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Genetic studies of glomerulonephritis
with special focus on IgA nephropathy
and Lupus nephritis

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Stockholm 2010
To my family
ABSTRACT

IgA nephropathy and nephritis in Systemic Lupus Erythematosus (SLE) are two common forms of glomerulonephritis with unknown etiology. As for many other complex diseases, both genetic and environmental factors could be of importance for disease development. However, immunological and biochemical similarities between SLE and IgA nephropathy demonstrate a direct link to impaired immune function in both diseases. In our study we tested the hypothesis that there is an overlap in genetic susceptibility between Lupus nephritis and IgA nephropathy and we aimed to identify specific biomarkers associated with the severity of nephritis. We addressed this question in cohorts of 212 individuals with IgA nephropathy, 272 individuals with SLE, including 106 with nephritis and up to 1569 individuals from a healthy control population, by analysis of genetic variants in genomic DNA and by investigation of plasma from patients and controls.

Our analysis of distribution of HLA-DRB1 variants showed a significant association with IgA nephropathy, with the HLA-DRB1 *03, and *15 revealing a strong protective effect for IgA nephropathy. In contrast, the HLA-DRB1 *03, and *15 indicated a risk effect to SLE. We found a similar contrast in respect to non-HLA risk factors for these two types of nephritis. While TGFB1 gene variants are associated with IgA nephropathy, this was not demonstrated for Lupus nephritis. On the other hand, several genetic polymorphisms previously found in association with SLE, like IRF5, STAT4 and TRAF1-C5, were not demonstrated to associate with Lupus nephritis or with IgA nephropathy in our cohort. Additionally, we found no evidence for an association of FCAR (CD89) gene polymorphisms in the investigated nephritis groups. No genetic factors associated with the progress of IgA nephropathy were detected in these genetic association studies.

Two biomarkers were tested in nephritis patients: mannose-binding lectin (MBL) and soluble CD89 receptor (sCD89, Fc alpha receptor). A new method for detection of sCD89-IgA complex in human serum/plasma was developed and applied in the IgA nephropathy cohort. Our study does not suggest that it is possible to predict development of nephritis based on these biomarkers. However, a significant association between low levels (less than 10 relative units) of sCD89-IgA complex in sera of IgA nephropathy patients and disease progression was detected. In a disease control group of patients with other forms of glomerulonephritis, including Lupus nephritis, who had similar renal function and proteinuria as the IgA nephropathy group, no correlation to disease progression was observed. When sCD89 analysis was performed on individuals, with repetitive samples during 5-15 years of follow-up, we found that serum levels of sCD89 remained stable and low in IgA nephropathy patients with disease progression and were continuously high (more than 40 relative units) in IgA nephropathy patients without disease progression. Thus, the sCD89 level could be a valuable prognostic marker of progressive renal failure in IgA nephropathy patients.

In our study we identified several genetic factors and a specific biomarker, which are different for IgA nephropathy and Lupus nephritis risk or progression. These findings point to a difference in the possible mechanisms of renal failure and suggest detection of HLA-DRB1 alleles for differential diagnostics of IgA nephropathy and Lupus nephritis at early stages of the disease. The discovery of a prognostic factor for disease progression in IgA nephropathy suggests that earlier and more aggressive therapy should be instituted, as well as opening the possibility of developing new methods of treatment for severe IgA nephropathy cases.
LIST OF PUBLICATIONS


# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACE inhibitors</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>ACR</td>
<td>American college of rheumatology</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II receptor antagonist</td>
</tr>
<tr>
<td>bp(s)</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>C5; C3; C1q</td>
<td>Complement component 5; or 3; or 1q</td>
</tr>
<tr>
<td>CNVs</td>
<td>Copy number variations</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
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<tr>
<td>FcAR</td>
<td>Fc alpha receptor</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
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<tr>
<td>HLA-DRB1</td>
<td>Human leukocyte antigen class II DR beta 1</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IRF5</td>
<td>Interferon regulatory factor 5</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
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<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>STAT4</td>
<td>Signal transducers and activator of transcription 4</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>TRAF1</td>
<td>TNF receptor-associated factor 1</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
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Kidney diseases have been studied for many years and have attracted a lot of interest. Glomerulonephritis has attracted particular attention due to the fact that patients with this disease are not expected to regain healthy kidneys because the damage is persistent. This could be said to be true of any chronic disease; however, the very serious influence on the quality of the life of these patients, and the fact that they may progress to end-stage renal disease with numerous cardiovascular and metabolic complications and a need for dialysis or renal transplantation is of particular importance.

Medication and replacement therapy, including dialysis and renal transplantation, are expensive and could be a major public health problem in any country. In particular, the high medical costs are a challenge for developing countries. In Vietnam, when performing the examination of 8,505 participants aged > 40 years in 2006, we identified 3.1% with Chronic Kidney Disease (stages 3–5). In the Nephrology Department, Bach Mai Hospital, Hanoi, there are around 150 in-patients daily and most of the patients are in a very late stage of chronic kidney disease.

My medical career started with six years at medical school, followed by four years training as a hospital resident physician, living and working 24/7 in hospital, doing duty in the areas of emergency and internal medicine. After graduation, I was immediately employed to be a lecturer at Hanoi Medical University and a nephrologist belonging to the Nephrology Department, Bach Mai Hospital, Hanoi, Vietnam, I have been deeply involved in both teaching students about nephrology and clinical treatment for kidney disease patients. The experience of seeing severely ill patients never fades.

On 15th November 2006, I registered as a PhD student at Karolinska Institutet in Stockholm, Sweden. The PhD project introduced me to molecular medicine, a very exciting area, which I have enjoyed. I have developed a link between my clinical knowledge and in-depth genetic studies, allowing me to combine my role as a clinician with the performance of various techniques in the laboratory. In addition, these studies have introduced me to the field of statistics and I now enjoy performing statistical analysis in the process of studying genetics.

I hope that my effort at undertaking PhD level research will contribute to the understanding of the pathogenesis of kidney disease development and be helpful for the future discovery of a more scientific basis to support the new strategies for chronic kidney disease management.
1 BACKGROUND

1.1 GENETIC STUDIES OF HUMAN COMPLEX DISEASES

There is much evidence that human complex diseases, including glomerulonephritis, are due to a combination of environmental and genetic risk factors. Since our study concerns the genetic background of glomerulonephritis, we would like to present the main challenges in this area of research. In deciding to perform genetic study of a complex disease, it is important to consider the issues of study design, genotyping procedure, phenotype definition, and statistical analysis.

1.1.1 Design of genetic studies

In general, many genetic studies have been performed on samples, which consist of a small number of subjects from the total population of interest. In fact, the selected population sample may differ greatly with respect to important factors involved in the development of diseases. The goals of study design are to find the population of interest, to choose selection criteria, which will correspond to the goal of the study, and to identify the necessary statistical power to address the hypothesis. Proper selection of the criteria will generate a dataset with lower informational noise and with fewer biases towards known confounders.

The tools for practical experimental design were developed to help researchers. In a power analysis one may consider parameters such as sample size, disease prevalence, gender influence and also unknown parameters such as effects from environmental factors, linkage disequilibrium level, etc. Clearly, a large effect with rare risk factor will be easier to detect in association with the disease in a sample of small size. On the other hand, a small effect from common risk factors will need a much larger sample size, the typical situation for common complex diseases.

1.1.1.1 Family linkage study

A family study is designed to collect participants from several pedigrees with similar phenotypes and to take advantage of phenotype heterogeneity, due to gene segregation in offspring. This genetic analysis can use the candidate gene approach or cover the entire genome in the search for genetic risk factors.

Familial linkage studies have been successful in mapping genes for rare Mendelian diseases. However, linkage studies can usually only locate loci, which may cover several megabases of DNA. Therefore, the second step should be association studies to identify the frequency of specific markers within these loci in cases and controls. It is important to be aware that the affected familial members may share the same environment factors that might interact with genetic factors in the development of disease. Linkage analysis in families could be based on comparisons of genotypes and phenotypes between parents and offspring, or between siblings only.
1.1.1.2 Twin study
In general, twin studies are a special kind of family study. The twins may be monozygotic and share 100% of their genetic variations, and most of the environment because they have developed from only one fertilized egg. The twins may also be dizygotic, and come from two fertilized eggs, and share most of the environment, and at least 50% of their genetic variations. The discrepancy between genotypes and phenotypes in monozygotic and dizygotic twins illustrates the effects of genetic and environmental variations on the disease development. At the moment, there are very few twin studies of glomerulonephritis, with very low numbers of observations.

