Identification of candidate genes involved in Mercury Toxicokinetics and Mercury Induced Autoimmunity

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Faculty of Medicine and Health Science
You can try but never stop me
This is what I'm made of
I will never ever let go
This is what I'm made of
No one can control me
Cause this is what I'm made of
You can hate but never break me
This is what I'm made of

Nause – “Made of”
(2011)

To my beloved Family Lina, Sabrina and Oliver

My Mother Aidah, my Father Hazim

and my Brothers Yazan and Wusan
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ABSTRACT

BACKGROUND: Autoimmune diseases require the involvement and activation of immune cells and occur when the body builds up an immune response against its own tissues. This process takes place due to the inability to distinguish self-antigen from foreign antigen. Systemic autoimmunity represents an important cause of morbidity and mortality in humans. The mechanisms triggering autoimmune responses are complex and involve a network of genetic factors. Genome wide association study (GWAS) is a powerful method, used to identify genetic risk factors in numerous diseases, such as systemic autoimmune diseases. The goal of GWAS is to identify these genetic risk factors in order to make predictions about who is at risk and investigate the biological process of disease susceptibility. There are several valuable mouse models to investigate the underlying mechanisms causing systemic autoimmune diseases in which mercury induced autoimmunity (HgIA) is a well-established and relevant model. HgIA in mice includes development of autoantibodies, immune complex glomerulonephritis, lymphocyte proliferation, hypergammaglobulinemia and polyclonal B cell activation. In humans, mercury exposure accumulates with considerable concentrations in kidney, liver, and brain. Toxicokinetics of Hg has been studied extensively but the key for inter-individual variation in humans are largely unclear. Differences in accumulation of renal Hg between inbred mouse strains suggest a genetic inter-strain variation regulating retention or/and excretion of Hg.

OBJECTIVES: To find loci and candidate genes associated with phenotypes involved in the development of autoimmunity and find candidate genes involved in the regulation of renal Hg excretion.

METHODS: MHC II (H-2b) mice were paired (A.SW x B10.S) to achieve F2 offspring exposed to 2.0 or 4.0 mg Hg in drinking water for 6 weeks. Mercury induced autoimmune phenotypes were studied with immunofluorescence (anti-nucleolar antibodies (ANoA)), ELISA anti-DNP/anti-ssDNA (polyclonal B cell activation), anti-chromatin antibodies (ACA) (4.0 mg Hg), and serum IgG1 concentrations. Mercury accumulation in kidney was performed previously and data was included as phenotype. F2 mice exposed to 2.0 mg Hg were genotyped with microsatellites for genome-wide scan with Ion Pair Reverse Phase High Performance
Liquid Chromatography (IP RP HPLC). F2 mice exposed to 4.0 mg Hg were genotyped with single nucleotide polymorphisms for genome-wide scan with SNP&SEQ technology platform. Quantitative trait loci (QTL) was established with R/QTL. Denaturing HPLC, next generation sequencing, conserved region analysis and genetic mouse strain comparison were used for haplotyping and fine mapping on QTLs associated with Hg concentration in kidney, development of ANoA and serum IgG1 hypergammaglobulinemia. Candidate genes (Pprc1, Bank1 and Nfkb1) verified by additional QTL were further investigated by real time polymerase chain reaction. Genes involved in the intracellular signaling together with candidate genes were included for gene expression analysis.

**RESULTS:** F2 mice exposed to 2.0 mg Hg had low or no development of autoantibodies and showed no significant difference in polyclonal B cell activation in the B10.S and F2 strains. F2 mice exposed to 4.0 mg Hg developed autoantibodies and significantly increased IgG1 concentration and polyclonal B cell activation (anti-DNP). QTL analysis showed a logarithm of odds ratio (LOD) score between 2.9 – 4.36 on all serological phenotypes exposed to 4.0 mg Hg, and a LOD score of 5.78 on renal Hg concentration. Haplotyping and fine mapping associated the development of ANoA with Bank1 (B-cell scaffold protein with ankyrin repeats 1) and Nfkb1 (nuclear factor kappa B subunit 1). The serum IgG1 concentration was associated with a locus on chromosome 3, in which Rxfp4 (Relaxin Family Peptide/INSL5 Receptor 4) is a potential candidate gene. The renal Hg concentration was associated with Pprc1 (Peroxisome Proliferator-Activated Receptor Gamma, Co-activator-Related). Gene expression analysis revealed that the more susceptible A.SW strain expresses significantly higher levels of Nfkb1, Il6 and Tnf than the less susceptible B10.S strain. The A.SW strain expresses significantly lower levels of Pprc1 and cascade proteins than the B10.S strain. Development of ACA was associated with chromosomes 3, 6, 7 and 16 (LOD 3.1, 3.2, 3.4 and 6.8 respectively). Polyclonal B cell activation was associated with chromosome 2 with a LOD score of 2.9.

**CONCLUSIONS:** By implementing a GWAS on HgIA in mice, several QTLs were discovered to be associated with the development of autoantibodies, polyclonal B cell activation and hypergammaglobulinemia. This thesis plausibly supports Bank1 and Nfkb1 as key regulators for ANoA development and HgIA seems to be initiated by B cells rather than T cells. GWAS on renal mercury excretion plausibly supports Pprc1 as key regulator and it seems that this gene has a protective role against Hg.
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    Hammoudi Alkaissi, Jimmy Ekstrand, Aksa Jawad, Jesper Bo Nielsen, Said Havarinasab, Peter Söderkvist, Per Hultman

    *Environmental Health Perspective, 2016, Jul; 124(7):920-6*

II. **Bank1 and NF-kappaB as key regulators in anti-nucleolar antibody development**

    Hammoudi Alkaissi, Said Havarinasab, Jesper Bo Nielsen, Peter Söderkvist, Per Hultman

    *PLOS, One, 2018, Jul: 13(7): e0199979*

III. **IgG1 Hypergammaglobulinemia in Mercury Induced Systemic Autoimmunity Maps to Chromosome 3**

    Hammoudi Alkaissi, Said Havarinasab, Jesper Bo Nielsen, Peter Söderkvist, Per Hultman

    *Manuscript*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibodies</td>
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<tr>
<td>ANoA</td>
<td>Anti-nucleolar antibodies</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Bank1</td>
<td>B-cell scaffold protein with ankyrin repeats 1</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
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<td>CDC-like kinase 2</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
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<tr>
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<td>Centimorgan</td>
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<tr>
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<tr>
<td>dHPLC</td>
<td>Denaturing high performance liquid chromatography</td>
</tr>
<tr>
<td>DMSA</td>
<td>meso-2,3-dimercaptosuccinic acid</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenyl</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FO</td>
<td>Follicular</td>
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<td>GCL</td>
<td>Glutamyl-cysteine ligase</td>
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<tr>
<td>GSTM1</td>
<td>Glutathione S-transferase Mu 1</td>
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<td>GWAS</td>
<td>Genome Wide Association Study</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------------------</td>
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<tr>
<td>Hg⁰</td>
<td>Elemental mercury</td>
</tr>
<tr>
<td>HgIA</td>
<td>Mercury induced autoimmunity</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigens</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>INSL5</td>
<td>Insulin-like peptide 5</td>
</tr>
<tr>
<td>IP RP HPLC</td>
<td>Ion pair reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of odd</td>
</tr>
<tr>
<td>LRR</td>
<td>Extra-cellular leucine-rich repeats</td>
</tr>
<tr>
<td>MeHg</td>
<td>Methyl mercury</td>
</tr>
<tr>
<td>MG</td>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td>MGI</td>
<td>Mouse Genome Informatics</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MRP</td>
<td>Multidrug resistance-tolerated protein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Msto1</td>
<td>Misato 1, mitochondrial distribution and morphology regulator</td>
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<tr>
<td>Muc1</td>
<td>Mucin 1, transmembrane</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>Nfkb1</td>
<td>Nuclear factor kappa B subunit 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor NF-kappa-B</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
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<td>Nrf1</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear factor-erythroid 2-related factor 2</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular pattern molecules</td>
</tr>
<tr>
<td>Pprc1</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator-related protein 1</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>RIG</td>
<td>Retinoic acid–inducible gene</td>
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<td>RLN3</td>
<td>Relaxin-3</td>
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<td>RIG–like receptor</td>
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<td>Rxfp4</td>
<td>Relaxin family peptide receptor 4</td>
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<td>Scamp3</td>
<td>Secretory carrier membrane protein 3</td>
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<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>Ssc</td>
<td>Systemic Sclerosis</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TD</td>
<td>T cell dependent</td>
</tr>
<tr>
<td>TI</td>
<td>T cell independent</td>
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<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
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<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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INTRODUCTION

1.0 Genetics
Genetics is an important field and influences all life on earth. All animals and plants are developed by instructions based on our genes. Our genes control how we and how our body work. All human beings are unique and differ from each other and these diversities are also observed in other animals and plants. These genetic diversities may also lead to the susceptibility to developing various diseases. Two main factors control the diversity: inherited genes and environmental exposure. Genes underlying observations such as eye, skin, hair color and complex diseases, are discovered by the use of genetic mapping [1].

1.1 Inherited genes
Somatic cells of animals contain two copies of the genome that consist of DNA organized into chromosomes. Humans have 23 chromosome pairs (46 chromosomes) [2] and mice have 20 chromosome pairs (40 chromosomes) [3]. During somatic cell division, chromosomes are replicated and then separated, so that each daughter cell receives the full complement of chromosomes. During germ cell division however, the chromosome number in gametes are reduced in half and are the carriers of genes for reproduction. Each parental gamete will enter meiosis in which recombination occurs. Homologous chromosomes (1 from each parent) pair by length and exchanges of alleles occurs at certain positions [4].

1.2 Environmental exposure
A number of environmental factors can affect structures and functions in our body. Tobacco smoke [5, 6], air pollution [7, 8], phalates [9, 10] and metals such as arsenic [11], mercury (Hg) [12, 13] and nickel [14, 15] are all associated with DNA methylation. These epigenetic modifications are associated with numerous number of diseases such as cardiovascular disease [16], autoimmune diseases [17], neurological disorders [18], and cancer [19].
1.3 Phenotype
Phenotypic traits are observed traits that include both macro (such as eye color) and microscopic (physiological) properties [20]. Many phenotypic traits can be measured and therefore called quantitative traits. Quantitative traits in common and complex diseases are measured for investigating genetic risk factors in association studies. In this thesis, we studied various quantitative traits in a model for systemic autoimmunity such as autoantibodies, hypergammaglobulinemia and polyclonal B cell activation in mice. We further studied the accumulation/excretion of Hg in mouse kidney.

1.4 Genotype
This is the inherited material transmitted by gametes and consists of DNA sequences, a double helix composed of the four nucleotides adenine (A), thymine (T), guanine (G) and cysteine (C) [21]. The combination of these four nucleotides determines our unique genetic code. A genetic variation of a nucleotide in a sequence will lead to a polymorphism. Polymorphism can also be changes in repeated elements at a specific position. Humans, mice and all other mammals might have changes at these locations spanned all over the genome. These changes are very useful when performing genetic mapping by genotyping, to study recombination. Numerous markers have been discovered as tools for genetic mapping, such as RAPD (Random Amplification of Polymorphic DNA), RFLP (Random Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), Microsatellites and SNPs (Single Nucleotide Polymorphisms) [22]. Vast amounts of DNA sequences from different species have been determined and stored in databases and are continuously updated and available on the internet. This is an excellent tool for genetic studies such as association studies, homologous sequence comparison, protein-coding regions and mutations [23]. In this thesis, we applied these databases for genetic mapping and association studies.

1.5 Genetic Mapping
Genetic mapping is implemented when studying locations of genetic susceptibility for a phenotypic trait. During recombination, sets of alleles tend to cross over as blocks (haplotypes) through a pedigree. These haplotypes can be tracked through pedigrees and populations but can be broken up by further recombination at later offspring (Fig 1). The further away two loci are located from each other, the higher chance it will be separated from each other by crossover.
By the use of centimorgan (cM), the genetic distance of a crossover can be measured. 1 cM equals to a recombination fraction of 0.01 (1%) recombination between two loci. The genetic map does not correspond to the physical distance. The genetic map show the distance of the probability there will be a recombination, whereas the physical map show the distance in kilo-/megabases. A rule of thumb is used, in which 1 cM equals to 1 megabase, but it is important to know that there are recombination that occur in less than 0.3 cM/Mb and more than 3cM/Mb [1].

