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GENETIC FACTORS PREDISPOSING TO MYASTHENIA GRAVIS AND RELATED AUTOIMMUNE DISORDERS

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Stockholm 2011

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ISBN 978-91-7457-200-1

ABSTRACT

Myasthenia gravis (MG) is an autoimmune disorder in which patients experience weakness of the skeletal or ocular-bulbar muscles. In most patients, anti-AChR antibodies affect the signal across the neuromuscular junction necessary for muscular contraction. It is a complex disorder, wherein multiple genes plus environmental factors are likely necessary for disease manifestation.

IgA deficiency (IgAD) is defined as serum IgA levels less than 0.07 g/l with normal levels of IgG and IgM. Due to the role of IgA in mucosal barriers, some patients experience recurrent infections, although many individuals are asymptomatic. Both MG and IgAD patients experience concomitant autoimmunity more often than in the background population, and IgAD may caused by autoimmune mechanisms.

Investigations into genetic associations with MG have targeted genes implicated previously in autoimmune disorders. The *PTPN22* rs2476601 polymorphism was genotyped in 409 MG patients and 1557 controls, and was associated with the entire cohort (p=2.7x10^{-4}, OR=1.52), as well as with hyperplasia (p=1.4x10^{-4}, OR=1.87), anti-AChR positivity (p=4.9x10^{-4}, OR=1.52), and patients exhibiting both (p=6.6x10^{-5}, OR=1.96). Furthermore, after antigen activation, a significant increase in IL-2 producing cells (p=0.002) and IgG secreting cells (p=0.004) in patients carrying the T allele was observed, indicating that the allele may be a gain-of-function variant in MG.

Genotyping of 446 MG patients and 1866 controls for the *CIITA* rs3087456 was conducted without a statistically significant difference observed between MG patients and controls for either allele frequencies (p=0.092) or genotypes (p=0.251). The control material differed significantly from the original study reporting association of the SNP with several disorders (0.266 and 0.216, respectively, $p<10^{-5}$). A lack of association in replication cohorts (4 of 26 reported association) may indicate that the SNP does not predispose to autoimmunity. The *CD45* SNP rs17612648 was genotyped in 446 MG patients and 2303 matched controls, with no association with MG (p=0.199). A lack of replication in subsequent studies (4 of 24 reported association), as well as the identification of a homozygous blood donor without obvious disease, indicates that this SNP's role in autoimmunity may have to be reconsidered.

The HLA A1, B8, DR3, DQ2 (8.1) haplotype has been associated with several autoimmune disorders, and homozygosity for this haplotype has been reported to impart high risk for IgAD development (RR=77.8). Using 117 identified homozygous individuals, 2 were found to be IgAD (1.7%), indicating that the RR for IgAD for such carriers is estimated to be 11.89. Despite overlapping 8.1 association in IgAD and MG, IgAD is not elevated in MG patients (p=0.14), although concomitant autoimmunity is higher than expected in MG (15.9%). The overlapping effects of the haplotype may be due to an independent association of B8 in MG. Examining overlapping genes in autoimmunity has thus proved to be a valuable method of identifying patterns of predisposition.

Using the summary associations to determine complex disease predisposition has proven to be difficult. A method was created to use complex disease MZ and DZ twins concordance to estimate the frequency of the predisposition (1:5240 in MG) and number of overlapping genetic regions contributing to disease (2-4 in MG). This information has made it possible to formulate a model of complex disease which is flexible to the number of underlying genetic subgroups of disease, and future genetic information can be used to predict the probability of disease development.

LIST OF PUBLICATIONS

I. Lefvert AK, Zhao Y, **Ramanujam R**, Yu S, Pirskanen R and Hammarström L. PTPN22 R620W promotes production of anti-AChR autoantibodies and IL-2 in myasthenia gravis. *J Neuroimmunol*. 2008 Jul; 197(2); 110-3.

II. **Ramanujam R**, Zhao Y, Pirskanen R and Hammarström L. Lack of association of the CIITA -168A/G promoter SNP with myasthenia gravis and its role in autoimmunity. *BMC Med Genet*. 2010 Oct 13;11:147.

III. **Ramanujam R**, Pirskanen R and Hammarström L. The CD45 77C/G allele is not associated with myasthenia gravis – a reassessment of the potential role of CD45 in autoimmunity. *BMC Research Notes*. 2010 Nov 10;3(1):292.

IV. Mohammadi J, **Ramanujam R**, Jarefors S, Rezaei N, Aghamohammadi A, Gregersen PK and Hammarström L. IgA deficiency and the MHC: assessment of relative risk and microheterogeneity within the HLA A1 B8, DR3 (8.1) haplotype. *J Clin Immunol*. 2010 Jan;30(1):138-43.

V. **Ramanujam R**, Piehl F, Pirskanen R, Gregersen PK and Hammarström L. Concomitant autoimmunity in myasthenia gravis – lack of association with IgA deficiency. (Manuscript)

VI. **Ramanujam R**, Pirskanen R, Ramanujam S and Hammarström L. Utilizing twins concordance rates to infer the predisposition to myasthenia gravis. *(Twin Research and Human Genetics*, Accepted)

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LIST OF ABBREVIATIONS

AAD	autoimmune Addison's disease
abs	antibodies
AChR	acetylcholine receptor
AIH	autoimmune hepatitis
BLS	bare lymphocyte syndrome
CD	celiac disease
CD45	cluster of differentiation 45
CHRNA1	cholinergic receptor, nicotinic, alpha 1
CIITA	class II, major histocompatibility complex, transactivator
CNS	central nervous system
CTLA-4	cytotoxic T-lymphocyte antigen 4
CVID	common variable immunodeficiency
DLE	discoid lupus erythematosus
DNA	deoxyribonucleic acid
DZ	dizygotic
EMG	electromyography
EOMG	early onset myasthenia gravis
GWAS	genome wide association study
HLA	human leukocyte antigen
IBS	irritable bowel syndrome
Ig	immunoglobulin
IgAD	immunoglobulin A deficiency
IL	interleukin
JIA	juvenile idiopathic arthritis
LD	linkage disequilibrium
LOMG	late onset myasthenia gravis
MAF	minor allele frequency
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MG	myasthenia gravis
MHC	major histocompatibility complex
MHC2TA	MHC class II transactivator
MI	myocardial infarction
MS	multiple sclerosis
MZ	monozygotic
NMJ	neuromuscular junction
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCR-SSP	polymerase chain reaction – sequence specific primers
PTPN22	protein tyrosine phosphatase, non-receptor type 22
PTPRC	protein tyrosine phosphatase, receptor type C
RA	rheumatoid arthritis
RR	relative risk
SF-EMG	single fiber electromyography
SLE	systemic lupus erythematosus

SNP	single nucleotide polymorphism
SSc	systemic sclerosis
T1D	type 1 diabetes
TNF-α	tumor necrosis factor-alpha
WG	Wegener's granulomatosis

1 INTRODUCTION

1.1 MYASTHENIA GRAVIS

Myasthenia gravis is an autoimmune disorder in which the presence of autoantibodies leads to muscular weakness [1]. It was first described by Thomas Willis 1672 in a patient exhibiting dysarthia. It was hypothesized to be autoimmune in nature by Simpson in 1960 [2], who observed a higher prevalence in females, concomitant autoimmunity, and transient neonatal transfer of symptoms. Patrick and Lindstrom demonstrated the role of anti-AChR abs in 1973 by observing experimental autoimmune MG in rabbits immunized against purified AChRs [3].

The main symptom of MG is weakness of either skeletal or ocular/bulbar muscles, resulting in general weakness, double vision, drooping eyelids, and may result in breathing difficulties. Approximately 20% of patients experience a myasthenic crisis, defined as the need for mechanical ventilation due to affected breathing muscles, within two years of diagnosis [4].

MG occurs in 14.1 per 100,000 individuals in Sweden [5], and affects females at approximately a 2:1 ratio to males. Clinical classifications of MG symptoms by Oosterhuis [6] have been reviewed by Jaretzki [7], and are presented in Table 1.

Myasthenia gravis classification scale	
Class I	Ocular
Class II	Mild generalized
IIa	Predominantly skeletal muscle symptoms
IIb	Predominantly bulbar symptoms
Class III	Moderate generalized
IIIa	Predominantly skeletal muscle symptoms
IIIb	Predominantly bulbar symptoms
Class IV	Severe generalized
IVa	Predominantly skeletal muscle symptoms
IVb	Predominantly bulbar symptoms

Table 1. Osserman-Oosterhuis classification of MG modified by Jaretzki

Patients may have progressive disease or reduction of symptoms with treatment. Remission of symptoms is often observed with treatment; however, relapses exist and most patients experience fluxuating symptoms throughout their lives.

1.1.1 Disease pathogenesis

The pathophysiology of myasthenia gravis is located at the neuromuscular junction (NMJ), in which nerve impulses are delivered from the CNS to muscle fibers. The transmission of the impulses are achieved through secretion of acetylcholine from nerve endings, which travel across the junction and bind to receptions situated on the muscle fibers. This creates the action potential for muscle contraction. Acetylcholine is then rapidly destroyed by acetylcholinesterase, resetting the NMJ for future muscle contraction [8].