1.1.1.3 Cohort study
Cohort studies employ data for a subset of individuals from the population of interest with phenotype-related observations during a certain period of time. In practice, the most common aim of a cohort study, besides measure of risk due to studied factors, is estimation of an incidence rate or prevalence. Performing cohort studies is time consuming and expensive, and in the case of rare events it may not be very efficient, since statistical power will be relatively low.

1.1.1.4 Case-control association study
The aims in the case-control study are similar to in cohort studies but it is more cost-effective because of a simpler sampling procedure. This type of study includes separately collected cohorts of cases and controls. When case-control studies have a nested design or are carried out within a well-defined cohort, it is the most efficient approach to address associations between phenotypes and genotypes in complex diseases. In fact, the majority of genetic association studies at the moment were designed as case-control studies.

1.1.1.5 Genome-wide association study (GWAS) and candidate gene approach
Both linkage studies in families and association studies in cohorts are valuable approaches for detecting genetic risk factors for complex diseases. The family studies are based on the assumption of relatedness of genetic factors between different families, and these families have different rate of individuals with and without disease. In association studies the comparison groups usually consist of unrelated individuals, selected with certain criteria, likely from the same geographical population. Regarding the selection of genetic markers, it could be done in the narrowest way in a certain part of the genome, usually in a region and close to the gene of interest (candidate gene approach) or in a genome-wide manner, when multiple markers are selected across the genome with a certain density. The requirements for these two types of studies are different. Modern technology permits simultaneous genotyping of hundreds of thousands of markers. However, due to massive multiple comparisons, the significance threshold is very low in comparison with univariate associations and only very strong effects could be detected unambiguously in a single study. Genome-wide design gives a unique opportunity to perform “hypothesis-free” studies in the absence of any knowledge about the biology of the disease. On the other hand, the candidate gene approach requires less stringent significance levels, but the selected candidate genes
Background

should have strong biological evidences in the development of specific disease\(^5\). The size of the study could be determined by the statistical power needed to detect risk factors and ideally both GWAS and candidate gene studies demand replication in the independent cohorts.

**Table 1: Strategies and objectives for genetic studies of complex diseases**

<table>
<thead>
<tr>
<th></th>
<th>Case-control studies</th>
<th>Linkage studies</th>
</tr>
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<tbody>
<tr>
<td><strong>Objective</strong></td>
<td>Identify common susceptibility variants underlying disease</td>
<td>Identify a single gene with large effect on phenotype</td>
</tr>
<tr>
<td><strong>Assumptions</strong></td>
<td>Common variants confer susceptibility in the general population</td>
<td>Rare variants with large effect confer disease susceptibility to rare patients</td>
</tr>
<tr>
<td><strong>Cohorts</strong></td>
<td>Singleton patients and controls</td>
<td>Members of rare families with Mendelian forms of disease</td>
</tr>
<tr>
<td><strong>Targets</strong></td>
<td>Genome-wide or candidate gene analysis</td>
<td>Genome-wide or candidate gene analysis</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Examination of a multifactorial trait possible</td>
<td>Causal evidence often obtained</td>
</tr>
<tr>
<td></td>
<td>Findings will apply to the general population</td>
<td>Disease-relevant biological pathways could be identified</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Large cohorts required</td>
<td>Identification of rare families required</td>
</tr>
<tr>
<td></td>
<td>Clinical significance of statistical association might be unclear</td>
<td>Findings may only apply to rare patients or families</td>
</tr>
</tbody>
</table>

Adapted from Beerman I. et al.\(^7\)

**1.1.2 Genetic markers in human genome**

Genetic variance is a fundamental feature of the genome, and is essential for understanding connections between the genotype and the phenotype. The genetic differences among individuals can be in the number of gene copies, microsatellites (short repeated sequences), insertions/deletions, and single nucleotide changes. Only a minority of these variations is functionally important, but due to the linkage disequilibrium between functional and non-functional polymorphisms, any genetic variation could be used as a marker for a study. Due to the progress of human genome analysis during the last decade, several millions of genetic variations have become available for association studies and for comparisons of different populations.

**1.1.2.1 Copy number variations (CNVs)**

Historically, CNVs were first discovered during a study of karyotypes in 1959 by Jérôme Lejeune\(^8\), and became very important for the clinical diagnosis of diseases such as Down’s syndrome, and particularly cancer. Because of urgent requirements in this area, the methods for CNV detection in cancer were developed quickly and became very accurate. Today, array-based comparative genomic hybridization is able to detect small CNVs in a normal population\(^9\). While in tumours they represent non-inheritable, somatic changes, there are plenty of CNVs in a germline DNA with a usual inheritance pattern. In these cases CNVs may represent stretches of DNA larger than 1000 bp,
which are different with respect to the numbers of copies normally found in the general population. Furthermore, CNVs may cover the entire gene, thus influencing protein expression and overall function of the complex phenotype, a phenomenon known as “gene dosage”10, 11.

1.1.2.2 Variable number of tandem repeats (VNTR)
VNTR is a short nucleotide sequence (at least two nucleotides in a row), which may be of different length in individuals (Figure 1). Identification of VNTR, or microsatellite alleles, at different locations is a standard procedure used in forensics for personal identification by DNA profile. These markers are distributed all over the genome, but not regularly, and can be used for low-resolution genome-wide screening.

Figure 1: SNP represents a difference in a single nucleotide between individuals and is usually biallelic. VNTR varies in number of repeats (TAC in this example) and differs between individuals in its length, representing multiallelic variation.

1.1.2.3 Single nucleotide polymorphisms (SNPs)
Single nucleotide changes are found in all parts of the genome and may be either neutral or functional. A SNP may affect protein structure, when it is in exon, transcription activation, if it is in the promoter region, alternative splicing, when it is in intron, or mRNA stability, when it is in the 3′- or in 5′-UTR regions (untranslated regions). Previously, the main focus was on non-synonymous SNPs, which change the codon and affect aminoacid composition. Most GWAS studies of common diseases
have not shown an association with SNPs in exons, however. Undoubtedly, SNPs, are the most abundant and useful genetic markers, with roughly one variant in every 100-1000 bp, with a total number of approximately 12 million in the human genome.\textsuperscript{12-14} Due to the relatively easy methodology for detection of SNPs, these variations are in use for different types of genetic studies, from locating a single individual mutation to genome-wide screening in association studies. It is possible to use a combination of SNPs for personal identification and this profile as the foundation for a new generation of studies in population genetics.

### 1.1.3 Genotyping methods

The methods for detection of different variations in the human genome are usually specific to certain types of variations. Historically, these methods were first developed in relation to certain practically relevant phenotypes; later, more general methods for basic research were introduced.

#### 1.1.3.1 Methods for detection of chromosomal abnormalities

About 1 in 500 individuals in a population is a carrier of a balanced chromosome rearrangement and most of these carriers do not have an abnormal phenotype. However, if both healthy parents carry the chromosome abnormality, it may increase the risk for abnormalities in their children. Particularly, an imbalanced “gene dosage” in a chromosome may influence a specific phenotype.\textsuperscript{15}

Several molecular and cytogenetic methods are used to detect abnormal chromosomes, such as GTG-binding, fluorescence \textit{in situ} hybridization (FISH), Spectral Karyotyping/multicolor FISH (M-FISH), Multiplex Ligation-dependent Probe Amplification, and copy number analysis by hybridization at microarray. Each method usually has both advantages and limitations.\textsuperscript{15}

#### 1.1.3.2 Detection of Copy Number Variations

Quantification of the number of copies for CNVs with > 1000 bp was for a long time based on low throughput methods, like restriction endonuclease mapping. The progress in this area came from implementation of comparative hybridization (CGH) methods based on arrays for the whole genome or for selected loci. Due to generation of SNP-based genotyping data, additional analysis of stretches of homozygosity was employed to detect CNVs. Previously, low resolution array platforms were used, but with the development of new technologies, medium to high resolution array platforms were applied in these analyses.\textsuperscript{14} However, this approach remains complementary, since it is not based on direct detection of CNVs. Targeted arrays for known CNVs were also successfully applied, with occasional discovery of new CNVs.\textsuperscript{14} Recently, qPCR-based techniques, similar to the original TaqMan PCR, were introduced for all known genes and are now available from Applied Biosystems (Life Technologies).