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**Figure 1. Illustration of recombination.**

*Homolog chromosome from pure breeding strains (F0) paired together to receive heterozygote chromosome pairs in the F1 offspring. During meiosis, recombination occurs in the F1 offspring, leading to 1 chromatid in each gamete with haplotypes inherited from each parental strains. The F1 offspring are paired together to achieve an F2 offspring that contain the recombination in their somatic cells as well.*
1.6 The Human and Mouse Genome

The mouse genome and biology are key tools for understanding the contents of the human genome and biology. After sequencing the human genome in 2001 [24], the mouse (Mus Musculus) was the second mammal in 2002 [25], and is a frequently used model to understand human diseases. Mammals such as human and mouse shared common ancestor for around 80 million years ago [26]. The human genome is $3.3 \times 10^9$ base pair (bp) long [27] and contains approximately 21,000 protein-coding genes [28]. The mouse genome is $2.8 \times 10^9$ bp long [29] and contains approximately 24,000 genes [30]. 80% of the human genes and 75% of the mouse genes are in 1:1 orthologous relationship [31]. Genomic comparison between these two species are therefore very informative.

1.7 Genome Wide Association Study

Genome Wide Association Study (GWAS) is a powerful method, used to identify genetic risk factors in numerous diseases such as Asthma [32], Allergy [33], Multiple sclerosis (MS) [34], Systemic Lupus Erythematous (SLE) [35], Rheumatoid Arthritis (RA) [36] and Systemic Sclerosis (Ssc) [37]. The goal of GWAS is to identify these genetic risk factors in order to make predictions about who is at risk and investigate the biological process of disease susceptibility for developing new prevention and treatment strategies. Performing an association study requires the genetic map of the species population and the quantitative traits of interest. The genetic map is traditionally used with microsatellites or SNPs spread out over the genome. Microsatellites are highly polymorphic DNA sequences with a number of tandem repeats. These tandem repeats are found throughout the genome composed of di-, tri-, tetra- or bigger repeats (Fig 2). Genes are co-segregated with the highly polymorphic microsatellites, which make them useful markers for mapping studies [38-41].
Figure 2. Microsatellites.

Illustration of trinucleotide tandem repeats in two mouse strains of same microsatellite. The A.SW strain contains seven tandem repeats (21 bp size) whereas the B10.S strain contains eight tandem repeats (24 bp size).

SNPs occur naturally in the human population and is a variation of a single nucleotide replaced with different nucleotide. In humans, a SNP is defined when more than 1% of the population does not carry the same nucleotide [42]. In mouse, a SNP is defined when two strains differ by a single base pair [40]. SNPs can be located on non-coding regions (intron), coding regions that do not result in an amino acid change (synonymous change), coding regions with an amino acid change (non-synonymous/missense) or untranslated region (UTR)/regulatory region (Fig 3) [39]. Microsatellites are spaced at intervals of approximately 10-20 cM across the genome, whereas SNPs are spaced approximately every 5kb. Microsatellite markers are more precise, due to the higher levels of heterozygosity [43], but SNP markers have higher density and less error rate [44].
Figure 3. Single Nucleotide Polymorphisms.

Illustrates positions of SNPs on intron and exon (UTR and coding region). SNPs on coding region are further divided in synonymous and non-synonymous. An example of synonymous SNP with the codon GCT and reference GCA of which both codes for the same amino acid, alanine. An example of non-synonymous SNP with the codon CGT and the reference TGT that codes for different amino acids, arginine and cysteine respectively.

Discovering genomic regions associated with the quantitative trait of interest, is performed with statistic association/correlation software. There are various software tools for genome-wide association study analysis, based on input-data [45]. The result is presented as a plot of the test statistic, presented as the likelihood ratio or the logarithm of odd score (LOD-score), against the chromosomal map position presented in recombination unit (cM). The quantitative trait locus (QTL) is the chromosomal region associated with the variation of the phenotypic trait.
2.0 The Immune System - mouse and human

The evolution of immunity occurs at several timescales: to adapt to pregnancy, to tackle viruses, bacteria, parasites and fungi, for tissue repair and wound healing, for healthy gut microbiota. All these factors have built up a complex immune system of innate and adaptive immunity [46].

The innate immunity is our ancient system that protects us from surrounding environment by natural barriers (skin and mucosa), innate lymphoid cells, natural killer cells and inflammatory cytokine producing cells such as monocytes/macrophages, dendritic cells and cells with the ability to present antigens (Ag). The activation initiates by soluble pattern recognition molecules bound to pattern recognition receptors (PRRs) on surface and/or in the cytoplasm of innate immune cells. PRRs are divided in 4 subclasses: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid–inducible gene (RIG)–like receptors (RLRs) and NOD-like receptors (NLRs) [47].

The adaptive immunity is highly specific to Ag, mediated by B and T lymphocytes and characterized by immunological memory. Adaptive immunity is further divided into humoral (Ab production) and cell-mediated immunity (Ag presenting) [48].

2.1 T cells

T cells originate in the bone-marrow and migrates to the thymus for positive and negative selection. Negative selected cells die and the positive cells carry T cell receptor (TCR) and become naïve thymic CD4+ or CD8+ T cells. CD4+ T cells are activated by antigen presenting cells (APCs) presenting Ag on its MHC class II. MHC class II interact with the TCR on CD4+ T cell. Co-stimulatory molecules on the surfaces of APC (i.e. CD80) and CD4+ T (i.e. CD28) cell are expressed and interact. This initiate an intracellular cascade leading to Ca^{2+} influx to the cytoplasm and expression of cytokines and cell surface molecule CD40L, necessary for B cell activation. The expression of cytokines acts on developing T cells and initiate CD4+ T cell specific lineage. CD4+ T cells can become a numerous amount of subclasses: T helper 1 (T_{H1}), T_{H2}, T_{H3}, T_{H9}, T_{H17}, T_{H22}, follicular T helper cell (T_{FH}) and T regulatory cell (T_{REG}) [49].

T_{H1} cells are characterized by IFN_{γ} production [50] and targets pathogens [51]. In humans, T_{H1} cells activate plasma cells to produce Immunoglobulin (Ig) G1 and IgG3 leading to
complement activation and FcγR-mediated phagocytosis [52]. In mouse, it is involved in inducing cell-mediated immunity and class switching to IgG2a [53, 54].

TH2 cells are characterized by Interleukin (IL) 4 [55] and involved in humoral immune responses in both human and mouse. However, in human it provides help to B cell class switching antibodies (Abs), mainly IgE mediated [56]. In mouse, it provides help to B cell class switching Abs to IgG1 [53, 54].

2.2 B cells

In mammals, the development of B cells begins in the fetal liver as hematopoietic stem cells (HSCs) [57, 58]. HSCs are seeded to the bone marrow (BM) and B cells develop there throughout life [59]. Rearrangement processes of immunoglobulin gene segments take place leading to expression of one IgM of the cell surface, displayed as a B cell receptor (BCR) of an immature B cell [60]. Checkpoint of self and non-self-Ag occurs at this stage and BCRs recognizing self-Ag will be deleted [61]. Immature B cells migrate to spleen, lymph nodes, peyer’s patches, tonsils and mucosal tissues for finalizing the development into three main B cell subsets: B-1, follicular (FO), and marginal zone (MZ) B cells. B-1 cells are the main source of circulating Abs. Antigens such as lipopolysaccharides (LPS) and stimuli such as IL-5 and IL-10 cytokines activate B-1 cells. They respond fast to Ags and transforms into plasma cells. MZB cells are located in the marginal zone of the spleen. They express high levels of TLRs and are activated by T cell independent (TI) and T cell dependent (TD) signals and become Ab producing plasma cells. TI Ag are able to initiate B cell activation in the absence of T cells, whereas TD Ag initiate B cell activation that requires T cells as well. FO cells are the largest subpopulation of B cells and located as naïve B cells secondary lymphoid organs and the circulation. FO B cells are activated by TD signals through BCR, CD40 and TLRs. [62, 63]. The Ag recognition by BCR induces an endocytosis, leading to degradation of Ag that MHC class II recognize, and presents on the cell surface together with CD40 co-stimulatory molecule to T cells (Fig 4). This allows intracellular signaling to occur in the B cell and permitting the activation of several transcription factors such as NF-kB, AP-1, and NF-AT. BCR also induces the TLR signaling pathway as well that is dependent on the T cell permission of transcription factors. [48]. The cell will further produce cytokines, based on TH cell, such as IL-4 (TH1) and IFNy (TH2). These processes lead to expression of pro-inflammatory cytokines, B cell differentiation, proliferation and production of Abs.
Figure 4. T cell-Dependent activation of B cells

*B cell* recognizes and internalizes an antigen and presents it to a helper *T cell* with its *MHC II*. *T helper cell* recognizes the foreign antigen with *TCR* followed by an interaction between *CD40L* on the *T helper cell* and *CD40* on the *B cell*. This linked recognition leads to secretion of cytokines by the *T helper cell* and activation of the *B cell*.

### 2.3 Toll-like receptors

One of the mechanisms in the immune system is to recognize and inform against pathogenic molecules, and TLRs play a crucial role here. They are involved in both innate and adaptive immunity and recognize both pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) leading to ligand mediated signaling and an immunological response back [64]. In human, there are 10 types of TLRs in which TLR 1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR11 are located on the cell surface, and TLR3, TLR7, TLR8 and TLR9 are located in the endosomal/lysosomal surface inside the cell. Twelve murine TLRs have been characterized, TLR1-TLR9, TLR11, TLR12, TLR13 [65]. Each receptor recognize distinct ligands:
- TLR1/TLR2 - bacterial triacylated lipoproteins
- TLR3 - Double-stranded RNA from virus
- TLR4 - bacterial LPS
- TLR5 - bacterial flagellin
- TLR6 - Bacterial diacylated lipopetides
- TLR7/TLR8 – single stranded RNA (ssRNA)
- TLR9 - unmethylated CpG DNA

Toll-like receptors initiate their signaling through adaptor proteins. Adaptor proteins interact with the cytoplasmic domains of TLRs through hemophilic interactions between Toll/IL-1 receptor (TIR) domains present in each TLR and each adaptor protein. The most well known adaptor proteins are MYD88 (myeloid differentiation primary response protein 88), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR-domain-containing adapter-inducing interferon-β, also known as TICAM1) and TRAM (TRIF-related adaptor molecule, also known as TICAM2 (Fig 5) [64].

![Toll-like receptor family](image)

**Figure 5. Domain structure of TLR and adaptor protein**

TLRs are composed of a type I transmembrane (TM), an extra-cellular leucine-rich repeats (LRRs) that mediate recognition of PAMPS and a cytoplasmic TIR domains that interact with downstream adaptors. One downstream adaptor is MYDD88 that consists of two domains, the dead domain (DD) and TIR domain that interact with TLRs.
TLRs induce different cascade signaling responses based on adaptor protein in which TLR4 and TLR2 occurs through the adaptors TIRAP and MyD88. TLR3 requires the TRIF and TLR4 requires the adaptors TRIF, TRAM, TIRAP and MyD88. The intracellular TLRs, TLR3, TLR7, TLR8 and TLR9 acts through MyD88. MyD88 activates NF-κB and MAPKs pathways, leading to induction of pro-inflammatory cytokines such as IL-6, TNFα and IL-1β. Activation of intracellular TLRs will also lead to the expression of Type 1 IFN via the activation of IRF7 [64].

2.4 Autoimmunity

There are over eighty identified autoimmune diseases [66] with an accumulated prevalence of 5-10% on a global scale [67]. The autoimmune disease requires the involvement and activation of immune cells and occurs when the body builds up an immune response against its own tissues. This process takes place due to the inability to distinguish self-Ag from foreign Ag. The phenomenon originates from the activation of self-reactive T and B cells generating cell-mediated or humoral immune responses directed against self-Ag [68]. Defects in genes controlling normal immune responses, Ag processing and presentation are all linked to develop an autoimmune reaction. Autoimmune responses may be triggered through altered proteins and molecular mimicry [69] due to pathogens, leading to immune responses that direct at antigenetic determinants on pathogens having similar epitopes in normal host tissue [69]. The pathological consequences of this reactivity constitute of several types of autoimmune diseases such as thyroid disease, type 1 diabetes and myasthenia gravis (MG), referred to as organotypical illness. Systemic illness includes diseases such as RA, SSC and SLE [70].

SLE is a chronic rheumatic systemic disease that may affect multiple organs, including skin, joints, kidney, lungs and nervous system. There is a great diversity of prevalence between ethnic groups and ranges from 20-150 cases per 100 00 people [71]. In Sweden, the prevalence of SLE is 65-80 cases per 100 00 inhabitants [72]. Its pathogenesis is complex and include polyclonal B-cell activation, lymphocyte proliferation, hypergammaglobulinemia, autoantibody production, and immune complex (IC) formations [73-75].

