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Summary, general discussion and future perspectives

The current thesis reports on novel developments in diagnosis and treatment, as well as novel insights into the pathogenesis of Refractory Coeliac Disease (RCD). RCD may now be recognized as a relevant clinical entity, associated with a poor prognosis in a specific subgroup of patients. A key role for flow cytometry of intestinal lymphocyte populations has emerged, allowing accurate discrimination between both RCD subgroups. Given the association of aberrant intestinal T lymphocytes with the development of Enteropathy-Associated T-cell Lymphoma (EATL), the therapeutic challenge in RCD II is effective elimination of these cells, either chemotherapeutically or by means of stem cell transplantation. Although the underlying mechanisms of RCD development remain ill defined and require further investigation, key players have been indicated at cellular, molecular and genetic levels involved in intestinal homeostasis. The individual contributions described in this thesis relevant to the above mentioned novel insights and developments will be summarized and discussed below.

Chapter 1: Overview of RCD

Over the past 2 decades major advances have been accomplished in the field of RCD. This first chapter provides and extensive overview of RCD pathogenesis, involving genetic factors and immunological mechanisms. In addition, the work-up in establishing the diagnosis of RCD is postulated. In the distinction between RCD I and II, intraepithelial lymphocytes (IELs) play a key role. Flow cytometry and clonality analysis represent the diagnostic parameters required to make this distinction, which has important implications for prognosis and subsequent treatment. An outline of the therapeutic options thusfar reported for RCD as well as EATL patients is provided at the end of this chapter.

Chapter 2: Survival in RCD and EATL

Chapter 2 provides further insight into the prognosis of RCD and the development of EATL, by reporting on long-term survival and risk of transition of RCD into EATL in a large cohort of patients with complicated coeliac disease. When normal expression of T-cell surface markers is present in RCD patients (type I), the prognosis appears to be less dismal than when an aberrant IEL population is present (RCD type II); 50-60% of the latter patients develops EATL within 4-6 years, after which the 5-year survival is only 8-20%. Furthermore, patients with EATL may present in two different clinical patterns. Firstly, patients with well-established coeliac disease who deteriorated because of the development of RCD II and eventually progressed to secondary EATL. In the other group patients develop EATL without a preceding history of complicated coeliac disease, often presenting with perforation or obstruction (primary or de novo EATL). Interestingly, no CD-related mortality was recognized in the RCD I group and none of our RCD I patients has progressed to RCD II during follow-up. More aggressive and targeted therapies seem mandatory in RCD II and EATL.
Chapter 3: Incidence of EATL

The third chapter provides the epidemiology of EATL in the Netherlands, by studying the incidence of EATL as well as the demographic characteristics of patients with EATL by searching the nation-wide network and registry of histo- and cyto-pathology reports in the Netherlands (abbreviated as PALGA). Clinico-pathological data were obtained for 116 cases of EATL, diagnosed between 2000 – 2006. EATL appeared to be a rare disease with a crude incidence of 0.10 per 100,000 inhabitants per year; and an incidence of 2.08/100,000 over 50 years of age, with a peak incidence in the 7th decade. The tumour was mainly localised in the proximal small intestine. Although uncomplicated CD is twice as frequent in female patients, EATL was more prevalent in males.

Chapter 4: Flow cytometry in the diagnosis of RCD

Early detection of those patients actually at risk to develop EATL is of utmost importance for curative intervention. A cut-off point between acceptably normal and pathologically increased percentages of aberrant T-cells in RCD is defined and validated in this chapter. This reference range now allows accurate distinction between RCD types I and II, having a very different prognosis as described earlier in Chapter 2. To establish an optimal cut-off value for this percentage, reference ranges for aberrant T-cells in the duodenal mucosa of different CD patient and control groups were generated. Furthermore, the predictive value of this cut-off was compared with intestinal T-cell clonality, as prognostic parameter for EATL development in RCD. Quantification of aberrant T-cells by flow cytometry was preferable to T-cell clonality analysis for identification of RCD patients at risk for EATL development. A cut-off value of 20% appeared reliable for risk stratification, therapeutic options and subsequent follow-up of RCD patients. Interestingly, the aberrant T-cells in primary EATL patients as well as ulcerative jejunitis appeared to be largely confined to tumour mass and ulcerations and could not be found in such high percentages diffusely throughout the small intestine as in RCD II and secondary EATL patients. The latter may suggest a differential pathogenesis, requiring further investigation.