Re-sequencing is the best, but relatively expensive, technique and it could suit small sample sizes. The Fosmid End Mapping and Paired End Mapping were applied to
detect small CNVs. In general, detection of CNVs in disease association studies is a growing area with many new findings anticipated in the near future.

1.1.3.3 Whole-genome genotyping arrays
Rapid development in our knowledge of the human genome and new technical solutions made it possible to develop arrays for identification of massive numbers of genetic variants simultaneously in a single experiment. The absolute cost for such experiments remains relatively high, but since hundreds of thousands or millions of variations could be detected in few nanograms of DNA, the relative cost for genotyping is very low and allows generation of enormous sets of data, without pre-selection of genetic loci to study. Two commercial platforms are available: Affymetrix arrays based on synthetic oligonucleotides and Illumina beadchips. There are similar arrays with more specific collection of variants (SNPs and CNVs) across the genome based on preliminary selection of genes related to certain types of disease, like metabolic disorders, cardio-vascular diseases and autoimmune diseases, with dense coverage of genetic loci.

The next step in these developments would be whole-genome sequencing. The value of the whole-genome sequencing is in the possibility of providing the most complete genetic variance at individual level.

1.1.3.4 Genotyping of Single nucleotide polymorphisms
Developing new methods for the detection of SNPs has been a major focus in genetics during recent years. These methods are all based on the progress and reagents for polymerase chain reaction, and use different modifications of this technique. Historically, allele-specific PCR, Restriction fragment length polymorphisms (RFLPs) or Restriction endonuclease mapping and Allele-specific hybridization were employed first, followed by many other methods, including Invasive oligonucleotide cleavage, Oligonucleotide ligation assay and Primer extension assays etc.

Different genotyping platforms provide different capacities regarding the number of simultaneously tested variants (SNP throughput) and the number of DNA samples (sample throughput) in a single experiment. There are several choices: Small scale: 10 SNPs-100 individuals, Medium scale: 10 SNPs-1000 individuals, Medium scale: 100 SNPs-100 individuals and Large scale: 100 SNPs-1000 individuals. Selection of the method is also dependent on the experimental design and the availability of multiple DNA samples. TaqMan allelic discrimination assays have become one of the standard solutions for high sample throughput genotyping. However, since specificity of allelic discrimination is based on probe hybridization, genotyping of SNPs with the TaqMan technique in loci with repeats and with close multiple variants, remains problematic.
1.1.4 Functional genetics: investigation of gene polymorphism influence on phenotype

Genetic association and linkage studies have been successful in the detection of genetic loci, which correlate with disease or any disease-related subphenotype. However, due to low resolution of genotyping (when only a few known variants are investigated), infrequent study of rare variants and non-homogeneous linkage disequilibrium patterns in human genome, it is very rare for such studies to find definite functional variations to explain disease phenotypes. Environmental factors introduce additional complications and the picture becomes an intricate network of interaction from both known and unknown parameters.

It is generally accepted that most genetic variants are neutral and do not influence any biological function. But those that generate phenotype heterogeneity may illuminate disease pathways and may help to develop a target for individual therapy. There is a wide spectrum of biological activities connected with genetic polymorphisms.

Previously identified functional contexts for sequence variations are available from reference sequence (RefSeq) and GenBank mRNAs, including nine dbSNP variation functional classes: locus region, coding, coding-synonymous, coding-non-synonymous, mRNA-UTR, intron, splice-site, contig-reference, and coding-exception. A variance may have different functional relationships to the gene that has multiple transcripts or to local gene neighbours, if multiple genes are in a contig region. The position of the variation may influence transcription activation (in the promoter region), splicing mechanisms (in intron) and mRNA stability (in 3’ or 5’UTR), while exonic non-synonymous SNPs affect the protein structure. Most of these relations are annotated for SNPs and short indels, while long stretches of CNVs will most likely generate natural knockouts, best known for the Glutathione S-transferase Mu 1 (GSTM1) gene.

Table 2: Useful annotation resources for characterization and hypothesizing of SNP function

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<th>Protein</th>
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Adapted from Mooney, S.D. et al.
1.2 IGA NEPHROPATHY

1.2.1 Clinical presentation

IgA nephropathy is a complex disorder with disease manifestations ranging from mild mesangial matrix expansion and hematuria to rapidly progressive glomerulonephritis with endocapillary proliferation and crescent formation\textsuperscript{25}. Patients with IgA nephropathy usually present at a relatively young age, although any age group may be affected. Patients have frequently experienced symptoms over a long period of time before a definitive diagnosis is made. Recurrent macroscopic hematuria, often accompanied by loin pain and constitutional symptoms, and occurring within 48 hours of an upper respiratory tract, or other, infection, is a classic feature of IgA nephropathy and is seen in over 40\% of patients. Persistent microscopic hematuria is present in the majority of patients, often accompanied by proteinuria and, when specifically looked for, increased numbers of white blood cells and casts on centrifuged urine microscopy\textsuperscript{26}. Albuminuria of more than 1 gram per day is present in nearly 50\% of patients and exceeds 3 gram per day in approximately 10\% of patients. An increased serum creatinine at presentation is found in approximately 1/3 of patients and severely impaired renal function at presentation occurs in about 5\% of patients. About 25\% of patients have hypertension at presentation and another 25\% develop hypertension over time.

1.2.2 Definition

Although an increased serum IgA/C3 ratio, combined with more than five red blood cells per high-power field on urinalysis, persistent proteinuria of more than 0.3 gram per day, and a serum IgA level greater than 315 mg/dl, can distinguish many IgA nephropathy patients from those with other forms of glomerulonephritis, a renal biopsy remains the standard for diagnosis\textsuperscript{27}. A renal biopsy should be examined at least by light microscopy and by immunofluorescence microscopy for identification of mesangial IgA deposits. Light microscopy findings range from mild mesangial proliferation, to a more diffuse pattern with focal segmental scarring, to crescentic glomerulonephritis. Immunohistological features include mesangial polymeric IgA1, frequently accompanied by C3, and IgG or IgM, or both and C5b-9\textsuperscript{28}. 
Table 3: Definition of pathological variables used in the classification of IgA nephropathy according to Cattran, D.C. et al.29.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial hypercellularity</td>
<td>&lt;4 Mesangial cells/mesangial area=0</td>
<td>M0≤0.5</td>
</tr>
<tr>
<td></td>
<td>4–5 Mesangial cells/mesangial area=1</td>
<td>M1&gt;0.5(a)</td>
</tr>
<tr>
<td></td>
<td>6–7 Mesangial cells/mesangial area=2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;8 Mesangial cells/mesangial area=3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The mesangial hypercellularity score is the mean score for all glomeruli</td>
<td></td>
</tr>
<tr>
<td>Segmental glomerulosclerosis</td>
<td>Any amount of the tuft involved in sclerosis, but not involving the whole tuft or the presence of an adhesion</td>
<td>S0 – absent</td>
</tr>
<tr>
<td>Endocapillary hypercellularity</td>
<td>Hypercellularity due to increased number of cells within glomerular capillary lumina causing narrowing of the lumina</td>
<td>E0 – absent</td>
</tr>
<tr>
<td>Tubular atrophy/interstitial fibrosis</td>
<td>Percentage of cortical area affected by tubular atrophy or interstitial fibrosis, whichever is greater</td>
<td>0–25% – T0</td>
</tr>
</tbody>
</table>

(a) Mesangial score should be assessed in periodic acid-Schiff-stained sections. If more than half the glomeruli have more than three cells in a mesangial area, this is categorized as M1. Therefore, a formal mesangial cell count is not always necessary to derive the mesangial score.

Picture 1: Images from a male IgA nephropathy patient, 29 years old. The light microscopy picture (1a) of a glomerulus showed mesangial expansion and deposition of IgA in the glomerular mesangium was demonstrated by immunofluorescence (1b).
1.2.3 Epidemiology of IgA nephropathy

IgA nephropathy remains the most predominant form of primary glomerulonephritis. It is frequent in Caucasian and Asian populations, in contrast to its apparent rarity in African populations, especially in African Americans\(^{30}\).

