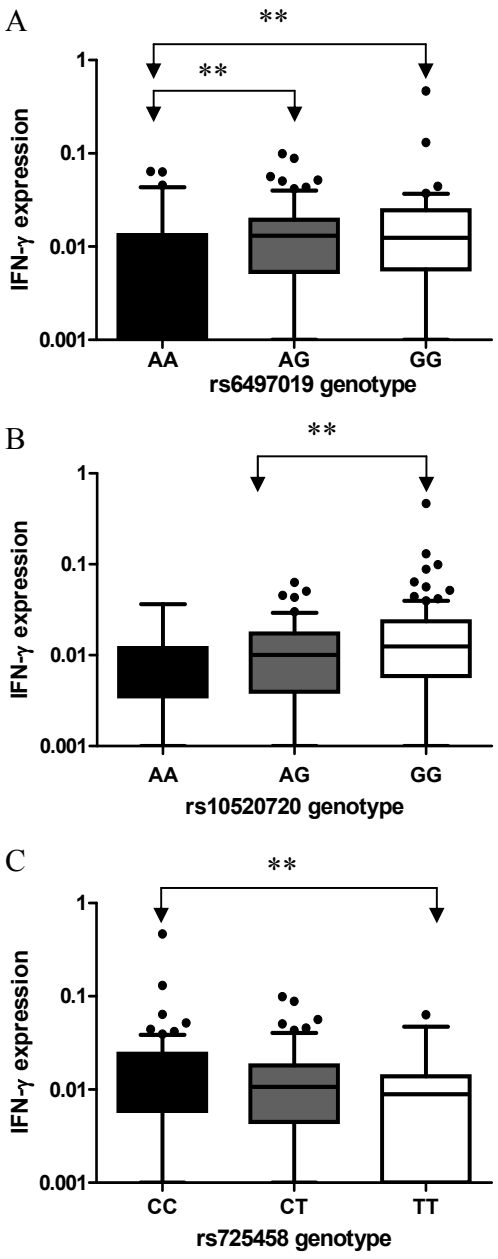

Figure 8: *Association of the disease associated markers A) rs6497019; B) rs10520720 and C) rs725458 to IFN-γ production.*

For *RGMA*, we found a correlation between three markers acting independently on higher levels of IFN-γ expression: a G allele of rs6497019, a G allele of rs10520720 and T allele of rs725458 ($p<1.8x10^{-3}$; $p< 2.0x10^{-2}$ and $p< 8.6x10^{-3}$ respectively) (figure 8 A, B and C respectively).
In addition, we found a correlation between the disease-associated AGG haplotype of rs997941-rs6497019-rs10520720 and a higher IFN-γ expression ($p< 4.0 \times 10^{-3}$) in the CSF of MS patients (Figure 9 A).
In contrast to the positive impact on IFN-γ expression, lower IL-6 expression in CSF of MS patients was associated with the protective rs34925346-rs6497019-rs10520720 GAG haplotype ($p<1x10^{-3}$) (Figure 8 B). We even tested for any gender effect in this context, but since the material used is small on itself, and stratifying for the males resulted in even fewer samples in this subset, we could not find any significant correlation in males.

Although the association in SWE I was not confirmed in SWE II, we still aimed on testing any particular correlation of *IL4R* genotype with the expression levels of TNF, IFN-γ and IL-6. We could not find any correlation between markers of *IL4R* and expression of any of the cytokines mentioned above.

For *IL21R*, the markers rs8060368-rs2214537-rs961914-rs12934152 forming the protective TGCT haplotype, displayed an association to a lower IFN-γ expression in PBMCs of MS patients ($p<7x10^{-3}$) (Figure 9C).
Moreover, we found a strong negative correlation between a non-disease associated rs80603688-rs2214537-rs961914-s12934152 CCTT haplotype of *IL21R* and TNF expression in PBMCs of MS patients with the ($p<5x10^{-5}$) (Figure 9D).

To our knowledge, this is the first evidence of association of *RGMA* and *IL21R* with MS, supported by data from experimental models. Furthermore, our data suggest a new role of *RGMA* in neuro-immunomodulation. *IL21R* on the other hand has been previously linked to EAE and to its contribution in inflammatory regulation of autoimmune diseases. Our data suggests its role in regulating inflammation in general.

Future confirmation of both *RGMA* and *IL21R* in larger materials of MS patients, both Nordic and non-Nordic, in addition to association studies of *IL21R* in a Nordic RA material are of importance to validate the effect of these genes. A better characterization of *RGMA*- and *IL21R*-driven pathways in inflammation might aid development of selective therapies.

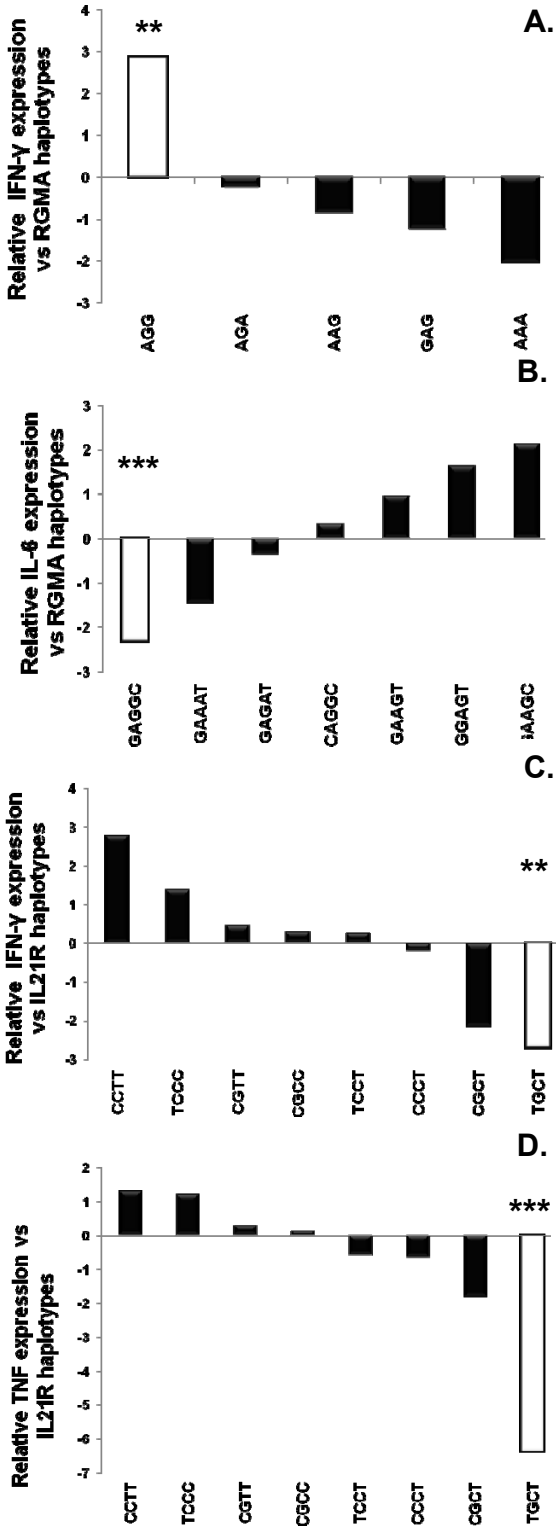

Figure 9: *Correlation of RGMA haplotypes with expression levels of:* **A**. *IFN-γ;* **B**. *IL-6; and correlation of IL21R haplotypes with expression levels of:* **C**. *IFN-γ and* **D.** *TNF.*