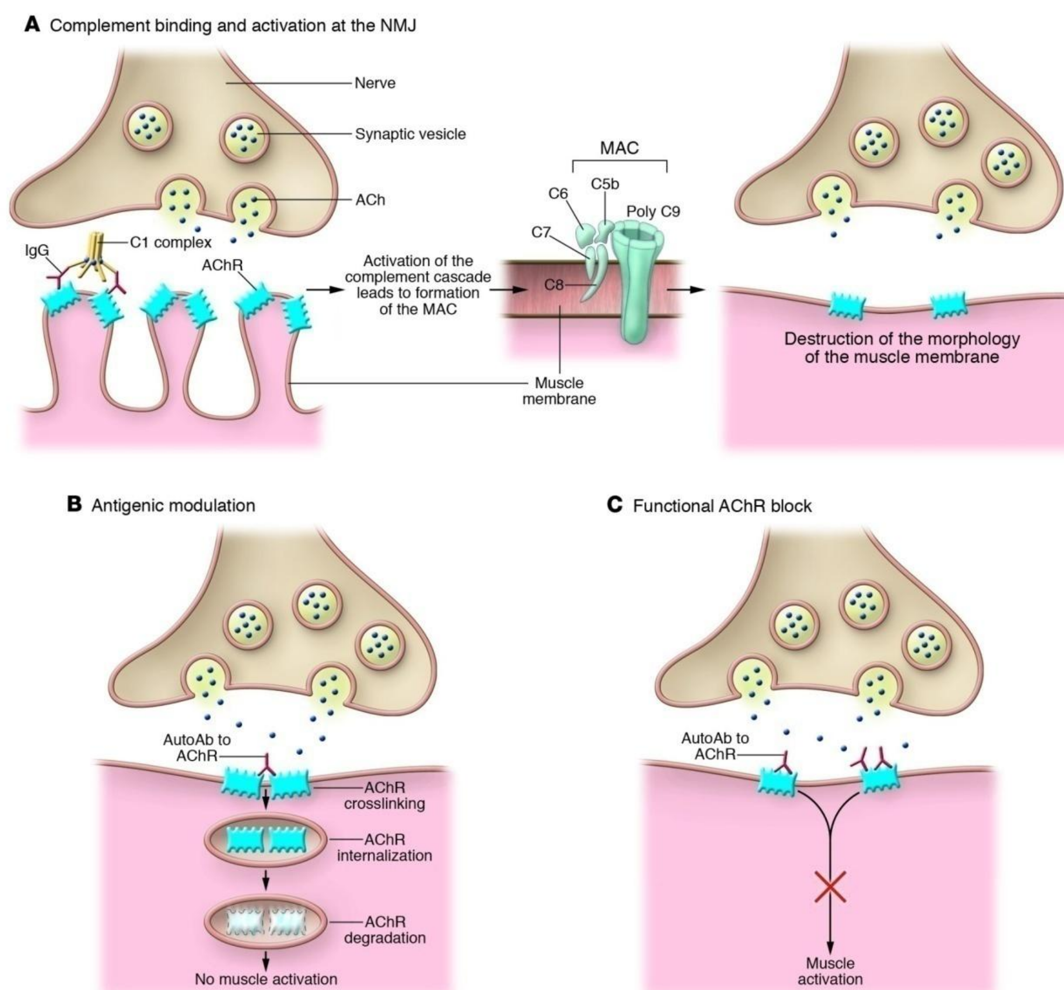

Figure 1. The pathophysiology of anti-AChR abs in MG at the neuromuscular junction. Reprinted with permission from [9].

The presence of auto-antibodies against the AChR (anti-AChR abs) may reduce this activity by several mechanisms (Figure 1). First, they can cause complement dependent lysis of the postsynaptic muscle membrane, leading to a flattening of the postsynaptic folds and a reduction in AChR concentration [10]. Second, they cross-link AChRs on the membrane surface, which can lead to internalization and degradation, reducing the number of AChRs [11]. Last, they can directly block the binding of acetylcholine to the receptor [12].

Anti-AChR abs are detected via radioassay [13]. They occur in approximately 80-90% of all MG patients, and antibody titers do not correlate to the severity of MG, although there is a higher proportion of antibody positivity in more severe forms of the disease [13][14]. There is evidence that patients who do not test positive for anti-AChR abs may have low affinity antibodies present [15]. Other antibodies found in anti-AChR negative patients include anti-MuSK abs, found in approximately 50% of anti-AChR abs negative patients, which target another membrane bound receptor in the NMJ [16]. However, patients of Swedish origin who are anti-MuSK positive are extremely rare [R. Matell]. Antibodies against the striated muscle protein titin are found commonly in MG patients with thymoma, and late onset MG patients without thymoma [17].

1.1.2 Role of the thymus

The thymus functions as an organ for shaping T cell repertoires, including negative selection of autoreactive thymocytes. Thymic abnormalities frequently occur in MG patients, with about 75% of patients having hyperplasia, 10-15% with thymoma, and 10% with normal histology [18]. Thymic hyperplasia is observed more commonly in younger female patients [5], whereas thymoma occurs more frequently in older patients and often represents more severe disease [19].

Thymectomy, surgical removal of the thymus, is frequently performed on patients with generalized MG or severe symptoms. Thymic histopathology is examined post-operatively. Thymectomy improves patient condition through unknown mechanisms [17], and reduces the frequency and severity of myasthenic crisis [20].

1.1.3 Diagnosis

The diagnosis of myasthenia gravis is made after the presence of muscular weakness of the skeletal muscle or ocular-bulbar region and confirming criterion. Repetitive supramaximal nerve stimulation at 3 Hz resulting in an increased decrement of the muscle action potential evoked is often observed in myasthenic individuals [21]. A

more sensitive test is a single fiber EMG (SF-EMG) investigation developed by Stålberg and Ekstedt [22]. An edrophonium test, wherein a short acting intravenous anticholinesterase results in a rapid but brief improvement in muscle strength in MG patients, is indicative of the disease [23]. Measurement of anti-AChR abs confirms the diagnosis. Cases with decremental response to repetitive nerve stimulation or abnormal SF-EMG without anti-AChR or anti-MuSK abs are classified as seronegative MG.

1.1.4 Treatment

Treatment for MG usually begins with oral cholinesterase inhibitors, such as pyridostigmine bromide, which prolongs the activity of acetylcholine. Thymectomy is performed most commonly in early onset MG patients with generalized symptoms who are anti-AChR positive [17]. Immunosuppressive therapy is indicated in severe or progressive cases. Corticosteroids are commonly used, although often side effects are evident. Azathioprine may be used alone or with prednisone, and requires long term usage to achieve maximum effect [4]. Also, cyclosporine A, mycophenolate mofetil, tacrolimus, rituximab and other modern immunomodulating drugs have been prescribed. Plasma exchange and intravenous human immunoglobulins (IVIg) are more expensive treatments producing faster benefits, and are used in severe or life threatening cases of MG, or prior to surgery to increase patient strength [4].

1.1.5 Subgrouping of patients

Bimodal peaks of MG onset are observed, with the first being younger patients who are anti-AChR positive with thymic hyperplasia [24]. Female patients are most prevalent in this group (3:1). The second peak, in patients with later onset disease, frequently with thymoma, has a slightly higher ratio of male patients (1.5:1) [8]. The age of onset of patients has been increasing in the last two decades [25]. Division of patients using age of onset into early onset MG (EOMG) and late onset MG (LOMG) can be used. There is a lack of consensus on the age which most accurately divides these groups, with some publications using 50 years of age [26][27] and others using 40 years of age [28].

Clinical classification can also be made solely based on disease class (ocular, generalized/severe) or thymic histopathology (normal, hyperplasia, thymoma). The presence of anti-AChR abs, and HLA alleles, are also used to create subgroups to measure association.

1.1.6 Genetic associations

HLA associations are common in MG, with the HLA-B*08 (B8) and DRB1*03 (DR3) being associated as well as the HLA A1, B8, DR3, DQ2 “8.1” haplotype [29-32]. The

aforementioned EOMG group and thymic hyperplasia are most strongly associated with these alleles/haplotype, and LOMG is associated with HLA-B*07, DRB1*02 (B7, DR2) [33][34]. Other genetic associations to MG include IL-1 [35][36], CTLA-4 [37][38], IL-10 [39][40], TNF-α [41], the acetylcholine receptor subunit alpha (CHRNA1) [42][43] and PTPN22 [44].

1.2 SELECTIVE IGA DEFICIENCY

Selective IgAD is defined by serum IgA levels lower than 0.07 g/l with normal levels of IgG and IgM. It is the most common primary immunodeficiency in Caucasian populations, with a frequency of 1:600. A large scale screening of blood donors for IgA in Sweden occurring over many years [45] has found 47 IgAD of 28,413 samples measured, indicating a background rate in Sweden of 1:604 individuals.

IgA comprises the largest proportion of immunoglobulins, and is secreted in mucosal linings. Involved in innate immunity, IgA prevents microbes entering the body from multiplying, particularly in the respiratory and gastrointestinal tracts [46]. Many IgA deficient individuals may be asymptomatic, while others may suffer from recurrent infections, allergies or autoimmunity [47]. Normal levels of IgA are approximately 2-3 g/l in serum [46].

An increase in the HLA-B*08, DRB1*03, DQB1*02 (B8, DR3, DQ2) haplotype has been reported in IgAD patients [48][49], as well as increases in DR7, DQ2 and DR1, DQ5 [50]. It has further been previously suggested that the prevalence of IgAD in HLA B8, DR3, DQ2 homozygous individuals is approximately 13% (RR=77.8) [51-53]. The DR15, DQ6 haplotype confers strong protection against the development of the disease [50][54][55].

IgAD occurs concomitantly with autoimmunity, such as RA [56][57], SLE [58] and juvenile diabetes mellitus [59], possibly due to overlapping HLA associations. It has also been suggested that IgAD may be caused by autoimmune mechanisms [60].