The incidence of IgA nephropathy has been reported from different regions and is estimated in France to be between 26 and 30 new cases per million population, in Japanese children 45 cases per million population, and in the United States 12 cases per million population\(^{31, 32}\). The incidence is influenced by the renal biopsy policy and whether it is liberal in cases of isolated microscopic hematuria or restricted to patients with albuminuria greater than 1 gram per day. The male predominance of IgA nephropathy is well established in Europe and America, but appears not to be so characteristic in Asian countries.

1.2.4 Pathogenesis of IgA nephropathy

A number of factors can contribute to the development of IgA nephropathy, and the extent to which each is operational determines the severity and outcome of the disease. Examples of such factors are the synthesis and release into the circulation of polymeric IgA1, with characteristics that favour mesangial deposition. Another important factor is the responsiveness of the glomerular mesangium in dealing with the inflammatory response by resolution of inflammation, rather than ongoing sclerosis. Another important element is the tendency of the whole kidney to respond to injury by mounting a response-favouring progressive renal failure, including hypertension, proteinuria, tubular atrophy and interstitial fibrosis. It is also important to remember that the disease entity called IgA nephropathy is defined by a pattern of glomerular morphology, and it cannot be assumed that it will eventually prove to be one entity, with a single pathogenic mechanism. It seems more probable that several mechanisms or combinations of mechanisms can produce IgA nephropathy. The pathophysiologic mechanisms that predispose to the deposition of IgA complexes in the glomerular mesangium are largely unknown. Elevated levels of IgA1 and IgA-containing circulating complexes are found in sera of most patients with IgA nephropathy\(^{33, 34}\). Recent studies have demonstrated that polymeric IgA complexes with reduced glycosylation at the hinge region of the molecule, exhibit higher avidity for binding to mesangial cells. This reduction in glycosylation at the hinge region not only facilitates formation of polymeric IgA1, but also contributes to its recognition as a neoantigen and the subsequent formation of IgG autoantibodies against Gal-deficient IgA1 molecules. Gal-deficient IgA1 or IgG autoantibodies against IgA1, bind to CD89 (FcR) expressed on the surface of mesangial cells, leading to production of pro-inflammatory cytokines, recruitment of circulating cells, and overt inflammation\(^{34}\).
1.2.5 Genetic predisposition to IgA nephropathy

Genetic factors are likely to influence the pathways contributing to the pathogenesis of IgA nephropathy. Familial forms of IgA nephropathy are common in some regions. Several genetic loci are strongly associated with IgA nephropathy, usually as an autosomal-dominant trait with highly variable penetrance. Population studies have failed to show a consistent association with any single genetic marker, suggesting that IgA nephropathy does not have classic Mendelian inheritance attributable to a single gene locus, but is a complex polygenic disease probably involving both major histocompatibility complex (MHC) and non-MHC susceptibility alleles. Most population studies in IgA nephropathy have been relatively small case-control genetic association studies, examining single nucleotide polymorphisms (SNPs) in single candidate genes. Genome-wide linkage analysis has demonstrated linkage of IgA nephropathy to 6q22-23. Two further loci (4q26-31 and 17q12-22) have also been linked to familial IgA nephropathy.

1.2.6 Natural history of IgA nephropathy

Early reports of the natural history of IgA nephropathy demonstrated an overall benign course, with approximately 10% of patients reaching end stage renal disease (ESRD) within 10 years. Recent studies have, however, shown that approximately 20-25% of patients with IgA nephropathy will have developed ESRD when followed up after 10 years. Clinical risk factors linked to progressive disease include presence of hypertension, proteinuria >1.0 gram per day, male gender and persistent microscopic hematuria. Severe histopathologic lesions, such as the presence of glomeruli with focal or segmental glomerulosclerosis or hyalinosis and/or with crescents are additional markers of progression. Other risk factors are hypertriglyceridaemia, overweight/obesity and age at onset.

Figure 2: The 18 amino acids specific for the hinge region of human IgA1 (not in IgA2). The upper formula shows the undergalactosylation of IgA nephropathy patients and the lower displays the normal galactosylation of IgA1 in healthy subjects. Adapted from Beerman I. et al.
1.2.7 Treatment of IgA nephropathy

The management of IgA nephropathy has advanced over recent decades, largely as the result of randomized clinical trials. For patients at risk of developing ESRD, the two most critical goals of treatment are to control blood pressure rigorously, preferably with an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin II receptor antagonist (ARB), or both, and to reduce albuminuria to less than 500 mg per day. If these two goals can be met without undue side effects, and if the patient remains compliant in the long term, many patients can avoid advanced chronic kidney disease. Patients who cannot achieve these goals despite vigorous attempts, become candidates for adjunctive therapy with prednisone or cytotoxic drugs combined with prednisone.

Tonsillectomy as a treatment is controversial. While no controlled studies have been performed to date, observational studies suggest a higher rate of clinical remission with tonsillectomy, than with steroid treatment alone. Omega-3 fatty acids may also be considered as an add-on therapy, particularly for patients with heavy proteinuria and reduced glomerular filtration rate (GFR).45-48.

1.3 LUPUS NEPHRITIS

1.3.1 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune rheumatic disease. The disease is characterized by involvement of various organs including joints, skin, and central nervous system, together with immune complex-mediated glomerulonephritis (Lupus nephritis), combined with the presence of autoantibodies against a series of nuclear antigens. Lupus nephritis is a serious organ manifestation and affects approximately one third of the patients with SLE. Genetic factors, hormones, cytokines and other signaling molecules, and cellular changes in the immune system are of importance, both for the initiation and perpetuation of the disease.

1.3.2 Epidemiology of SLE

The prevalence of SLE varies in different geographic areas and ethnic groups, and is higher in patients with African and Asian ancestry.49, 50. The annual incidence of SLE varies between different regions and is estimated to be 4.8/100,000 habitants in Southern Sweden.51 In the UK, the annual incidence of SLE was reported to be 6.5/100,000 in women and 1.5/100,000 in men.52

The disease is more prevalent in females (9:1 female: male ratio) and disease onset is usually observed in ages younger than 40 years. Despite the over-representation of women suffering from SLE, Lupus nephritis is highly prevalent in men with SLE, and men also develop severe renal disease more often compared to female patients.53

The diagnosis is based on classification criteria for SLE and shown in detail below (ACR 1982).54
Table 4: Classification criteria for SLE according to Tan et al.54.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malar rash</td>
<td>Fixed erythema, flat or raised, over the malar eminences</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Non-erosive arthritis</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>a) Pleuritis OR b) Pericarditis</td>
</tr>
</tbody>
</table>
| 7. Renal disorder  | a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed, OR  
                           b) Cellular casts—may be red cell, haemoglobin, granular, tubular, or mixed |
| 8. Neurologic disorder | a) Seizures, OR b) Psychosis                                                 |
| 9. Hematologic disorder | a) Hemolytic anemia, OR b) Leukopenia, OR c) Lymphopenia, OR d) Thrombocytopenia |
| 10. Immunologic disorder | a) Positive LE cell preparation, OR b) Anti-DNA, OR c) Anti-Sm, OR d) False positive serologic test for syphilis |
| 11. Antinuclear antibody | An abnormal titre of antinuclear antibody, ANA                        |

* The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

1.3.3 Clinical presentation

Proteinuria has been reported to be present to various degrees in all Lupus nephritis patients and up to 45-65% have the nephrotic syndrome at presentation. The presence of microhaematuria is approximately 80% but macrohaematuria seldom occurs.55, 56 SLE patients who develop nephritis usually have increased levels of anti-dsDNA antibodies and low complement levels of the classical cascade.56

1.3.4 Pathogenesis of Lupus nephritis

Lupus nephritis is characterized by glomerular immune deposition with the presence of immunoglobulins and complement components in kidney biopsy specimens. The etiology of this deposition is still unknown; however, antibodies specific to dsDNA, Sm, SSA, SSB, and C1q have been shown in glomerular despositions of proliferative Lupus nephritis.57-59

Immune complex depositions in the glomeruli attract inflammatory cells such as monocytes, macrophages or lymphocytes that in turn may mediate further tissue damage. This may influence the filtration process in the glomeruli and lead to kidney dysfunction.56
1.3.5 Genetic predisposition to SLE and Lupus nephritis

Genetic factors have been shown to have an important role for development of SLE. The evidence for genetics being involved in the pathogenesis of disease has been demonstrated from family studies\textsuperscript{60-62} and twin studies\textsuperscript{63}. The results from a twin study in which 107 SLE twin pairs were included indicated a higher disease concordance of monozygotic twins (24\% of 45) compared to dizygotic twins (2\% of 62 pairs)\textsuperscript{64}. Apart from results obtained from twin studies, different approaches such as candidate gene association, linkage analysis and GWAS studies have been carried out and several important genetic risk factors were discovered for SLE development.