Ssc is a rheumatic disease that is characterized by pathological thickening of the skin and involvement of internal organs, including, kidneys, heart, lung and gastrointestinal tract [76]. The world-wide prevalence vary substantially and estimates <150 per million people [77]. It’s pathogenesis involves development of autoantibodies, lymphocyte proliferation and fibroblast proliferation [76].
2.5 Gender and Autoimmunity

Female to male ratios for systemic autoimmune disease such as SLE and Sjögrens syndrome are estimated to be 9:1 meaning an underlying mechanism to the female susceptibility for development of autoimmune disease [78, 79]. By using animal models for gender autoimmunity, studies have shown increased levels of sex hormones (primarily estrogen and progesterone) [78, 80], removal of sex glands and treatment with agonistic or antagonistic agent related to sex hormones affects the incidence of autoimmune phenotypes [78, 79]. Rodent models have shown that basic immune responses differ between males and females. T cell activation is more vigorous in female mice and they produce more Abs [78, 80]. In addition to sex hormones, males and females differ in sex chromosomes, which also play a role in the female predisposition for autoimmune diseases. A number of studies have been carried out to understand the role of sex chromosomes in autoimmunity, but not been able to succeed due no clear understanding of the regulation involved in X and Y chromosomes biology. Over 1000 genes are unique to the X chromosome, that are not found in the Y chromosome and about 70 % of X chromosome linked functions are directly associated with human diseases [80].

2.6 Animal models for Autoimmunity

There are several valuable mouse models to investigate the underlying mechanisms causing systemic autoimmune diseases, which are either spontaneous or induced. Each mouse model represents features of phenotypic traits in patients, but there is no model that represents the entire clinical spectrum. Models for systemic autoimmune diseases are divided in 4 categories: (i) direct immunization, (ii) spontaneous, (iii) gene mutation and (iii) exposure to exogenous agents. Direct immunization is used when auto-Ag on the cell or extracellular elicit autoantibody responses. For example, MG-like disease which is produced in rodents because of immunization with purified acetylcholine receptor [81]. Spontaneous model does not require manipulation at all. Certain murine strains develop diseases that serve models of the Ab specificity and pathology in human diseases [81]. For example, female New Zealand Black/New Zealand White (NZB/NZW) mice develop spontaneously autoantibodies with specificity to nucleic acid Ags [82, 83]. In order to influence the expression of autoimmunity, gene mutation models are based on deleting a gene (“knockout”) or adding a gene (“transgenic”). These types of modifications can be used to study the influence of single genes on animal models [81]. Exposure to exogenous agents includes mediators such as drugs and environmental agents such as the heavy metals Hg, silver (Ag) and gold (Au). The toxicity of
heavy metals in animals and humans are dependent on dose, frequency, individual susceptibility and genetic predisposition. Xenobiotics such as Hg, Ag and Au have been used as experimental models for systemic autoimmune disease [84-87]

2.7 Mercury induced autoimmunity
Mercury induced autoimmunity (HgIA) is a well-established and relevant model to study systemic autoimmunity. HgIA in mice includes antinuclear antibodies (ANA), and more specifically, anti-nucleolar antibodies (ANoA). Some ANoA [88] targets the protein fibrillarin [89] which are also same ANoA in Ssc patients [90]. This model also includes IC glomerulonephritis, lymphocyte proliferation, hypergammaglobulinemia and polyclonal B cell activation [91-96]. The two most related diseases to HgIA, are SLE and SSc.

2.8 Genetics in HgIA
Exposure to heavy metals such as Hg leads to the development of immunoreactions in some rodents controlled by genes residing in the MHC region (referred to HLA in human and H-2 in mice), mapping to the I-A region of H-2 [97]. Strains with haplotype H-2^a are most susceptible for production of ANoA, while H-2^q and H-2^f mice have intermediate susceptibility and H-2^a, H-2^b, and H-2^d mice are found to be resistant (Table 1) [98]. Both genes of MHC class II and non-MHC genes control the susceptibility to Hg in mice, to develop systemic autoimmunity [97]. In a genetic study by Kono et al 2001, to define genes responsible for resistance to HgIA by performing genome wide searches using F1 and F2 intercrosses involving the resistant DBA/2 (H-2^d) strain to the susceptible SJL (H-2^q) strain. By comparing the locations of QTL, there was genetic linkage between induction of IC deposits in the glomeruli and chromosome 1 (designated Hmr1), and weakly to chromosome 7 [99]. Another genetic study, by Alkaissi et al 2018, we examined the differences in the serum levels of anti-nucleolar antibodies (ANoA) caused by non-H-2 genes in HgIA. By performing GWAS using H-2 congenic mouse strains (H-2^q) and their F1- and F2-hybrids, followed by fine mapping the QTL, there was a linkage between ANoA and the two genes, Bank1 and Nfkb2, involved in the intracellular pathway of BCR activation [41]. Knockout studies in HgIA in mice have shown that IL-6^{−/−}, CD28^{−/−}, and IFN^{−/−} H-2^q mice do not develop ANoA [99, 100].
Table 1. H-2 haplotypes in different mouse strains.

<table>
<thead>
<tr>
<th>Prototype strain</th>
<th>Other strains with the same haplotype</th>
<th>Haplotype</th>
<th>K</th>
<th>IA</th>
<th>IE</th>
<th>S</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA</td>
<td>AKR, C3H, B10.BR, C57BR</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
</tr>
<tr>
<td>DBA/2</td>
<td>BALB/c, NZB, SEA, YBR</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>C57BL/10</td>
<td>C57BL/6, C57L, CH3.SW, LP, 129</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>A</td>
<td>A/He, A/Sn, A/Wy, B10.A</td>
<td>a</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>A.SW</td>
<td>B10.S, SJL</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>A.TL</td>
<td>STOLI, B10.Q, BDP</td>
<td>t1</td>
<td>s</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>d</td>
</tr>
<tr>
<td>DBA/1</td>
<td></td>
<td>q</td>
<td>q</td>
<td>q</td>
<td>q</td>
<td>q</td>
<td>q</td>
</tr>
</tbody>
</table>

*Prototype strains of different mouse strains and the designation of haplotypes on H-2. Also shown are some other strains with the same haplotype [101].

2.9 *Mechanisms in HgIA*

Several observations have demonstrated that T-cells are essential for induction of ANoA production using Hg exposure [98, 102]. CD4+ T cells become polarized into T_H cell types after activation, such as T_H1 and T_H2. T_H1 cells produce cytokines such as IFNγ and promote cellular responses, whereas T_H2 cells produce IL-4, IL-5 and IL-13 and promote humoral responses. It was first suggested, that Hg induction in susceptible mice leads to an activation of the T_H2 CD4+ T helper cell subset and expression of cytokines such as IL-4 [85]. However, other studies have been unable to demonstrate the critical role for T_H2 cells. Kono and colleagues studied the role of T_H1 and T_H2 subset in susceptible deficient B.10S (H-2s) mice of IL-4 and IFNγ which were exposed to HgCl2, and demonstrated that IL-4 deficient mice were as susceptible as wild type mice, whereas IFNγ knockout mice were resistant to HgIA [103]. More recent studies have focused on the innate immunity and intracellular pathways, suggesting that endosomal TLRs, IL-1α and IL-6 but not type I IFN are the major innate factors that drive autoimmunity following exposure to mercury [104].
3.0 Mercury

Mercury is a highly toxic metallic element that can be found naturally in the environment. Natural sources of Hg are volcanic eruptions and decay of Hg containing sediments. It can be transported through the atmosphere and circulate in the air globally for years. Humans and animals are exposed to Hg through anthropogenic sources such as mining activities, combustion of fossil fuels, waste disposal and industrial activities which are now assumed to be the main source of Hg in the environment [105, 106].

3.1 Types and Sources of Mercury Exposure

Mercury circulates in three forms; elemental Hg (mercury vapor), inorganic Hg (HgCl₂) and organic Hg (methyl- ethyl- mercury) [107]. The toxicological profile and metabolic fate depends on the form of Hg, the dose to which the organism is exposed, age and the exposure route [108]. Elemental Hg is found in dental amalgam, which is considered to be the largest source of Hg exposure to general population in industrialized countries. Studies have shown an association between the number of amalgam filling and the concentration of inorganic Hg in blood and urine [109]. Elemental Hg can also be found in private homes (thermometers), thermostats, chlorine-alkali manufacture, electronic switches and fluorescent lamps [110]. Organic Hg is considered to be the most toxic form of Hg exposure in which methyl-Hg (MeHg) is the most common form to which humans and animals are exposed. It is formed by methylation of inorganic Hg by aquatic microorganisms in oceans, lakes and rivers and bioaccumulates in the aquatic food chain leading to high concentrations in fish. Ingestion of contaminated fish and seafood is the major source of human exposure to methyl-Hg [111, 112]. The main sources of inorganic Hg compounds can be found in cosmetic and medical products, antiseptics, skin-lightning creams and teething powders [113, 114]. Renal proximal tubular cells are the main targets in which inorganic Hg accumulates and induces cell injury [111]. In vitro studies have shown that Hg has a cytotoxic effect and can induce cell death by apoptosis or necrosis in a time- dose- and cell-dependent manner [115].
3.2 Route of Exposure
Elemental Hg (Hg⁰) is inhaled as Hg vapor and about 80% is retained in the body. Hg⁰ dissolves and accumulates in erythrocytes and transported to all tissues in the body. Hg⁰ mainly accumulates in kidney but can cross the blood-brain barrier and the placental barrier. When Hg⁰ enters the cell, it becomes inorganic Hg through oxidation by the catalase enzyme [116, 117]. Organic Hg travels mainly through gastrointestinal tracts after fish consumption, in which 95% retains in the body. It is mainly accumulated in brain and liver. Conversion of organic Hg to inorganic Hg occurs partly through metabolizing in the microflora in the intestine. Organic Hg crosses the blood-brain barrier and placental barrier and accumulates in liver and brain [111, 112, 118]. Inorganic Hg is mainly derived from Hg⁰ and organic Hg. About 10% of consumed Hg retains via the gastrointestinal tract. It accumulates mainly in the kidney and cannot cross the blood-brain barrier or the placental barrier [117].

3.3 Mechanisms of Excretion
The exact mechanisms of how Hg accumulates in organs and excreted from the body are becoming clearer. Mercury has a high capacity to bind to thiol-containing proteins, which gives it the ability to bind to a wide range of proteins and affect their function [119]. Thiol-containing proteins are both targets for toxicity but also play a role in defense against toxicity. Glutathione (GSH) is a thiol-containing protein that plays a central role in the cytotoxic effect of Hg [111, 120, 121]. Hg binds to GSH to form glutathione-Hg complexes and exports Hg out to extracellular space and this way eliminating it from the body. GSH and Hg complexes have been identified in liver, kidney and brain and appear to be the primary form in which Hg is transported out of cells [107]. GSH and Hg complexes have also been identified in the bile and urine [122]. Studies have shown that polymorphisms in proteins glutamyl-cysteine ligase (GCL) and glutathione S-transferases (GST) that regulate the production of GSH can influence the accumulation of Hg in the tissue [123, 124] and polymorphisms in the GSH-related genes glutathione S-transferase Mu 1 (GSTM1) and glutamate-cystein ligase modifier subunit (GCLM) may modify MeHg metabolism [125]. Multidrug resistance-tolerated proteins (MRPs) play an important role in transporting GSH-Hg complex into the extracellular space [126, 127]. Toyama et al 2007 proved that not only GCL and GST, but also MRP1 and MRP2 proteins are involved in decreasing MeHg concentration in cells and this process was regulated by the transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2) [128]. Human and rat studies have demonstrated that the thiol containing chelator meso-2,3-dimercaptosuccinic
acid (DMSA) significantly reduces Hg concentration in the body [129, 130] and latest findings have shown that MRPs act as a primary transporter of secreting DMSA S-conjugates of inorganic Hg from proximal tubular cells [131, 132]. Nuclear respiratory factor 1 (Nrf1) is a transcription factor that is important in the transcriptional regulation of human and mouse GCL subunits and GSH levels. Fetal hepatocytes from Nrf1 knockout mice exhibit lower GSH levels and Nrf2 deficient mice that received MeHg showed an increase in Hg accumulation in brain and liver [133]. Alkaissi et al 2016 discovered Pprc1 as key regulator in the excretion of Hg from the kidney by GWAS and fine mapping on QTL. Pprc1 and two genes Nrf1 and Nrf2 coactivated by Pprc1 had significantly lower gene expression in the strain that accumulated more Hg in the kidney [38].
THE AIM

Elucidate genomic factors responsible for differences in mercury induced autoimmunity (HgIA) and excretion of mercury (Hg) from the body.