6.5. Unpublished results

6.5.1 Rat chromosome 8, a genetic hotspot for regulation of experimental autoimmunity

A previous study on a (LEW.av1 x PVG.av1)F_2 cross of a MOG-EAE model showed linkage to clinical EAE phenotype for a region on rat chromosome eight (RNO 8)(Becanovic 2003). We aimed at confirming this finding and investigating the presence of this QTL in a different combination of rat strains. We therefore performed a linkage study in a MOG-EAE model of a (DA x PVG) AIL-G_7 and G10.

Linkage analysis in AIL-G_7 confirmed this QTL in another strain combination and further resolved the region into what looks like several adjacent QTLs (figure 10A). It also pointed to an interaction between the gender and this QTL(s) in regulation of all clinical phenotypes of EAE (data not shown).

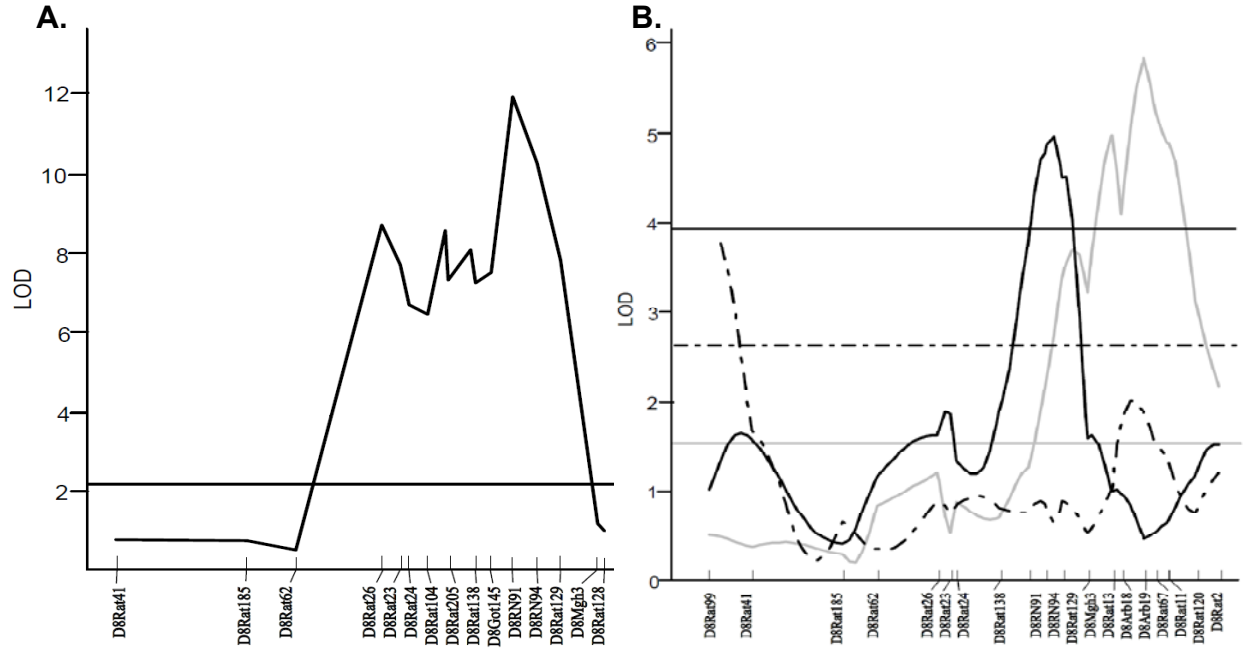

Figure 10: *Log-likelihood (LOD) plots for the QTL identified regulating:* ***A.*** *Cumulative EAE score in MOG-EAE in AIL-G_7 with sex as an interactive covariate.* ***B.*** *Chronicity of PIA AIL-G_{12} with sex as an interactive covariate (thick black line); TNF (thick grey line) and IL-6 (dashed black line) production after WB-LPS stimulation in rats of AIL-G_{12} with sex as an interactive covariate. Significance thresholds calculated from the residual threshold for respective phenotypes are depicted as horizontal lines with the corresponding color to the QTL line. (Not all markers genotyped are depicted).*

A congenic strain PVG.LEW-*D8Rat87-D8Rat75* (hereafter named C8) was created based on the F2 data by selectively breeding the EAE-susceptible LEW strain into a background of the

EAE-resistant PVG strain. The resistant PVG strain required stronger immunization protocols in CFA (IFA with Mycobacterium tuberculosis) and made it difficult to show a statistical significance on clinical course of EAE following MOG/CFA immunization. We could however detect a contribution on EAE-regulation of this region with higher susceptibility in females of the C8 congenic strain, which is in line with F2 and AIL findings and with the gender influence in AIL (Figure 11).

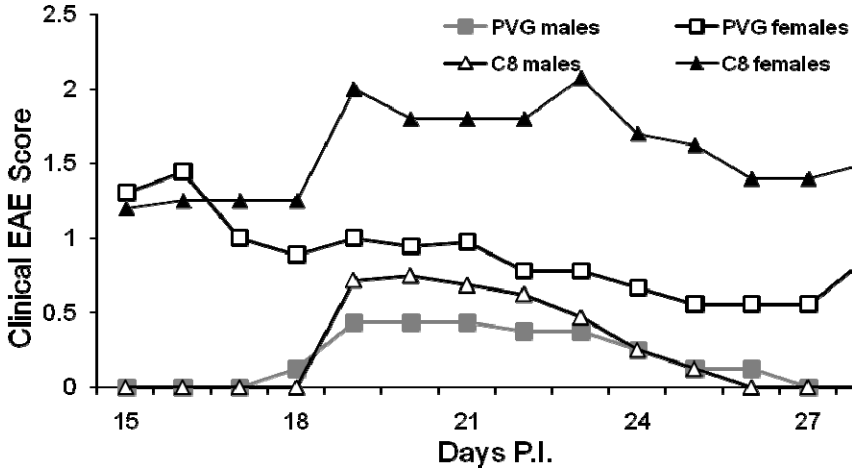

Figure 11: *Clinical EAE course of MOG-EAE in the C8 congenic and the background strain PVG used as controls for the EAE experiment.*

Atemps to further fine-map this region in AIL-G_{10} did not result in same high significance for the EAE-severity phenotypes (maximum EAE score, cumulative score and duration) as for AIL-G_7 although suggestive QTLs were mapped overlapping all above mentioned QTLs (data not shown). One explanation for these data would be the difference in EAE severity in AIL-G_7 and AIL-G_{10} reflected by an incidence of ~15% in AIL-G_7 and ~30% in AIL-G_{10}. The stronger disease induction in AIL-G_{10} could potentially override the effect coming from this QTL (the threshold model is discussed in the discussion section).