According to current understanding, the genetic locus with the strongest association with SLE is located at the HLA locus on chromosome 6p21.3. Previously, HLA was shown in association with numerous autoimmune diseases and specifically in SLE the class II \textit{HLA-DRB1}, allele *03 with strong linkage disequilibrium with the null alleles for C4A was reported; additionally, *15 allele was implicated\textsuperscript{65, 66}. However, several hundred genes are located in HLA class I, II and III loci, with the majority of them are of importance for the immune system\textsuperscript{67, 68}. By using a family-based approach in 314 SLE trios, two independent associations with SLE, \textit{HLA-DRB1}\*0301 in class II and rs419788 in intron 6 of class III were recently demonstrated in a Caucasian population\textsuperscript{69}. The risk for development of lupus is also substantially increased in patients deficient for components of the early part of the classical complement pathway, including C2, C4A, C4B, and C1q\textsuperscript{65, 66, 70-73}.

During the last two decades, new candidate genes associated with susceptibility to SLE have been identified, including \textit{IRF 5}\textsuperscript{74-76}, \textit{STAT4}\textsuperscript{77}, the programmed cell death 1 gene \textit{(PDCD1)}\textsuperscript{78} and B-cell scaffold protein with ankyrin repeats 1 gene (\textit{BANK1})\textsuperscript{79}. The genetic locus C8orf13-BLK, integrin alpha M (\textit{ITGAM}) and integrin alpha X (\textit{ITGAX}) variants have also been confirmed in association to SLE\textsuperscript{80}.

Among the identified susceptibility genes for SLE, very few genes have also been found to associate with the development of Lupus nephritis. The programmed cell death 1 gene (\textit{PDCD-1}) in particular in PD-1.3A variant, showed an association with Lupus nephritis in European patients\textsuperscript{81}. Genes encoding for \textit{ITGAM} have also been found to associate with Lupus nephritis patients in both a European population study\textsuperscript{82} and an Asian ancestry study\textsuperscript{83}.

However, the role of the identified genes in determining severity or long-term prognosis has not been investigated.

1.3.6 Renal biopsy and classification of Lupus nephritis

Renal biopsy including routine examination by light microscopy, immunofluorescence, and electron microscopy is necessary for diagnosis and is of help in selecting appropriate therapy for Lupus nephritis patients. To classify the renal pathologic lesions, the World Health Organization (WHO) has presented classification criteria,
Background

first published in 1974 and revised in 1982\textsuperscript{84} and 1995\textsuperscript{85}. Since 2003, a new classification from the International Society of Nephrology/Renal Pathology Society (ISN/RPS)\textsuperscript{86} has been applied for classification of Lupus nephritis.

\textbf{Picture 2:} Lupus nephritis class V+ III an otherwise typical membranous nephropathy displays focal mesangial expansion and hypercellularity (left PASM, right PAS) (x400).

Reproduced from Giannakakis, K. Histopathology of Lupus nephritis. 2010. Clinic Rev Allerg Immunol\textsuperscript{87}, with the permission from Springer. Licence number 2544280404602.

\textbf{Picture 3:} Double immunofluorescence staining with antibodies against IgG (red) and C3 (green); confocal microscopy analysis of the immune deposits and 3D rendering. The outer surface of the deposits is made mostly of C3; (left side of the picture deposits seen from above, right side vertical view) (x600).

Reproduced from Giannakakis, K. Histopathology of Lupus nephritis. 2010. Clinic Rev Allerg Immunol\textsuperscript{87}, with the permission from Springer. Licence number 2544280404602.
Picture 4: Lupus nephritis class IV direct immunofluorescence double staining with FITC-conjugated antibodies against C3c (green) and TRITC-conjugated antibodies against IgG (red). In picture 4a, the combined signal is seen; picture 4b shows the C3 deposition and picture 4c shows the IgG deposition. The co-localization of immunoglobulins and complement is evident (x600).

### Table 5: Classification from ISN/RPS

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td><strong>Minimal mesangial Lupus nephritis</strong></td>
<td>Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence</td>
</tr>
<tr>
<td>Class II</td>
<td><strong>Mesangial proliferative Lupus nephritis</strong></td>
<td>Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits.</td>
</tr>
<tr>
<td>Class III</td>
<td><strong>Focal Lupus nephritis(a)</strong></td>
<td>Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving ≤50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations</td>
</tr>
<tr>
<td>Class III (A)</td>
<td>Active lesion: focal proliferative Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class III (A/C)</td>
<td>Active and chronic lesions: focal proliferative and sclerosing Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class III (C)</td>
<td>Chronic inactive lesions with glomerular scars: focal sclerosing Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class IV</td>
<td><strong>Diffuse Lupus nephritis(b)</strong></td>
<td>Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) Lupus nephritis when ≥50% of the involved glomeruli has segmental lesions, and diffuse global (IV-G) Lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft.</td>
</tr>
<tr>
<td>Class IV-S (A)</td>
<td>Active lesions: diffuse segmental proliferative Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class IV-G (A)</td>
<td>Active lesions: diffuse global proliferative Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class IV-S (A/C)</td>
<td>Active and chronic lesions: diffuse segmental proliferative and sclerosing Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class IV-S (C)</td>
<td>Chronic inactive lesions with scars: diffuse segmental sclerosing Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class IV-G (C)</td>
<td>Chronic inactive lesions with scars: diffuse global sclerosing Lupus nephritis</td>
<td></td>
</tr>
<tr>
<td>Class V</td>
<td><strong>Membranous lupus nephritis</strong></td>
<td>Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations Class V Lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed</td>
</tr>
<tr>
<td>Class VI</td>
<td><strong>Advanced sclerosis Lupus nephritis</strong></td>
<td>≥90% of glomeruli globally sclerosed without residual activity</td>
</tr>
</tbody>
</table>

(a) Indicate the proportion of glomeruli with active and with sclerotic lesions
(b) Indicate the proportion of glomeruli with fibrinoid necrosis and/or cellular crescents Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.
1.3.7 Treatment of Lupus nephritis

The recommended therapy for severe Lupus nephritis is high dose of corticosteroids in combination with cytotoxic drugs as induction therapy, followed by maintenance therapy given for longer periods of time. The alkylating agent cyclophosphamide (CYC) has for many years been the gold standard of therapy, either given as a monthly high-dose regimen or a low dose given every other week for shorter periods of time. More recently, mycophenolate mofetil (MMF; Cellcept) has become more widely used, although randomized controlled studies have failed to prove superiority compared to cyclophosphamide. Cyclosporin A (CyA) has also been shown to reduce proteinuria and preserve kidney function, but is not generally used for induction therapy.

Therapies targeting B cells have in recent years been used in the treatment for Lupus nephritis. The anti-CD20 antibody, rituximab, has in uncontrolled studies shown beneficial results, although preliminary reports from randomized controlled studies in Lupus nephritis have failed to meet the primary endpoint.

Even though advances in treatment have been achieved, around 10-30% of Lupus nephritis patients progress to ESRD. As in other cases of ESRD, the renal replacement therapies available are haemodialysis, peritoneal dialysis or renal transplantation.
2  AIMS OF THE STUDY

To identify biomarkers, including genetic markers, for susceptibility and severity of IgA nephropathy and to compare these with findings in patients with Lupus nephritis.