SPECIFIC AIMS

- Investigate systemic autoimmune phenotype characteristics in the Hg susceptible congenic strains A.SW, B10.S and their F1 and F2 offspring in HgIA.
  - IgG anti-nucleolar antibodies (ANoA).
  - IgG1 hypergammaglobulinemia
  - Anti-chromatin antibodies (ACA)
  - Polyclonal B cell activation

- Genome Wide Association Study to combine systemic autoimmune phenotypes to genomic regions in HgIA.
  - Identify gene(s) involved in the development of ANoA.
  - Identify genomic region involved in the development of serum IgG1 hypergammaglobulinemia.
  - Identify genomic region involved in the development of ACA.
  - Identify genomic region involved in the development of polyclonal B cell activation

- Genome Wide Association Study to Hg excretion from different organs to genomic regions in HgIA.
  - Identify gene(s) involved in the excretion of Hg from kidney.
MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Phenotype and genotype data is required in order to perform a GWAS. The main phenotypic data were autoimmune parameters triggered by Hg in a F2 mouse generation by crossing two susceptible strains A.SW (H-2^s) and B10.S (H-2^s). The A.SW strain is more susceptible compared to the B10.S strain. Mercury concentration in kidney was the non-immunological parameter in this thesis. The experimental design was divided in two main experiments (Fig 6).

In **the first experimental study**, F2-hybrids were obtained by crossing female A.SW and male B10.S mice followed by crossing their F1 generation. Mice were exposed to 2.7 mg HgCl\(_2\)/L (Fluka) in drinking water (**2.0 mg Hg/L**) at age 8–10 weeks, for 6 weeks before sacrifice.

**The second experimental study** was based on a new setup of mice. Serological studies and GWAS were performed on F2 mice (A.SW x B10.S) exposed to a 5.71 mg HgCl\(_2\)/L (Fluka) in drinking water (**4.0 mg Hg**), for 6 weeks before sacrifice. Gene expression studies were performed on A.S W and B10.S mice exposed to 8 mg HgCl\(_2\)/L (Fluka) in drinking water (6 mg Hg), for 0, 4, 8 or 12 days.

**Figure 6. Experimental Study**

*This thesis is divided in two main experiments based on Hg exposure on F2 mice, for GWAS. Both experiments are composed of two separate breedings of F2 derived by crossing A.SW and B10.S. Mice in first experiment received a dose of 2 mg Hg/L, and mice in the second experiment received a dose of 4 mg Hg/L. Phenotypic traits and experimental methods are presented. Hg accumulation in kidney data was obtained from Ekstrand et al. 2010 [134].*
FIRST EXPERIMENTAL STUDY (2 MG Hg/L)

The first experiment was performed in order to measure Hg concentration in kidney and autoimmune parameters were included. HgCl$_2$ was mixed with 203Hg isotope and 1 mL drinking water contained 35,000–45,000 counts per minute. Blood, tail, spleen and kidney were obtained from A.SW, B10.S, F1 and F2 mice.

MERCURY CONCENTRATIONS

Accumulation of Hg in kidney was performed by Ekstrand et al. 2010 [134]. The radioactivity of the kidney obtained at sacrifice was measured using a gamma counter. In this thesis, we used the data of renal Hg concentration in F2 mice (n = 334) and classified it as “high” (> 5,836 ng/g wet weight, the highest concentration in F1 mice), “low” (< 2,990 ng/g wet weight, the lowest concentration in F1 mice) and “intermediate” (2,990–5,836 ng/g wet weight, the range of concentrations observed in F1 mice). 28 F2 mice selected at random from each group using the randomized function RANDBETWEEN in Microsoft Excel, for a total of 84 mice. For detailed description, see paper I.

SEROLOGICAL ANALYSIS

The randomly selected 84 mice were further studied with serological methods. Unexposed F2 mice (n = 14) were included as control. Serum antinuclear antibodies of polyclonal IgG was assessed by indirect immunofluorescence. Result which resulted in a specific ANoA staining was scored from 0 – 3 (0, no specific staining; 1, slight staining; 2, moderate staining; 3, strong staining).

Polyclonal B cell activation was assessed by enzyme-linked immunosorbent assay (ELISA) by detecting antibodies targeting DNP albumin (dinitrophenyl) and ssDNA [135]. The optical density (OD) was measured at 405 nm. Serum IgG1 hypergammaglobulinemia was assessed by ELISA and OD was measured at 492 nm. IgG1 in the serum samples were determined using IgM standard curve [136]. See appendix for methodological description of ELISA anti-DNP, anti-ssDNA.
SECOND EXPERIMENTAL STUDY (4 MG HG/L)

The second experiment was performed in order to measure autoimmune parameters in HgIA. Autoantibodies were measured by two methods: I) Serum antinuclear antibodies of polyclonal IgG, assessed by indirect immunofluorescence. Instead of scoring, a titre (diluted 1:80 – 1:20480) was defined on specific ANoA staining. This phenotype was included in paper II, containing detailed description. II) Anti-chromatin antibodies were measured by ELISA and the OD measured at 405 nm. Polyclonal B cell activation was assessed by ELISA anti-DNP and anti-ssDNA. Serum IgG1 hypergammaglobulinemia was assessed by ELISA and this phenotype was included in paper III, containing detailed description. See appendix for methodological description of ELISA anti-DNP, anti-ssDNA, anti-ACA.

GENETIC ANALYSIS

In the first experimental study with 2 mg Hg/L, GWAS was only performed on Hg accumulation in kidney (paper I). Serological data was not included.

In the second experimental study with 4 mg Hg/L, GWAS was performed on all serological data in which ANoA was included in paper II, and serum IgG1 hypergammaglobulinemia was included in paper III.

BIOINFORMATICS

Sequences, polymorphisms, Single Nucleotide Polymorphisms (SNPs), microsatellites and conserved region, were identified and studied using Ensemble [23] and Mouse Genome Informatics (MGI) [30]. The data base NCBI/Primer-Blast (using Primer 3 and BLAST) was used to design primers [137]. The background strains of A.SW (A) and B10.S (C57BL/6) were used to study DNA sequences since the genome of the A.SW and B10.S strains are not in the database.
DNA EXTRACTION
AND QUALITY CONTROL

Briefly, DNA was extracted from tail tips, spleen or kidney of the mice, diluted to 20ng/μL. Purity control of the DNA was established using microsatellite marker with PCR, and verified by gel electrophoresing. Detailed description in paper I-II.

GRADIENT PCR
THE CORRECT ANNEALING TEMPERATURE

There are several phases occurring during a PCR in order to amplify the fragment of interest: i) Denaturing is the first phase, which the DNA is heated up to a temperature (around 95°C) to separate the double stranded DNA into two single strands. Then the DNA becomes two single strands and ii) annealing occurs were the temperature is lowered to a specific degree to enable the primers to bind in to the single stranded DNA. The temperature needed for the primers to bind depends on the primer length and the primer melting temperature (T_m) which is the DNA-DNA hybrid stability. It is based on how many G’s and C’s the primer has (GC content). The GC´s content should be 40-60%. Wrong temperatures leads to no binding or unspecific binding. Once the right annealing temperature is settled and primers bind, iii) extension starts, in which the temperature is raised (around 95°C) so the Taq polymerase binds to each primer and begins adding nucleotides and amplify the fragment.

In order to find the right annealing temperature, a calculation can be made based on GC content followed by a PCR test run of the primer with several annealing temperatures. Starting from low temperature on the left side of the PCR plate/strip to higher temperatures on the right side. Running the samples on an agarose gel will give you the information needed on what temperature gives the best fragment.
GENOME WIDE GENOTYPING

FIRST EXPERIMENTAL STUDY (2 mg Hg/L)

Genome wide genotyping was based on 330 microsatellites covering autosomes and the X chromosome. Microsatellites were genotyped using PCR amplified fragments with Ion pair reverse phase high performance liquid chromatography (IP RP HPLC) and agarose gels. Sizing of DNA fragments with microsatellites that differed 2-10bp between strains were detected with IP RP HPLC on Transgenomic WAVE system. Percentage of triethylammonium acetate (TEAA) solution, column temperature and flow rate (mL/min) was optimized for every microsatellite.

SECOND EXPERIMENTAL STUDY (4 mg Hg/L)

Genome wide genotyping was based on the SNP&SEQ technology platform at Uppsala University. Samples were genotyped using the Illumina mouse medium density linkage panel that contained 1449 SNP markers.

QUANTITATIVE TRAIT LOCI 1

Quantitative trait loci (QTL) were identified based on the logarithm of odds (LOD) score profiles derived from a genome-wide single-QTL scan by Haley-Knott regression [138] with a hidden Markov model (HMM) using R language based software with the qtl addon package (v.2.15.3) [139]. Regression was based on the data from F2 offspring for genotypes covering 19 autosomes. The genome-wide significance threshold was calculated based on 10,000 permutation replicates.
HAPLOTYPING

PAPER I-III

Additional microsatellites were used to narrow down the region by haplotype analysis in which the QTL was found. Haplotype analysis was performed by selecting a genotype with homozygote inheritance from one of the parental strains or heterozygote, followed by genotyping F2 generation upstream and downstream from the QTL with microsatellites. Microsatellites were used to amplify the selected regions with PCR and fragments run on agarose gels.

FINE MAPPING

PAPER I

Fine mapping was based on investigating SNPs on genes within the haplotype block. SNPs were genotyped with designed primers amplified with PCR and run on denaturing high performance liquid chromatography (dHPLC). Before analysis, PCR products of F2 mice were pooled with either A.SW or B10.S and denatured and then gradually cooling. PCR products were loaded on the DNAsep column and eluted on a linear ACN gradient. The gradient start and endpoint were optimized according to the size of PCR amplicon. Selected Tm’s for optimal separation of amplified DNA products were calculated using the WAVE maker software, Version 3.3.3 and tested for optimal resolution. A.SW, B10.S and their F1 offspring were used as control samples. Some genes with SNPs between A and C57BL/6 in Ensemble/MGI were not detected on the A.SW and B10.S strains. Sanger sequencing was performed to verify the dHPLC data. Sequencing is carried out to predict SNPs in a sequence and performed in four steps before running on capillary electrophoresis: I) PCR amplification is carried out to amplify the DNA fragment of interest. Primers are designed to cover the fragment and cannot exceed 1000 bp. One should also add 50 bp on each end because the capillary electrophoresis instrument needs some bp to start correctly. II) The next step is performed to clean your fragment form all unnecessary parts that was used to amplify your fragment and this was done with ExoProStar 1-Step. III) Preparations of sequencing reactions is the second PCR run to prepare labeling the fragments and this is performed for each primer separately. PCR product was used in a standard protocol for fluorescently labeled dideoxynucleotides (BigDye). IV) Before running on capillary electrophoresis, a second cleanup of fragment from all unnecessary
parts that was used to amplify the fragment. This was performed with washing and drying upside down. v) Samples ran on a capillary electrophoresis instrument (ABI 3500) for separation and detection. Sequences obtained were compared between A.SW, B10.S and the reference strain C57BL/6J.

**PAPER II**

Fine mapping was based on sequencing entire genes with next generation sequencing (NGS), within the haplotype containing differences between the two strains used. Design of target sequences was performed using the web-based application SureDesign (Agilent) for coding exons and UTRs (5’UTR and 3’UTR) for 11 genes. The genomic DNA (gDNA) library was prepared from 30 F2 mice (homozygous for A.SW strain on marker rs3676039), one A.SW mouse and one B10.S mouse (used as controls) using SureSelect QXT Target Enrichment for Illumina kit in accordance with the manufacturer’s protocols. Briefly, 32 DNA samples (n = 30 for F2 mice, n = 1 for A.SW mice, n = 1 for B10.S mice) were enzymatically fragmented, and adaptors were added to the ends of the fragments (350 bp fragment size). gDNA libraries were amplified and purified, followed by hybridization and capture the next day. Libraries were indexed and pooled into 4 groups (8 libraries per group) for multiplex sequencing. Sequencing was performed with a MiSeq Benchtop Sequencer using 500 cycles paired-end reads and a MiSeq v2 reagent kit. All data were analyzed using the command line in the Linux operating system. Quality score of raw data (FASTQ files) were analyzed with FastQC [140]. Sequence data were aligned with the mouse reference gene, *Mus musculus* USCS Mm10 [141], using the Burrows-Wheeler Aligner (BWA) software package [142]. Aligned sequencing data (SAM files) were converted into BAM files with SAM tools [143]. Variant calling was performed with the Genome Analysis Toolkit (GATK) [144].
**Paper III**

Fine mapping was based on investigating SNPs on genes within the haplotype block. The haplotype consisted of 63 genes and sorted in two waves. In the first wave, all SNPs (between the two background strains) located in the associated haplotype were identified. In the second wave, the identified genes were sorted based on the location of SNPs. Genes with SNPs of non-synonymous variants were selected whereas genes with SNPs located on untranslated regions (UTRs) and of synonymous variants were sorted out. Bioinformatics tools (Clustal X [145], Ensembl database [23]) were used to localize SNP positions (UTR’s, synonymous, non-synonymous) and to estimate evolutionary conservation of SNPs and amino acids.