This region also co-regulated all clinical phenotypes of PIA following the induction of PIA in AIL-G_{12}, with a higher contribution on disease severity and chronicity than on disease incidence (figure 10 B). Even here we noticed an interactive effect from the gender on the outcome of all clinical phenotypes. Morover, a region overlapping EAE/PIA QTLs, which also regulates TNF and IL-6 production in PBMCs stimulated with LPS was identified in AIL-G_{12} (figure 10 B).

Future plans for this region are to perform confirming EAE experiments on the congenic strains and intra-recombinants bred between the EAE-resistant PVG strain into a background of the EAE-susceptible DA strain. Regulation of cytokine production in these congenics and functional studies in these strains will further contribute to a better understanding on the regulation of experimental AIDs, particularly EAE and PIA. Once few candidate genes are chosen based on further mapping in the EAE and PIA models, association studies performed in humans would be crucial.

7. Discussion

7.1 The rat and its importance in disease-mapping research

Although the rat was the first mammalian species to be exploited in medical research already 150 years ago (reviewed in (Jacob 1999)), it has lagged behind the mouse that has been the model of choice in evaluating genetic impacts on diseases. By the time I started by PhD studies five years ago, the rat genome was still not completely sequenced and an absolute coverage of some chromosomal regions with microsatellite markers was not available. This made it difficult for us to map some of the regions as extensively as we would have wished for. In paper I for instance, the "ghost" peak lying between D15Rat154 and D15Got93, would not be shown as a potential QTL adjacent to what we believe is the actual QTL between D15Mgh4 and D15Rat154. This problem was also reflected in paper III of *Vav1* on rat chromosome 9 (D9MJ280 and D9MJ2170) and in the chromosome 8 region mentioned in the section of unpublished data (D9RN91 and D9RN94). For these two regions, we designed a number of microsatellite markers based on the incomplete rat sequence that was available back then. By genotyping these markers, we choose the polymorphic ones between our used rat strains in order to achieve a denser mapping of these QTLs. This task would have been more fruitful and achieved more efficiently without these tedious moments.

In spite of these difficulties, a number of factors have contributed to the popularity of the rat in the use of medical research. Since the first inbred rat strains were established by Helen Dean King in 1909 (reviewed in (Aitman 2008)) more than 500 different strains have been developed to be used as models for a broad variety of disease research, all of them developed from the founder Brown Norway (BN) strain (BN/NsdMcwi). The abundant variety of inbred rat strains in addition to the ease in handling these animals raised the attractiveness of the rat as an experimental animal. In our case, a plausible reason for focusing on the rat as an experimental model has been the milder immunization protocols required for induction of diseases with no requirements of strong adjuvants in most cases. The variety of immunization protocols that result in different disease courses that reflect MS are additional reasons for why we chose to use the rat as our experimental model.

With the growing interest in the rat as a model organism, joint efforts from collaborating resources worldwide have been fruitful for completing the sequence of the rat genome and enhancing bio-informatic tools for a better mapping of disease QTLs. Starting from the Rat Genome Project and the Rat Expressed Sequence Tag (EST) project that were initialled from an initiative of The US National Institutes of Health (NIH) in 1995 and 1997, respectively, more databases and resources have been developed. A community of researchers from the United States, Japan, Germany, Great Britain and other countries from the European Union gathered their forces and made a priority list of goals for a better profit of the rat in genetic research (Aitman et al. 2008).

The ambitions aiming at increasing the number of genomic resources and interaction with mouse resources for a better development of rodent models of human diseases have at the present exceeded the expectations. For instance, a new upgrade of the sequence from the BN rat strain was published in late 2008. Additionally, a progressive increase in the genomic

markers was noticeable (Kwitek 2004) (Wilder 2004) (Zimdahl 2004) reaching up to three millions SNPs identified by the STAR consortium from various rat strains (Saar 2008). With the advances in rat microarray platforms such as Affymetrix, Agilent, Illumina, Ambion, NimbleGen, Solexa and Exiqon, the measure the expression of thousands of genes and microRNA is one further step in the developing rat era. The gathered knowledge about all these versatile genetic rat tools, from markers to QTL information, is gathered in open access rat genomic databases. Examples of such databases with focus on rat resources are the Rat Genome Database (RGD; http://rgd.mcw.edu); The US National Center for Biotechnology Information (NCBI); University of California, Santa Cruz (UCSC); Ensembl (www.ensembl.org) and the Whitehead Institute. With the increasing number of rat strains, genetic resource centres were also established to collect and preserve these strains for the benefit of all researchers around the world. The largest of these centres is the Japanese the National Bio Resource Project for the Rat (NBRP-Rat; http://www.anim.med.kyoto-u.ac.jp/NBR/), followed in size by the NIH Rat Resource Research Center (RRRC; http://www.nrrrc.missouri.edu/) from the USA and two European repositories namely, the Hannover Medical School (http://www.mh-hannover.de/2652.html) and the Czech Academy of Sciences by the EURATools (http://www.euratools.eu).

All these available resources were to guarantee optimal benefits to the entire community of rat researchers to study phenotypic effects of genomic variations. It is therefore not surprising the amount of genes positionally cloned in rat models of complex diseases since 1999. In our group, we mapped the *Mhc2ta* gene that was lately associated to MS, RA and myocardial infarction (Swanberg et al. 2005). Papers of this thesis also point to three other examples of genes mapped rat models of EAE: *Vav1* that was lately associated to MS and RA in addition to *RGMa* and *IL21R* that we associated to MS.

