CHAPTER 11

Discussion and future perspectives
The aetiology of autoimmune hepatitis (AIH) is still unknown, but is believed to occur as the consequence of an aberrant immune response towards an unknown trigger in a genetically susceptible host. In the absence of a gold standard, the diagnosis of AIH is based on the combination of clinical, biochemical and histopathological criteria for which two scoring systems have been developed. Immunosuppressive treatment with corticosteroids has been the cornerstone of treatment since the earliest description of the disease in 1950 by Waldenström. These regimens are often successful at inducing remission and generally lead to normal life expectancy, but are associated with side effects and are sometimes unsuccessful in inducing remission of disease. Hence, there remain areas of unmet aetiological and clinical needs including fundamental insight in the pathogenesis, diagnosis and management of autoimmune hepatitis, some of which are addressed in this thesis.

Autoimmune hepatitis is a rare disease in itself and familial occurrence is extremely rare (<1%). In addition, there is virtually no twin data on AIH, which does not suggest a monogenetic cause for AIH development. However, the female preponderance as well as the clinical overlap with other autoimmune diseases in up to one-third of cases does support genetic involvement in disease pathogenesis. In addition, up to 15% of AIH cases presents with an overlap with primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), both autoimmune liver conditions in which genetic susceptibility loci have been identified. In the early nineteen-nineties, AIH has been associated with different human leukocyte antigen (HLA) class-II genotypes in different populations. Although these associations show that that susceptibility to AIH is in part determined by genetic predisposition, the absence of these alleles does not preclude AIH development.

Over the years several candidate gene studies, targeting one genetic locus or single nucleotide polymorphism (SNP), aimed but have failed to establish independent and reproducible associations outside the major histocompatibility complex (MHC) region. One such SNP, at position +49 in the cytotoxic T lymphocyte antigen-4 (CTLA-4) gene, has been described as a potential determinant of increased susceptibility to autoim-
mune diseases such as multiple sclerosis, type-1 diabetes and autoimmune thyroiditis.\textsuperscript{13} The CTLA-4 molecule is expressed on the surface of activated T-cells and regulatory T-cells (T-regs) and acts as an inhibitory signal receptor in T-cell activation through binding to the B-7 ligands 1 and 2 (CD80 and CD86) on antigen presenting cells (APC) in competition with CD28. The $\text{CTLA-4 +49 A/G}$ SNP results in an amino acid substitution of Threonine with Alanine at position 17 in the CTLA-4 protein, affecting expression and function of the protein.\textsuperscript{14-16} This SNP has been described as a non-HLA susceptibility determinant in small study cohorts of Caucasian and Chinese AIH patients.\textsuperscript{17, 18} Chapter 3 describes the first large genetic study of the Dutch AIH Group in 667 Caucasian patients and 498 population control subjects, which aimed to assess the frequency of the previously associated $\text{CTLA-4 +49 G}$ AIH risk allele. In this large study, we did not identify a significant association between the $\text{CTLA-4 +49 A/G}$ SNP and AIH, disproving previous reports on the involvement of this polymorphism as a major susceptibility risk allele for AIH in Caucasians.\textsuperscript{19} In addition, no associations between this SNP and AIH patient characteristics or outcome were identified. The previously reported association in Caucasians was most likely a false positive result due to the relatively small control cohort that was used in the first study in AIH which rendered an unusual low prevalence of the $\text{CTLA-4 +49 G}$ allele.\textsuperscript{17} However, it must be noted that our study also focused on just this single nucleotide polymorphism, leaving other potential susceptibility loci for AIH inside and outside the $\text{CTLA-4}$ gene unremarked.

