Immune-mediated enteropathies

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The current thesis reports on novel insights in the pathogenesis, diagnostic developments and clinical aspects of (refractory) coeliac disease. It further provides an overview of other immune-mediated enteropathies with a focus on adult-onset autoimmune enteropathy. The novel insights will be summarized and discussed per chapter.

SUMMARY AND DISCUSSION

Chapter 1. *Immune-mediated enteropathies: from bench to bedside*

This first chapter provides an overview of immune-mediated enteropathies and discusses clinical, diagnostic and therapeutic aspects. There is considerable overlap between various immune-mediated enteropathies in clinical, histological and serological features. This means further research is needed to reveal the exact link and possible partly shared pathogenesis between them, but it also underlines the challenge it sometimes imposes in clinical practice to differentiate between these enteropathies, which is important to determine optimal therapy and prognosis.

Chapter 2. *Outcome of referrals for non-responsive coeliac disease in a tertiary center: low incidence of refractory coeliac disease in the Netherlands*

Previous studies showed inconsistent results regarding the prevalence of refractory coeliac disease (RCD) in Europe and North-America, which has led to much debate about population characteristics, diagnostic workup and definition of RCD.[1] Our data show that RCD is indeed an extremely rare disease, with a slightly higher prevalence of RCD type 1 over RCD type 2, and an incidence of RCD in the Netherlands that is more similar to other European and North-American populations than previously thought.

In CD patients that fail to improve upon a gluten-free diet (GFD), RCD was responsible for these persisting symptoms in only 23%. In a similar percentage of patients the symptoms were caused by inadvertent gluten ingestion. Others have found similar high percentages of inadvertent gluten ingestion responsible for persisting symptoms, and our data shows it currently is still a common problem which underlines the need to refer patients that fail to respond to the GFD to an expert dietitian in an early stage and carefully review the dietary approach.[2,3] In one third of patients that fail to respond to a GFD duodenal mucosa recovered completely, and in some of these patient diseases associated with CD such as microscopic colitis or, to a lesser extent, inflammatory bowel disease were found. The majority of these patients were however diagnosed with irritable bowel syndrome, which indeed appears to be more common in CD patients, even when following a GFD.[4] Whether these symptoms are related to inadvertent minor gluten intake, undetected by our current serologic tests and dietary review, or whether CD patients are prone to IBS is unclear. In patients with persistent symptoms and duodenal abnormalities despite a GFD, one has to be aware of an alternative diagnosis other than (R)CD, such as autoimmune enteropathy, common variable immune deficiency disorder (CVID) or olmesartan-associated enteropathy (OAE), in which patients will also not respond to dietary

measures. In our study this was only the case in a minority of patients, probably due to strict inclusion criteria in which the original diagnosis of CD was revised based on serological tests, HLA-genotype and histological findings. It is not unlikely that this is an underestimation of patients with an enteropathy other than CD that in daily clinical practice would be erroneously classified as CD.

Chapter 3. *Adult onset autoimmune enteropathy: limited need for long-term immunosuppressive therapy*