A noticeable effect of the collaborative work of rat scientists to accomplish the above mentioned achievements is the increasing number of genes discovered in proportion to the increasing amount of established resources and powerful tools. Besides that, the mapped loci during the latest couple of years were more for complex disorders while the mapped genes from earlier part of the research conducted in the rat were mostly for monogenic diseases. Exceptions to this observe were the *Ncf1* gene that was mapped in a model for RA (Olofsson 2003), the *Cd36* gene for insulin resistance, the Fcgr3 gene that predisposes to glomerulonephritis (Aitman et al. 2006) as well as *Cblb* and *Gimap5* that were linked to autoimmune diabetes (Aitman et al. 2006) and (reviewed in (Aitman et al. 2008)).

With all these records of success in the genetic research conducted in the rat during the past couple of years, we have no doubts that more genes will be positioned very soon. Likewise, we have great hopes that the still not achieved goal of site-specific gene-targeting in the rat will be a reality in the very near future.

7.2 Finding genes in MS – why is it so complicated?

Mapping heredity of autoimmune diseases (AIDs) came across difficulties due to their multifactorial character. Already in 1933, multifactorial inheritance was described as a

"threshold liability model" in a study on variability in number of digits in an inbred strain of guinea pigs (reviewed in (Wandstrat and Wakeland 2001)). The model stated that the more the number of susceptibility alleles increases in an individual, the higher is the disease liability by means of an increased chance of developing a phenotype. In the case of complex disorders such as MS where environmental factors also take part in the aetiology, these will provide additional steps towards the threshold liability (figure 12). Thus, each additional factor gathered in an individual, whether it is genetic, environmental or any stochastic event such as penetrance is a further step towards disease development.

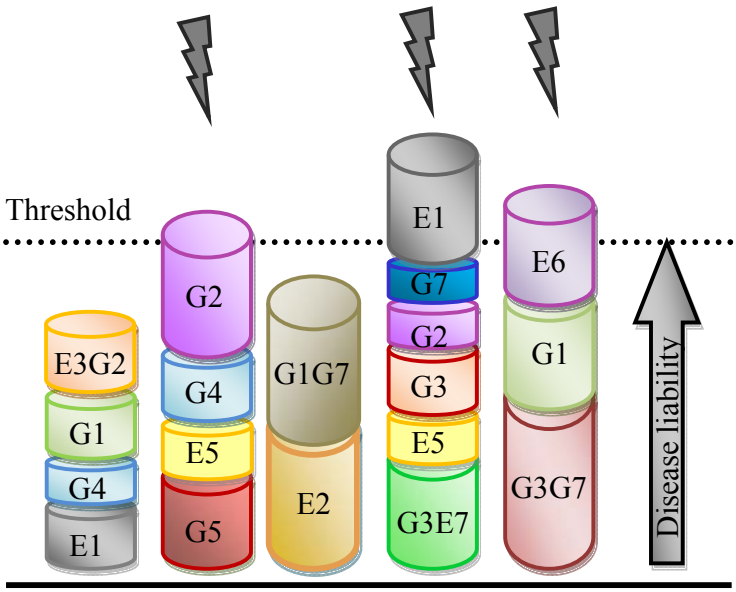

Figure 12: *The threshold model describing the susceptibility to complex diseases such as MS. G= Genetic factor; E= Environmental factor; a combination of different G and E in the same section indicates epistatic action between these two factors. The thunder-like arrows show the cases where a disease is developed by the gathered effect of underlying factors.*

The primary method of choice for testing disease-associated genes has been linkage analysis. Attempts of finding QTLs in MS by linkage analysis were not as rewarding as we had hoped for. Instead, the almost exclusive hit to the HLA complex overshadowed any contribution from other loci with much smaller effects. With the low rate of success, the glory of this strategy started to fade and the spotlight was directed towards association studies with the hope of reaching better results with this approach.

The original idea of association studies was to find disease associated alleles where allelic variation at a particular SNP was thought to be accounting for a certain phenotype. But is that really the entire truth? Do alleles act independently or do they require an orchestrated work with other alleles? In reality, a fast expansion in mapping genetic basis was more flourishing in Mendelian disorders due to strong heritability of an investigated trait within families. Another reason for this success is a strong genotype-to-phenotype correlation as a consequence from a single mutation of strong effect with a principal contribution to disease pathogenesis. Instead, this goal was more difficult to achieve in the genetic research of complex disorders like MS.

With the current unclear idea about genetic basis of complex disorders, it is not surprising if allelic combinations in form of haplotypes and/or epistatic interactions might not only be suggestive contributors to diseases, but also necessary grounds for a developing phenotype (Plenge and Rioux 2006). It is though naive to think that association studies will provide the complete ideas about correlations of genotypic variance to phenotypic differences in complex diseases.

An additional level of intricacy in mapping QTL of complex disorders is defined by *epistasis* that infers a further source of variance. Therefore, in-depth linkage studies are not accomplished solely by an estimation of single QTL mapping and marginal effect of particular QTL, but also by taking into consideration inter-loci interactions (Lark 1995) (Zeng 1999). Epistatic variance can be separated into two major components; the "*additive-by-dominance component*", defined as the interaction between an additive effect of one QTL and a dominance effect of a second locus, and the "*dominant-by-dominance component*" which is the contribution of a dominant effect of both loci (Yi and Xu 2002) (Yi 2005).
Epistatic interactions have been predicted to be a consequence of numerous functional polymorphisms influencing immunological responses and will therefore be part of AIDs in most species (reviewed in (Wandstrat and Wakeland 2001)). Examples of epistatic interactions are the results pointed to in this thesis reflect the influence on disease outcome. The extent of epistatic contribution in human autoimmunity is still not fully understood. It is though reasonable to assume that immunological responsiveness of AIDs will be defined by numerous functional polymorphisms working in an epistatic manner to increase disease susceptibility both in humans and other species.

The haplotype structures constructed from genomic regions in high LD inherited together more often than chance would allow, opened a new possibility for scientist in association studies. In paper III and IV of this thesis, we found associations of *Vav1*, *RGMA* and *IL21R* both on a single-marker level and on haplotype-based form. A widely spread idea among scientists have always been to rather aim for single disease-causing alleles rather than haplotype strategies. This bias towards single allele associations is mainly due to the assumption that these might hint for some missense mutations leading to an amino acid change and hence, pointing directly towards a functional contribution. The existence of haplotypes is though not to be disregarded, but rather taken advantage of. For instance, haplotypes could be reflecting functional units of genes that will, in some cases, increase the power to detect a QTL in conjunction to a particular SNP and hence, minimize the number of tests to be employed (Clark 2004). An increase in power is also in need when a mutation at one locus is noticed on numerous haplotypes. In this case, the lack of power resulting from the low degree of allelic heterogeneity is avoided through haplotype-based association studies.