Chapter 5: RCD is a disseminated disease

Chapter 5 describes whether aberrant T-lymphocytes in RCD II could be detected in other parts of the small intestinal mucosa besides the intraepithelial compartment. Additionally, the presence of aberrant T-lymphocytes was analyzed in two RCD II patients that developed ill-defined skin lesions. Multiparameter flow cytometric immunophenotyping was performed on both IEL and lamina propria lymphocyte (LPL) cell suspensions, isolated from fresh small bowel biopsy specimens of RCD II. In RCD II the aberrant T-lymphocytes may also reside in the extracellular layer of the small intestinal mucosa, and even in extraintestinal localizations including the skin. Whether this phenomenon represents a passive overflow from the intestinal epithelium or active trafficking towards other anatomical localizations remains to be elucidated. RCD II appears to be a disseminated disease, which may impose the risk of EATL development outside the intestine.
Chapter 6: Aberrant IELs at the molecular level in RCD
Aberrant IELs and EATL cells are typically cytCD3+, but lack expression of the T-cell receptor (TCR)-CD3 complex on the cell surface. It is currently unknown what causes the loss of surface TCR-CD3 expression. In this chapter we report on the generation and molecular characterization of a IEL cell line, derived from a RCD II patient, with the characteristic immunophenotype of EATL. This study provides the first evidence that loss of TCR-CD3 surface expression on IELs in RCD II is due to defects in the synthesis and/or assembly of T-cell receptor chains providing a first step in understanding the process leading to the development of RCD II and subsequent progression to EATL.

Chapter 7: Do TCRγδ+ IEL protect against EATL?
TCRγδ+ IELs play an important role in mucosal repair, homeostasis and tumor surveillance. Recently, human small intestinal TCRγδ+ IELs were shown to have regulatory potential in uncomplicated CD. In Chapter 7 we investigated whether TCRγδ+ IELs are decreased in RCD II, providing a possible explanation for persisting mucosal damage and inflammation, and the emergence of aberrant T-cells with clonal expansion to EATL. A significantly lower percentage of TCRγδ+ IELs was found in RCD II as compared to all other CD groups. Overall, there was a clear negative correlation between the presence of TCRγδ+ IELs and aberrant IELs. This may imply that TCRγδ+ IELs play a crucial role in the disease process. Interestingly, TCRγδ+ IELs increased again in RCD II after therapy aimed at elimination of aberrant IELs. These cells could be important markers in flow cytometric analyses, in addition to aberrant T-cells, to differentiate between disease categories and to evaluate the effectiveness of therapeutic strategies.

Chapter 8: The role of peripheral regulatory cells in RCD
In this chapter, we investigated whether a lack of circulating homeostatic T-cells, such as Treg, Tγδ or iNKT cells would be associated with the development of RCD or EATL. In summary, our study demonstrates that only the iNKT cell numbers are selectively reduced in RCD I and II. With respect to other circulating T-cells with regulatory potential, including Treg and Tγδ cells, we did not find unusual levels, neither in responsive nor in refractory CD. CD patients treated with a gluten free diet (GFD) displayed a significantly increased fraction of CD4+ iNKT cells. This indicates that regulatory cell numbers can increase during a GFD, or that individuals with higher frequencies of regulatory cells are more likely to respond to a GFD. Follow up studies are necessary to determine whether CD4+ iNKT cells control the immune response against gluten and if their absence contributes to the progression to RCD and EATL.