More specifically, the goals were to test a set of genes immunologically relevant to the IgA pathways of the immune system and to assess related proteins:

- Transforming growth factor-β1 (TGFβ1), regulator of B cell isotype switching;
- Fc-alpha receptor (FCAR/CD89), cellular and soluble receptor for IgA;
- STAT4 (signal transducer and activator of transcription 4), T cell transcription factor involved in T cell contribution in isotype switching regulation;
- IRF5 (interferon regulatory factor 5);
- TRAF1 (TNF receptor-associated factor 1);
- HLA-DRB1 (major histocompatibility complex, class II, DR beta 1);
- Mannose-Binding Lectin (MBL2), complement activating protein involved in deposition of IgA complexes in kidney.
3 PATIENTS AND HEALTHY CONTROLS

3.1 IGA NEPHROPATHY COHORT

Two hundred and twelve unrelated patients (146 males and 66 females), mean age 38.5±14.4 (range 17 to 77 years) with biopsy-proven IgA nephropathy and 477 healthy Swedish Caucasians (321 males and 156 females), mean age 44.8 ± 13.0 (range 18 to 80 years) were included in the study.

The patients were recruited from the Department of Nephrology at the Karolinska University Hospital (n=117), Danderyd Hospital (n=31), Skövde Hospital (n=36) and Linköping Hospital (n=28), representing mainly a population from the central part of Sweden.

Patients with Henoch-Schönlein purpura and other forms of glomerulonephritis were not included in the cohort.

3.2 SYSTEMIC LUPUS ERYTHEMATOSUS COHORT

A total of 579 participants were included in the study. The cohort of patients with SLE, consisted of 272 SLE patients, all self-reported Caucasians from 18 to 80 years of age (mean age 45±14 years). 106 SLE patients had biopsy-proven nephritis (39%) and 166 SLE patients had no clinical or laboratory signs of nephritis (61%). The control group for SLE patients consisted of 307 healthy age- and gender- matched individuals from the same population in Sweden, who were 17 to 70 years old, mean age 44±13 years.

3.3 HEALTHY CONTROLS FOR HLA STUDY

1569 healthy controls were included in the HLA study, coming from the Epidemiology Investigation of Rheumatoid Arthritis (EIRA) study.

3.4 ETHICAL APPROVAL

All included individuals gave informed consent. The studies were approved by the Ethics Committee of the Karolinska University Hospital, and/or Regional Ethics Committee of Stockholm, Sweden.
4 METHODOLOGY

4.1 DNA EXTRACTION AND QUALITY CONTROL

High molecular weight DNA was extracted from whole EDTA blood samples or from nucleated cell fraction after blood centrifugation by the “salting-out” method. The concentration and quality of DNA solution was assessed by optical density at 260/280 nm with spectrophotometer DU600 series (Beckman Coulter, Bromma, Sweden). All solutions were normalized to 30 ng/ul (for HLA typing and restriction endonuclease mapping) or to 2 ng/ul (for TaqMan allelic discrimination). For TaqMan genotyping DNA samples were prepared in 384-well plates, 5 ul per well.

4.2 THE TAQMAN ALLELIC DISCRIMINATION

Allele-specific TaqMan assays (Applied Biosystems, Foster City, USA) were used to detect most of the investigated SNPs in our study. Genotyping was performed according to the standard protocol in 384-well plates with 10 ng of DNA per sample. PCR was run in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, U.S.A.) and the fluorescent signals from the hybridization probes were detected by a 7900 Sequence Detector (Applied Biosystems, Foster City, USA). Positive detection rates were between 99.3 and 99.9% for different SNPs.

![Figure 3: TaqMan SNP Genotyping Assay (Applied Biosystems)](image_url)

V: VIC dye fluorescence indicates homozygosity (AA) F: FAM dye fluorescence indicates homozygosity (GG). Simultaneous fluorescent signals indicate heterozygosity (AG) (A) Probe for A allele; (G) Probe for G allele.
4.3 THE RESTRICTION ENDONUCLEASE MAPPING

The restriction endonuclease mapping method was used to detect Codon 10 and Codon 25 polymorphisms in the TGFBI gene\(^6, 7\). Positive rates of genotype detection were 99.6% for codon 25 and 100% for codon 10 in our experiments. PCR was performed with two primers built on the flanking region for the SNP of interest. For codon 25 the upper primer for the generation of 317 bp fragments from the TGFBI gene was GCCTCCCGATGCCGCCCTCCGGGCTGCGGCGGCGGC and the lower was GTAGTCGGCCTCAGGCTCGGGCTCC\(^7\). The codon 25 polymorphism of the TGFBI gene was recognised by Bgl II digestion of amplified fragments with generation of 243, 60 and 14 bp bands for homozygote GG, 303, 243, 60 and 14 bp bands for heterozygote CG and 303 and 14 bp bands for homozygote CC. These bands were identified by electrophoresis in polyacrylamid gel with silver staining.

For codon 10 polymorphism of TGFBI gene, the PCR forward primer was TTCCCTCGAGGCCCTCCTCTTA and the reverse primer was
Methodology

GCCGCAGCTTGGACAGGATC\textsuperscript{96}. The PCR products were digested with MspAI (10,000 units/ml) (New England Biolabs, Hitchin, U.K), run on Novex TBE gels (Invitrogen, CA, USA), and then visualized. The TT homozygotic state yields fragments of 161, 67, 40, and 26 bp; the CC homozygotic state yields fragments of 149, 67, 40, 26, and 12 bp; the CT genotype simultaneously yields fragments of 149 and 161, which specify heterozygotic state.

![Diagram of the restriction endonuclease (RE) mapping workflow]

**Figure 5: The restriction endonuclease (RE) mapping workflow**

4.4 THE MULTIPLEX SNPSTREAM

The fluorescent single base extension using the multiplex SNPstream system (Beckman Coulter Inc) was used in genotyping of seven SNPs in the IRF5 gene in our study, with a positive rate more than 97%. This analysis was performed at the Department of Medical Sciences, Uppsala University, Sweden, in collaboration with Professor Ann-Christine Syvänen.

4.5 POLYMERASE CHAIN REACTION WITH SEQUENCE SPECIFIC PRIMERS FOR HLA TYPING

SSP-PCR (Sequence specific primers PCR) HLA-DR low resolution was used in our study, according to the producer’s protocol (Olerup SSP, Saltsjöbaden, Sweden). In brief, PCR reaction with sequence specific primers was performed in separate wells in 9700 GeneAmp PCR System and PCR products were run at agarose gel electrophoresis with ethidium bromide and the genotype was identified according to the interpretation table. Primers for the linkage control (HLA-DRB3, DRB4 and DRB5) are included in the kit providing proof for most of the known allelic groups for HLA-DRB1.
Figure 6: Representation of the gel after electrophoresis for a single DNA sample by SSP-PCR in DR low-resolution kit. In this example HLA-DRB1*04 and *13 allelic groups were identified.

4.6 PROTEIN ELECTROPHORESIS, WESTERN BLOT

Recombinant CD89 (rCD89) and plasma proteins from plasma of IgA nephropathy patients and controls were separated on 14% Tris-Glycine polyacrylamide gels in NOVEX electrophoretic system (Invitrogen Life Technologies, Carlsbad, CA, USA), with either reducing by mercaptoethanol or in non-reducing sample buffer, together with the broad range protein molecular weight marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen).

After electrophoresis, the gel was either stained by Coomassie Blue for total protein staining or the proteins were transferred onto nitrocellulose (NC) membrane (0.45 nm, Invitrogen) for Western blotting. This was performed by electric transfer in NOVEX transfer module (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions.

After the transfer, the NC membranes were blocked with 5% gelatine from cold water fish skin (Sigma), followed by washing and incubation with one of anti-CD89 antibodies at the time: MIP8a, A3 or pIDEAE, in concentration of 1 mg/ml at 4°C overnight. After incubation with corresponding species-specific AP-conjugated secondary antibodies (rabbit anti-mouse IgG (Abcam), goat anti-mouse IgG (Southern Biotech) and goat anti-rabbit IgG (Sigma-Aldrich)) the bands were visualized with AP Conjugate Substrate Kit (Bio-Rad Labs Inc., Hercules, CA, USA).
4.7 THE INDIRECT ELISA

Indirect ELISA was used for determination of soluble CD89 in serum/CD89-IgA complex by sandwich ELISA with a first layer of monoclonal mouse anti-human CD89 (A3) antibody (Abcam) and a second rabbit anti-human IgA antibody conjugated to alkaline phosphatase (Dako, Glostrup, Denmark). The serum samples from IgA nephropathy patients and controls and the reference serum (a patient sample with high levels of sCD89) were used in two dilutions (1:10 and 1:1000) and in four 10-fold dilutions (1:10-1:10000), respectively.