1.3 MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocomptability complex (MHC) is a 3.6 Mb, gene rich region of chromosome 6 which plays a crucial role in the recognition of self and non-self. In humans termed human leukocyte antigen (HLA), class I and class II MHC genes encode antigen presenting proteins expressed on cells of the immune system [61]. HLA-A, B and C belong to Class I, and HLA-DPA1, DPB1, DQA1, DQB1, DRA1 and DRB1 belong to Class II. Class I molecules are expressed on most somatic cells,

whereas Class II molecules are expressed on antigen presenting cells. The highly polymorphic nature of the MHC and the number of HLA allele combinations makes each individual immunologically unique (with the exception of MZ twins) [62].

In response to the existence of a pathogen, antigen presenting cells load digested peptides onto Class II molecules after phagocytosis for display to T-cells. When a T-cell recognizes a peptide, B-cells are stimulated to produce antibodies against the pathogen. Since the antigen recognizing capabilities of T-cells are immense, autoreactive T-cells may arise, which are normally negatively selected; however, in autoimmunity, thymocytes which strongly bind to self antigens escape this process [63].

HLA alleles have been associated to virtually all forms of autoimmunity. In particular, the HLA B8, DR3, DQ2 haplotype, both with and without A1 (8.1 haplotype), has been associated with MG [64], IgAD [48][49], Graves' disease [65], CD [66], and SLE [67].

1.4 COMPLEX DISEASE

A disease is general termed complex if its development requires both multiple genetic factors as well as environmental interaction [68]. The genetic factors are typically not monogenic in nature; that is, there are usually many genes which act together to predispose the individual to a disorder. For this reason, genes are rarely discovered with large ORs, whereas in monogenetic disorders, such as cystic fibrosis, a single gene mutation is frequently responsible for causing the disorder [69].

The environmental component(s) necessary to develop the disease are often unknown, although risk factors related to the environment exist, such as smoking in RA [70]. Exposure to the requisite environmental factor(s) necessary to develop the disease has been termed the environmental trigger, which may be a single event or an aggregate effect. Often this underlying genetic predisposition causes no disease manifestation for many years, until either a threshold of environmental exposure or a triggering event occurs, after which the disease manifests.

Often it is unclear whether a complex disease is a single disorder or a collection of different disorders with overlapping symptoms. Rheumatoid arthritis (RA), for example, is often referred to as a "syndrome of diseases" due to both the various forms of the disorder, and the variety of genetic associations that have been found to contribute to the disease [71].

Despite advances in genotyping and sequencing technologies, with increases in throughput and decreases in costs, new methods to determine the manner in which genetics predispose to complex disease have largely not been undertaken.

1.4.1 Threshold model

The threshold model of complex disease attempts to explain the interaction between genetic and environmental effects (Figure 2) [72]. Individuals are assumed to fit a normal distribution, which shifts toward a threshold based on the number of susceptibility genes present in the individual. This threshold, which represents disease development, is itself influenced by environmental factors. An increase in genetic factors predisposing to the disease increases the individual's chances of developing the disease accordingly, whereas exposure to environmental factors reduces the threshold [72]. This assumes an additive effect of susceptibility genes, wherein each gene has a fixed effect and do not interact [73]. A purely additive model assumes that the risk is constrained by contributions from risk alleles and environmental elements, and not probabilistic [74].

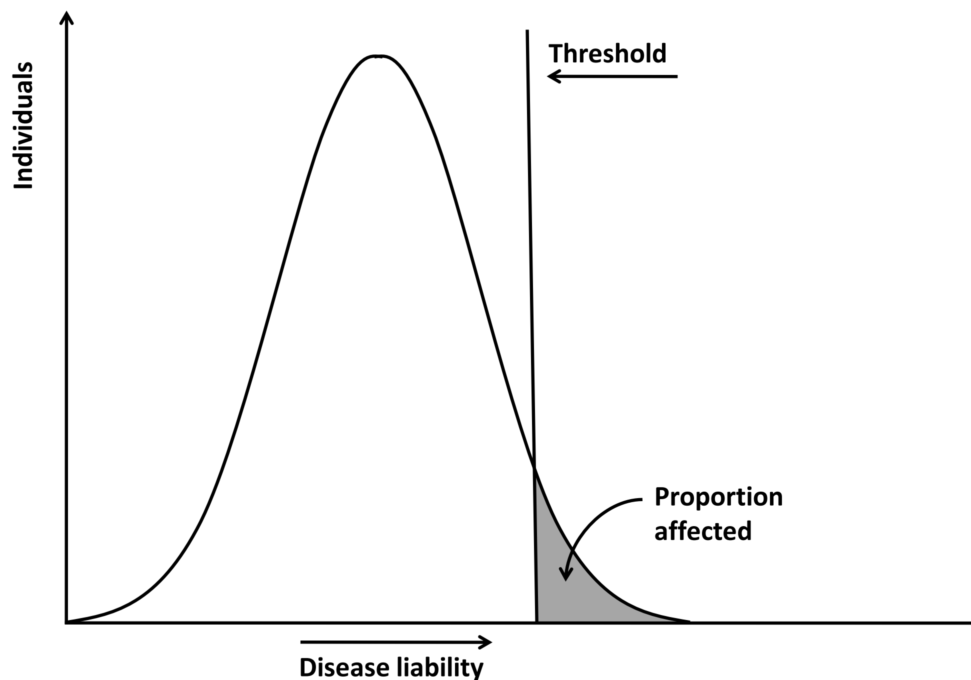

Figure 2. The threshold model of complex disease

This model has the weakness of assuming that the genetic contribution to disease development is additive; that is, the presence of risk alleles shift the distribution either

toward or away from the threshold. Furthermore, the risk of developing disease is stochastic, and all individuals with some risk allele(s) may have a small probability of disease development. Both of these conditions may be false if predisposition requires a precise combination of genetic factors.

Multiplicative models attempt to correct for this lack of gene-gene interaction, and thereby account for epistasis present in measured genes/loci [73]. However, these models have failed experimental validation [75], and often explain only a small proportion of overall genetic predisposition to a disorder.

1.4.2 Rothman pie diagram

A model which alleviates concerns with the threshold model was created by Rothman, which he referred to as a pie diagram of sufficient disease causes (Figure 3) [76]. This model presumes that disease which appears in an individual is the result of several sufficient causes, where each cause is denoted by a letter. An individual who accumulates the correct number/combination of sufficient causes will develop disease, and one who does not will not develop disease.

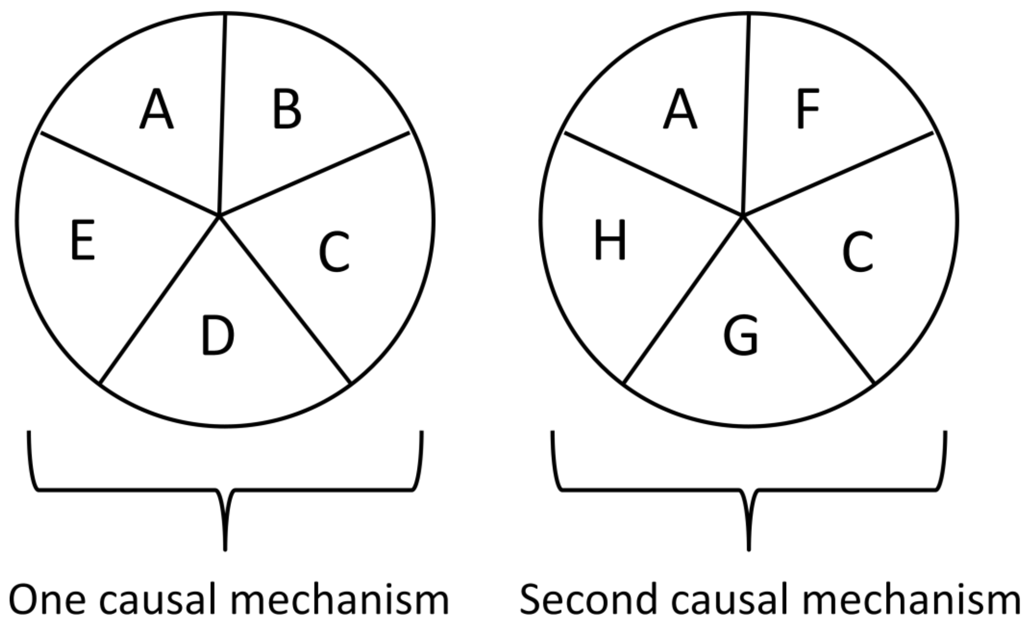

Figure 3. Rothman pie diagram of sufficient causes of disease

The pies are therefore different for various underlying forms of disease, and each complex disease may be caused by many different pies. Furthermore, these causes may be genetic in nature, or may contain external factors, such as the environment.

1.5 TWINS DATA

Twins data has mainly been used in the context of classical twins studies (CTD), in which the pairwise concordance is given by C/(C+D), where C indicates a concordant twin pair and D indicates a discordant twin pair [77].

The twin concordance rate in monzygotic (MZ) twins is defined as an additive genetic effect (V_A) plus an environmental effect (V_C). The dizygotic twin (DZ) concordance rate is given as $\frac{1}{2}V_A+V_C$, due half of all genes shared, and solving for V_A yields a genetic component equal to twice the difference in concordance between MZ and DZ twins [2 * (MZ-DZ)]. This is often used as a proxy for the maximum genetic component expected to be observed in case-control studies [78], and is also termed "heritability". This, however, does not yield any information about the underlying predisposition, nor is it informative if the difference between MZ and DZ concordance is greater than 50%.

1.6 CONCOMITANT AUTOIMMUNITY

Autoimmune disorders are caused by the production of auto-antibodies directed against various structures of the body. The severity and symptoms of these diseases are diverse, and frequent diagnosis of concomitant autoimmune disorders suggests interplay between their various causes. Although the means by which the diseases manifest themselves are largely unknown, many key genes conferring predisposition to various forms of autoimmunity have recently been discovered. Due to the overlap of some genetic factors predisposing to autoimmune disorders, it is common to test a gene or SNP associated with one disorder to other disorders, as this provides an immediate target for measuring if a similar effect exists.