Chapter 9: Genetic predisposition in RCD
Genes play a key role in the pathogenesis of CD. The class II human leucocyte antigen (HLA)-DQ2 and HLA-DQ8 loci are the most important genetic contributors identified so far. Furthermore, uncomplicated CD has been linked to genetic variants in the MYO9B gene on chromosome 19. HLA-DQ2 homozygosity is associated with compli-
cations of CD such as RCD II and EATL. We investigated whether certain MYO9B variants also predispose to RCD II and EATL. One single nucleotide polymorphism (SNP) in MYO9B showed a significantly different allele distribution in RCD II and EATL patients compared to controls ($p=0.00002$). The rs7259292 T allele was significantly more frequent in RCD II and EATL patients (11%), compared to controls (2%) and CD patients (3%). Both MYO9B rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to CD patients without evidence for interaction between these two risk factors. This study shows that both MYO9B variants and HLA-DQ2 homozygosity might contribute to a complicated course of CD.

Chapter 10: Targeted treatment of RCD?
This chapter describes the treatment of a RCD II patient with Alemtuzumab (Campath®, monoclonal anti-CD52). CD52 is expressed by all T- and B-cells and targeting of CD52 by antibodies has proven to be successful in eradicating overt lymphoma. Although the patient showed a clinical response as illustrated by an increase in bodyweight and decrease in diarrhea, the mucosal lesions persisted and the intestinal aberrant IELs even increased from 60% to 91%. Eventually this patient developed EATL. A possible explanation may be that IELs are not sufficiently reached and/or targeted by alemtuzumab, given the fact that in our patient virtually all aberrant T-cells in the intestinal mucosa still expressed CD52, whereas in peripheral blood barely any B- and T-cells could be detected.

Chapter 11: ASCT in RCD – future promise
Autologous Hematopoietic Stem Cell Transplantation (ASCT) is an increasingly accepted treatment for refractory autoimmune diseases. This study reports on the feasibility, safety and efficacy of ASCT in RCD type II, with the ultimate goal of resetting the immune response to prevent or delay development of overt EATL. Seven RCD II patients were transplanted after conditioning with fludarabine and melphalan. Engraftment occurred in all patients. No major non-hematological toxicity or transplantation-related mortality was observed. There was a significant reduction in the amount of aberrant T-cells in duodenal biopsies associated with clinical improvement, and normalization of hematological and biochemical markers (mean follow-up 15.5 months, range 7-30 months). One patient died 8 months post-transplant from progressive neuroCD. High-dose chemotherapy followed by ASCT seems feasible and safe, and may result in long-term improvement of disease activity in RCD patients with aberrant T-cells not responsive to available treatments. However, extended follow up and additional pilot studies with larger groups of patients are needed to confirm the efficacy of this therapy.

Chapter 12: ASCT in EATL – yet to be successful
Despite treatment, EATL has a very poor outcome, as described in Chapter 2. In this study we report on the feasibility, safety and efficacy of high dose chemotherapy
followed by ASCT in 4 patients with EATL (3 upfront and 1 at relapse), with or without prior partial small bowel resection. One patient had ongoing complete remission up till 32 months after transplantation. Three patients died from relapse within few months after transplantation. ASCT for patients with EATL seems unsatisfactory. More intensive conditioning and aggressive chemotherapy with/or without targeted immunotherapy as well as allogeneic SCT should be explored.

**Future perspectives**

Although the diagnostic work-up of RCD has evolved considerably, at least in part as a result of the implementation of flow cytometry, further identification of prognostic parameters for EATL development appears required. Since approximately half of the RCD II patients actually develops EATL, the mere presence of increased numbers of aberrant intestinal T-cells, and decreased numbers of intraepithelial TCRγδ+ cells, may only partly predict the development of overt lymphoma. The search for novel prognostic parameters, based on for instance specific immunophenotypic (de)differentiation of aberrant T-cells, needs to be continued. Differentiation within the RCD II patient group, between those patients that will and those that will not develop EATL, represents an ultimate target. Applying flow cytometric analysis of molecules associated with proliferative capacity (Ki-67, IL-15Ra), monitoring of further dedifferentiation (loss of CD7, CD52, CD103) and monitoring of the acquisition of EATL associated phenotype (gain of CD30) are included in current and future investigation. In addition, analysis of cytokine production profiles as well as evaluation of apoptosis resistance profiles may provide a significant contribution. Importantly, further increase in the awareness of complicated CD, with respect to both incidence and clinical presentation, is warranted.