Various sources of errors skewing findings of association studies leading to either false positive or false negative findings will still have to be adjusted for and taken care of in order to achieve more accurate findings. Taking care of all steps of pre- and post association studies, starting from gathering of ethnically homogenous cases and controls to correct statistical methods employed is therefore a necessity for this purpose.

7.3 Shared genetic factors of AIDs - the light at the end of the tunnel?

A well observed fact is the co-occurrence of AIDs, reflected in epidemiological studies by an increased susceptibility to AIDs if an individual already suffers from another AID (Vyse and Todd 1996; Sloka 2002). Evidence of this theory have been presented already a decade ago through various genome-wide linkage scans of both human and mouse models where several susceptibility loci of many AIDs were proven to co-occur in some regions (Becker et al. 1998) (Becker 1999) (Griffiths 1999) (Jawaheer 2001). For instance, MS patients show increased risk of developing other AIDs (Henderson 2000) (Barcellos 2006) (Midgard 1996) such as Grave's disease (Broadley 2000) inflammatory bowel disease (Gupta 2005) and T1D (Marrosu 2002) (Pitzalis 2008) ("The expanding genetic overlap between multiple sclerosis and type I diabetes" 2008).

This co-localization hypothesizes that many AIDs are controlled by shared genetic factors either reflected by allelic differences ("Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls" 2007) ("The expanding genetic overlap between multiple sclerosis and type I diabetes" 2008) or on expression levels of genes or even environmental factors affecting the outcome of these disorders. In fact, studies in non-affected first degree relatives of patients with AIDs and a comparison of gene expression levels between patients with MS, RA, T1D and SLE point to intersecting expression patterns between AIDs and advocate for universal genetic regulation among them (Mandel 2004) (Maas, 2002) (Maas, 2005). Analogously, studies on environmental factors and AIDs pointed to shared correlations between disease predisposing environmental factors and AIDs (Alonso 2008), such as Epstein–Barr virus infection with MS (Alotaibi 2004) (Ascherio and Munch 2000) (Levin 2003) (Levin 2005), and SLE (James 1997) (James 2001), and disease protecting effect of Vitamin D on MS (Munger 2004), RA (Merlino 2004) and T1D (Hypponen 2001). On a gene level, several genes have been associated to more than one autoimmune disorder e.g *CLEC16A*, *CD226* and *PTPN22* (Zoledziewska 2008) (Criswell 2005) (De Jager 2006) (Hakonarson 2007) (Rubio et al. 2008).

In this thesis we show a clustering of QTLs along our *Eae19* (paper I) that overlapped with QTLs for adjuvant-induced arthritis QTL 4 (*Aia4*) (Kawahito et al. 1998) (Becker et al. 1998) (Becanovic et al. 2003a), serum cholesterol QTL 1 (Kato et al. 2000), blood pressure QTL cluster 12 (Stoll et al. 2000) and gastric cancer susceptibility QTL 1 (Ushijima et al. 2000).
Eae4 also overlaps with a number of autoimmune diseases, namely *Cia15* and *Ciaa3* in arthritis (Furuya et al. 2000) (Griffiths et al. 2000), *Aiid3* that regulates gold salt-triggered glomerulonephritis (Mas et al. 2004) and $CD45RC^{high}/CD45RC^{low}$ ratio in T cell subsets (Subra et al. 2001) (Butterfield et al. 1998) (Xystrakis et al. 2004).
The overlapping QTL on *Eae30* and *Eae31* of paper IV are additional illustrations of the discussed mechanistic similarity between autoimmune disorders that enabled us to narrow down the candidate loci.

Many hypotheses have been suggested to explain the clustering of QTLs from different AIDs. One reason proposed a lack in the statistical power of the conducted linkage studies. For instance, in the cases where numerous QTLs with smaller effect of their own are physically

adjacent, linkage is then expected to be detected almost exclusively as a reflection from the combined phenotype of all QTLs together (reviewed in (Wandstrat and Wakeland 2001)).

A second interpretation for this clustering refers to the organizational feature of genomes and genes' functionality. These clusters are supposed to be the consequence many genes acting in the same signalling cascade and resulting in similar immunological responses. This co-localization of genes is then a consequence of diseases associated genes from the same cluster rather than a correlation to a common allele.

The co-occurrence of genetic foundations of AIDs is with no doubt an invaluable asset worth to profit from. Identifying shared genetic foundations will most certainly augment our understanding not only to particular diseases, but even to some universal mechanisms that lead to AIDs. Great hopes are then build on this strategy being the long awaited "*light at the end of the tunnel*" to unravel mechanistic enigmas of complex disorders and discover new therapies.

7.4 Genes associated to MS in this thesis

In this thesis we point to an association of three genes to MS. These genes seem to be, individually, crucial enough for their genetic manipulation of immunological pathways leading to MS. Of course the possibility of them being independently acting is not impossible. A rather more possible action of these genes is based on a speculative net of interaction where all three genes collaborate in one way or other. Common links between these genes hint for this hypothesis. For instance, Rho-GTPases, the link between RGMA and VAV1, the TNF and IFN-γ regulation by all three genes, in addition the site of immunological action of these genes suggest a more collaborative role of these genes. This also hypothesize an interaction that, once understood, may lead to a better understanding of mechanistic involvement of these genes in MS and inflammation in general.

7.4.1 *VAV1* and its potential relevance in MS/EAE.

In paper III of this thesis, we mapped a region on rat chromosome 9 harbouring the *Vav1* gene, a member of the Vav of protein family. Three homologues have been found in this family (*Vav1*, *Vav2* and *Vav3*) mainly located in the cell cytoplasm. Upon phosphorylation of their tyrosine residues by cell surface receptors and growth factor receptors, such as PDGF and EGF, Vav proteins are activated and get involved in numerous immunological responses (Moores 2000).

Vav1 is a 95 kDa protein originally considered to be exclusively expressed in haematopoietic cells (Bustelo 1992) (Katzav 1995) (reviewed in (Cantrell 1998)) (Moores et al. 2000), but lately, Vav1 was also reported in neuronal cells (Betz 2003). In contrast, Vav2 and Vav3 are expressed ubiquitously (reviewed in (Cantrell 1998)). *Vav1* was initially identified as an oncogene in gene transforming assays in fibroblasts (Katzav 1989). Alongside its occurrence in the cytoplasm, *Vav1* has been also found in the nucleus, suggesting its involvement in

regulation of gene expression as a crucial component of the NFAT and NFκB signalling pathways (Romero 1998) (Houlard 2002).