**Quantitative Trait Loci 2**

**Paper I-II**

QTL2 analysis was performed similar to QTL1 but the regression was instead based on data from F2 offspring for genotypes covering SNPs within the haplotype block.

**Conserved Region**

**Paper I-III**

Comparison of SNPs between mammals was performed using the Ensembl database [23]. The conserved region of the amino acid sequences was analyzed using Clustal X (version 2.1) multiple sequence alignment software [145]. Amino acid sequence alignment was performed together with mouse strains A (background strain for A.SW) and C57BL/6 (background strain for B10.S). Specific species were selected because they have a sequenced gene of interest that can be used for alignment using the Ensembl database.


**Candidate Genes**

Identified candidate genes in paper I and paper II were further analyzed by performing expression analysis and secondary structure prediction.

**Housekeeping Gene**

There has never been any tests to confirm housekeeping genes that are unaffected by Hg. 10 housekeeping genes were selected in order to discover what genes that are unaffected by Hg and use it for normalization in gene expression (Table 2).

Table 2. Housekeeping genes

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>Beta Actin</td>
</tr>
<tr>
<td>Ppia</td>
<td>Peptidylpropyl isomerase A (cyclophilin A)</td>
</tr>
<tr>
<td>18s</td>
<td>Eukaryotic 18S ribosomal RNA</td>
</tr>
<tr>
<td>Gapd</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Pgkl</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>B2m</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>Tfrc</td>
<td>Transferrin receptor (P90, CD71)</td>
</tr>
<tr>
<td>Tbp</td>
<td>TATA box–binding protein</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hypoxanthine Phosphoribosyltransferase</td>
</tr>
<tr>
<td>Ywhaz</td>
<td>Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein</td>
</tr>
</tbody>
</table>

Ten housekeeping genes tested on mice in HgIA.
RNA EXTRACTION AND GENE EXPRESSION

**Paper I, II**

Total RNA was extracted from kidney using RNeasy Mini Kit. Quantity and purity were measured with NanoDrop ND-1000 spectrophotometric absorption at A260/A280 value of 1.8-2.0 and diluted to 20ng/µL. cDNA was synthesized by reverse-transcription of 0.2 µg total RNA using High-capacity cDNA Archive Kit. Analysis was performed in duplicates using Applied BioSystems 7500 Fast Real-Time PCR System with applied BioSystems Taqman Gene Expression Assays. Target gene expression for \textit{Pprc1}, \textit{Nrf1}, \textit{Nrf2}, \textit{Btrc}, \textit{Njkb2}, \textit{Bank1}, \textit{Nfkb1}, \textit{Tlr9}, \textit{Il6} and \textit{Tnf} were measured with reporter dye FAM (6-carboxyfluorescein) labeled probes. \textit{Gapdh} and \textit{Ppia} were selected as endogenous controls after determination of several genes [146]. Results are presented as relative transcription using the comparative Ct method. \(\Delta\text{Ct}_1\) was calculated for each of the target genes in every mouse by subtracting the endogenous control using geometric mean for each sample between \textit{Gapdh} and \textit{Ppia}. \(\Delta\text{Ct}_2\) was calculated by subtracting reference genes in untreated F1 mice (since parental strains are examined). \(\Delta\Delta\text{Ct}\) was calculated by subtracting \(\Delta\text{Ct}_2\) with \(\Delta\text{Ct}_1\) and relative quantification was finally calculated as \(2^{-\Delta\Delta\text{Ct}}\).

**Splice Variant Expression**

**Paper II**

cDNA was amplified for splice variant detection. Fragments were amplified by 30 cycles of PCR under following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s. PCR products were separated on 1% agarose gel for 30 minutes at 120 Volts and measured with the GeneFlash gel documentation system. Bands were quantified based on their relative intensities using ImageJ software 1.x [147].
SECONDARY STRUCTURE PREDICTION

PAPER II

Prediction of the secondary structure of protein was performed using the Chou & Fasman Secondary Structure Prediction (CFFSP) server. The cDNA sequences of strains were used to obtain protein sequences that were used to predict the secondary structures by the Chou & Fasman algorithm [148]. The cDNA sequences were obtained from the Ensembl database [39].

STATISTICAL ANALYSIS

Phenotype data were tested for normality using the D’Agostino–Pearson omnibus normality test, which computes a p-value for the combination of the coefficients of skewness and kurtosis. Data that did not pass the normality test are presented as medians ± interquartile ranges. Comparisons of phenotypes between two groups were performed using the Mann–Whitney U-test. Comparisons of phenotypes within a group consisting of 3 or more parameters, were performed using the Kruskal-Wallis and Dunn's multiple comparisons tests. Data that did pass the normality test are presented as the mean ± SD, and comparison between two groups was performed using Welch’s t-test. Differences with p < 0.05 were considered significant.
RESULTS

PHENOTYPIC ANALYSIS

FIRST EXPERIMENTAL STUDY

Only the Hg accumulation was selected for GWAS in the first experimental study because the other phenotypes showed a weak Hg induced activation of the immune system.

MERCURY ACCUMULATION

PAPER I

In our previous study, which compared the two mouse strains A.SW and B10.S (Fig 7), A.SW mice accumulated more Hg than B10.S mice. In terms of sex, male A.SW mice showed significantly greater accumulation of Hg than females of this strain, whereas the B10.S mice showed the opposite trend [134]. Renal Hg measurement data of F2 mice from Ekstrand et al. (2010) were used to find candidate genes associated with regulation of renal Hg$^{2+}$ accumulation in mice.

![Figure 7. Mercury accumulation in kidney.](image)

Kidney mercury concentrations. Mercury deposition in kidneys of male and female A.SW and B10.S mice exposed to 2 mg Hg/L drinking water for 6 weeks. Data obtained from previous study [134]. Figure is presented as mean ± SD, **p = 0.0041, ***p < 0.0001 (Welch’s test).
SECOND EXPERIMENTAL STUDY
SEROLOGICAL ANALYSIS

Paper II

ANoA corresponds to a staining of the nucleoli with clumpy nucleolar pattern, with 2-6 brightly
staining dots in the nucleoplasm (Fig 8A). The F2 generation (n = 129) showed significantly
higher IgG ANoA titer (n = 0.0001) compared to control F2 mice (n =14) (Fig 8B)

Figure 8. Serum anti-nucleolar antibodies (ANoA).

Serum IgG ANoA in F2 mice control (n = 14) and F2 mice exposed to 4 mg HgCl2/L (n =
129) after 6-week exposure. A) ANoA assessed by indirect immunofluorescence using HEp2
cells. Arrows show strong clumpy staining of the nucleoli. B) Y-axis represents the ANoA titer
(0–20,480). Graph is presented as the median ± interquartile range, ****p = < 0.0001
(Mann–Whitney test).
Paper III

Serum IgG1 (Fig 9) was significantly increased (p < 0.05) in Hg-exposed F2 mice (n=129) compared to control mice (n= 14). A large inter-individual variation was seen on exposed F2, indicating a genetic variation.

Figure 9. Serum IgG1 concentration on F2 mice.

*Serum IgG1 concentration in 4 mg Hg/L exposed F2 mice (n = 129) mice and control F2 mice (n = 14). Y-axis represents the serum IgG1 concentration. Graph is presented as median ± interquartile range, *p = < 0.05 (Mann–Whitney test).
Anti-chromatin antibodies

ACA was significantly increased (p < 0.05) in Hg-exposed F2 mice (n=129) compared to control mice (n= 14) (Fig 10).

Figure 10. Anti-chromatin antibodies

Anti-chromatin antibodies in 4 mg Hg/L exposed F2 mice (n = 129) mice and control F2 mice (n = 14). Y-axis represents the serum IgG1 concentration. Graph is presented as median ± interquartile range, *p = < 0.05 (Mann–Whitney test).

Anti-DNP antibodies

Polyclonal B cell activation measured with ELISA anti-DNP (Fig 11) was significantly increased (p < 0.05) in Hg-exposed F2 mice (n=129) compared to control mice (n= 14).

Figure 11. Anti-DNP antibodies

Anti-DNP antibodies in 4 mg Hg/L exposed F2 mice (n = 129) mice and control F2 mice (n = 14). Y-axis represents the serum IgG1 concentration. Graph is presented as median ± interquartile range, *p = < 0.05 (Mann–Whitney test).
GENOME WIDE GENOTYPE ANALYSIS

FIRST EXPERIMENTAL STUDY

All selected microsatellites for the genome wide scan, were based on the MGI database [40] to be non-polymorphic between the background strain for A.SW (A) and the background strain for B10.S (C57BL/6). 234 out of 330 microsatellites were excluded because of non-polymorphic pattern between the A.SW and B10.S strain. The X chromosome did not contain any polymorphic microsatellites between the two strains. In total, 96 polymorphic microsatellites were used, covering 19 autosomes with an average spacing of 20 cM (Fig 12).

Figure 12. Genetic Map

Genetic map of F2 population showing physical location of informative autosomal microsatellites. For a microsatellite to be informative, it should vary between the genotypes of the two strains, A.SW and B10.S.
SECOND EXPERIMENTAL STUDY

The Mouse MF Linkage Panel consists of 1449 SNPs between ten common background strains (129S7/SvJ, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6, CBA/J, DBA/2J, FVB/NJ, NOD/LtJ, SJL/J). Raw data achieved by SNP&SEQ technology platform (Uppsala University) was analyzed for separation between polymorphic and non-polymorphic genotype between A.SW and B10.S. 819 SNPs were polymorphic spanning all chromosomes (Fig 13).

![Genetic Map](image)

**Figure 13. Genetic Map**

Genetic map of F2 population showing physical location of informative autosomal SNPs. For an SNP to be informative, it should vary between the genotypes of the two strains, A.SW and B10.S.
A single, highly significant (p < 0.0002) QTL located at 38.46 cM (D19Mit53), on chromosome 19 had a LOD score of 5.78. Chromosome 8 (12.59 cM), 13 (27.48 cM) and 17 (55.48 cM) showed a LOD score of ≥2 but no significant difference after 10,000 permutations (Fig. 14A). Effect plot for renal accumulation and genotype was significantly higher (p < 0.0001) in F2 mice that were homozygous for the A.SW allele of D19Mit53 than in heterozygotes or in mice that were homozygous for the B10.S allele (Fig. 14B).

**Figure 14. Quantitative trait loci on autosomes and effect plot.**

(A) Genome-wide scan (n = 44 male, 40 female F2 mice) on autosomes was performed to identify quantitative trait loci (QTL) associated with Hg accumulation in kidney. Logarithm of odds (LOD) scores (y-axis) indicate a high association with microsatellite D19Mit53 on chromosome 19; LOD score = 5.78, ***p = 0.0002. (B) Mean ± SD renal Hg concentration (ng/g wet weight) according to D19Mit53 genotype. AA, homozygous for the A.SW allele; BB, homozygous for the B10.S allele; AB, heterozygote; ****p < 0.0001 (Mann–Whitney test).
SECOND EXPERIMENTAL STUDY

ANTI-NUCLEOLAR ANTIBODIES

PAPER II

QTL with the highest LOD score of 3.05 was located at 128292534 (rs3670168) on chromosome 3 (Fig 15A). An effect plot was performed to detect if ANoA phenotype was linked to high or low autoantibody production. F2 mice homozygous for the A.SW allele (AA) on rs3670168 (chr 3), had a significantly higher ANoA titer than heterozygous (p < 0.001) or in mice homozygous for the B10.S allele (p < 0.001), suggesting an autosomal recessive inheritance (Fig 15B).

Figure 15. QTL and effect plot.