**Genome-wide association study**

To better study the genetic background of disease, particularly in autoimmunity, genome-wide association studies (GWAS) have been successful in identifying new candidate genes.\textsuperscript{20} Hence in chapter 4 we describe the analysis and results of the first GWAS of 300,739 SNPs in 649 AIH type-1 patients and 13,436 population controls, which showed that AIH type-1 is associated with variants in the major histocompatibility complex (MHC) region as well as variants in the $\text{SH2B3}$ and $\text{CARD10}$ genetic regions.\textsuperscript{21} The $\text{SH2B3}$ locus did not exceed the stringent threshold for genome-wide significance ($P < 5.0 \times 10^{-8}$), but it yielded a consistent result in both the discovery and replication analysis and thus most likely represents a true positive association. This SNP then represents the first genetic risk locus for AIH outside the MHC. It encodes a missense variant in exon 3 of the Scr homology 2 adaptor protein 3 ($\text{SH2B3}$) gene located on chromosome 12 (12q24 region). $\text{SH2B3}$ is a negative regulator of T cell activation, tumor necrosis factor (TNF) and Janus kinase 2 and 3 (JAK2/3) signalling and plays an essential role in normal hemapoiesis.\textsuperscript{22, 23} The AIH risk allele $\text{rs3184504*A}$ results in replacement of the basic polar arginine with the nonpolar tryptophan at position 262 (R262W) in the pleckstrin homology domain of the SH2B3 protein. Expression quantitative trait locus analyses in 5,311 healthy individuals has established that the AIH risk allele $\text{rs3184504*A}$ is associ-
ated with higher expression levels of several genes involved in interferon-γ production, suggesting that the risk allele leads to an increased adaptive immune response. The associated risk of the rs3184504*A allele for concomitant autoimmune diseases in this study is consistent with previous reports that identified this allele as a risk factor in PSC, PBC, type 1 diabetes mellitus (T1DM), hypothyroidism, rheumatoid arthritis (RA) and celiac disease (CeD). This finding and the marked inflation of association results in this study in autoimmune and immune associated SNPs, specifically with PBC and PSC, further supports involvement of pleiotropic loci increasing the risk of developing an autoimmune disease, but fails to explain why a certain individual would develop AIH in particular.

As an exception, a recent case study showed a relationship between a mutation in the GATA2 gene and AIH with lower levels of T-reg transcription factor FOXP3 in a patient with monocytopenia. Another example of monogenic involvement in autoimmune hepatitis is the autosomal recessive disorder of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is characterized by immune-mediated destruction of endocrine tissues, chronic candidiasis and ectodermal disorders. This syndrome is associated with mutations in the autoimmune regulator gene (AIRE), which regulates the transcription of organ-specific self-antigens in thymic epithelial cells and is involved in the negative selection of organ-specific thymocytes. However, a form of hepatitis is only observed in up to 20% of cases, suggesting a non-selective role for AIRE in the pathogenesis of AIH.

It should be noted that genetic variation explains only 20% of the risk for development of AIH. Exposure to thus far unknown environmental factors, such as viral infections, drugs, and possibly the (gut-)microbiota are likely to be important contributors to AIH development. So far there are no experimental studies focusing on the biological effects of the identified genetic loci in AIH, PBC and PSC, explaining the mechanism of how this genetic risk results in AIH development. Hence, this first GWAS in AIH presented in this thesis unequivocally established AIH type-1 as complex genetic disorder with strong involvement of the MHC region. Our findings support that part of the genetic susceptibility for AIH type-1 overlaps with other immune-mediated diseases, including PBC and PSC. In other conditions, some of the newly identified genetic loci have helped to generate new hypotheses for basic immunological research, contributing to the development of new therapies biological acting agents. Further studies in larger AIH cohorts and denser genotyping techniques are mandatory to improve statistical power, whereas meta-analyses and combination analyses with clinically and genetically overlapping autoimmune traits will likely result in the identification of more AIH susceptibility loci as well as severity or immunomodulating markers. This may lead the development of early markers of disease progression and potential cirrhosis development, thus impacting on individual patient care.
Immunomodulation

To further investigate the potential immunomodulating effect of the important AIH susceptibility loci HLA-DRB1*03:01 and HLA-DRB1*04:01, Chapter 5 describes the effect of these genotypes in the MHC region on clinical characteristics and outcomes of AIH patients. Three-quarters of the AIH type-1 patients were HLA-DRB1*03:01/HLA-DRB1*04:01 positive and a clear, independent associations between these alleles and the IAIHG score was observed. As both these alleles encode the amino acid lysine at position 71 (71K) in the binding groove sequence 67 to 72 (LLEQKR) of the HLA-DR beta chain, this finding supports the hypothesis of molecular mimicry. In this hypothesis (unknown) exogenous antigens trigger an immune response which is also directed at similar but endogenously expressed antigens. Apart from this shared motif, we also found differences between the HLA-DRB1*0301 and HLA-DRB1*0401 alleles in relation to the clinical phenotype. HLA-DRB1*03:01 was independently associated with higher IgG levels, the exposure to immunosuppressive medication and liver transplantation whereas the HLA-DRB1*04:01 allele was independently associated with presentation at older age and a female predominance. These combined observations reaffirm the likelihood that disease severity and treatment response in type-1 AIH have a genetic basis and that it may be possible to define genetic indices of prognosis. Some, targeted genetic testing has proven to be useful in the delivery of personalized care in other conditions. Single nucleotide polymorphisms in the IL-28B gene as well as HLA-genotypes were identified as clinically relevant predictors of clinical response to interferon based therapies in chronic hepatitis C and hepatitis B respectively.35, 36 The search for similar immunomodulating polymorphisms or loci, genetic variations that do not necessarily cause or contribute to disease development, but are markers of clinical outcome or therapeutic effectiveness, is of interest as current clinical practice lacks validated predictors of outcome. In clinical practice the assessment of HLA-DRB1 alleles can help to identify individuals with AIH who may have an unfavourable prognosis and need closer monitoring. This study emphasizes the importance of novel genetic factors in occurrence, clinical expression, and behaviour of AIH.