Vav1 is a guanine nucleotide exchange factor (GEF) for the family of Rho-GTPases of the Rho/Rac family (Rho, RhoG, Rac, and Cdc42) that are activated by tyrosine-phosphorylation following stimulation of T cell receptors (TCRs) (Crespo 1997; Han 1997) (Hall 1998) (Bustelo 2000) (Lopez-Lago 2000). Rho-GTPases are known as molecular keys with a conformational change between two states, an active guanosine triphosphate (GTP) state and an inactive guanosine diphosphate (GDP) state. Three classes of protein groups regulate this conformational change between the GPD and the GTP bound forms among them: the GTPase activating proteins (GAPs) that push the switch towards the active GTP form, the guanine nucleotide dissociation inhibitors (GDIs) that keep the GTPases in the inactive GDP form and the GEFs, the group to which Vav1 belongs to, that activate the RhoGTPases by facilitating the exchange of GDP for GTP (Katzav 1995) (Hall 1998) (Bustelo 2000). Rho-GTPases play numerous roles from mediating cytoskeletal reorganization to actin-based motility processes in the nervous system and involvement in different immunological pathways (Hall and Nobes 2000) (Etienne-Manneville and Hall 2002) (Meyer and Feldman 2002).

Vav1 encloses a unique combination of domains, each of them with a particular contribution to facilitate the protein-protein interactions and aiding in the signalling pathways where *Vav1* is involved. These domains include: a Calponin-homology (CH) domain, an acidic domain (Ac), a Dbl-homology (DH) domain flanked by a Pleckstrin homology (PH) domain, a zink finger (ZF) domain and a Src-homology 2 (SH2) domain flanked by two SH3 domains (reviewed in (Krawczyk and Penninger 2001)). The CH domain binds to F-actin and provides the oncogenic property to Vav when 67 amino acids from its N-terminus are deleted (Katzav 1991). The DH domain is a characteristic of all GEFs as it promotes the exchange activity for Rho family GTPases from GDP to GTP (Tybulewicz 2005). The function of the PH domain remains unclear and speculations about various roles of this domain in different Dbl-family proteins have been suggested (Hoffman and Cerione 2002). It has been proposed that PH enables transportation of *Vav1* from the cytoplasm and facilitates localization in the inner cell membrane by binding to phospho-inositides (reviewed in (Krawczyk and Penninger 2001)). Other data suggest that binding of phospholipids to the PH domain of *Vav1* regulate the GEF activity of the DH domain in vitro (Prisco 2005). For instance, an inhibitory GEF activity of *Vav1* is noticed when the PH domain binds to the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) while an enhancing activity of *Vav1* comes into action upon binding to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Han et al. 1997). The ZF mediates protein-protein interactions and binding of *Vav1* to the SH3 domains, while the SH2 domain binds to phosphor-tyrosine motifs and SH3 domains bind to proline-rich motifs (reviewed in (Krawczyk and Penninger 2001)).

Vav1 appears to be involved in a variety of regulatory processes with the best studied roles of this gene in the development and activation of B- and T-cells (Moores et al. 2000) (Zhang 1995). For instance, *Vav1* plays a role in transducing T cell signals, data reflected by an abolished positive and negative selection of thymocytes in Vav1 deficient mice (Prisco et al. 2005). Furthermore, experiments in *Vav1* knock-out mouse models (*Vav1*$^{-/-}$) pointed to an essential role in the development and function of T cells (Tedford 2001), in TCR signaling

(Kong 1998) (Turner 1997) (Tybulewicz 2003) and TCR-induced IL-2 secretion and proliferation in Vav1-deficient T cells (Fischer 1995) (Tarakhovsky 1995) (Zhang et al. 1995). RNA interference experiments also pointed to a contribution on TCR-mediated IL-2 promotor activity (Zakaria 2004). In our congenic strain of rat chromosome 9, we could point to a regulation on IL-2 expression and proliferation and effect on T cell regulation, linking this effect to the previously known functions of *Vav1*.

A noticed expression of Vav1 in cells of the cerebellum suggests a role of *Vav1* in the nervous system by mediating the activation of Rho-GTPases as a result of receptor activation (Betz et al. 2003). A role in cell cycle progression is suggested for VAV1 since it regulates cytoskeletal reorganization, Ca^{2+} flux and TCR clustering that are required for T-cell maturation, production of IL-2 and cell cycle progression (Fischer 1998). Furthermore, Vav1/Rac1 pathway shows a contribution in lipid raft clustering, Rho-GTPase and Ca^{2+} dependant actin polymerization, activation of stress activated protein kinases (SAPK/c-Jun N-terminal kinases) and cytoskeletal reorganization (Crespo et al. 1997) (Villalba 2001). In fact, mutations in the PH domain of Vav1 results in a perturbation of Rac1 activation, calcium flux and activation of NF-kB, NFAT and ERK-MAP in $CD4^+$ T cells (Prisco et al. 2005). Although a role of *Vav1* in EAE regulation has been reported following a noticed resistance to EAE in *Vav1* knock-out mice (Fischer et al. 1995) it is intriguing to draw conclusions from *Vav1* gene deletion experiments. This noticed protection to the disease might be related to a general dysfunction of lymphocytes since the depletion of *Vav1* leads to reduced numbers of T and B cells and severely impairs their function (Korn 2003). On the other hand, the inflammatory regulation and impact on the disease outcome of the naturally occurring genetic variant of *Vav1* is a better proof of the contributive effect of this gene to disease regulation.

7.4.2 The Repulsive guidance molecule A (*RGMA*) in MS and EAE.

In paper IV of this thesis, we mapped a QTL on rat chromosome 1, *Eae30*, which harbors one member of the RGM family, namely RGMa. Repulsive guidance molecules (RGMs) consist of a family of membrane-bound proteins. The first member of this family was originally cloned and characterized in chicken as a membrane-associated glycoprotein involved in axonal guidance of retinal temporal axons (Monnier 2002). Four isoforms of the RGM gene family have been found in vertebrates; *RGMA* is predominantly expressed in the central nervous system, *RGMB* mostly in the peripheral nervous system and *RGMC* exclusively expressed in all striated muscle and in the myocardium (Brinks 2004) (Brinks et al. 2004) in addition to a forth isoform, *RGMD*, only present in fish (Camus and Lambert 2007).