Sofar, already a substantial amount of insight into the pathogenesis of RCD has been acquired. The impact of genetic factors involved in intestinal permeability has been recognized. At the molecular level, the underlying mechanism of defective TCR/CD3 expression by aberrant IEL has been elucidated. It is to be expected that further analysis of RCD patients at the genetic and molecular level will shed additional light onto the pathogenesis of RCD. Interestingly, T-cells with regulatory potential, including iNKT and TCRγδ+ cells, appear to be involved in the RCD disease process, both systemically and in situ. Further dissection of such regulatory cells, including in situ analysis of FoxP3+ regulatory T-cells in the small intestinal mucosa, will be of interest. In addition, the potential involvement of the recently identified pro-inflammatory Th17 subset should be considered and will open up novel research areas. Th17 cells have been identified as key players in the pathogenesis of the inflammatory bowel disease M.Crohn, an entity with obvious analogies to RCD, both being a chronic inflammatory condition. The identification of aberrant T-cells outside the intestinal epithelial layer, and even outside the gut itself, is an intriguing finding. Whether it represents passive overflow from the epithelium, or active dissemination is currently unknown. However, it could impose a serious threat with regard to the appearance of EATL in diverse anatomical localizations. Mapping the expression profiles of different adhesion molecules and
homing receptors of aberrant IEL and/or EATL cells, presenting outside the intestine could help to predict disseminating potential of aberrant IEL still residing in the epithelial compartment.

The treatment of RCD patients remains a challenge, especially in case an aberrant IEL population is present (RCD II). The RCD I patients respond well to general immunosuppression (Azathioprine and Prednisone) and do not seem to be at risk for EATL or RCD II. However, currently there is no established treatment available for the RCD II (and UJ) patients aimed at prevention of EATL. Fortunately, therapeutic strategies are evolving rapidly. Therapies directed at eradicating the aberrant IEL population, such as upfront Cladribine, are promising. In addition, the combination with ASCT should be further explored, as these treatments have proved to significantly reduce number of aberrant IELs. In view of the increasingly successful application of monoclonal antibody based therapies, targeting of aberrant intestinal T-cells by anti-CD52 (Alemtuzumab, Campath®) was explored. However, single-agent therapy with Alemtuzumab did not appear to sufficiently eradicate aberrant T-cells and thus failed to be effective in RCD II. Its use in combination with chemotherapy and ASCT, may be still promising. Regarding future treatment of RCD II / UJ patients, optimization of therapy may imply fine-tuning the ASCT-protocol by using T-cell depleted grafts and exploring novel preconditioning regimens. Importantly, to attain a significant impact on improving the dismal prognosis of these patients (which now have a 5-year survival of 58%), multi-center collaboration and pooling of data are mandatory.

Despite treatment with combination chemotherapy and high dose conditioning regimens followed by ASCT, the survival of EATL remains very poor. Results of ASCT for EATL are unsatisfactory so far as patients often present with an advanced stage of disease and relapse occurs regularly. Therefore, instituting therapy at an earlier stage (if possible RCD II / UJ), development of more effective combined/targeted treatments, and improved conditioning regimens are needed. More importantly, the use of allogeneic SCT with reduced intensity conditioning may hold considerable promise in these EATL patients and should be further explored. If patients are not suitable for allogeneic SCT, new options, such as a so-called “Sandwich-treatment” with Cladribine, CHOP, Cladribine (C,CHOP,C) may have a future role. To facilitate an optimal international collaboration, in order to set up multicenter trials for the treatment of RCD II / UJ and EATL patients, diagnostic criteria for these disease entities should be determined in a similar way to that done for CD in 2001. It is anticipated that efforts for this purpose will take place at the next International Coeliac Disease Symposium in Amsterdam 6-8 April 2009.
Samenvatting voor niet-ingewijden