Adult-onset autoimmune enteropathy (AIE) is extremely rare and therefore much is unknown about this disease. This chapter describes clinical, serological and histological features of thirteen adult onset AIE patients that have been diagnosed at our center over a 13 year timespan. While this study underlines the rarity of the disease, it is the second largest case-series of adult AIE described so far. Patients in our cohort were more often female (62%), median age at diagnosis was 52 years (range 23-73), and 46% had previously been diagnosed with an autoimmune disease. In 85% circulating auto-antibodies were found, again underlining the susceptibility for autoimmunity in these patients. The coeliac disease associated HLA-DQ 2.5 haplotype was overrepresented in our cohort (79% vs general population 35%) while CD had been excluded, which draws attention to the role of HLA-DQ 2.5 in non-CD enteropathies given it has also been associated with olmesartan-associated enteropathy. The latter mimics the histological abnormalities found in AIE, and anti-enterocyte antibodies (AEA) are found in up to 30% of patients.[5] Histologic features of AIE encompass active chronic enteritis, deficiency of goblet and Paneth cells, as well as apoptotic cells, and histological patterns can vary between patients and therefore have been divided into four subtypes.[18] AEA are associated with AIE, but are no longer absolutely required for the diagnosis AIE according to the modified AIE criteria.[6] The role of anti-enterocyt antibodies (AEA) in the pathogenesis of AIE remains unclear. In agreement with previous studies, the levels of AEA in our study did not correlate with the degree of villous atrophy nor clinical presentation, suggesting AEA's to be secondary to T-cell mediated intestinal tissue damage that leads to release of (auto)antigens that can lead to (auto)immune response by (auto)antibody production.[7] For this purpose we tested AEA's in other patients with enteropathies with various severity. We found no AEA's in healthy controls, but in patients with an enteropathy these antibodies could be identified in a minority and even more so, presence of AEA's appeared to increase with the severity of the enteropathy, again supporting the idea of being a secondary phenomenon. Adult-onset AIE shows to be a severe disease as it requires hospitalization for intravenous fluid and potassium suppletion in more than half of the patients, and total parenteral feeding in more than two thirds of patients. Three patients (23%) died as a result of therapy-refractory malabsorption. Steroids induced clinical remission in 62% of patients. Maintenance therapy with thiopurines or budesonide was effective in these patients. When patients were refractory to steroids several therapies showed only moderately effective. One patient with severe therapy-refractory AIE was eventually successfully treated with autologous stem cell transplantation and has been clinically well ever since, without medication for over 10 years now. Remarkably, three other

patients are in a long-lasting drug-free clinical remission after being treated for 3-7 years with immunosuppressive therapy, which shows that adult-onset AIE is curable in some patients.

Chapter 4. *Novel variant of EATL evolving from γδ T-cells in a RCD patient*

This chapter describes a patient with RCD type 1 that developed a novel variant of an enteropathy-associated T-cell lymphoma (EATL) originating from mucosal γδ T-cells in the duodenum. Typically, patients with RCD type 2 harbor premalignant monoclonal αβ T-cells with an aberrant phenotype that are located in the small intestine, and these patients are at risk to develop an EATL. Numbers of γδ T-cells are elevated in the intestinal mucosa of CD patients compared to healthy controls, during both active disease and after recovery upon a GFD. Their exact role remains unclear, but despite their increased activity in CD malignant transformation has not been reported in the literature so far. In contrast, peripheral γδ T-cells lymphoma's are well known especially in hepatosplenic T-cell lymphomas, but our patient had dissimilar clinical, phenotypical and chromosomal characteristics. This unique case of EATL with distinctive features and originating from mucosal γδ T-cells, together with another recently described new EATL variant that arose in an AIE patient, underlines the need for a new classification for EATL, and raises awareness of EATL development in these patients.[21]

Chapter 5. *Optimal strategies to identify aberrant intraepithelial lymphocytes in refractory coeliac disease*

Clonal intraepithelial lymphocytes with an aberrant phenotype are the hallmark of RCD type 2. These cells are thought to be the precursor cells from which EATL originate, which occurs in about 50% of RCD type 2 patients.[8,9] Accurate identification of this premalignant cell population is therefore crucial in order to timely initiate aggressive therapy such as cladribine, and for those eligible, autologous stem cell transplantation.[10,11] Phenotypical analysis using flow cytometry has previously been shown to be superior to TCRG clonality analysis for this purpose.[12] The most commonly used technique is CD3 /CD8 staining using immunohistochemistry, but this technique has theoretical flaws, as discussed in chapter 5a. In order to test this theory, in chapter 5b immunohistochemistry was compared to flow cytometric analysis in its ability to correctly identify this premalignant IEL population, and whether the use of this test would influence diagnostic outcomes in clinical practice. In RCD type 2 patients with a dominant aberrant IEL population (>50% of total IELs) immunohistochemistry performed well, but when the aberrant IEL population was only moderately increased (> 20% < 50% of total IELs) half of the patients were erroneously classified as RCD type 1 instead of RCD type 2. It should be noted that once the threshold of an abnormally high percentage of aberrant IEL is surpassed the risk to develop an EATL is increased regardless the exact height of this percentage.[10] Taken together, our data shows a limited sensitivity of immunohistochemistry to adequately identify RCD type 2 patients, which leads to the misclassification of patients and an undesirable delay in onset of treatment. Based on this data we propose that at time of RCD diagnosis all patients should undergo flow cytometric analysis of IELs.