Many autoimmune disorders have high prevalence of concomitant autoimmunity, possibly due to similar genetic factors resulting in common mechanisms. In MG, SLE has been known to occur in patients more than expected by chance [79-81], and the appearance of SLE in MG patients who have undergone thymectomy has been reported [82][83].

Other autoimmune disorders reported at a greater rate in MG patients include thyroiditis/Graves' disease [84-89], and RA [86].

1.7 PTPN22

The PTPN22 (Protein tyrosine phosphatase, non-receptor type 22) gene is located on chromosome 1p13.2, and contains a functional SNP in exon 14 (1858C>T, R620W, rs2476601) which replaces an arginine with a tryptophan. This polymorphism has been associated with several forms of autoimmunity, such as T1D [90][91], RA [92][93][94], SLE [95][96] and Graves' disease [97]. The polymorphism was also reported to be overrepresented in a subgroup of MG (patients without thymoma or anti-titin abs) [44]. However, studies on several other autoimmune diseases, such as MS [98][99], Crohn's disease [98], and primary Sjögren's syndrome [100], reported no association with the SNP.

PTPN22 consists of an N-terminal phosphatase domain and C-terminus with several proline-rich motifs [101]. The missense mutation rs2476601 alters the proteins interaction with the negative regulatory kinase Csk, thereby altering the T-cell response [102]. A recent study which reported association of this mutation with T1D suggested that higher catalytic activity of the phosphatase (gain-of-function) resulted in more efficient inhibition of TCR mediated activation of T lymphocytes, and therefore reduced IL-2 production [103].

The large number of autoimmune disorders association with the rs2476601 SNP makes it a candidate autoimmune gene which might underlie common disease mechanisms. This may be in part due to its role as a functional variant without largely deleterious consequences.

1.8 CIITA

The MHC class II transactivator (MHC2TA, CIITA), located on chromosome 16p13, contains four alternative promoters which exhibit cell-type specific activity [104]. The gene is a transactivator of MHC class II genes, expression of which is crucial for cell collaboration and induction of immune responses, whereas lack of expression is associated with the severe immunodeficiency disease bare lymphocyte syndrome (BLS) [105].

The type III promoter (-168A/G, rs3087456) was reported to be associated with rheumatoid arthritis (RA), multiple sclerosis (MS) and myocardial infarction (MI) [106]. This SNP was shown to reduce transcription levels of CIITA *ex vivo* in human leukocytes stimulated with interferon-γ. Further, the same study also demonstrated

lowered expression of MHC class II molecules in rats based on CIITA polymorphisms [106].

Due to the direct effect on MHC molecule expression, as well as association to well characterized autoimmune disorders (MS and RA), the polymorphism is interesting for association studies in autoimmunity, including replications studies in additional cohorts. Despite this, the role of the SNP in MG has not been established.

1.9 CD45

CD45, also called protein tyrosine phosphatase, receptor type C (PTPRC), is located on chromosome 1q31-32. The receptor belongs to the protein tyrosine phosphatase family, which has been shown to be involved in cell growth, differentiation and signaling [107]. Heavily expressed on T-cells, it has been shown to play a role in T-cell receptor signal transduction and activation as well as in thymic selection of T-cells, both important features in the development of autoimmunity. A lack of CD45 expression results in severe immunodeficiency [108][109].

The gene produces eight known isoforms via complex alternative splicing which is cell specific. One such isoform, containing exon 4 (CD45RA+), is expressed mainly by naïve T-cells, while an isoform with exons 4-6 spliced out (CD45RO+) is expressed by most memory T-cells [107].

A low frequency SNP, rs17612648 (77C/G), has been reported to disrupt an exonic splicing factor, thereby causing the failure of exon 4 to splice out. This leads to higher levels of CD45RA expression on memory T-cells of homozygous individuals [110]. It has been suggested that this may provide a mechanism to alter the T-cell activation threshold, providing a mechanism for the development of autoimmunity [111]. CD45 is therefore a potential common autoimmunity gene, although its role in MG has not been determined.

2 AIMS

2.1 SPECIFIC

The most specific aim of the thesis is to investigate genetic factors which may predispose individuals to autoimmune disorders, in particular MG, and IgAD. The overlap between the two disorders and the role of HLA associated genes is a direct goal.

2.2 GENERAL

A more broad aim of the thesis is to use the results in MG and IgAD, together with published information, to further understand complex disease predisposition. Using autoimmune disorders, most of which have complex etiology, a common basis for autoimmunity may be investigated.

Furthermore, these studies yield insight into the inherited basis of complex disease, and a model of complex disease can be created to aid in future research.

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS

3.1.1 MG

The myasthenia gravis patient cohort used in these studies included samples accumulated over many years. The diagnosis of MG was made by the neurologist, and blood and/or serum was collected during visits at the Neurology department. Patients were analyzed for anti-AChR abs routinely at the first patient visit, and occasionally during subsequent visits at the physician's discretion. A total of 547 MG patients were used in these studies. Healthy controls consisted primarily of self-reported healthy blood donors. All patients gave informed consent and use of patient and control materials was approved by the Karolinska Institutet and The Regional Ethical Review Board.

Clinical subgroups of MG were made according to the Oosterhuis-Jaretzki scale, thymic histopathology, age at onset of disease, etc. as reported by the primary physician.

3.1.2 IgAD

Five hundred thirty-three IgAD subjects from the immunodeficiency unit at the Karolinska University Hospital were included in the study for determination of HLA types. All patients were identified or referred by fulfilling the criterion for IgAD (IgA < 0.07 g/l with normal IgG and IgM).

3.1.3 8.1 haplotype

Three groups of Caucasian subjects (n=117), homozygous for the B8, DR2, DQ2 haplotype, were obtained from the following sources: 78 from the Swedish volunteer bone marrow registry (www.tobiasregistret.se), 30 from routine tissue typing of transplantation relative screening and 9 population based controls from the Rheumatology Unit, Department of Medicine at the Karolinska University Hospital. All subjects were chosen solely on the basis of the presence of the haplotype in homozygous form, and without any prior knowledge of IgA levels.

3.2 DNA EXTRACTION

Genomic DNA was extracted from whole blood using the phenol-chloroform or salting out method.

3.3 ELISPOT

Peripheral blood mononuclear cell (PBMC) isolation was performed as previously described [112]. ELISpot was conducted on 100 randomly selected MG patients without previously knowledge of genotype to enumerate the number of cells secreting IL-2, IFN-γ or IgG against the human AChR receptor [112].

3.4 GENOTYPING

Measurements of genotypes were performed at the Mutation Analysis Facility at the Karolinska Institutet, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [113] (SEQUENOM Inc., San Diego, California, USA). Primers specific to the genotypes were synthesized, and genotyping utilized IPLEX chemistry.

Confirmation of genotypes, when utilized, were performed by direct "Sanger" sequencing at Macrogen, South Korea. For rs17612648, the primers used were CTGGGAGGAGCATACATTTAGG and AGCACTAGCATTATCCAAAGAG.

3.4.1 IMAGEN genotyping

As part of the IMAGEN project, genotyping was conducted on 1535 SNPs for 453 MG samples, 270 IgAD samples, and 673 healthy controls using a custom array [114]. After quality control, this panel contained 1288 SNPs, of which 1230 were in the extended MHC region.

3.4.2 Illumina genome wide genotyping

Genome-wide scans for both patient cohorts and controls were conducted as part of a separate study. Five MG patients and 6 controls homozygous for the HLA B8, DR3, DQ2 haplotype were typed as part of a larger material using the Illumina HumanHap300 (317K) genotyping BeadChip. A further 18 IgAD patients and 9 controls homozygous for the same haplotype were genotyped using this platform. An additional 8 IgAD patients and 15 healthy controls, also homozygous for the HLA B8, DR3, DQ2 haplotype were genotyped using the Illumina Human610-Quad (620K) genotyping BeadChip array.

Quality control was conducted using PLINK [115] to remove SNPs which were above a given missingness in cases or controls (>10%), not in Hardy-Weinberg equilibrium ($p<0.001$) or below a minor allele frequency threshold (<1%). As a last control, the number of missing samples for each of cases and controls were compared to those

successfully typed (for the entire cohorts), and a *p*-value of missingness between cases and controls of less than 0.001 were discarded.

3.4.3 SNP mapping

For IgAD and control comparisons, two separate SNP maps were constructed using genotyping data. The first map used 1,116 SNPs across the MHC from upstream of HLA-A to downstream of HLA-DP. These SNPs were extracted from an Illumina custom array panel described previously [114]. An additional 896 SNPs across the same region from the GWAS data were included. Merging the data sets and removing duplicate SNPs left a total of 1,718 unique SNPs across the MHC. Swedish IgAD patients, homozygous for the HLA B8, DR3, DQ2 haplotype (n=18), were compared to population based controls (n=9) with normal IgA levels who were homozygous for this haplotype.

The second IgAD SNP map consisted of a total of 3,874 SNPs across the same region using the higher density array. This SNP map included additional HLA B8, DR3, DQ2 homozygous Swedish IgAD patients (n=8) and IgA sufficient donors from the Swedish volunteer bone-marrow registry (n=15).

For MG, a single SNP map consisting of 5 patients, 6 healthy controls and 10 IgAD individuals was created, using 1,719 SNPs, including 897 SNPs from merged GWAS data and 1,116 SNPs from the custom panel, with duplicate entries removed.

The data were extracted in PED/MAP format and merged using custom PERL scripts (www.perl.org). Conversion to genotype format (0,1,2) was conducted with EIGENSTRAT [116], and the data were color coded for visualization with MS Excel.