Coeliakie is een veel voorkomende ziekte die wordt veroorzaakt door een overgevoeligheid voor gluten. Gluten is een belangrijk bestanddeel van o.a. tarwe en wordt in veel voedingsmiddelen verwerkt. Bij het eten van gluten krijgen de meeste patiënten een ontstekingsreactie in de darm en beschadigt de darmwand, hierdoor ontstaan vaak buikklachten, diarree en gewichtsverlies. De ontstekingsreactie in de darmwand wordt veroorzaakt door bepaalde cellen in de darm die verantwoordelijk zijn voor de afweer, genaamd T-lymfocyten. De laatste jaren worden er, mede dankzij de verbeterde diagnostiek, meer en meer coeliakiepatiënten gediagnosticeerd bij wie maag-darmklachten niet op de voorgrond staan. Het betreft hier patiënten met o.m. groei-achterstand, bloedarmoede, chronische vermoeidheid, hormonale stoorlijnen of osteoporose.

De therapie van coeliakie bestaat uit het instellen van een glutenvrij dieet, dat levenlang gevolgd moet worden. Bij vrijwel alle coeliakiepatiënten normaliseren de klachten dan en herstelt de darm. Bij een zeer gering aantal patiënten echter, veelal op volwassen leeftijd gediagnosticeerd, herstelt de darm niet en blijft de ontstekingsreactie in de darm bestaan. Er kunnen complicaties optreden, ook al volgen de patiënten een strikt glutenvrij dieet. Bij deze mensen spreekt men van refractaire coeliakie (RCD).

Een zeer ernstige complicatie van refractaire coeliakie is een bepaalde vorm van lymfeklierkanker in de dunne darm, enteropathie geassocieerd T-cel lymfoom (EATL) genoemd. Deze vorm van kanker ontstaat uit een deel van de hierbovengenoemde T-lymfocyten. Deze specifieke T-lymfocyten kenmerken zich door een afwijkend eiwitpatroon op het celoppervlak. Een EATL (enteropathie geassocieerd T-cel lymfoom) wordt vrijwel uitsluitend gevonden bij patiënten bij wie op volwassen leeftijd coeliakie werd vastgesteld, meestal voorafgegaan door een stadium van RCD (refractaire coeliakie). Op dit moment kan niet goed voorspeld worden welke RCD patiënten EATL zullen ontwikkelen en welke niet.

In dit proefschrift worden de ontwikkelingen op het gebied van de diagnose en de daaropvolgende behandeling van RCD en EATL besproken, daarnaast wordt inzicht gegeven in factoren die een rol spelen in het ontstaan van RCD en EATL. Er wordt onderscheid gemaakt in 2 verschillende typen RCD patiënten, type I en type II, waarvan alleen type II het risico heeft op het ontwikkelen van een lymfoom. Een duidelijk criterium om onderscheid te maken tussen deze 2 subtypen ontbrak nog in de literatuur. Aan het ‘aanwezig zijn’ van de hierboven genoemde afwijkende T-cellen in de dunne darm (type II) zou mogelijk een voorspellende waarde hebben bij het uiteindelijk ontwikkelen van EATL. Zoals al eerder gezegd hebben deze T-cellen een afwijkend ofwel ‘aberrant’ eiwitpatroon aan het oppervlak, ook wel celoppervlakte ‘markers’ genoemd, aan de hand waarvan verschillende afweercellen gekenmerkt en onderscheiden kunnen worden. De gevolgen die het aantonen van een bepaalde hoeveelheid van deze afwijkende cellen zou hebben voor het type behandeling van RCD patiënten waren ook nog onduidelijk. Daarom was het doel in dit proefschrift om, nadat een overzicht gegeven wordt van de beschikbare literatuur over RCD en EATL (in deel 1):
1. een 'afkapwaarde' (grenswaarde) voor deze afwijkende T-cellen te bepalen, te valideren en te onderbouwen op basis van klinische gegevens met betrekking tot de overleving van RCD en EATL patiënten (hierover gaat deel 2 van dit proefschrift).

2. deze afwijkende T-cellen in de dunne darm verder te karakteriseren en andere factoren te zoeken die betrokken zijn bij het ontstaan van refractaire coeliakie (dit staat beschreven in deel 3).