The reaction was developed with alkaline phosphatase substrate (Sigma-Aldrich) and absorbance was read as optical density (OD) at 405 nm in the Multiscan MS spectrophotometer (Labsystems, Finland) after the appropriate time. Results were expressed as % of reference.

Figure 7: Sandwich ELISA for detection of soluble CD89/IgA complex. 1) Mouse anti-CD89 monoclonal antibody A3; 2) sCD89-IgA complex; 3) Anti-human IgA labelled with alkaline phosphatase.
4.8 STATISTICAL ANALYSIS

4.8.1 Nonparametric tests and logistic regression

Since our population studies were referred to as distribution-free tests, non-parametric tests were used. Pearson Chi-square and/or Fisher’s Exact tests were used for genotype, allele and haplotypes frequencies; Mann-Whitney or Kruskal-Wallis tests were used to compare the levels of proteins in serum from patients and controls. These tests were also used to analyse the differences between groups of individuals with different genotypes and in progression or non-progression groups.

Estimated odds ratios (ORs) and 95% confidence interval (95%CI) were calculated by Mantel-Haenszel test. Unconditional logistic regression was used to calculate ORs with 95%CI with adjustment for age and gender. All of these analyses were performed by SPSS/PASW statistics 18.0 Software.

4.8.2 Meta-analysis

Two publications related to $TGFB1$ gene polymorphisms in IgA nephropathy and matched to our criteria were included in our meta-analysis. The Mantel-Haenszel method was used with a fixed effect and 95%CI for odds ratio.

Power calculations were performed for two-tail or one-tail tests when appropriate for 5% threshold of significance.

4.8.3 Linkage disequilibrium and haplotype analysis

Linkage disequilibrium (LD) and haplotype analysis were carried out by Haploview 4.0, and the permutation tests were set to 100,000 permutations for single markers and haplotype.
5 RESULTS AND DISCUSSION

5.1 SELECTION OF CANDIDATE GENES

Since the pathophysiology of IgA nephropathy is likely to be tightly bound to the biology of IgA, we decided to test several candidate genes from the immune system, which are known to be involved in IgA pathways.

The major histocompatibility complex (MHC) or Human leukocyte antigen (HLA) class II DR beta 1 (DRB1) molecules play an important role in the immune system by presenting peptides to T lymphocytes. Because the specific MHC class II alleles define peptide binding specificities this may cause a risk of disease development. This locus has multiple alleles related to different autoimmune diseases.\(^\text{101}\)

Transforming growth factor-β1 (TGFβ1) signalling is an essential mechanism in isotype switching to immunoglobulin A in B lymphocytes. This cytokine up-regulates IgA production in general, contributes to the development of fibrosis and to cell proliferation both in animal models and in human diseases, particularly in the kidneys. We investigated 5 SNPs from TGFBI locus at human chromosome 19q13.1.

Genetic polymorphisms in interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4) and TNF receptor-associated factor 1-Complement component 5 (TRAF1-C5) loci have been shown to be risk factors for the development of SLE, rheumatoid arthritis, multiple sclerosis, Sjögren’s syndrome and inflammatory bowel disease. IRF5 and STAT4 are members of the signalling system in lymphoid cells. TRAF1 is involved in TNF signalling, while C5 is a complement system component.

FcαRI/CD89 is a type I receptor glycoprotein, expressed on myeloid cells, which binds with high affinity to the Fc part of IgA. It was shown previously that CD89 transgenic mice develop spontaneous IgA nephropathy.\(^\text{102}\) We investigated five SNPs from FCAR locus at human chromosome 19q13.2-q13.4.

5.2 IG A NEPHROPATHY COULD BE A COMPLEX POLYGENIC DISEASE, INVOLVING BOTH MHC AND NON-MHC SUSCEPTIBILITY ALLELES (PAPER I AND PAPER II)

5.2.1 Paper I

The allelic frequencies for HLA-DRB1 in human populations are known to be significantly different, which may directly or indirectly influence the susceptibility to autoimmune diseases. Our study revealed an association of variations in the HLA-DRB1 gene with IgA nephropathy. More specifically, we indicated a protective effect of HLA-DRB1*03 and *15 in Swedish Caucasians.
The distribution of allele frequencies in the present study showed a susceptibility effect with regard to IgA nephropathy for the HLA-DRB1*01, *04, *10 and *14 alleles, and a protective effect for the HLA-DRB1*03, *07 and *15 alleles. Since strong risk factors may generate non-random biases in the analysis of odds ratios, the group of individuals without risk and protective alleles was chosen as a reference group. We found a protective effect of the cumulative “protective” alleles HLA-DRB1*03, *07 and *15, that remained significant in any analysis together with correction for multiple testing. Further specific analysis of the different protective alleles demonstrated statistical significance both for *03 and *15, while the influence from *07 was less clear, possibly due to the small number of observations. These associations were independent from the found risk alleles, while risk alleles, individually or in the group, did not represent independence from protective alleles, which indicates a statistical bias in the susceptibility effect.

It is well known that 8.1 ancestral haplotype (8.1 AH), where HLA-DRB1*03 allele is represented, is commonly associated with different autoimmune diseases. Individuals carrying this allele have been shown to have less dense expression of TCR/CD3, and more profound capacity to express pro-inflammatory cytokines. Interestingly, the HLA-DRB1*03 and *15 alleles are susceptibility alleles for SLE, which often coincide with the development of Lupus nephritis. This opposing effect emphasizes the difference between IgA nephropathy and Lupus nephritis with regard to genetic risk factors and may indicate discrepancy in subsequent pathways of disease development.

5.2.2 Paper II

In our study, genotyping for five various polymorphisms in the TGFB1 gene was performed in 212 unrelated patients with biopsy-proven IgA nephropathy and in 477 healthy subjects. A significant difference between the genotype distribution in male IgA nephropathy patients and male healthy controls was found in a co-dominant model for all five SNPs. When comparing the frequency of alleles, we found significant differences for frequencies of rs2241715, rs1982073 and rs1800469 between male patients and male controls (p<0.02). To check for possible false positive associations, we performed a permutation test with 100,000 permutations, simultaneously for single markers and haplotypes, which resulted in empiric p=0.04 and p=0.02 for rs1982073 and rs1800469, respectively. In addition, a meta-analysis with rs1982073 was performed for this, and two other, studies of IgA nephropathy. This analysis confirmed the association and supported the role of TGFβ1 as an important factor in development of IgA nephropathy.
Results and discussion

Figure 8: Human TGFB1 gene structure and investigated SNPs (Chromosome 19q13.1)

Two out of five haplotypes, TGGCG and CTGTA, showed a significant difference between male patients and male controls (p=0.0012 and p=0.0018 respectively), and difference in frequency of these two haplotypes passed the permutation test with empiric p=0.006 and p=0.008, respectively. These haplotypes represent either protective (TGGCG) or susceptible (CTGTA) variants and are opposite in four out of five alleles. The protective TGGCG haplotype comprises 53% of all chromosomes in the control male population and we achieved 90% power of analysis to detect significant differences at 0.05 threshold in two-tail tests.

Our finding of association in male IgA nephropathy patients may demonstrate important gender influence in the involvement of TGFBI in pathogenesis. However, we cannot totally exclude its importance for disease in females due to relatively low representation of women in the IgA nephropathy group and subsequent low statistical power to detect the effect.

We propose that the TGFBI gene is an important contributor for susceptibility to IgA nephropathy.

5.3 THE CONTRAST IN IGA NEPHROPATHY AND LUPUS NEPHRITIS (PAPER III)

Genetic findings in IgA nephropathy and Lupus nephritis at TGFBI, STAT4, IRF5, and TRAF1-C5 loci (Paper III)
Immunological and biochemical similarities between SLE and IgA nephropathy demonstrate a direct link to impaired immune function in both diseases. Lupus nephritis and IgA nephropathy are both chronic renal diseases that are classified in the "predominant" inflammatory group, based on morphological similarities. By investigation of the frequency of polymorphisms in genes that have recently been shown to associate with different autoimmune diseases, particularly with SLE, we tested the hypothesis that there may be an overlap in genetic susceptibility between Lupus nephritis and IgA nephropathy. On the other hand, there could be specific genetic makers associated with the development of nephritis in SLE patients and with IgA nephropathy.