3.5 HLA TYPING

Anonymized control data for HLA alleles was obtained from the Swedish volunteer bone marrow registry (www.tobiasregistret.se). The data consists of 40,789 participants, HLA typed for HLA-B (n=40,789), HLA-DR (n=23,609) and HLA-DQ (n=1193). HLA-A data has not been collected in this database; therefore, the frequency of HLA-A alleles in Sweden from a previous publication using 252 unrelated Swedish individuals was used [117].

3.5.1 Imputation

A method of HLA imputation based on 3 SNP tagging was used to infer the HLA type of samples. As part of the IMAGEN project [118], most MG samples were typed at

1288 SNPs, 1230 of which were tag SNPs across the MHC [118]. Using this information, imputations of HLA-A, B, DR and DQ were obtained using a previously validated method shown to have a 95% accuracy in imputing 4 digit types in European populations [114][119], of which 2 digit types were used.

HLA types shown to have mismatches from previously typed samples via PCR/serology were repeated with PCR-SSP. These included HLA-A*3101, B*3501, B*4002, DRB1*401, DRB1*403 DRB1*701, and DRB1*1401.

3.5.2 PCR-SSP

PCR by sequence specific primers (SSP) is a highly accurate method to determine HLA types [120]. This technique utilizes standard PCR with pre-formulated plates containing primer pairs specific to certain HLA alleles. Different numbers of wells/primer pairs are required for the determination of HLA types. In these investigation, 2 digit types were obtained for HLA-A (24 wells), HLA-B (48 wells), HLA-DR (24 wells), HLA-DQ (8 wells) and HLA-DR-DQ (32 wells).

The PCR was conducted according to the manufacturer's specification (Olerup SSP AB, Saltsjöbaden, Sweden). The PCR products were then run in 2% agarose gel stained with ethidium bromide for 10 minutes at 150 mA, 300 V, visualized by a UV illuminator and photographed. Each well has a positive control and may contain amplified PCR products, and HLA-A, B, DR, and DR-DQ have a negative control. Determination of the HLA type was made by matching wells positive for PCR products to interpretation tables.

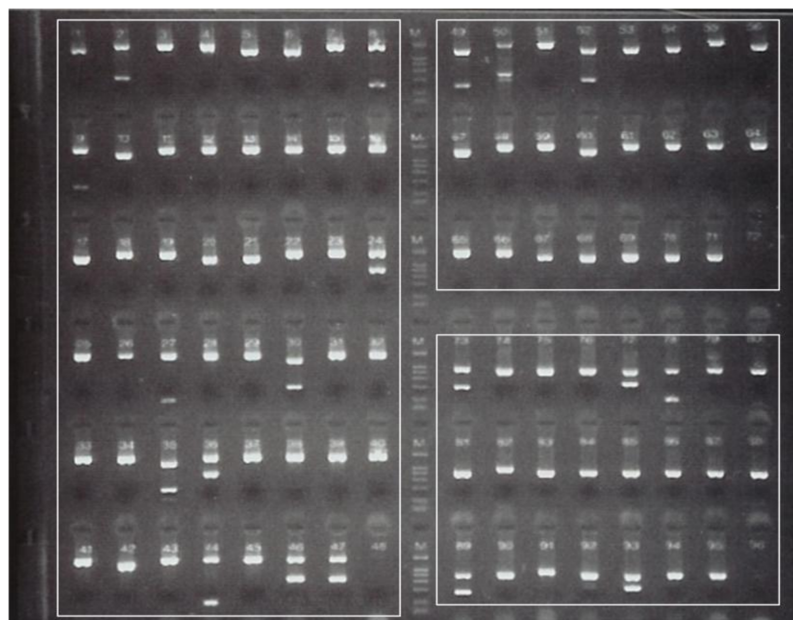

Figure 4. A typical SSP-PCR gel. The left half of the gel contains products from the wells (ie. primer pairs) for HLA-B low (48 wells), and is positive for bands in wells 2, 8, 9, 24, 27, 30, 35, 36, 44, 46 and 47. The upper right quadrant contains HLA-A low, positive for bands in wells 1, 2, and 4. The lower right quadrant is HLA-DR low, and has positive bands in wells 1, 5, 6, 17, and 21). The corresponding HLA type is A1,2 B8,51 DR1,3

The kits used in these studies included the HLA-A low resolution (26F, 62G), the HLA-B low resolution (L21, M26, J82, N80, R56, X13, X82, 90F, 63G), the HLA-DR low resolution (40E, 83F), the HLA-DQ low resolution (41E, 91F) and the HLA- DQ-DR SSP Combi Tray (K88, R60, V95, M01, M84).

3.6 SERUM IMMUNOGLOBULIN LEVELS

IgA, IgG and IgM serum levels were determined by nephelometry at the Karolinska University Hospital Clinical Chemistry Laboratory. Samples were considered to be IgAD if serum IgA was under 0.07 g/l with normal levels of IgG and IgM.

3.7 STATISTICAL ANALYSES

Association for case-control studies was determined using the χ^2 test, using one degree of freedom for allele counts and two degrees of freedom for genotype ratios. When the expected value for some data was low (n<5), Yates' correction for continuity was used.

For all studies, a significance threshold α=0.05 was used. In the case of testing multiple SNPs or clinical subgroups of MG, correction for multiple testing error was conducted. The Bonferroni correction approximates the α required for a similar significance level when simultaneously making multiple measurements. The accepted approximation α/n for n tests, following from $[1-(1-\alpha)^n] = \propto_0$, was used.

One-way ANOVA test for comparison of three groups and Mann-Whitney *t* test were used to analyze ELIspot data, with significance indicated by a *p*-value below 0.05.

The odds ratio (OR) gives the ratio of the occurrence of an event in one group to the occurrence of the event in a second group. In case-control studies, the event is the genetic factor, given by allele frequencies: $OR = ad/bc$

	allele 1	allele 2
case	*a*	*b*
control	*c*	*d*

The relative risk (RR) is defined as $\frac{p(exposed)}{p(non-exposed)}$. In genetic case-control studies, the relative risk describes the ratio of the proportion that develop the disease with the genetic factor (exposed) to the proportion who develop disease without the genetic factor (non-exposed). This requires an unbiased measurement from the population of

those with and without the genetic factor, followed by subsequent examination for clinical diagnosis of disease.

3.8 POWER CALCULATIONS

The statistical power of a study is defined as the probability of rejecting a false null hypothesis, and is therefore equal to (1-β), where β is the false negative rate. The calculation of power is highly dependent on the sample size as well as the minimum effect size that is expected to be detected. In genetic studies, this is represented by the allelic odds ratio observed. The calculation is typically reported as the OR corresponding to an 80% power in case-control association studies at a given α.

For the studies presented, power was calculated by the Power Calculator for Two Stage Association Studies" (http://www.sph.umich.edu/csg/abecasis/CaTS/) [121]. The background allele frequency was estimated by the aggregate control MAF reported, with significance level α=0.05. The frequency of MG in the population for calculations was 1:10,000.

4 RESULTS AND DISCUSSION

4.1 PTPN22 IS ASSOCIATED WITH MG

We genotyped the PTPN22 1858C>T mutation (R620W, rs2476601) in 409 MG patients and 1557 matched healthy controls. The allele frequencies were associated to all MG patients (n=409) (p=2.7x10^{-4}, OR=1.52) as well as anti-AChR abs positive patients (n=350) (p=4.9x10^{-4}, OR=1.52).

In patients with thymic hyperplasia (n=142), a similar association of the polymorphism was observed (p=1.4x10^{-4}, OR=1.87), with an increase in the OR in patients with both thymic hyperplasia and anti-AChR abs (n=128) (p=6.6x10^{-5}, OR=1.96).

We furthermore examined IL-2 and IgG producing cells, indicative of anti-AChR abs secretion, after antigen activation in 100 randomly chosen patients with different rs2476601 genotypes. Enumeration of cells secreting IL-2, IFN-γ, or IgG did not differ between PTPN22 genotypes in medium alone. However, in cells incubated with auto-antigen, there were increases in IL-2 producing cells (p=0.002) and IgG secreting cells (p=0.004) in patients carrying the T allele.

These results confirm the association of rs2476601 with MG, and suggest that the gene may play a role in autoimmune disorders through a common mechanism. While previous findings suggested that the mutation may be a gain-of-function variant, our data indicate that the mutation may be a loss-of-function variant in MG, resulting in IL-2 production and T-cell proliferation. This effect may indicate a disease specific mechanism of the mutation, or that other genetic factors may influence expression through epistasis.

4.2 CIITA IS NOT ASSOCIATED WITH MG

Genotyping of 446 MG patients and 1866 controls for rs3087456 was conducted, and patients were stratified according to common clinical subgroups. No statistically significant difference was observed between MG patients and controls for either allele frequencies (p=0.092) or genotypes (p=0.251). The strongest associations were in the ocular and LOMG subgroups, with uncorrected p-values of 0.040 and 0.010, respectively. However, they yielded non-significant p-values when compared with the multiple testing corrected significance threshold of 6.25x10^{-3}, corresponding to 8 tests.

Furthermore, when examining replication studies conducted on the SNP, only 4 of 26 reported significant association, including 2 of 10 in RA cohorts (Table 2). One of the positive RA replication studies further reported association with an increase in the major (A) allele [122] in a Japanese population, drawing the proposed mechanism into question.

Disease	Number of cohorts	Positive assoc.
RA	10	2
SLE	3	0
MS	4	0
WG	1	0
MI	1	0
AAD	2	2
JIA	1	0
IBS	1	0
CVID	1	0
IgAD	1	0
CD	1	0
Total	**26**	**4**

Table 2. A summary of replication studies of the CIITA SNP rs3087456 in autoimmune disorders

Our control cohort had a significantly higher frequency of the minor (G) allele that the original study reporting association of the SNP to autoimmunity [106] (0.266 and 0.216, respectively, $p<10^{-5}$). We also compared MG allele frequencies to this control material. However, the MG group did not show a statistically significant association when using the original study's control frequency.