DIAGNOSIS

Autoimmune hepatitis is characterised by chronic destructive inflammation within the liver parenchyma and elevated serum immunoglobulin G (IgG) levels as well as the presence of serum autoantibodies in the absence of other liver disease.5 Since there is no single test to diagnose this condition, the international autoimmune hepatitis group (IAIHG) has developed (1992) and revised (1999) a set of diagnostic criteria to standardise the diagnostic work-up for research purposes.37 In 2008 the same group devised
a more comprehensive ‘simplified’ score intended to help the clinician at the bedside of the patient. In the diagnostic work-up of AIH liver biopsy is used to support the clinical diagnosis of AIH and assess potential other entities, concurrent liver disease, as well as overlap syndromes. In addition, liver biopsy provides valuable information of prognosis, such as the severity of inflammation, the presence of fibrosis and cirrhosis. The presence of features as portal inflammation, fibrosis, portal neutrophils and plasma cells as well as intracellular cholestasis have been shown to effectively differentiate cholestatic drug-induced liver injury (DILI) from AIH. In both the 1999 and the 2008 simplified IAIHG criteria histological proof of (interface) hepatitis is mandatory to make a definite diagnosis of AIH, but they differ with respect to other histological features such as emperiploesis and the presence of rosettes. Unfortunately, the diagnostic IAIHG criteria do not describe a clear definition of these key features. In chapter 6 we developed a practical, descriptive method to ascertain the presence of emperiploesis and rosettes as diagnostic hallmarks of AIH to improve the histological diagnosis of AIH. We identified these features as superior markers to interface hepatitis and plasma cell rich infiltrates of AIH in chronic hepatitis. The assessment of rosettes as well as other typical features so far has been based on standard haematoxylin-and-eosin staining, whereas in this thesis we propose a method using reticulin staining to assess the degree of rosette formation. Similarly, a recent study showed the potential of improving histological characterisation in AIH using specific cell markers such as multiple myeloma-1 (MUM-1) stains for plasma cells in children. In AIH emperiploesis has been shown to be mostly mediated by CD8 T-cells which induced apoptosis of affected hepatocytes, suggesting a potential mechanism of hepatotoxicity in AIH. Although confirmatory studies are still needed for the application of these types of immunohistochemical markers (MUM-1, CD-8 and reticulin), they may help to establish the diagnosis for AIH with a higher degree of certainty. In addition, the presence of moderate to severe lymphocytic cholangitis, often reported as an atypical feature of AIH, is present in over one-fourth of AIH liver biopsies and does not preclude a definite diagnosis of AIH. The emergence of non-invasive fibrosis assessment by measuring liver stiffness, although promising during follow-up, is unreliable at diagnosis and does not exclude concomitant aetiologies of liver injury. Given the lack of new non-invasive diagnostic AIH markers, liver biopsy remains a necessary tool in the diagnostic work-up of AIH. A new diagnostic AIH score can benefit from adjusted histological criteria using the semi-quantitative assessment methods for emperiploesis, rosettes and biliary inflammation that as proposed in this thesis. New studies using immunohistochemistry markers may improve diagnostic accuracy of histopathological key-features in autoimmune hepatitis.
Autoimmune hepatitis may be difficult to differentiate from idiosyncratic drug-induced liver injury (DILI). Drug-induced liver injury (DILI) occurs in approximately 19 per 100,000 persons per year.\(^52, 53\) Liver injury may occur due to direct toxicity but is more often idiosyncratic, due either to metabolic or immunologic factors. It is hypothesized that liver cell damage can trigger a sensitization response to nuclear and actin auto-antigens, resulting in B-cell mediated auto-antibody production and cytotoxic T-cell responses which resembles autoimmune hepatitis (AIH). The presence of autoimmune features such as anti-nuclear antibodies (ANA), smooth muscle antibodies (SMA), elevated immunoglobulin G (IgG) levels and liver histologic features of AIH are not uncommon in DILI.\(^37, 38, 43, 54\) Nitrofurantoin, minocycline, hydralazine and methyldopa are known to cause this type of DILI, but evidence has so far been anecdotal.\(^54\) Patients with drug-induced liver injury may have a rapid, beneficial response to corticosteroid therapy, but most can eventually be successfully withdrawn from therapy\(^55\), whereas those with idiopathic AIH require long-term if not life-long therapy.\(^56\) Differentiating DILI with concomitant autoimmune features from pre-existing or new-onset AIH can therefore be diagnostically challenging but clinically relevant.\(^57-59\) Chapter 7 describes the analysis of the prevalence and the clinical relevance of an autoimmune phenotype in a cohort of patients with nitrofurantoin, minocycline, hydralazine and methyldopa-induced liver injury.\(^60\) To standardize for differences in autoantibody and IgG tests between different clinical centers, stored serum samples of patients with DILI due to nitrofurantoin, minocycline, methyldopa and hydralazine were used to determine baseline autoantibody and IgG status using ELISA based tests for ANA and SMA. It must be noted that ELISA assessment for these markers allowed for the assessment of an autoimmune phenotype, but is inferior to immunofluorescence techniques, which are the gold standard for ANA and SMA assessment.\(^61\) An autoimmune-like hepatitis occurred in most but not all patients with nitrofurantoin and minocycline induced liver injury and in at least half of those with methyldopa and hydralazine injury. The autoimmune phenotype was not associated with clinical outcome and appears to resolve after cessation of the culprit drug and recovery from the acute hepatocellular liver injury. Furthermore, the presence of autoimmune features is not associated with the typical HLA alleles found in idiopathic AIH, \textit{HLA-DRB1*03:01} and \textit{DRB1*04:01},\(^52\) indicating that it does not represent drug-induced injury occurring in patients with a pre-disposition to AIH. We hypothesize that DILI with autoimmune features represents a human model of AIH with a known, but different trigger to idiopathic AIH, which resolves after eliminating the culprit agent.
Herbal and dietary supplements
The use of herbal and dietary supplements (HDS) has increased over the last decades, which has been accompanied with an increase in the reports of HDS associated hepatotoxicity. Chapter 8 describes a review of literature with the current insight into the increase in the reports of HDS associated induced liver injury, which may present with autoimmune features. Limited regulatory oversight, inaccurate product labeling, adulterants and inconsistent sourcing of constituent ingredients may all contribute to the potential for toxicity. The spectrum of HDS induced liver injury is diverse and the outcome may vary from transient liver test abnormalities to acute hepatic failure requiring liver transplantation, or resulting in death. The most commonly implicated products include bodybuilding and weight loss products. There are no validated standardized tools to establish the diagnosis, but some HDS products do have a clear clinical signature that can make diagnosis almost certain. The keys to diagnosis are a high level of suspicion and a comprehensive workup to eliminate competing etiologies. Management is generally supportive and nonspecific.