3. nieuwe therapieën te evalueren, die specifiek gericht zijn tegen deze afwijkende T-cellen, met als doel om de overleving te verbeteren en het ontstaan van lymfeklierkanker te voorkomen of vertragen (dit kunt u lezen in deel 4).

Samenvattend hebben we gevonden dat bij RCD op basis van het aankleuren van de verschillende ‘celoppervlakte-markers’ op de T-cellen in de dunne darmwand onderscheid gemaakt kan worden tussen RCD type I en II. RCD type I heeft een afkapwaarde van minder dan 20% ‘aberrante’ T-cellen, RCD type II heeft een afkapwaarde van meer dan 20% ‘aberrante’ T-cellen. De prognose van refractaire RCD type I (zonder afwijkende cellen) lijkt goed. Sterfte gerelateerd aan deze vorm van coeliakie, of de ontwikkeling van RCD II of EATL, werd niet waargenomen. Daarentegen bestaat er bij de groep patiënten met RCD type II een sterk verhoogde kans op het ontwikkelen van EATL. Binnen 4-6 jaar ontwikkelt 50-60% van de patiënten EATL. De 5-jaarsoverleving is dan slechts 8-20%. EATL is in Nederland een zeldzame aandoening, met 0.10 nieuwe gevallen per 100.000 inwoners per jaar. Bij mensen ouder dan 50 jaar ligt dit aantal aanzienlijk hoger, namelijk 2.08 nieuwe gevallen per 100.000 inwoners per jaar (met name tussen 60 en 70 jaar).

De afwijkende cellen bij RCD II kunnen diffuus verspreid door de darm maar ook buiten de darm en zelfs in de huid gevonden worden. We hebben het moleculaire mechanisme beschreven dat ten grondslag ligt aan het afwijkende markerpatroon van een van de oppervlakte-kenmerken van de ‘aberrante’ T-cellen bij RCD II, alsmede een genetische factor die een rol lijkt te spelen bij het ontstaan van RCD (MYO9B). Verder is er in het bloed en in de darmwand gekeken naar de aanwezigheid van bepaalde afweercellen die een dempende werking hebben op de ontstekingsreactie. Deze afweercellen, γδ-T-cellen genaamd, bleken met name bij RCD type II verminderd te zijn in de darm, maar wel weer toe te nemen na effectieve therapie zodra de afwijkende cellen afnemen.

De behandeling van RCD blijft een uitdaging, met name wanneer er ‘aberrante’ T-cellen aanwezig zijn in de darm (RCD type II). De RCD I patiënten reageren goed op therapie die de ontstekingsreactie onderdrukt (Azathioprine en Prednison) en lijken geen verhoogd risico te hebben op EATL. Voor RCD II echter, is momenteel nog geen vaststaande behandeling om EATL te voorkomen. Gelukkig ontwikkelen therapeutische strategieën zich snel. Behandeling specifiek gericht op het uitroeien van de ‘aberrante’ T-cel populatie in de darm, zoals met Cladribine therapie, is veelbelovend. Verder wordt autologe stamcel transplantatie (ASCT) na hoge dosis chemotherapie verder geëxploreerd, met als doel om de ‘ontspoorde’ ontstekingsreactie te doorbreken door de immuunrespons te ‘resetten’. Alleen Cladribine en ASCT hebben bewezen
het percentage afwijkende cellen significant te reduceren. Het uiteindelijke doel van therapie bij RCD II is om EATL te voorkomen of uit te stellen. Want er is momenteel nog geen goede behandeling voor EATL en dit lymfoom heeft een zeer sombere prognose. Omdat het om een zeldzame aandoening met kleine groepen patiënten gaat is optimale internationale samenwerking nodig. Grote studies dienen opgezet te worden, om de verschillende behandelingen te vergelijken en te komen tot een gestandaardiseerde, optimale behandeling voor RCD en EATL patiënten. Voor deze samenwerking zijn duidelijke diagnostische criteria en afspraken nodig, deze zullen hopelijk in 2009 bij het Internationale Coeliakie Congres in Amsterdam vastgesteld gaan worden, zodat de prognose van deze patiënten zal verbeteren.