These findings indicate that the *CIITA* SNP rs3087456 is not associated to MG, that its role in autoimmunity may not be as strong as previously thought, and that other factors may regulate CIITA expression in patients with autoimmunity.

4.3 CD45 IS NOT ASSOCIATED WITH MG

The *CD45* SNP rs17612648 was genotyped in 446 MG patients and 2303 matched controls. Allelic variants of rs17612648 were not associated with myasthenia gravis in the patient group as a whole (p=0.199), although the minor (G) allele appears slightly more frequently in MG patients than in controls (1.91% compared with 1.35%). No subgroup of MG associated with the SNP (p>0.260, corrected), despite a slightly elevated minor allele frequency in most subgroups. In patient subgroups, the LOMG

HLA B7 (0.91% MAF) and DR2 (0.81% MAF) subgroups had a lower frequency of the G allele than the control population (1.35% MAF), although, due to the low allele frequency, each group contained only one heterozygous case.

A blood donor control sample was found to be homozygous for the G allele, and confirmed by sequencing. Due to the presumed deleterious effect of homozygosity of this allele [123], a serum sample was tested in order to determine if autoantibodies were present. ANA antibodies (Anti-Nucleosome, Ribosomal P, RNP68, RNP A, Scl-70, Sm, SmRNP, SS-A(Ro52), SS-A(Ro60), SS-B, Centromere, Jo-1 and dsDNA) and ANCA antibodies (Anti-PR3, MPO and GBM) were not demonstrated. Serum immunoglobulin levels were also normal (IgM=0.7 g/l, IgG=9.7 g/l, IgA=1.7 g/l). Due to restrictions in the ethical permission of the study, requesting anonymous control samples, material for additional analysis, including CD45 expression, could not be obtained.

Disease	Number of cohorts	Positive assoc.
RA	9	2
CVID	3	0
IgAD	3	0
TID	2	0
Graves	1	0
SSc	2	1
SLE	1	0
MG	1	0
AIH	2	1
Total	**24**	**4**

Table 3. Summary of replication studies of the CD45 SNP rs17612648 in autoimmunity

Only 4 of 24 replication cohorts of rs17612648 with autoimmunity have reported association of the SNP with a disorder, including 2 of 9 in RA (Table 3). In a recent MG study, although the SNP was not present in the cohort, ratios of CD45RO to CD45RA CD8+ T-cells were found to be significantly lower in patients with late onset MG (LOMG) as well as in T-cells in patients with thymoma [124]. These differences suggest an alteration in CD45 expression independent of the rs17612648 SNP, and provide evidence that CD45 splicing may be regulated by other factors. Furthermore, it has been demonstrated that human/mouse somatic cell hybrids carrying only the mutant (G) allele are still able to generate CD45RO [125].

The discovery of a homozygous blood donor without obvious autoimmunity suggests that the mutation may not have strong deleterious effects. The lack of association of the SNP with MG, as well as a lack of replication, indicates that the mutation's role in autoimmunity may have to be reevaluated.

4.4 8.1 HAPLOTYPE IN IGAD

Of 117 individuals homozygous for the B8, DR3, DQ2, 2 were found to be IgAD (1.7%), far less than previous studies have reported. The approximate relative risk of homozygosity for the haplotype for the development of IgAD can be estimated using a previous population based screening for IgAD, which found 10 IgAD individuals (9 measured and all non-homozygous) in 6955 blood donors in Sweden. Based on these data, the relative risk has been calculated to be 11.89. The large discrepancy between our results and previous studies (RR=77.8) may be due to inaccuracies in serological typing, low samples sizes or publication bias.

The frequency of the haplotype was much higher in our cohort of 421 IgAD patients (25.9%) than in the background in Sweden using data from the volunteer registry (8.2%) (p=5.1×10^{-74}, OR=3.90). Among homozygotes, The frequency of the haplotype was 6.9% in the IgAD group compared with 0.7% in the background population (p=1.4×10^{-47}, OR=11.12). SNP maps were unable to elucidate any differences in homozygosity between IgAD individuals and controls.

Concomitant haplotypes were examined, with DR7 found to be more frequently present in heterozygotes (21.3%) compared to controls (8.5%) (p-val = 3.5x10^{-6} with Bonferroni correction for 10 tests), with the result possibly due to independent association of DR7 with IgAD.

We also addressed the hypothesis that a rare, more highly penetrant variant of the 8.1 haplotype might explain the data. Assuming that the Swedish volunteer bone marrow registry represents a cross section of the Swedish population, the frequency of a putative disease predisposing rare variant of the B8, DR3, DQ2 haplotype was estimated to be 18 of 3,880 copies (0.46%). Sequencing this haplotype in homozygous patients and controls is a logical extension of these experiments, which could determine the region(s) of association more definitely.

4.5 AUTOIMMUNITY IN MG – NO ASSOCIATION WITH IGAD

In 547 MG patients, concomitant autoimmunity was diagnosed in 87 (15.9%), far great than in the general population (approximately 5-8% in developed countries [126]) (**Paper V**, Figure 1a).

Two of 482 MG patients were found to be IgAD, not significantly increased from the background prevalence (p=0.14). Assuming that all individuals with one B8, DR3 and DQ2 contain this haplotype, the frequency of the haplotype is 9.8% in controls, 25.5% in IgAD patients, 15.1% in MG and 23.8% in EOMG patients. This corresponds to p-values of 1.5×10^{-33} in IgAD, 8.1×10^{-6} in MG and 1.9×10^{-16} in EOMG. The haplotype was not significantly increased in the 87 MG patients with concomitant autoimmune disorders (13.0%, p=0.22) as compared with MG patients without concomitant autoimmunity.

In heterozygous HLA B8, DR3, DQ2 patients with MG and concomitant autoimmunity, an increase in DR1, DQ5 (33.3% compared with 8.9% of controls; $p=2.5 \times 10^{-3}$) occurs, although the number of patients with this haplotype is low (n=5). This haplotype does not occur in the two MG patients with IgAD (DR13, DQ3 and DR11, DQ3).

Despite borderline significant p-value (0.049), thymectomy does not appear to increase susceptibility of MG patients to developing other autoimmune disorders, as 22 of 57 thymectomized patients developed their disorder before thymectomy. Since the rate of EOMG is somewhat higher in patients with additional disorders (57.5% and 48.2%, respectively), it is possible that EOMG may be influenced by common autoimmune mechanisms which increase the risk of additional disorders. No evidence was found that thymectomy increases predisposition to SLE, as 2 cases occurred before surgery and 3 after.

The SNP map for MG patients (n=5), controls (n=6) and IgA patients (n=10) homozygous for the B8, DR3, DQ2 haplotype did not display any appreciable differences, with all individuals being homozygous through the region with some slight heterozygosity.

In order to visualize the overlap of genetic factors in autoimmunity, several classical autoimmune and related disorders were selected for assessment of published genetic association using “A Catalog of Published Genome-Wide Studies”, a public database of

all GWAS results to date (www.genome.gov/gwastudies) [127]. Autoimmune and inflammatory disorders examined included celiac disease, Crohn's disease, IgAD, MS, psoriasis, RA, SLE, systemic sclerosis, T1D and ulcerative colitis, and SNPs with reported association of $p<10^{-5}$ in at least one study were cross referenced in all studies and charted. Regions of strong association to large number of autoimmune disorders, particularly the HLA, are visible. The full map of results is given in Table 1 of **Paper V**. The patterns of genetic association highlighted by this map illustrate that several regions of strong association to multiple disorders exist, such as the HLA, PTPN22, etc. However, for some diseases, very few strongly associated non-HLA genes are visible (ie. IgAD and systemic sclerosis). Investigating the overlap in genetic contributing to autoimmune using novel methods for multi-gene analysis may therefore be necessary to explain the nature of disease predisposition.

4.6 TWINS CONCORDANCE RATES AND COMPLEX DISEASE PREDISPOSITION

Although many genes are associated to complex diseases, the frequency of the predisposition and environmental component to such disorders is unclear. A logical and useful step is to infer information from twins data that can be used to estimate this information. It is possible to estimate the predisposition and environmental trigger frequencies by first assuming that MZ twins share the underlying predisposition for the disorder. The probabilities of twin concordance can be given as:

Twin 1	Twin 2	Probability
0	0	$(1- P_T) * (1- P_T)$
0	1	$(1- P_T) * P_T$
1	0	$P_T * (1- P_T)$
1	1	$P_T * P_T$

where 0 denotes a twin without MG, and 1 denotes a twin with MG, with P_T being the probability of an MZ twin with genetic predisposition developing MG and the probability of an MZ twin not developing MG is $1- P_T$.