TREATMENT
Expert management
Autoimmune hepatitis patients often present with fibrosis or even cirrhosis. If left untreated 6 month mortality ranges up to 40% of newly presented cases. Current treatment strategies for AIH consist of an induction course with prednisone and frequently include subsequent addition of azathioprine (AZA) 1-2 mg/kg/day as corticosteroid-sparing maintenance therapy. These regimens are based mainly on the results of randomised trials published over four decades ago. Unfortunately, in 10-20% of AIH patients this therapeutic strategy proves ineffective, due to lack of clinical response or intolerable side effects. Decisions regarding the use of second-line therapies are based on small series or even case reports, mostly reporting the experience of a limited number of centres with a special interest in AIH. Societies such as the American Association for the Study of the Liver (AASLD, 2010), the British Society of Gastroenterology (BSG, 2011), and more recently the European Association for the Study of the Liver (EASL, 2015) published guidelines based on the limited available data. Thus, expert-opinion rather than evidence-based medicine remains the most important factor in the management of patients with autoimmune hepatitis. Chapter 9 describes a study that was designed to explore the current practices on the management of AIH by a panel comprising 37 international expert hepatologists, all members of IAIHG. This investigation showed that prednisolone remains the preferred agent for induction of remission in newly diagnosed patients with autoimmune hepatitis in which cirrhosis
was absent. This is remarkable, as budesonide has been included as a therapeutic option in treatment naïve patients in both British and European guidelines and the presence of randomized data in non-cirrhotic patients.\textsuperscript{40, 41, 69} Moreover, there is a lack of consensus among expert hepatologists regarding both the initial management and follow-up of patients with autoimmune hepatitis. The survey also showed that there is considerable experience within the field in relation to second- and third-line therapies for difficult-to-treat autoimmune hepatitis patients, which is mostly unpublished data. All in all, this analysis emphasizes the need for standardised definitions for therapeutic endpoints and biomarkers as well as new prospective clinical studies to improve the management of AIH.