Expression of *RGMa* was noticed in the neural tube before the formation of retinal axons suggesting an important role of *RGMa* in CNS development (Matsunaga and Chedotal 2004) (Matsunaga et al. 2004). Over-expression of *RGMa* was shown to promote neuronal differentiation while down-expression of *RGMa* by small interference RNA had an opposite effect (Matsunaga 2006). Knockout mice models of *RGMa* confirm these data as they show defects in neural tube closure (Niederkofler 2004). *RGMa* and its receptor, a transmembrane protein called neogenin, have mainly been implicated in nervous system development and apoptosis {Rajagopalan, 2004 #70}(Brinks et al. 2004) (Matsunaga and Chedotal 2004;

Matsunaga et al. 2004) (Metzger 2005) (Matsunaga et al. 2006) (Wilson and Key 2006) (Fitzgerald 2007) (De Vries and Cooper 2008). For instance, when acting independently, Neogenin was shown to induce apoptosis when unbound to *RGMa* but once bound to *RGMa* , the apoptotic action of neogenin is blocked ((Mehlen and Thibert 2004).

RGMA also acts as a bone morphogenesis co-acting protein through involvement in the bone morphogenesis proteins (BMP) signaling pathways (Babitt 2005). BMPs represent a large sub-family of the transforming growth factor beta (TGF-β) superfamily that includes many cytokines playing a crucial role in cell differentiations, proliferation, apoptosis, migration and patterning during embryonal adult tissue development. Even though *RGMA* enhances the activity of BMPs in bone morphogenesis, it shows no effect on TGF-β, leading to the conclusion that *RGMA* acts independently to generate BMP signals via the classical BMP pathway and not through the TGF-β signaling (Babitt et al. 2005).

In the adult CNS, injured axons show a limited regeneration ability due to the presence of myelin-associated axonal growth inhibitors such as myelin-associated glycoprotein (MAG), Nogo and MOG(Yamashita T 2005). Recently, *RGMA* was also included to this list and considered as a potent myelin-derived neurite outgrowth inhibitor both *in vitro* and *in vivo* (Hata 2006) following experiments in rats with spinal cord injury (SCI) (Schwab 2005a; Schwab et al. 2005b) (Yamashita 2007). In the SCI model expression of *RGMA* was noticed around injury sites in oligodendrocytes, macrophages and/or microglia, infiltrating leukocytes, neurons and white fibers positive for myelin basic protein (Schwab et al. 2005a; Schwab et al. 2005b). In addition an interactive role between axons and myelin has been suggested for *RGMA* leading to a similar inhibitory activity on myelin as for MOG and MAG (Schwab et al. 2005b). This central role in limiting axonal regeneration is shown both in rats after SCI and in humans after CNS injuries such as focal cerebral ischemia and traumatic brain injury (Schwab et al. 2005b) (Hata et al. 2006; Yamashita et al. 2007).

Furthermore, *RGMA* acts through the activation of a RhoA-ROCK (RhoA/Rho kinase dependent) pathway by binding to its receptor and activation of the downstream effector, myosin II (Hata 2009) (Conrad 2007) (Kubo 2008). Activation of RhoA was noticed in neurons and glial cells around lesion sites of SCI suggesting an inhibitory role of activated RhoA in CNS regeneration (Dubreuil 2003) (Madura 2004). Decreased tissue damage and neuro-protection upon Rho-ROCK inactivation has been reported, suggesting an important role in CNS regulation (Fournier 2003). Moreover, inhibition of Rho family functions ameliorates EAE in rats by promoting myelin repair, inhibiting leukocyte infiltration into the CNS and reducing axonal damages of the CNS (Paintlia 2008) (Greenwood 2003; Hendriks 2004) (Walters 2002; Zhang 2008). The strong expression of RhoA in active MS lesions in comparison to a lower expression in chronic MS lesions and rare expression in normal brain suggests that RhoA also plays a role in MS (Zhang et al. 2008).

We believe that the regulation of RhoA and its contribution to the outcome of MS and EAE is the result from an upstream effector molecule with a great effect on Rho activation, such as RGMA. Nonetheless, the continuum of biological functions displayed by *RGMA* on neurons and the neuronal system is still to be defined with the hope of a better characterization of the inflammatory mechanisms where it is involved. The cellular and organ-specific site of action

of RGMA in addition to its partaking in CNS injury models and contribution in inflammatory pathways suggest a potential function of similar CNS diseases and inflammation in general and a potential role in MS in particular.

7.4.3 Interleukin 21 receptor (*IL21R*)

Interleukin 21 receptor (IL21R) is a relatively newly discovered member of the type I cytokine superfamily of receptors. It was identified based on homology to other receptors of this family after sequence alignment with the closest homology to IL-2Rβ (Ozaki 2000). IL-21R shares a common γ-chain together with IL-2R, IL-4R, IL-7R, IL-9R and IL-15R, as a part of their receptor complex (Habib 2003).

IL21R is expressed by resting and activated B- cells, resting and activated T-cells of both $CD4^+$ and $CD8^+$ type, resting natural killer (NK) cells and NK-cells activated with IL-15 (Kasaian 2002) (Parrish-Novak 2000) (Parrish-Novak 2002). The site of expression of IL21R suggests therefore a role of its ligand, IL-21, in lymphoid organs.

The IL-21R complex comprises the common cytokine receptor γ-chain in addition to IL21R chain that is specific for IL-21 binding. Although IL-21 shows a significant structural homology to IL-2, IL-4 and IL-15 that share the common γ-chain (γc) in addition to the β-chain from the IL-2R (IL-2Rβ), IL-21R seems to bind exclusively to IL-21 (Asao 2001) (Habib 2002) (Habib et al. 2003). The γc shows an indispensable role in IL-21R that, upon binding to IL-21, forms a complex that leads to activation of Janus kinase (JAK)1, JAK3, STAT1, and STAT3 (Asao et al. 2001) (Habib et al. 2003).

IL-21R and its have a modulating role on B-, T- and NKT-cells with various contribution in maintaining the immune system suggesting a regulation of both innate and adaptive immune responses (Habib et al. 2003). Knocking out IL-21R in mice does not seem to worsen these mice's life quality nor fertility nor the development of their lymphoid and myeloid organs (Kasaian et al. 2002). Although no impact of IL-21R was observed on the development or hematopoeisis of these mice, it shows a function in immune-modulation with knockout mice exhibiting an impaired production of serum IgG2b revealing a Th1 response, (Kasaian et al. 2002) IgG1 reflected by a Th2-like response and increased IgE responses (Habib et al. 2003). Although IL-21 on its own enhances the survival of NKT-cells in vitro and is efficient in sustaining survival of human NK cells (Skak 2008), it is unable to support both human and murine NK-cell growth and proliferation but rather requires the support of other cytokines (Coquet 2007) (Kasaian et al. 2002) (Parrish-Novak et al. 2000). Even though IL-21 inhibits the expansion of IL-15 activated NK-cells, it enhances the cytolytic activity and production of IFN-γ from NK-cells stimulated with IL-15.