The probabilities then simplify to:

p((0,0))	No twins with MG	$(1- P_T)^2$
p((1,0),(0,1))	Exactly one twin with MG	$2 P_T * (1- P_T)$
p((1,1))	Both twins with MG	$P_T^{\ 2}$

The rate of MZ twins concordance is given by the proportion of both twins with MG p((1,1)) to the total MZ twin pairs observed p(1,1) + p((1,0),(0,1)), which is equal to

$$MZ\ concordance = \frac{P_T^{\,2}}{P_T^{\,2} + 2P_T(1-P_T)}$$

P_T can be solved and is given as follows:

$$\mathrm{P_T} = \frac{2{*}MZ}{1+MZ}$$

The number of regions which contribute to the predisposition can be estimated by assuming that the same inherited regions predispose to disease in DZ twins. We assign the probability of dizygotic twins (Di) inheriting the disease to be equal to that of monozygotic twins (Mo) multiplied by an inheritance ratio (β) and a recombination probability (RF) for each of n regions.

$$p(Di) = p(Mo) \times [\beta \times (1-RF)]^n$$

where the term RF represents the chance of recombination within the given region disturbing required genetic components. The previous equation can be modified by including separate values of β and RF per inherited disease haplotype, since these may have different inheritance patterns:

$$p(Di) = p(Mo) \times \prod_{i=1}^{n} [\beta_i \times (1-RF_i)]$$

and then including genetic modifiers, denoted as ξ with effect sizes ($0<\xi<1$), occurring m times:

$$p(Di) = p(Mo) \times \prod_{i=1}^{n} [\beta_i \times (1-RF_i)] \times \prod_{j=1}^{m} \left[\beta_j \times (1-\xi_j)\right]$$

The monozygotic twin concordance rate in MG, based on a comprehensive literature review, is 11 of 31 pairs, or 35.5%. Of 16 dizygotic twin pairs, none were concordant for MG. However, due to the low number of studies, the familial rate, measured to between 3.8 and 7.2% [128],[129], is assumed to be an accurate estimate.

The result of the predisposition calculation in MG using an MZ pairwise concordance rate of 35.5% is that P_T, or the rate of the environmental trigger, is estimated to be 52.4% in predisposed twins. A consequence of this calculation is that this rate can be used to approximate the frequency of genetically predisposed individuals in the general

population, by dividing the rate of incidence by P_T. In the case of MG, this would make the overall frequency of genetic predisposition approximately 1 in 5240.

When estimating the inherited regions in MG, for simplicity the term *p(Di)* can be approximated from the familial rate to be approximately 2.5% and that of *p(Mo)* is approximately 40% (MZ rate). We can thereafter determine the expected minimum and maximum values under the assumption that all regions must be inherited from both parents (β=0.25) under a recessive model or that a single copy is required (β=0.50) using a dominant model. Solving the equation for n using these βs (and assuming RF=0) gives a solution that between two and four regions are required, which are inherited in their entirety.

The implications of these calculations are that fewer regions than previously anticipated appear to be required to confer predisposition to MG. These regions may be long haplotypes with multiple SNPs/mutations present, and it is therefore of practical importance to identify these haplotypes by sequencing regions of association in case/control studies.

4.6.1 Consideration of potential sources of error

There are several possible sources of error in estimations for MZ twins rates. First, MZ twin publications occur if it is known that a patient has a twin, and this is very likely to be reported if the twin is concordant for MG. Therefore, publications are likely to bias toward MZ concordance, and over report this rate. Second, studies observe only a static time point in patients and their families' lives, and non-affected MZ twins might develop disease later in life. It is thus difficult to estimate the effects of these factors without a large set of data collected over time. However, publication bias is likely to overestimate the MZ concordance while future diagnosis of disease in a twin pair can only decrease the reported rate from the true rate. Although it is certain whether these are equal in their effects, they are most certainly opposite in misclassification. Without further estimation methods, we will therefore disregard these potential sources of error.

4.6.2 Effect of MG subgroups

Myasthenia gravis has important clinical subgroups which may represent distinct disease entities, some with stronger underlying genetic components. A notable example is the aforementioned associations of HLA B8, DR3 with early onset MG and HLA B7, DR2 with late onset MG. Since nearly all reported affected MZ twins were EOMG, regardless of concordance or discordance for MG, it is very difficult to determine if this

is a result of publication bias or if EOMG may be overrepresented among MG twin pairs who are concordant for MG. In order to determine the effect of potential subgroup disparity in concordance rates of MZ twins, we examined the strongest possible example and for the moment assumed that all MZ twins concordant for MG are EOMG, while discordant twins have similar proportions of EOMG as we observe in our cohort of 542 MG patients (30% EOMG). The MZ concordance rate for EOMG will change from $\frac{C}{C+D}$ to $\frac{C}{C+D*(0.3)}$, due to a 30% proportion of EOMG. Thus, the previous data gives an MZ concordance rate of 64.7% and increases P_T to 0.79. While this represents an extreme case, the net result would be an increase in the proportion of the population with disease predisposition who develop the disease, and a more frequently encountered environmental trigger. This effect is likely to be more modest, however, since the lack of reported MG twin pairs with late age of onset does not definitely bias the twin calculation toward EOMG.

4.6.3 Epigenetic effects

Epigenetic changes are commonly defined as those that are not stably inherited through DNA alterations, and often refer to chromatin remodeling due to DNA methylation and histone modifications such as acetylation, methylation, phosphorylation and ubiquitination [130]. These changes have been hypothesized to be a potential underlying component of complex disease, that may or may not work in tandem with genetic predisposing factors. Since monozygotic twins have similar genetics, but may vary in epigenetic patterns, this could be a potential cause of variation explaining twin concordance/discordance. However, a recent study investigating the epigenetic patterns of three pairs of MZ twins discordant for MS, using high density SNP profiling and sequencing of mismatching polymorphism, determined that epigenetic patterns did not vary significantly between identical twins [131]. In fact, only 2 to 176 differences were noted in the methylation of 2 million CpG dinucleotides, whereas unrelated individuals contain approximately 800 such changes. It is therefore likely that at least DNA methylation is not a primary cause of MZ twin disease discordance.

Epigenetic changes may be determined, as least partially, by genetic regulation mechanisms. An individual may inherit certain, as yet unidentified, genes which stimulate an increase or decrease in methylation or histone modification in certain tissues. These regulation patterns may affect levels of gene expression involved with disturbed equilibrium in the development of complex disorders. Should such a mechanism occur independently of the genetic predisposing elements, the effect would

be comparable in all twin pairs, and could therefore be considered similar to environmental factors. However, if epigenetic factors occur in tandem with certain required genetic predisposition, they can be considered to be genetic modifiers, thereby increasing the risk of a threshold of activation occurring. Thus, the requirement of epigenetic factor(s) for the development of MG would similarly reduce the number of genetic predisposing regions present.

5 TOWARD A MODEL OF COMPLEX DISEASE

5.1 BASIS AND MODEL ASSUMPTIONS

It is clear from previous investigations that associations of genes with complex disease are few in number relative to their effects. A genome-wide study which found 20 loci which influence adult height estimated that these variants explain 3% of the population variation [132], and it was recently estimated that based on this data, 93,000 SNPs would be required to explain 80% of height variation [133]. This highlights the potential pitfalls of assuming an additive effect of risk loci to explain phenotype, and the need for more advanced models of complex disease which can combine genetic risk factors.

The association of a particular SNP with a disorder also yields little information about the increased risk for the individual to develop the disease. While the relative risk can be calculated, due to the effects explained above, there remain many individuals with the risk allele who do not develop disease; conversely, many individuals with disease do not possess the risk allele. Therefore, inclusion of the SNP into an explanation of disease pathogenesis is incomplete without further information about the network or pathway affected. A model of complex disease will therefore be formulated which considers genetic predisposition, gene-gene interaction, environmental factors, disease subclasses and genetic modifiers.

Assuming subclasses based on clinical disease phenotypes is unsuitable, particular for a disease such as MG, wherein classification can change with treatment, and overlap of subgroups exists. The predisposition is therefore assumed to fit pie model of underlying genetic predisposition, wherein the environmental factor(s) are considered separately. This is valid if certain factors are required together; that is, if they are a necessary but not sufficient condition to develop disease. It is unknown how many genetic subclasses, or "pies" exist, in MG. These could number a few, or in the extreme case, hundreds. However, we will construct the model of complex disease to be insensitive to the number of subclasses present. The presence of genetic modifiers, which may influence disease development, will also be added.

5.2 GENETIC FACTORS INCLUDED IN A MODEL

The genetic predisposition of complex disease and the environmental trigger are necessary to occur in tandem within an individual in order to cause disease development. A model of complex disease must consider both of these for each

subclass, and construct a union of all subclasses. The genetic predisposition will be represented by the presence of all required factors for a particular subclass in an individual, while the environmental component is a continuous probability of encountering the event over an individual's life.

The term for environmental component, including both genetic modifiers and a trigger, is given as

$$\textit{Genetic modifiers} * \textit{Trigger}$$

The genetic modifiers have previously been denoted in **Paper VI** as ξ_j occurring m times with effect sizes $0<\xi<1$. The environmental trigger can be represented by a hazard rate, h(t), which is used to describe the probability of encountering an event at time t, given that the event has not been encountered from time 0 to time t. In this case, the probability is that the triggering event has not been encountered in an individual devoid of modifier genes. In order to include the effect of modifier genes, a Cox proportional hazard model can be used to represent the environmental components

$$h(t, \mathrm{X}) = h_0(t) e^{\sum_{j=1}^{m} \beta_j \mathrm{X}_j}$$

where $h_0(t)$ is the baseline hazard rate in the absence of modifiers, and the coefficients β provide weighting for each of j genetic modifiers, which are now denoted by X and are static over time. The cumulative hazard rate function is given by

$$H(t, \mathrm{X}) = H_0(t) e^{\sum_{j=1}^{m} \beta_j \mathrm{X}_j}$$

where $H_0(t)$ denotes the baseline cumulative hazard rate function and

$$H_0(t) = \int_0^t h_0(s) ds$$

The survival function is given by

$$S(t, \mathrm{X}) = e^{-H(t, \mathrm{X})}$$

which signifies the probability of not having encountering the trigger from time 0 until time t, while the probability of encountering the trigger up to time t is $1 - S(t, \mathrm{X})$.