(A) A genome-wide scan (n = 129) on autosomes was performed to identify quantitative trait loci associated with anti-nucleolar antibodies (ANoA) in mice exposed to Hg. LOD scores (y-axis) demonstrate curves over the autosomal chromosomes. X-axis demonstrates SNP markers on 19 autosomes. Arrow indicates the top peak on chromosome 3. (B) Effect of different alleles in F2 offspring at peak marker rs3670168 on chromosome 3. Allele effects in the F2 offspring (X-axis), homozygous for A.SW (AA) or B10.S (BB) or heterozygous (AB) for ANoA titer (y-axis). The plot displays the mean ± SD. p < 0.01 (Mann-Whitney test).
QTL located at SNP rs13477251 (3: 88854680), on chromosome 3 had a LOD score of 4.36 (p = 0.017) (Fig 16A). The concentration of serum IgG on position rs13477251 on F2 mice homozygous for the A.SW allele (AA) was significantly higher compared to heterozygous (p < 0.05) F2 mice (AB) and F2 mice homozygous (p < 0.01) for the B10.S allele (BB). No significant difference between AB and BB (Fig. 16B).

Figure 16. QTL and effect plot.

A) A genome-wide scan (n = 129) on autosomes was performed to identify quantitative trait loci associated with serum IgG1 hypergammaglobulinemia in mice exposed to Hg. LOD scores (y-axis) demonstrate curves over the murine autosomal chromosomes. X-axis demonstrates SNP markers on 19 autosomes. Medians ± interquartile ranges of IgG1 concentration according to rs13477251 genotype. AA, homozygous for the A.SW allele; BB, homozygous for the B10.S allele; AB, heterozygote; *p < 0.05, ** p < 0.01 (Mann–Whitney test).
HAPLOTYPE DATA
PAPER I-III

The region in which QTL was detected was narrowed down by performing microsatellite genotyping to identify haplotype linked to phenotype (Fig. 17).

Paper I indicated that 32 of 84 F2 mice were homozygous for the A.SW allele on marker D19Mit53. Additional genotyping with 20 microsatellites spaced between 20.18 and 56.28 cM, identified a DNA block between microsatellites D19Mit67 (37.98 cM) and D19Mit9 (38.97 cM).

Paper II indicated that 30/129 F2 mice were homozygous for the A.SW allele on marker rs3670168. Additional genotyping with 9 microsatellites spaced between 54.48 and 61.32 cM, identified a DNA block between D3Mit247 (128 110 214 bp) and rs3676039 (136 217 610 bp).

Paper III indicated that 39/129 F2 mice were homozygous for the A.SW allele on marker rs13477251. Additional genotyping with 5 microsatellites spaced between 37.88 and 43.03 cM, identified a DNA block between D3Mit175 (38.30 cM) and D3Mit49 (39.02 cM).
Figure 17. Haplotypes with genes polymorphic between background strains.

(A) Haplotype position between 37.98 and 38.97 cM on chromosome 19 associated with Hg accumulation in kidney (B) Haplotype associated with ANoA in the range 128 110 214 - 136 217 610 bp on chromosome 3. (C) IgG concentration associated with haplotype on chromosome 3 between D3Mit175 (38.30 cM) and D3Mit49 (39.02 cM).
Three microsatellites and eight SNPs polymorphic between background strains were selected within the haplotype. A.SW and B10.S strains. Lbx1, Tlx1, and Poll genes within this haplotype are polymorphic between the background strains [23, 30]. dHPLC did not detect any difference and this was further verified by sanger sequencing and were therefore excluded (data not shown). Fine mapping narrowed the region to 19:45630547–19:46384795 with a LOD score of 1.94 (Fig 18). SNP analysis revealed three genes segregated between background strains; Btrc, Pprc1, and Nfkb2. of the SNPs for Btrc and Nfkb2 were located in untranslated regions: Btrc had 12 SNPs (5´-UTR) and Nfkb2 had one SNP (3´-UTR). Pprc1 had seven nonsynonymous SNPs, all of which resided on exon 5.

**Figure 18. Quantitative Trait Loci 2 - fine mapping data on Hg concentration in kidney**

*QTL associated with Hg accumulation in kidney based on fine mapping results on chromosome 19, on 32 F2 offspring homozygous for A.SW on D19Mit53. Btrc, Pprc1 and Nfkb2 all had a LOD score of 1.94.*

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Fine mapping analysis was performed by sequencing the entire haplotype block with NGS. Thirty F2 mice homozygote for A.SW allele on rs3670168, and the control mice A.SW (n =1) and B10.S (n =1) were sequenced on the coding region on 11 genes. Eleven genes were selected since they contain SNPs between background strains A (for A.SW) and C57BL/6 (for B10.S). Data passed several checkpoints for quality before variants were called out. Variants between B10.S and A.SW samples were extracted and compared with F2 variants. 136 SNPs were discovered within the haplotype, spread across 11 genes. QTL analysis revealed 3 peaks, one located on position on Nfkb1 gene (LOD 2.44) and two located on Bank1 gene (LOD 2.46 and 2.47) (Fig 19). Nfkb1 contains five SNPs (rs13477428, rs30771025, rs13472038, rs31054249, rs13472037) between background strains, all of synonymous variants. Bank1 contains three SNPs (rs30260564, rs50828248, rs47442962) between background strains in which all are of missense variants. rs30260564 resides on exon 2 and both rs50828248 and rs47442962 reside on exon 7.

Figure 19. Quantitative Trait Loci 2 - fine mapping data on ANoA

Fine mapping exons in 11 genes within the haplotype region performed with NGS to identify QTL associated with ANoA in 30 F2 mice homozygous for A.SW on rs3670168. LOD scores of 2.44 for Nfkb1, and 2.46 and 2.47 for Bank1.
**PAPER III**

Fine mapping was based polymorphic SNPs between background strains [23]. Fourteen informative genes within this haplotype are polymorphic and was further analyzed based on SNP position (synonymous, non-synonymous and UTRs). Selection of non-synonymous SNPs revealed five of the 14 genes with polymorphic amino acids between background strains of A.SW and B10.S, Relaxin family peptide receptor 4 (Rxfp4), Misato 1, mitochondrial distribution and morphology regulator (Msto1), CDC-like kinase 2 (Clk2), Secretory carrier membrane protein 3 (Scamp3) and Mucin 1, transmembrane (Muc1) (Table 3).

Table 3. SNPs of missense variants.

<table>
<thead>
<tr>
<th>Gene</th>
<th>ID (Ensembl)</th>
<th>A</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rxfp4</td>
<td>rs30709009</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>rs31334673</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>rs31696635</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Msto1</td>
<td>rs30809262</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>Clk2</td>
<td>rs30710443</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>rs13468134</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>Scamp3</td>
<td>rs13461067</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>Muc1</td>
<td>rs8259089</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>rs8259141</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>rs8259135</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>rs30987263</td>
<td>A</td>
<td>T</td>
</tr>
</tbody>
</table>

Single nucleotide polymorphisms on 5 genes between A/J and C57BL/6J according to Ensembl. Accession numbers followed by nucleotide are presented for each strain.
EVOLUTIONARY CONSERVATION

Nucleotides

Comparison of SNPs among mammals was performed using the Ensembl database.

Paper I

Comparison of SNPs on Pprcl among 15 mammals revealed three SNPs (rs30815571, rs30566249, and rs30352970) located on conserved region. Ensembl data indicated that none of the SNPs in Btrc and Nfkb2 were conserved.

Paper II

Two of the three SNPs in Bank1 (rs30260564 and rs50828248) are in a conserved region in the A strain but not in the C57BL/6 strain region in 33 mammalian species.

Comparison of SNPs between background strains on Nfkb1 among 21 mammals revealed three SNPs (rs13477428, rs31054249, and rs13472037) located on conserved region.

Paper III

The five genes having non-synonymous SNPs with polymorphic amino acids between background strains of A.SW and B10.S were investigated for conserved region among 21 mammals. Only two of the five genes have SNPs located on conserved region. Rxfp4 revealed two SNPs and Msto1 revealed one SNP.
AMINO ACIDS

To increase the strength of conserved regions, conservation of the amino acids they produce were investigated. Amino acid sequence alignment was performed on mammals that have a sequenced gene in the Ensembl database.

PAPER I

Pprc1 SNP rs30352970, has the codon AGT, on the A strain which codes for serine. C57BL/6 has the codon GGT, which codes for glycine. Mammals had the same amino acid (glycine), as the B10.S strain.

PAPER II

Bank1 amino acid sequence alignment was performed on 14 mammalian species. Rs30260564 has the codon TTA (Leucine) in the A strain and TTC (Phenylalanine) in the C57BL/6 strain. The A.SW strain was located on a conserved amino acid region.

PAPER III

Rxfp4 has two variants located on conserved region, rs30709009 and rs31696635. Rs30709009 corresponds to the codon TGC (cysteine) in the C57BL/6 strain and to the codon CTC (Leucine) in the A strain. Rs31696635 on Rxfp4 code for the codon CGT (arginine), in the B10.S strain and CAT (histidine) in the A.SW strain. The B10.S strain was located on a conserved amino acid region. The amino acid of the two other SNPs were not conserved.

Msto1 has one SNP (rs30809262) that corresponds to the codon GAT (Aspartic acid) in the B10.S strain and GAA (Glutamic acid) in the A.SW strain. The A.SW strain was located on a conserved amino acid region.
**MOUSE STRAINS**

Comparison of SNPs on candidate genes were performed between common strains in HgIA (B10.S A.SW DBA/2 BALB/c).

**PAPER I**

A.SW, BALB/c and DBA/2 share the same alleles for all seven SNPs on *Pprc1*. The B10.S strain had different allele on all SNPs compared to the other strains.

**PAPER II**

The *Nfkb1* gene revealed A.SW and BALB/c sharing the same alleles for all five SNPs whereas the B10.S and DBA/2 strains share the same allele.

A.SW, BALB/c and DBA/2 share the same alleles for all 3 SNPs on *Bank1*. The B10.S strain had different allele on all SNPs compared to the other strains. The *Bank1* gene has shown to express different splice variants in human and mouse.

**PAPER III**

B10.S BALB/c and DBA/2 share the same allele for all three SNPs on *Rxfp4*. The A.SW strain had different allele on all SNPs compared to the other strains. A.SW, BALB/c and DBA/2 share the same alleles for the SNP on *Msto1*. The B10.S strain had different allele on the SNP compared to the other strains.

**SPlice Variants**

**PAPER II**

In order to detect splice variants, the relative band intensities of amplified DNA fragment covering exon 2, where measured on A.SW and B10.S strains after 4, 8 and 12 days Hg exposure, with day 0 as control group. Both strains expressed DNA fragments of full-length (with exon 2), and short Δ2 isoform (without exon 2) of *Bank1*. The relative band intensity of the full-length of *Bank1* in the A.SW strain showed no significant. The B10.S strain showed a significant increase (p = 0.0095) after 4 days Hg exposure compared to day 0. The relative band intensity of the Δ2 isoform of *Bank1* was significantly higher in the A.SW strains after Hg exposure on all time points when compared to day 0 (day 4; p = 0.0043, day 8 and 12; p = 0.0079). The B10.S strain showed a significantly decrease of Δ2 isoform after 4 days of Hg exposure (p = 0.0381), followed by an increase between day 4 and 8 (p = 0.0022).
**Gene Expression – Housekeeping Gene**

Ten housekeeping genes were selected in order to find housekeeping genes that are unaffected by Hg. *Pgk1, B2m, Tfr1, Tbp, Hprt,* and *Ywhaz* had to high Ct variations between the samples, possibly because they are affected of the experiment, Hg exposure. *18S* had to low Ct value, since the Ct values of housekeeping genes are recommended to be close to the Ct value of target genes, it was excluded. We selected to improve with *Gapdh, Beta Actin* and *Ppia,* since they had Ct values close to target genes and Ct variation under 2 cycles. For improved precision we selected samples from paper I, in a single plate for each housekeeping gene (*Gapdh, Beta Actin, Ppia*), and repeated them twice. The Ct results of these 3 genes were plotted in to a software called Normfinder, which is a known algorithm used in order to identify optimal Housekeeping genes [149]. Finally, *Gapdh* and *Ppia* were selected as useful markers. Geometric mean was calculated for each sample using *Gapdh* and *Ppia.* The final result were used as Housekeeping gene in the delta delta Ct formula for all target genes (Table 4).

**Table 4. Housekeeping genes**

<table>
<thead>
<tr>
<th></th>
<th>Gapdh (Ct)</th>
<th>Ppia (Ct)</th>
<th>Geometric mean (Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.SW Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17,42669</td>
<td>19,368</td>
<td>18,37129</td>
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<tr>
<td>Standard deviation</td>
<td>0,170228</td>
<td>0,30706</td>
<td>0,206377</td>
</tr>
<tr>
<td><strong>A.SW Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16,0607</td>
<td>18,89317</td>
<td>17,41864</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0,529735</td>
<td>0,457512</td>
<td>0,475361</td>
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<tr>
<td><strong>B10.S Male</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>19,22829</td>
<td>21,55443</td>
<td>20,35791</td>
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<tr>
<td>Standard deviation</td>
<td>0,337812</td>
<td>0,28327</td>
<td>0,299126</td>
</tr>
<tr>
<td><strong>B10.S Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>18,1798</td>
<td>20,5966</td>
<td>19,3504</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0,426342</td>
<td>0,405469</td>
<td>0,414203</td>
</tr>
</tbody>
</table>

Mean +/- SD of Ct values of *Gapdh, Cycphilin A* and the geometric mean of them are shown for each group of mice.
**GENE EXPRESSION**

Gene expression was carried out on candidate genes together with genes enhanced by candidate genes or involved in the intracellular pathway of candidate genes.