Our study is the first investigation of the importance of IRF5, STAT4 and TRAF1-C5 gene polymorphisms in patients with IgA nephropathy and showed no evidence of an association of these genes with the development of IgA nephropathy or Lupus nephritis in the Swedish population. Moreover, the results from TGFB1, STAT4 and IRF5 loci in 1252 DNA samples from patients with biopsy-proven IgA nephropathy or with SLE (with and without nephritis), and healthy controls, did not suggest any overlap of genetic risk factors in the two diseases.

Our data demonstrate that different immune system-related genes may correlate with IgA nephropathy and Lupus nephritis.

5.4 FCAR IN IGA NEPHROPATHY, A NEW BIOMARKER FOR PROGRESSIVE RENAL FAILURE (PAPER IV)

In humans, two isotypes of IgA, IgA1 (mainly in plasma) and IgA2 (mainly secreted), are present in monomeric (mIgA) or polymeric (pIgA) forms. pIgA consists of two or more IgA molecules and a J chain. Circulated in plasma, IgA builds a complex with the soluble CD89, or with cellular receptors. The IgA system in mice does not have CD89 and mice IgA serum is not polymeric, lacking the hinge region with six potential O-linked glycosylation sites as in humans. We hypothesized that the amount of soluble receptor or the IgA-CD89 complex may be an important marker in IgA nephropathy susceptibility or severity.

First, we investigated the frequency of genetic variants of FCAR in IgA nephropathy and controls. We selected several gene variations for this analysis, based on our previous experience in genotyping of different gene loci, and chose SNPs distributed throughout the gene region and flanking regions. Our results reveal that a recombination inside the FCAR gene is rare in the Caucasian population: out of 32 expected haplotypes we recorded eight that were common for the investigated population. This is evidence for strong LD in this region, which in theory increases the chance for detection of an association by SNP or haplotype frequency analysis. However, we found no difference in frequency for selected common variations in the
Results and discussion

*FCAR* gene between patients with IgA nephropathy and the control group. Since the genetic risk for a complex disease is expected to be low, our genetic association study may represent a type II error. On the other hand, the haplotype analysis suggests that there is a low, if any, probability of less common genetic variation, which may strongly associate with the disease.

In this study we developed a new method for detection of soluble CD89 through identification of sCD89-IgA complex in human serum or plasma. Several previous attempts from other groups to quantify sCD89 directly in serum or plasma have failed, probably due to the fact that all sCD89 molecules are tightly bound to IgA within the CD89-IgA complexes and only a minor portion of the sCD89 molecule is exposed for antibody recognition. Alternatively, when these complexes were dissociated *in vitro* by strong detergents, such as SDS, this reaction in solution was hindered because of antibody denaturation. Western blotting was effective, but is, however, not an accurate quantitative method. We resolved the problem by detection of sCD89-IgA complexes instead of the free sCD89 molecules, through use of antibodies against IgA as a second type of antibody in sandwich ELISA. Capturing the sCD89 from the serum with a first type-specific monoclonal anti-CD89 antibody ensured the specificity of the reaction. This method could be used for detection of circulating sCD89 in serum/plasma in different pathological conditions.

The most important finding in this study is the significant association between the low levels of sCD89-IgA complex in sera of IgA nephropathy patients and the progression of severe IgA nephropathy. In a disease control group of patients with other forms of glomerulonephritis, who had similar renal function and proteinuria as the IgA nephropathy group, no correlation to disease progression was observed. Importantly, serum levels of sCD89-IgA remained stable and low in IgA nephropathy patients with progression of the disease and continuously high in IgA nephropathy patients without progression, during a follow-up period of 5-15 years. A considerable difference in levels of sCD89 in the circulation of patients with IgA nephropathy and in healthy controls was also observed. The mechanism behind this effect remains unclear. Based on our finding, we may speculate only that it may be possible that the lack of sCD89 is involved in aggravation of IgA nephropathy and it can be used as a biomarker for prediction of possible severe nephritis.

5.5 DETECTION OF SOLUBLE MANNOSE-BINDING LECTIN 2 IN PREDICTION IN IGA NEPHROPATHY (PILOT STUDY)

Mannose-binding lectin (MBL, also known as mannan- or mannose-binding protein, MBP) is a serum protein of the collectin family, and activates the lectin pathway of the classical complement system. The structure and functions of MBL are similar to C1q and it has an important role in innate immunity as a pattern recognition receptor in the complement system. The activity of MBL has been demonstrated in relation to many microorganisms, particularly in respiratory infections, for example influenza A virus107, pneumococci, *Haemophilus influenzae*108, and *Legionella pneumophila*109.
Therefore, MBL deficiency has been proposed to increase the risk of repeated respiratory tract infections\textsuperscript{110}. Patients with IgA nephropathy often have recurrent macroscopic hematuria, frequently accompanied by loin pain and constitutional symptoms, and occurring within 48 hours of an upper respiratory tract or other infection. This feature is seen in over 40\% of patients\textsuperscript{26}. We hypothesized that MBL levels in serum may associate with the susceptibility and/or severity of IgA nephropathy.

We analysed 39 serum samples from patients with IgA nephropathy (17 patients with progression and 22 in the non-progression group) from Karolinska University Hospital and 39 healthy controls by ELISA technique (Antibodyshop, DK-2820 Gentofte, Denmark). We did not find a difference (Mann-Whitney Test) in MBL levels between patients and controls. Additional stratifications by sex and disease progression did not show any trends for association. By using categorical analysis with different thresholds for MBL, no difference between groups was detected. We concluded that MBL is unlikely to be a valuable and useful biomarker for prediction of susceptibility or severity of IgA nephropathy.
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Many candidate gene association studies have been performed with the aim of identifying the susceptibility genes for IgA nephropathy and so providing an improved knowledge base for future therapy based on the disease mechanisms. We contributed to the understanding of these mechanisms by performing studies of immune system gene variants, soluble protein makers and clinical symptoms from IgA nephropathy and Lupus nephritis patients. Our intention was to analyze this data from different angles and to detect the true effects that may influence disease development and may serve as predictors for susceptibility, or for progressive renal failure, in patients with glomerulonephritis.

Current genetic studies of IgA nephropathy lack a genome-wide approach. Performing a GWAS to this disease is a most urgent task and there is a need for future developments in this area. Similarly, there is a lack of data regarding Lupus nephritis, though several GWAS have already been completed for SLE. In both diseases, only combined international efforts may provide satisfactory results, by increasing the numbers of cases and controls and by comparative analysis between different populations and ethnic groups.

*TGFB1* gene could be one of the important candidate genes in the development of IgA nephropathy and is a possible target for therapy. The data (paper II) revealed the role of *TGFB1* gene variants in association with IgA nephropathy. Since TGF beta 1 is a cytokine with a very broad spectrum of action, it will not be a simple task to modulate its function. There have been several attempts to interfere with the TGF beta 1 pathway through its receptors and associated proteins (integrins) in the treatment of systemic sclerosis and other diseases. Our study suggests a rationale for the use of similar approaches in IgA nephropathy.

Our study of several genetic polymorphisms demonstrated a contrast between IgA nephropathy and Lupus nephritis that was defined for *TGFB1* and *HLA-DRB1*. These data suggest that it is necessary to critically evaluate current approaches in the treatment of these two types of glomerulonephritis. Since the pathways of the diseases seem to be different or even contrasting, different treatment strategies may be required.

Finally, we suggested that the sCD89 could be a biomarker for progressive renal failure in IgA nephropathy. This finding is awaiting replication in independent cohorts and, if consistent, may be used for the identification of individuals at risk and therefore candidates for more aggressive therapy.

Genetic studies are a source for new hypotheses about disease development, but it is rare within the complex diseases that the cause of disease is convincingly identified. One of the possible reasons for this is our simplistic view of genetic associations. It has
been shown for other diseases that the interaction between susceptibility genes leads to a very high risk of developing a disease. Our discovery of important genetic associations suggests that gene-gene and gene-environment interactions should be investigated.

We should also recognise that the diseases we studied represent complex phenotypes. It will be important to perform further genetic analysis of large groups with regard to local renal events and systemic course of the disease. In addition, the study of the role of environmental factors, including infectious diseases, smoking, stress, etc. is vital in assessing the risk of glomerulonephritis.
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