Chapter 6. ***Serum parameters in the spectrum of coeliac disease: beyond standard antibody testing - a cohort study***

So far differentiation between RCD type 2 and other benign forms of CD can only be made with intestinal biopsies collected during upper endoscopy. Therefore we investigated whether levels of various cytokines in peripheral blood could differentiate between various forms of (complicated) CD. In addition to biochemical parameters such as C-reactive protein (CRP), and leukocyte count we determined serum levels of proinflammatory cytokines Il-6, IL-8, IL17a and IL-22, T-cell activation factors soluble (s)CD25 (IL2R-α) and sCD27, T-cell dysregulation factor sCTLA-4, that was previously shown to be up-regulated in various autoimmune disease, and a cytotoxic T-cell parameter granzyme B, and sMICA, previously shown to be associated with the presence of epithelial stress and malignancies. Statistical correction for multiple testing was performed. Our results underline that both RCD type 1 and RCD type 2 are characterized by an inflammatory disease status that shows resemblance to that observed in active CD (not on GFD). RCD type 2 however showed higher levels of IL-6 and granzyme B than patients with active CD. In contrast to IL-6 other proinflammatory parameters (e.g. CRP) did not differ between these groups. Receiver operator curve (ROC) unfortunately showed poor diagnostic characteristics, so that the value of IL-6 and granzyme B in clinical practice appears to be limited. Also, no distinctive levels of cytokine expression were found in the serum of patients with EATL.

Chapter 7. ***Antibody titers against food antigens decrease upon a gluten-free diet, but are not useful for the follow-up of (refractory) coeliac disease***

In this chapter a novel surrogate serological marker for histological recovery in RCD patient was evaluated. Anti-tissue transglutaminase IgA antibodies are used as indicator of mucosal recovery in patients with uncomplicated CD but these antibodies are of no use in RCD as patients adhere to a GFD. Follow-up of the healing of the intestinal mucosa is currently only feasible in these patients by means of an upper gastrointestinal endoscopy during which duodenal biopsies are collected. We hypothesized that due to loss of mucosal integrity antibody formation against common food antigens such as bovine serum albumin would increase. Indeed, titers of antibody titers against bovine serum albumin were higher in patients with CD and RCD as compared to healthy controls. The antibody titers did nonetheless not correlate well with the level of villous atrophy nor with mucosal recovery over time. Therefore, there currently is no clinical use for anti-bovine serum albumin antibodies in RCD.

Chapter 8. ***Origin and immunophenotype of aberrant IEL in RCDII patients***

This chapter focused on the hallmark of RCD type 2, the aberrant IEL, by studying the origin and phenotype of aberrant IELs that are thought to be responsible for the ongoing intestinal inflammation and are at risk to progress to an EATL. Understanding better where these cells originate from and how these cells develop will allow more specific treatment and hopefully better outcomes in the future. Aberrant IELs are characterized by the lack of expression of the TCR-CD3 complex on the cell surface, yet these cells do contain cytoplasmatic CD3 and display TCR rearrangements. Some have suggested that the TCR-CD3 complex is internalized due to overstimulation, implying that these cells originate from mature T-cells.[13] Alternatively, it has been hypothesized that a small unique $CD3^-CD7^+$ IEL population considered as NK/T-cell precursors that was observed in intestine of healthy individuals could be the physiological counterpart of aberrant IELs.[14,15] We studied TCR rearrangement patterns of the gamma, delta and beta chain in DNA isolated from duodenal biopsies of 18 patients. The results were remarkable heterogenic and four patterns could be distinguished. Notably, the only patients who developed an EATL were the three patients in the fourth group which was characterized by a mature stage of TCR(B) development. Considering the small numbers care must be taken with interpretation, but it might be the first clue in identifying more accurately which RCD type 2 patients develop an EATL. More recent data further supports a prognostic role for TCRB gene rearrangement patterns as an association was revealed between containing a high frequency of a dominant TCRB clone, that was determined using high throughput sequencing of TCRB gene rearrangement patterns, with the progression of RCD type 2 to EATL.[16] From our data we concluded that aberrant IELs, for the majority, originate from developing precursor T-lymphocytes but derail during different stages of maturation. The concept of the physiological counterparts of aberrant IELs has been further explored since then and four subsets of lineage negative $CD3^-CD7^+_{ic}CD3^+$ have been identified based on expression of CD127 and CD56 in both healthy controls and CD patients.[17] The massive expansion of aberrant IELs found in RCD type 2 appears to be the result of proliferation from both lineage negative $(Lin^-)CD127^-$ and Lin^-CD127^+ innate IELs.[17] The Lin^-CD127^- innate IELs in RCDII patients already seem defective, as these cells are able to give rise to both NK-cells and T-cells in CD patients, while when harvested from RCD type 2 patients these cells are unable to do so, most likely as a result of chromosomal aberrations found in these premalignant cells.[18]