**PAPER I**

The candidate genes *Pprc1*, *Btrc*, *Nrf1*, and *Nrf2* were examined. *Pprc1* acts as a co-activator for Nrf1 and Nrf2 (via CREB) [150, 151] and, therefore, was also analyzed. For males and females combined, *Pprc1*, *Nrf1*, and *Nrf2* mRNA expression was approximately 5 times higher in B10.S mice than in A.SW mice (all p < 0.0001). The gene expression of *Btrc* and *Nrf1* were not significant between A.SW and B10.S.

**PAPER II**

Gene expression of *Bank1*, *Nfkb1*, *Tlr9*, *Il6*, and *Tnf* were examined on female A.SW and B10.S mice at 0 days followed by 4, 8 and 12 days of Hg exposure.

The genes *Nfkb1*, *Tlr9*, *Il6*, and *Tnf* had significantly higher (p < 0.05) gene expression in A.SW mice compared to B10.S mice. *Bank1* gene expression was significantly lower (p < 0.05) in the A.SW strain compared to the B10.S strain.
**DISCUSSION**

The general setup of a GWAS is to achieve the phenotypic data and genotypic data in order to perform an association to find QTLs. This is a large experiment in total, and several aspects are essential to master.

**THE PHENOTYPIC DATA** can be anything of interest such as eye color, hair color and more interesting in the medical field, complex diseases. The ultimate material for studying complex diseases is human material. Unfortunately, it is very hard to achieve human material for linkage studies since a history record is needed from parents, grandparents, children etc. The bigger pedigree the better. This is when animal models are very handy, especially mouse models because of I) the rapid breeding. II) Mice live in a sterile atmosphere, which enable phenotypic specificity. III) Mice in general reach sexual maturity and developed immune system at 5-8 weeks of age. IV) Serum and organs can be extracted and studied. V) In mouse models, the genetic background can be controlled by using inbred strains [30, 40]. By using inbred mouse strains, we know the genome sequence. Crossing two inbred strains will give us specific known SNPs in the F1 generation and the positions of heterozygosity/homozygosity in the Fn generation [152].

We used the heavy metal Hg, to induce autoimmunity and by using Hg, we could study the accumulation of Hg in kidney as well. The phenotypic characteristics of HgIA in various doses, strains and types of Hg were studied intensively during the beginning of 1990 up to the beginning of year 2000. During this period, researchers published various results that did not always correlate between papers [153]. This is because Hg triggers an autoimmune manifestation that is dose, time, gender and strain dependent [41, 154]. This can be solved by calculating the internal Hg dose in mice, but the internal dose differs most of the time between studies. The HgIA model is so sensitive that the smallest change in dose will have a great impact on the phenotypic outcome [153]. This thesis is divided into two experiments. The first experimental study consists of mice exposed to 2.0 mg Hg and the second experimental study consists of mice exposed to 4.0 mg Hg.
In the first experimental study for GWAS

The lowest dose to trigger a development of ANoA in susceptible female A.SW mice is 1 mg Hg/L for 2.5 weeks whereas the female B10.S strain requires 4 mg Hg/L for 2.5 weeks. However, not all mice developed ANoA after 2.5 weeks. It required 10 weeks for all mice to develop ANoA. B10.S male requires even a higher dose in each strain [154]. In this thesis, we analyzed development of ANoA with immunofluorescence on A.SW, B10.S and their F2 generation exposed to 2.0 mg Hg/L, for 6 weeks. Most A.SW mice developed ANoA, whereas only a small fraction of B10.S developed ANoA. Further, 43% on the F2 mice did not develop any ANoA on this low dose (data not shown). These observations demonstrate the importance of dose and time. What could be changed in this case would be the exposure of Hg dose since a higher dose from 4 mg Hg/L triggered the ANoA development in B10.S mice, and longer time point of 1 mg Hg/L from 2.5 weeks to 10 weeks did only trigger the A.SW strain. We decided not to use the ANoA data from these F2 mice since a high percentage did not develop any ANoA at all. Nevertheless, it would be very interesting to investigate these mice. We have two strains that are both susceptible, and a dose that only triggers ANoA on one strain could still give us information of QTL. It would be similar to a crossing between a resistant and a susceptible strain. In this case, it might even be a more specific QTL associated with the beginning of the ANoA development and not only associated genes involved in the ANoA development.

We further analyzed polyclonal B cell activation with anti-ssDNA and anti-DNP with ELISA on the same setup of mice. The A.SW strain developed significantly higher antibodies against ssDNA but not DNP compared to control. The 2 mg Hg/L did not trigger enhanced antibody development in both B10.S and F2 strains (data not shown). Hypergammaglobulinemia has been shown to develop at even higher doses 4 < mg Hg/L for the A.SW strain and even lower (0.5 < mg Hg/L) for the B10.S strain [154] which is not in agreement with our findings. Johansson et al. 1998 exposed A.SW mice for 1.0 mg HgCl₂/kg body weight diluted in sterile 0.84% NaCl as a 0.1–ml subcutaneous injection every 3rd day for 27–47 days. Significant increase of serum anti-DNP and anti-ssDNA was detected after 2-3 weeks [155]. The internal Hg dose used in that study is however much higher than the internal dose in Hultman and Nielsen 2001 and in this thesis. Taken together, we decided not to go further with this data since both parental strains and their F2 did not develop any clear polyclonal B cell activation compared to control. The dose of 2 mg Hg/L is clearly a too low concentration and must be increased in order to observe an immunological reaction caused by Hg.
Serum IgG1 hypergammaglobulinemia was significantly higher (P < 0.01) in all exposed mouse strains (A.SW, B10.S and F2) compared to control. Hg triggers an increased IgG1 concentration on non-susceptible mice as well, but at a lower concentration compared to susceptible strains [155]. This data could be of use for a GWAS but we decided not to choose this phenotype since the other autoimmune parameters were only slightly induced.

Taken together, the dose, strain and time has a big impact on the development of autoimmunity and immunological parameters. We did not use the immunological data for GWAS since mice did not develop a strong immune reaction to Hg in general, and only a fraction of mice developed a weak reaction of autoimmunity.

Differences in accumulation of Hg in kidneys between inbred mouse strains [134, 156, 157] suggest a genetic inter-strain variation regulating the retention or/and excretion of Hg. Such differences between genders have been described in both humans and animals [134, 158, 159]. We used the Hg accumulation in kidney data to study what gene(s) are responsible for the excretion of Hg from the kidney by implementing GWAS and gene expression analysis (paper I).
In the second experimental study for GWAS

We exposed F2 (n = 129) mice for 5.71 mg HgCl₂/L in drinking water (4.0 mg Hg) and analyzed five phenotypic traits: ANoA (paper II), IgG1 (paper III), ACA, anti-DNP, anti-ssDNA.

A dose of 4 mg Hg/L for 2.5 and 10 weeks triggers a development of ANoA in 100 % exposed A.SW mice and 75 % exposed B10.S mice [154]. In an unpublished data by Alkaissi et al 2012, we observed that only 71 % of B10.S mice developed ANoA when exposed to 6 mg Hg/L for 30 days and 100 % ANoA on exposed A.SW mice. The F2 mice in this thesis showed significantly higher IgG ANoA titer (n = 0.0001) compared to F2 control mice. We observed a widespread titre and only 9 % of the F2 mice did not develop any ANoA. This implies a genetic underlying cause in which F2 mice with no/low titre have theoretically inherited the phenotype from the B10.S strain.

The model to induce autoimmunity in mice exposed to Hg includes activation of T- and B-lymphocytes, with subsequent increases in serum IgG1, IgG2a and IgE Ab production [154, 160, 161]. Susceptible H-2s mouse strain have shown diverse levels of IgG1 in serum when exposed to Hg [155, 162] and the A.SW strain has a higher B cell activation and serum IgG concentration compared to the B10.S strain [160, 163]. Our data have shown a significant increase on Hg exposed F2 mice compared to control (p < 0.05). Similar to the ANoA data, we observed an inter-strain variation on IgG1 concentration as well, which could be explained by the IgG1 differences between the parental strains.

ELISA ACA is used to study the levels of autoantibodies targeting chromatin. Chromatin is found in animals and is a histone-DNA complex organized into repeating series of nucleosomes. It is an antigen for T and B cells in SLE patients [164] and murine lupus [165]. In HgIA, the levels of ACA with ELISA, measured at OD 405 nm are extremely low, and most papers do not show the data. ACA is significantly increased in HgIA in A.SW (ethyl mercury) [166], C57BL/6 (HgCl₂) [167] and B10.S (6 mg Hg/L)[168]. In this thesis, F2 exposed to Hg had a significantly higher (p < 0.05) ACA compared to control but the levels were modest (0.03 – 0.24, measured at OD 405 nm). We did observe the same widespread pattern within the F2 mice as we did with the other phenotypes and a GWAS was performed on the ACA data.

ELISA anti-DNP and anti-ssDNA have been used to study antibodies of the IgM and IgG class, which are indicators for a polyclonal B cell activation, a hallmark in HgIA. Based on previous
observations, a 4 mg Hg/L should induce a polyclonal B cell activation in both A.SW and B10.S mice [154]. The F2 mice in this thesis had a significantly increased antibody levels of anti-DNP compared to control mice ($p < 0.0001$), but no difference between exposed and control in anti-ssDNA (data not shown). We decided to investigate loci associated with polyclonal B cell activation by using the data of anti-DNP in a GWAS.

**THE GENOTYPE DATA** was composed of mice from both experimental studies but performed at different time points. In this thesis, we used the two inbred strains, A.SW and B10.S as parental strains. These two inbred strains do not have a known genome sequence. In order to study bioinformatics, we studied the background strains instead. The background strain of the A.SW strain is A, and the background strain of the B10.S strain is the laboratory mouse strain, C57BL/6 [23]. Both are congenic strains in which H-2\* originated from an outbred Swiss strain. A problem that must be taken into consideration when using background strains for bioinformatics is mutations, due to the number of generations passing by, and the creation of these congenic strains. We did indeed demonstrate this in the first experimental study, with SNPs and microsatellites by Sanger sequencing and dHPLC, since these methods showed no difference between A.SW and B10.S in certain positions, whereas the database Ensembl [23] reported differences between the background strains A and C57BL/6.

We performed a GWAS on five phenotypes in total; Hg concentration in kidney (paper I) from the first experimental study and ANoA (paper II), IgG1 (paper III), ACA and anti-DNP on the second experimental study. This is the first time a GWAS has been performed on two susceptible strains the HgIA model. A linkage study was performed by Kono and colleagues, in which they crossed susceptible H-2\* mice (SJL) with resistant H-2\textsuperscript{d} mice (DBA/2) to identify loci associated with ANoA and IC. ANoA showed a QTL close to H-2 and IgG IC had a QTL on chromosome 1 which they designated *Hmr1* (Heavy metal resistance 1) but did not reach the threshold of significance [169]. We found a QTL on all five phenotypes:

- **Hg concentration in kidney** – LOD 5.78 on Chromosome 19
- **ANoA** – LOD 3.05, 2.9 and 2.9 on chromosomes 3, 4 and 7 respectively
- **IgG1** – LOD 4.36 on chromosome 3
- **ACA** – LOD 3.1, 3.2, 3.4 and 6.8 on chromosomes 3, 6, 7 and 16 respectively
- **Anti-DNP** – LOD 2.9 on chromosome 2
The QTLs of autoantibodies (ANoA and ACA) and hypergammaglobulinemia (IgG1) are all located on chromosome 3. The QTLs on ACA and IgG1 are relatively close to each other in which ACA is located at 87.15 Mb and IgG1 at 89.42 Mb. In general, 1 cM equals to 1 megabase but there are recombination that occur in less than 0.3 cM/Mb and more than 3cM/Mb [1]. This could mean that these two QTL are inherited together. Further, the QTL on ANoA is located further away in the chromosome and less likely to be inherited together, but they could be in linkage disequilibrium as well [170]. This can be calculated but candidate genes should be discovered first.