Allopurinol optimisation

As described earlier, immunosuppressive treatment is unsuccessful in 10-20% of AIH patients.\textsuperscript{5} In approximately half of these, this may be due to lack of clinical response or intolerable side effects from AZA or mercaptopurine (MP). Before exerting its immunosuppressive potential, AZA and MP need to be metabolised into the allegedly pharmacologically active 6-thioguaninenucleotides (6-TGN).\textsuperscript{70} As the thiopurine metabolism differs from individual to individual, the levels of 6-MMP and 6-TGN vary within the population.\textsuperscript{71} Higher 6-MMP levels have been associated with the development of hepatotoxicity but also therapeutic failure, mainly due to concomitant lower levels of the biologically active 6-TGN.\textsuperscript{72} In one study in AIH patients, a 6-TGN level of 220 pmol/8x10\textsuperscript{8} RBC has been reported as the optimal cut-off value as predictor for remission.\textsuperscript{73} The co-administration of allopurinol alongside low-dose thiopurine redirects the thiopurine metabolism towards 6-TGN formation instead of 6-MMPs, which has successfully been applied to a subgroup of inflammatory bowel disease (IBD) patients.\textsuperscript{74-76} Chapter 10 describes the results of a case series of eight AIH patients suffering from ineffectiveness or intolerance to AZA or MP due to an unfavourable thiopurine metabolism. This analysis showed that in these patients the combination therapy of low-dose thiopurine combined with allopurinol proved an effective and well tolerated alternative immunosuppressive maintenance strategy, allowing for steroid dose reduction in most patients. This strategy may represent a suitable alternative for long-term high-dose steroid or second-line alternatives such as mycophenolate mofetyl, ciclosporin or tacrolimus.

CONCLUSION

The aim of this thesis was to elucidate several key issues with regard to genetic risk, diagnosis and treatment of AIH. The immunogenetic studies in this thesis showed that the \textit{CTLA-4} \textit{+49 A/G} polymorphism does not represent a major susceptibility risk allele for
AIH in Caucasians and is not associated with disease severity at presentation. In the first GWAS in AIH we associated AIH type 1 with variants in the MHC region, and identified variants of \textit{SH2B3} and \textit{CARD10} as likely risk factors to AIH, suggesting a complex genetic basis for AIH pathogenesis which overlaps with other immune-mediated liver diseases. In a search for immunomodulating and disease outcome markers, we showed that the \textit{HLA-DRB1*03:01} and \textit{HLA-DRB1*04:01} alleles are both independently associated with the aggregate diagnostic IAIHG score in type-1 AIH patients, but these are not essential for AIH development. In addition, \textit{HLA-DRB1*03:01} is the strongest genetic modifier of disease severity in AIH, emphasizing the need for the identification of reliable (genetic) biomarkers and predictors of disease outcome. In an evaluation of histological criteria for AIH, we developed a novel, systematic assessment method for histopathological key-features and showed that emperipolesis and rosette formation are better histological predictors of AIH than the classic hallmark features of interface hepatitis and plasma cells. We reported that moderate to severe lymphocytic cholangitis should not preclude the diagnosis of AIH. In a study of patients with DILI, we showed that DILI due to nitrofurantoin or minocycline and about half of cases that were due to methyldopa and hydralazine have a phenotype of autoimmunity similar to that of AIH. These features decrease with recovery of the injury and are not associated with the typical HLA alleles found in patients with idiopathic AIH. In an evaluation of clinical practice methods among experts in in the field, we showed that there is a wide variation in the management of patients with AIH. Although good quality evidence is lacking, there is considerable experience with second-line therapies. Future prospective studies should address these issues, so that we move from an expert- to an evidence- and personalized-based care in AIH. In a small pilot study, we showed that allopurinol thiopurine combination therapy may be a safe and effective salvage strategy in AIH patients with intolerance and/or nonresponse due to an unfavourable thiopurine metabolism. There is a need for larger scale studies, improving the current first-line steroid based regimes as well as these and other second-line treatment options in AIH.
REFERENCES

Discussion and future perspectives

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