Predisposition for a subclass with n elements is determined by the product $y_1 * y_2 * \ldots y_n$, with $y_i=1$ indicating the presence of each genetic element i required to be necessary to develop the disease, and $y_i=0$ indicating a lack of element i. The product $\prod_{i=1}^{n} y_i$ equal to 1 therefore signifies disease predisposition for the subclass, and 0 a lack

thereof. It may follow that these y_i could be further classified according to membership criterion, for example $y_1=(y_{11}, y_{12},\ldots y_n)$ where at least one of $(y_{11}, y_{12},\ldots y_n)$ must be present in the individual for $y_1=1$, this case representing an "OR" rule wherein a network or pathway may be sufficiently affected in multiple ways. However, this would simplify the number of "pies" present while confounding determination of genetic elements. The current model also implicitly includes this case and it will therefore not be included.

5.3 A MODEL OF COMPLEX DISEASE

The joint probability of both predisposition and environmental components occurring in the individual for the subclass is given by the product of these terms

$$\prod_{i=1}^{n} y_i \times \left(1 - e^{-H_0(t)e^{\sum_{j=1}^{m} \beta_j X_j}}\right)$$

which is equal to the environmental probability for individuals with predisposition and zero for individuals without predisposition, thereby removing subclasses for which the individual is not predisposed. This equation is similar for all subclasses, so laws of probability can be used to combine these over all subgroups.

$$1 - \prod_{i=1}^{n} y_i \times \left(1 - e^{-H_0(t)e^{\sum_{j=1}^{m} \beta_j X_j}}\right)$$

gives the complement of disease development for a single subclass, that is, the probability of not developing the disease via this subclass. This is denoted *p(k)*, and the product of all such *p(k)* for all the subclasses of disease gives the overall probability of not developing disease. The complement of this product is

$$1 - \prod_{k=1}^{r} p(k)$$

and represents the combined probability of developing disease for all of r subclasses. Substitution yields the final probability of disease development:

$$1 - \prod_{k=1}^{r} \left[1 - \prod_{i=1}^{n} y_{ik} \times \left(1 - e^{-H_0(t)e^{\sum_{j=1}^{m} \beta_{jk} X_{jk}}}\right)\right]$$

This assumes that the baseline cumulative hazard rate function is proportional over time and similar across all subclasses. A further assumption is that modifier genes may have different weights in different subclasses, and that modifier effects are time independent.

5.4 MODEL IMPLICATIONS

This model is extremely flexible and the quantitative contributions of predisposition and environment are capable of varying according to empirical evidence. If epigenetic factors are largely responsible for disease pathogenesis, the predisposition component may be more frequent in the population, with a larger impact of epigenetic modifiers present.

The genetic elements and modifiers found to be acting in a particular subclass of disease can be used to determine how disease pathogenesis occurs. Gene-gene interactions in networks and pathways may be inferred, and the action of modifiers on these pathways may give important information about disease mechanisms.

Using this model of complex disease to resolve genetic predisposition, modifier effects, and the survival function requires a stepwise approach that must be explored further. First, the genetic subclasses must be identified, by determining all groups of SNPs, indels, etc. which are consistently present in a large proportion of cases. Partitioning these into subclasses will require a large sample size and the development of novel analytical tools. After the identification of relevant subclasses of predisposition, the inclusion of potential genetic modifier data may provide a means of determining the model parameters, including coefficients as well as the baseline survival function.

Confounding the issue of measuring the predisposition to complex disease is that SNPs associated to disease may be markers for the causative elements in linkage disequilibrium (LD) with them, thereby masking the true effects. A recent study determined that maximum allelic frequency variation between two SNPs with a squared correlation (r^2) of 0.8 is ±0.06, which is sufficient to reduce the power of a study from 80% to detect Type I error of $5x10^{-5}$ to 60% [134]. For r^2 =0.5, the maximum allele variation increases to 0.16, strongly reducing the ability to detect the SNP in case/control studies.

Furthermore, a low number of predisposing regions in each subclass **(Paper VI)** points to an association with haplotypes and not single SNPs. Future efforts should therefore focus on finding these genetic elements which comprise the predisposing subclasses, as well as genetic modifiers present, using sequencing of cases/controls and large scale computational analysis tools. An emphasis on identifying epistatic effects within haplotypes constituting the disease predisposing regions could greatly increase understanding of disease causing mechanisms.

6 CONCLUSIONS

- Myasthenia gravis is associated to the PTPN22 rs2476601 polymorphism, which stimulates T-cell proliferation and may therefore be a gain-of-function variant in MG. The mutation's effect may be disease specific.

- MG is not associated with the MHC2TA rs3087456 polymorphism, and this polymorphism does not appear to play a role in autoimmunity.

- MG is not associated with the CD45 rs17612648 polymorphism, and this mutation's role in autoimmunity may need to be re-evaluated.

- Although IgAD is strongly associated with the HLA-B8, DR3, DQ2 haplotype, the relative risk of developing IgAD for homozygotes has been overstated and is approximately 11.89.

- Autoimmunity disorders occur more frequently in MG patients, but this does not extend to IgAD. Overlapping effects in both HLA and non-HLA genes in autoimmunity suggest possible underlying mechanisms.

- Twin data can be used to estimate complex disease predisposition/environmental component as well as the number of inherited regions causing disease. In MG, the predisposition is roughly double the rate of incidence, and the number of co-inherited regions causing disease is 2-4. Sequencing associated regions is necessary to discern these haplotypes.

- It is possible to model the probability of developing complex disease using the approach of genetic subclasses. Determining genetic factors (including modifiers) is necessary to solve for the underlying function.

7 FUTURE WORK

The ultimate goal of these projects is to fully determine which genetic factors underlie myasthenia gravis, IgAD, and related autoimmune disorders. In order to accomplish this, it is necessary to 1) identify all important elements contributing to disease predisposition and 2) model how these factors interact.

There are several projects ongoing which will attempt to further characterize both 1) and 2). They are:

- Fine mapping of the HLA in MG patients via imputation using IMPUTE 2 and genome wide data. Association statistics using matched controls will then be used to pinpoint regions of the HLA in which disease predisposing polymorphisms occur.

- Sequencing of the MHC from upstream of HLA-B to downstream of HLA-DQ, initially in 4 MG patients, 4 IgAD patients without additional disorders, and 4 controls, all homozygous for HLA A1, B8, DR3, DQ2. This will be followed by additional sequencing of patients/controls as needed to clarify the role of any identified disease causing regions.

- A case-control GWAS to identify SNPs/genes associated with MG.

- A multi-SNP, multi-gene model that will create subgroups of complex disease directly from GWAS data, with high case/control prediction accuracy.

8 ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people:

Lennart Hammarström, my supervisor for guiding me on these projects. I am especially thankful for my adoption into the group, and for the opportunity to pursue projects which have broadened my research horizons. Thank you also for allowing me for working on my (sometimes farfetched) ideas while still keeping me on course, and also for the talks and advice on life, family and whiskey.

Ritva Matell, my co-supervisor, for all the time you have spent with me helping me understand the clinical aspects of the research we undertake, as well as compiling what must have seemed like endless clinical data on patients. You have provided me with a bridge between the lab and clinic, which has reminded me that improving the lives of patients is the goal of basic research.

Fredrik Piehl, for all the help in our collaboration in MG research. Hopefully we will have a chance to continue it and publish the result of ongoing projects in the future.

Qiang Pan-Hammarström, for the collaborations, reviewing manuscripts, and especially the dinner at Chopsticks Restaurant!

All other co-authors and collaborators, without whose contributions this thesis would be a lot thinner. Especially **Yaofeng Zhao**, **Priya Sakthivel**, **Javad Mohammadi**, **Sara Jarefors**, **Peter Gregersen**, and of course **S. Ramanujam** – we finally got a chance to publish together!

All my colleagues at the Division of Clinical Immunology. **Ingegerd**, for our MG work and endless energy, **Kerstin**, for help on many matters and a cheerful attitude, **Magda**, Soldaten Svek again soon! **Nina**, an HLA guru, **Alberto**, Panzeroto cook extraordinaire, **Marcel**, **Du**, **Gökce**, **Bea**, **Harold**, **Giuseppe**, **Connie**, **Kaspar**, **Sonal**, **Noel**, **Andrea**, **Renée**, and **Carin**. Former lab members **Javad**, **Neha**, **Lin**, **Yoko**. And of course **Naradja**. Where would we all be if you didn't help us out!

Also our neighboring groups, for sharing a fun environment: **Antony**, **Sepideh**, **Fredrik**, **Babi**, **Lars, Gustav, Anna, Kathrin**. Our IT gurus, **Fredrik** and **Thore**, for computer assistance and tech savvy. **Marita Ward**, for all the administrative assistance. And of course seminar master and Director of postgraduate studies **Andrej Weintraub** for guidance.

To my friends past and present at CMM, for all the great times. **Anna**, **Ida**, **Karin**, **Lollo**, **Malin**, **Philippe**, **Ame** – sorry I have missed several years of after works! My gratitude to **Selim** for friendship and helping me with the sequencing facility. To **Lars Klareskog** for supervision during the transition period, and to **Jesper Tegnér** for co-supervising my earlier studies. Also I am indebted for the contributions and friendship of former labmates at CMM, **Ricardo "The General" Giscombe**, **Gu Ming**, **Maria Kakoulidou**, and **Priya Sakthivel** – those were great times.

I would like to specially note the contributions of **Prof. Ann Kari Lefvert**, my original supervisor, who died tragically in 2007. Ann Kari welcomed me into her lab and guided my early research. In addition to being a genuinely kind person and excellent researcher, she impacted me most by believing in my ideas and potential. You had an enormous impact on your students, and are deeply missed.

Most importantly, I would like to thank my family for continual support. **Jenny** and **Viktor**, now I know what is most important in life.

This thesis was supported by the Swedish Research Council, the Palle Ferb Foundation and by a grant from the US National Institute of Allergy and Infectious Diseases.

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