Fine mapping was performed on haplotype data associated with Hg accumulation in kidney (paper I), ANoA (paper II) and IgG1 (paper III) and all three were analyzed in different ways. Before performing fine mapping, genes with SNPs between A.SW and B10.S were selected because the other genes have the same sequence between the two strains and cannot be associated candidate genes. In paper I, fine mapping was performed by genotyping 1< SNP from each gene within the haplotype and additional QTL analysis was performed. In paper II, all exons on genes with SNPs were sequenced with NGS and additional QTL analysis was performed. In paper 3, bioinformatics was performed to theoretically investigate what SNPs are more likely to be associated.

We discovered that Hg excretion is linked to Peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (Pprc1). The development of ANoA is linked to B-cell scaffold protein with ankyrin repeats 1 (Bank1) and nuclear factor kappa B subunit 1 (Nfkb1). Serum IgG1 hypergammaglobulinemia is linked to a loci on chromosome 3 in which Relaxin Family Peptide/INSL5 Receptor 4 (Rxfp4) is a potential candidate gene. The mutations on the associated genes on all three papers were linked to the most susceptible strain, the A.SW. Gene expression analysis in paper I and II, was performed on associated proteins and other proteins involved in the intracellular pathway.
**Paper I candidate genes**

*Pprc1* encodes PGC-1, a key regulator of the protein family of nuclear respiratory factors (Nrf) which co-activate these transcription factors [173]. PGC-1 interacts with both Nrf1 and Nrf2 and stimulates their activities [174, 175], enhancing intracellular GSH-levels, complexing with Hg and increasing MRP levels to stimulate the elimination of Hg [132]. Thus, the proximal tubular cells transport Hg into tubular lumen and out of the body (Fig 20) [107]. Conserved region analysis, gene expression and comparison of SNPs between mouse strains showed that the B10.S strain has a protective genetic sequence compared to the A.SW strain in eliminating Hg out of the body.

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**Figure 20. Schematic representation of the *Pprc1* (PGC1) during Hg exposure.**

Mercury enters the proximal tubular cells by the help of OAT-1, -3 [171] and binds to the enriched sulfhydryl protein GSH. MRP-1, -3 support GSH-Hg complex out of the cell into tubular lumen and out with the urine [128, 132]. *Pprc1* encodes the protein PGC1 that acts as a co-activator on proteins such as Nrf1 and Nrf2. The Nrf family serves as transcription factors in which Nrf1 regulates the production of GSH levels [133, 172, 173] and Nrf2 regulates the production of MRP-1, -2 [128].
Paper II candidate genes

Bank1 is an adaptor/scaffolding protein that is mainly expressed in B-cells but also a lesser extent to plasmacytoid dendritic cells and myeloid cells [174]. It seems that Bank1 regulates the intracellular pathway upon BCR signaling in two ways (Fig 21):

1) Bank1 regulates the Lyn, Syk, PLCγ2 pathway followed by calcium release via IP3R on endoplasmic reticulum stores [174, 175]. PLCγ2 also acts on PKC, leading to the activation of Nuclear factor NF-kappa-B (NF-κB) [176].

2) BCR undergoes endocytosis and activates Tlr9 [177] leading to p38 and JNK, and NF-κB activation (Yi et al 2003). B-cells from deficient Bank1 mice have shown that Bank1 controls Tlr9 signaling via the p38-MNK1/2 pathway. A significantly higher IL-6 expression was observed on Bank1 knock out mice when compared to wild type mice [178]. undergoes endocytosis and activates Tlr9, leading to p38 and JNK, and NF-κB activation [179] and expression of various cytokines, such as IL-6 and TNFα [180].

This thesis have shown that the more severe A.SW strain develops significantly higher gene expression of Tlr9, Il6 and Tnf upon Hg exposure compared to day 0, whereas the less severe B10.S had a significant decrease of Tlr9 only. Splice variants of Bank1 upon Hg exposure, significantly increased the Δ2 isoform of Bank1 in the A.SW and the full length Bank1 on the B10.S strain. Taken together, the more severe strain seems to develop a BCR signaling pathway associated with development of ANoA. The question rise about what activates the BCR to initiate the autoimmune manifestation in HgIA. Mercury has a high affinity to interact with cysteine, containing sulphydryl groups. Sulphydryl groups form disulfide bridges between cysteine and give a protein the three dimensional structure and Hg breaks the disulfide bonds leading to loss of structure [181]. Fibrillarin is known to be modified by HgIA in susceptible mice and they develop ANoA that seems to target fibrillarin [102], but also several other unknown proteins [88]. It has been suggested that cryptic epitopes of fibrillarin are shown as antigens by MHC class II in APC presented to T-cells. In our findings, it seems that ANoA development is initiated by a BCR rather than a TCR signaling since we have discovered that Bank1 and Nfkb1 are linked to ANoA in which Bank1 is highly expressed in B-cells and no expression in T-cells [174]. Nfkb1 is expressed in T- and B-cells but since Bank1 is B-cell specific, we believe that the severe ANoA production is initiated by APC on B-cells rather than T-helper cells. But the intracellular signaling permitting the activation of NF-κB is TD [48]. Another hypothesis could be that Hg, with its high affinity to sulphydryl, breaks the disulfide
bonds between Igα and Igβ on BCR, leading to structural modification. The BCR is composed of the antigen-binding chains heavy and light immunoglobulins with two proteins associated with the heavy chains, Igα and Igβ. Igα and Igβ are bound together by disulfide bonds [182]. Modification of BCR by Hg may initiate self-antigen detection and activation of the intracellular cascade, regulated by Bank1 and NF-κB.

**Paper III candidate genes**

*Rxfp4* codes for a G-protein coupled receptor with high affinity for the hormones Relaxin-3 (RLN3) and insulin-like peptide 5 (INSL5). This receptor is part of the Relaxin family and there are studies linking the other Relaxin hormones and receptors to the immune system [183, 184], but little is known regarding this specific Relaxin-3 receptor 2. According to UniProt (Q7TQP4), the function of Relaxin-3 receptor 2 is an intracellular signaling that inhibits cAMP accumulation and activates a strong calcium release in the cell when binding to its ligand (INSL5) [185]. Calcium is a vital second messenger that regulates downstream signaling upon TCR and BCR activation and these pathways are highly involved in antibody production and Ab class switching [186] [187]. More studies are needed to investigate associated candidate genes in IgG1 hypergammaglobulinemia and the role of *Rxfp4* in HgIA.

What would be the next step after genes have been identified by GWAS in a mouse model for systemic autoimmunity? First, a comparison of GWAS genes between mouse and human must always be performed. Genomic comparison between these two species are very informative since 80% of the human genes and 75% of the mouse genes are in 1:1 orthologous relationship [31]. When finding a good relation between mouse and human on candidate genes there are endless of experiments available for studying the structure and function *in vivo, in vitro*, and in computational models.
Figure 21. Intracellular pathway of BCR activation

BCR ligand incite the intracellular signaling cascade by: A) endocytosis of bound ligand and presented by BCR to endosomal Tlr9 for subsequent activation. Bank1 acts on Tlr9 downstream pathway via the p38-MNK1/2 and activation of NF-κB. B) Bound ligand on BCR activates directly intracellular proteins including Lyn, Blk, Syk, and Btk. These kinases phosphorylate adaptor proteins such as Bank1, which in turn activates the NF-κB pathway via PLCγ2/ PKC.
CONCLUDING REMARKS

By using a genetic approach for investigating what loci and genes that regulate phenotypic traits in a mouse model for systemic autoimmunity we have discovered:

- That the anti-nucleolar autoantibody production is linked to Bank1 and Nfkb2 genes in which Bank1 has shown to be highly expressed in B cells but no expression in T cells. The highest QTL in our GWAS was linked to the B cell, which also demonstrates the importance of the intrinsic pathway of B cells in autoimmunity.
- That serum IgG1 hypergammaglobulinemia was linked to a haplotype region on chromosome 3, where the Rxfp4 gene seems to be the highest potential candidate gene.
- That the development of anti-chromatin antibodies was linked to loci on chromosomes 3, 6, 7 and 16.
- That polyclonal B cell activation is linked to a locus on chromosome 2.

An increased understanding of the genetics rendering susceptibility to autoimmune diseases, and the linkage to conserved areas of the genes may give perspectives also for identification of susceptible human individuals and for potential pathways for developing new human therapies.

By using the mercury induced autoimmunity mouse model, we investigated what genes regulate the levels of mercury from the kidney. Regulation of Hg retention in the kidney is associated with the Pprc1 gene. The excretion process of Hg linked to the Pprc1 gene may also operate in other target organs, such as liver and brain, as well as different forms of Hg such as elemental and organic Hg. We have also discovered that highly conserved SNPs on Pprc1 may have a critical role for the susceptibility of excreting Hg out of the body. Mutations on the human PPRC1 could be the cause of why humans have shown interindivdual susceptibility in Hg toxicokinetic.
APPENDIX

METHODOLOGICAL DESCRIPTION

SERUM ANTI-DNP CONCENTRATION ASSESSED BY ELISA

Microtiter plates (Nunc) were coated over night with DNP-albumin (Albumin Human Dinitrophenyl, Sigma-Aldrich) at 4°C followed by repeated washes with PBS pH 7.35-Tween 20 (0.1%)-BSA (1%) [135]. Sera, diluted in PBS pH 7.35-Tween 20 (0.1%)-BSA (1%) were added to the wells (duplicate wells). Pooled serum from MRL mice (The Jackson Laboratory) was used as positive control and the blank was PBS pH 7.35-Tween 20 (0.1%)-BSA (1%). Plates were incubated for 1 hour in room temperature followed by repeated washes with pH 7.35-Tween 20 (0.1%)-BSA (1%). Goat anti-mouse-IgG-ALP (polyvalent IgG, IgA, IgM) (Sigma-Aldrich) was added to the wells and incubated for 1 hour in dark room temperature. Plates were washed before substrate was added and the optical density (OD) at 405nm was measured after 20 min and the reaction was stopped with 3 M NaOH. Background values in wells were subtracted.

SERUM ANTI-ssDNA CONCENTRATION ASSESSED BY ELISA

Microtiter plates (Nunc) were coated over night with single-stranded DNA from calf thymus (Sigma-Aldrich) kept in the 4°C, followed by repeated washes with PBS, pH 7.35. Blocked with PBS pH 7.35-Tween 20 (0.1%)-BSA (1%) for 1 hour in 37°C. plates were repeatedly washed first with PBS-Tween (0.2%) then with PBS ph 7.35. Sera, diluted in 1:150 in PBS pH 7.35-Tween 20 (0.1%)-BSA (1%) were added to the wells (duplicate wells). Pooled serum from MRL mice (The Jackson Laboratory) was used as positive control and the blank was PBS pH 7.35-Tween 20 (0.1%)-BSA (1%). Plates were incubated for 1 hour in 37°C followed by repeated washes with PBS pH 7.35-Tween 20 (0.1%)-BSA (1%). Goat anti-mouse-IgG-ALP (polyvalent IgG, IgA, IgM) (Sigma-Aldrich) diluted 1:1000 in PBS pH 7.35-Tween 20 (0.1%)-BSA (1%) was added to the wells followed by a 2 hours incubation in 37°C in dark. After repeated washes with PBS pH 7.35-Tween 20 (0.1%)-BSA (1%), the substrate was added to the wells followed by 20 minutes incubation in 37°C in dark. plates were washed before substrate was added and the optical density 405nm was measured after 20 min and the reaction was stopped with 3 M NaOH. Background values were subtracted.
SERUM ANTI-CHROMATIN ANTIBODIES CONCENTRATION ASSESSED BY ELISA

Microtiter plates (Nunc) were coated overnight with calf thymus chromatin kept in the 4°C, followed by repeated washes with PBS-Tween 20 (0.05%) blocked with 0.1% gelatin (Bio-Rad) in PBS over night at 4°C. After repeated washes with PBS-Tween 20 (0.05%), sera were diluted 1:400 in PBS and added to the wells (duplicate wells). Pooled serum from MRL mice (The Jackson Laboratory) was used as positive control and the blank was PBS. Plates were incubated for 1.5 hours in room temperature followed by repeated washes with PBS-Tween 20 (0.05%). Goat anti-mouse-IgG (gamma) ALK PHOS conjugate (Invitrogen) were diluted 1:16000 and added to the wells followed by 1.5 hours incubation in dark room temperature. Plates were washed repeatedly with PBS-Tween 20 (0.05%) and the substrate was added. The Optical density (OD) was measured at 405 nm.
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42. Brooks AJ. The essence of SNPs. Gene. 1999;234(2):177-86. doi: https://doi.org/10.1016/S0378-1119(99)00219-X.


Publications

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