Even if both $CD4^+$ and $CD8^+$ T cells express IL21R, IL-21 seems to have distinct effects on T cell activation. For instance, addition of IL-21 to $CD4^+$ cells upon differentiation inhibits IFN-γ production without affecting the production of other Th1 cytokines such as TNF and IL-2 (Wurster 2002). An opposite effect on IFN-γ production was noticed from $CD8^+$ T cells following the addition of IL-21, with an enhanced differentiation towards IFN-γ producing

cytolytic effector cells. The regulation of IFN-γ producing Th1 cells by the Th2 produced IL-21 has been assumed to serve as an amplifier for Th2 responses (Wurster et al. 2002). Thus, IL-21 and IL-21R appear to regulate cell-mediated immune regulation with effects on T-, B-, NK- and NKT- cells (Coquet et al. 2007) through a more complex effect than a simple explanation by a Th1/Th2 classification (Collins 2003). Accordingly, in paper IV of this thesis, we could show a correlation between variants of IL21R and higher production of IFN-γ in MS patients reflecting the above mentioned effects exerted by IL-21.

The immunological contribution of *IL21R* and its ligand together with influences on myeloid and lymphoid organs suggest the involvement of IL-21/IL-21R pathway in regulation of immunological diseases and autoimmune disorders in particular. Indeed, this enigmatic immunological couple has been associated to anti-tumour activity of various kinds of cancers such as colon carcinomas, follicular lymphoma and leukaemia (Ueda 2002) (Ugai 2003a) (Ugai 2003b) (Ueda 2005; Akamatsu 2007). In addition, a role has been shown in autoimmune disorders such as systemic sclerosis (Distler 2005), SLE (Mitoma 2005) (Herber 2007), T1D (Asano 2006) (Asano 2007), celiac disease (Garrote 2008) and psoriasis (Liu 2008b).

Since the shared QTL of *Eae31*/*Pia32* was proven to be linked to EAE and RA, perhaps the most relevant contribution of IL-21R and IL-21 would be a particular regulation in RA and EAE or MS. In fact, studies in RA regulation showed that IL-21R is expressed in synovial fluid of RA patients by synovial macrophages and fibroblasts (Jungel 2004) and a role in the animal model of RA (Young 2007). In addition, it associates to a positive production of TNF and IL-1β (Jungel et al. 2004) that were also linked to our QTL (Xu et al. 2002).

The impact of *IL21R* on EAE was illustrated in IL-21R$^{-/-}$ mice developing earlier EAE and more severe neurological impairment than control mice (Liu et al. 2008a). This EAE regulation is also associated to a down-regulation in the expression of Foxp3 and reduction of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) (Liu et al. 2008a) (Piao 2008). Upon expansion of Tregs, a clear recovery from EAE was noticed on the IL-21R$^{-/-}$ mice suggesting that IL-21R$^{-/-}$ mice are also defective in generating Th17 responses and a manipulative role of IL-21 on IL-17 and autoimmunity (Korn 2007). IL-21 can drive Th17 responses in conjunction with TGF-β whereas a T cells can be differentiated into Foxp3+ Tregs when IL-21 is combined with IL-6 (Coquet 2008) (Korn et al. 2007) (Nurieva 2007). In view of these findings, our rat data also showed that EAE susceptibility correlated with higher levels of LPS-induced IL-6 production, which could cause a stronger Th17 differentiation (Bettelli 2007), leading to more severe disease in both EAE and PIA.

8. Concluding remarks and future perspectives

Identification of genetic variants associated with complex diseases is the primary step to obtain knowledge and wider understanding of the mechanism behind these disorders. During the last decade, the increased development of bioinformatic tools and high-throughput methods was in the aim of facilitating the elucidation of genetic factors contributing to complex disorders. In spite of these improvements and the collaborative works of worldwide resources during the last decade, the complexity of AIDs has necessitated the complementing work in the animal models. Congenization techniques offer an invaluable tool for in vivo testing of QTL effects. The transition from the animal models to investigation of gene effects and associations to human diseases is more challenging.

In this thesis we used a combination of the AIL and congenization techniques to fine-map and narrow down QTL regions on four rat chromosomes, *Eae30-31*, *Eae20-22*, *Eae4* and *Eae19* on rat chromosomes 1, 4, 9 and 15 respectively. An AIL offers a possibility of narrowing down confidence intervals of QTLs combined with high precision in positioning linkage peaks and genomic locations with candidate genes. In addition, AIL offers the possibility of investigating epistasis, gene-gene interactions, which is not possible in congenic mapping. Epistatic effects have been previously described in congenic experiments where genotype at one allele was affecting the clinical outcome of disease depending on an allele at another locus (Morel 1999). AIL is the optimal tool for good mapping of an epistatic effect since F2 crosses are not considered to provide enough high resolution. The break of influence from QTLs outside the selected fragment in congenic strains also makes them less powerful in epistatic analysis. Based on data from AIL, congenic strains are created to confirm the exact relevance of QTLs and genes underlying them. In our case, an advantage we gained from interval-specific recombinant was the possibility of combining the clinical phenotype with *in vitro* approaches to further narrow down a QTL region on chromosome 9, *Eae4*, to less than 1cM.

We reached similar effects on narrowing down QTLs in the case of *Eae30 and Eae31* by applying the strategy of shared genetic factors between autoimmune diseases. This strategy will most certainly be employed more frequently in the near future as research will move from a disease-specific to a more inter-disciplinary format with focus on understanding mechanistic regulation rather than only mapping genetic variants of diseases.

With a lot of certainty, more QTLs and susceptibility genes for complex disorders such as MS will be discovered in animal models following further positional cloning of already discovered genes.

The candidate gene approach we employed for association studies based on the results found in animal models, will hopefully unravel one or several mechanisms behind MS, and open new doors for tailor-designed therapies. A still unclear fact is whether a found association is relevant only for the tested population and not any other. For this, the next step would be to additionally confirm the positive association findings in additional cohorts and/or more samples to test if the discovered susceptibility allele is actually both true positive and relevant for other populations.

Furthermore, testing for functional interpretations of the disease-associated alleles is highly interesting. For *IL21R*, much has already been done in understanding the immunological regulation of this gene. The advances in understanding *Vav1* is still lagging behind with much more remaining to be unravelled for *RGMA*. Understanding how the associated variants affect immunological pathways and understanding their effect on disease regulation is invaluable for developing new diagnostic tools and therapeutic agents.

Along with the discoveries of disease causing variants, characterization of the contribution of environmental factors and ethnicity on these alleles becomes more and more crucial. It is though not doubtful that identification of genetic causative variants for complex diseases and their downstream consequences will remain a perplexing task and challenging mission during the next decade.

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