Chapter 9. ***Differential IL-13 production by small intestinal leukocytes in active coeliac disease versus refractory coeliac disease***

In this chapter we assessed whether the local cytokine profile produced by mucosal intestinal cells from RCD type 2 patients would differ from the cytokine profile of CD patients. We hypothesized that this would be the result of comparing a gluten-mediated inflammatory response in CD, to an inflammatory response independent from gluten which is characteristic for RCD. CD patients with active disease while not yet adhering to a GFD as well as CD patients in remission upon a GFD were included. Furthermore, only patients with RCD type 2 were selected, because they contain, in contrast to RCD type 1, a distinctive IEL population with a unique T-cell

repertoire that has shown to be driven by gluten independent factors.[16] Cells from the lamina propria were isolated and cultured and stimulated with (PMA, ionomycin). No differences were observed between cells harvested from patients with active CD and RCD type 2 with regards to secretion of IFN-γ, TNF-α, IL-17A, IL-5 and IL-10. Only the levels of IL-13 differed between the groups in an unanticipated manner. Levels of IL-13 were higher in patients with RCD type 2 when compared to CD patients with active disease. IL-13 is known to play an important role in gut defense and inflammation, and is upregulated in ulcerative colitis, a chronic inflammatory disease of the colon.[19] IL-13 has shown to have direct cytotoxic effects on epithelial cells, and is produced by NK-cells as part of an innate response, which could be applicable in RCD type 2 where antigenic stimulation is lacking.[19] Somewhat confusing however is that levels of IL-13 were also higher in CD patients in remission when compared to CD patients with active disease, with the IL-13 levels of the former being similar to those found in RCD type 2. Based on our data we can only conclude that by the methods we used the immune response in RCD type 2 and active CD is surprisingly similar, with the exception of IL-13 production.

Chapter 10. *Genetic variations in interleukin 12 related genes in autoimmune disease*

In the era of genome wide-association studies (GWAS) large collaborations have brought us enormous amounts of data by identifying genetic polymorphisms that are associated with complex, multifactorial autoimmune diseases. Now the task awaits to elucidate what those genetic polymorphisms do, to slightly increase (or decrease) the chance to develop such a disease. GWAS have identified over 40 polymorphisms in CD, and in chapter 10 and 11 we have focused on a polymorphism that shows the strongest association with CD, excluding the HLA-system, that is located near the IL12A gene. The IL-12 cytokine family currently consists of heterodimeric cytokines, namely, IL-12, IL-23, IL-27 and IL-35. The IL12A gene codes for the IL-12p35 subunit, that together with IL-12p40 forms IL-12 cytokine, but can alternatively form a heterodimer with EBI3 to form the IL-35 cytokine. The IL-12 cytokine family exerts varying effects in the immune response, so to better understand the genetic associations we first focused on associations of all IL-12 related genes with immune-mediated diseases. Instead of reviewing the different genetic risk factors associated with a particular disease we reviewed the associated diseases with a specific gene cluster. By doing so we found that autoimmune diseases cluster in two groups. Remarkably, the diseases within these groups mirror to a certain extent the known clinical relationship between these diseases. The first group includes T helper 17 / T helper 1 pathway and includes ulcerative colitis, Crohn's disease, psoriasis, ankylosing spondylitis and rheumatoid arthritis. The second group encompasses the T helper 1 / IL-35 pathway and consists of primary biliary cirrhosis, multiple sclerosis, autoimmune thyroid disease and coeliac disease. In general it seems that the IL-12 cytokine family represents a key player in the immune response and genetic polymorphisms that affect its function will likely result in some alteration in the immune response. Nevertheless, several single nucleotide polymorphisms (SNP's) are associated with more than one autoimmune disease. To complicate matters the SNP involved may exert a different effect on their respective trait, meaning it can increase suscep-

tibility for one disease whereas it is protective for the other. The next question is how these polymorphisms alter the transcription and translation of the gene. In agreement with SNP's in other gene regions that are associated with autoimmune disease, only a small minority is located in a coding region (exon) where it alters the protein amino acid sequence and thereby possibly its biologic function.(30) The majority of SNP's are in fact located in introns (≈45%) or in-between genes (≈43%), and these non-coding may alter gene transcription in ways that are more complex to reveal such as affecting enhancers, microRNA's, or long-range transcription regulation.(31) The major challenge now is to elucidate the role of these disease associated gene loci in disease pathogenesis and to identify the functional consequences of these variants.

Chapter 11. *Coeliac disease associated SNP rs17810546 is located in a gene silencing region*

In follow-up of our study regarding genetic associations with IL12 and autoimmune disease in chapter 10, here we attempted to unravel the role of one of the SNPs (rs17810546) with the strongest association with coeliac disease that is also associated with multiple sclerosis, primary biliary cirrhosis and lupus erythematosus.[20] This SNP is located on chromosome 3 at 3q25.33, which is an intergenic region between the genes for SCHIP1 and IL12A.[21] First, we found that this SNP is located in distal enhancer sites of a number of immune regulatory cells known to transcribe the IL12A locus: monocytes, macrophages and neutrophils. The location in such regions make it conceivable that a SNP can alter the recognition site of a transcription factor. While the SNP is located in an intronic region of the long non-coding RNA gene IL12A-AS1 we were unable to find expression in duodenal samples from healthy controls nor CD patients. Next we explored the nature of the region and cloned the surrounding 500 base pares around the SNP. When comparing the DNA sequence of an individual homozygous for the G allele to that homozygous for the A allele another SNP rs7610082 was identified. Presence of the cloned region in transcription experiments showed major down-regulatory effect on the expression of the luciferase gene, which suggests that the cloned region contains sequences to which transcription down-regulatory factors can bind. We then tried to identify the transcription factors responsible for the observed downregulation. Using phylogenetic foot printing we selected four candidate transcription factors, that were subsequently silenced using microRNA (mRNA). None was however able to reverse the down regulatory effect of the cloned region, which means that other, currently unidentified, transcription factors are involved that are involved. Next we investigated whether the presence of the SNP influenced the expression of the IL12A gene in CD. The variant is located in between two genes, IL12A en SCHIP1 but we opted to focus on the IL12A gene because of its prominent role in the immune system. We found that expression of IL12A was strongly upregulated in duodenal biopsies taken from patients with active CD. Only EBI3 was abundantly present, while other candidate binding partners, e.g. IL12B, IL12-p27 were absent. Expression levels of IL12A and EBI3 also correlated well which is suggestive that IL-35 is involved in CD. We also correlated IL12A expression according to genotype which showed a trend towards higher expression in the presence of the risk (G) allele yet did not reach statistical significance which is probably due to the low frequency of the risk allele in

the European population. In conclusion we present evidence that SNP rs17810546 is located in an expression regulatory region and influences expression in a genotype dependent fashion. Furthermore, our data suggests that IL-35 is upregulated in the small intestinal mucosa in CD patients, where it probably exerts proinflammatory effects.