Tampere University

MINNA HIETIKKO

Characterising Disease-Specific IgA Responses in Coeliac Disease and Dermatitis Herpetiformis

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ACADEMIC DISSERTATION
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To my long-standing colleagues.

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Minna Hietikko

ABSTRACT

Coeliac disease is a common autoimmune-mediated disorder triggered by dietary gluten in individuals with genetic susceptibility. It is characterised by the inflammation and gradual destruction of the small-bowel mucosal structure and a variety of gastrointestinal symptoms. However, a wide spectrum of extraintestinal symptoms are also increasingly prevalent. One of the most well-known extraintestinal manifestations of coeliac disease is dermatitis herpetiformis, the coeliac disease of the skin, which presents with an itching and blistering rash particularly on the elbows, knees, and buttocks. In addition to skin symptoms, patients with dermatitis herpetiformis typically show coeliac-type structural and inflammatory changes in the small-bowel mucosa. The treatment of choice for both coeliac disease and dermatitis herpetiformis is a strict, life-long gluten-free diet, during which the small-bowel mucosa and skin heal and the clinical symptoms disappear.

A characteristic feature of both coeliac disease and dermatitis herpetiformis is an IgA class autoantibody reaction towards the self-antigen transglutaminase 2 (TG2). TG2-targeting antibodies can be found in the serum of untreated patients and as deposits in the small-bowel mucosa, where they are also known to be partly produced. In addition to the TG2 antibody reaction, patients with dermatitis herpetiformis show an IgA class autoantibody reaction towards epidermal transglutaminase, TG3. TG3 autoantibodies are found as deposits in the skin of dermatitis herpetiformis patients and are present in the serum of not only the majority of patients with dermatitis herpetiformis, but also in a minority of coeliac disease patients. During a gluten-free diet, the serum, small-bowel mucosal, and cutaneous antibodies disappear.

In this dissertation, the IgA class autoantibody responses towards TG2 and TG3 in coeliac disease and dermatitis herpetiformis were investigated. In study **I**, the frequency of small-bowel mucosal TG2 antibody-secreting cells at different stages of coeliac disease was determined. The results showed that TG2 antibody-secreting cells are present already in the early stage of coeliac disease when the small-bowel mucosal structure is still normal, and that their frequency increases along with the development of mucosal damage and the overt disease. After the initiation of a

gluten-free diet, the frequency of the cells decreased significantly within one year, and after long-term treatment, the cells were mostly absent if dietary adherence was strict. The frequency of the cells correlated with the levels of serum TG2-targeting antibodies and the intensity of small-bowel mucosal TG2-targeting antibody deposits, but it was not always parallel at the level of individual patients.

In study **II**, the TG2 and TG3 antibody responses were investigated exploiting the ex vivo organ culture method of patient-derived small-bowel mucosal biopsies. While biopsies from coeliac disease patients secreted primarily TG2 antibodies into the culture medium, those from dermatitis herpetiformis patients with the active disease were shown to secrete TG3 antibodies. In patients secreting high levels of TG3 antibodies, TG3 antibody-secreting cells were identified in the small-bowel mucosa.

In study **III**, the frequency and gluten-dependency of TG2 and TG3 antibody-secreting plasma cells in dermatitis herpetiformis patients undergoing a gluten challenge was investigated. Both cell populations were mostly absent in long-term dietary treated patients, but they appeared in more than half of the patients during the challenge. The frequency of the cells was shown to correlate with the levels of the corresponding serum antibodies, but it was not always parallel at the level of individual patients. TG3 antibody-secreting cells were generally not found in coeliac disease control patients.

In study **IV**, the disappearance of cutaneous IgA and TG3 deposits in dermatitis herpetiformis patients was investigated. IgA and TG3 were shown to disappear simultaneously during a strict, long-term gluten-free diet. The disappearance was not associated with the recovery of the small-bowel mucosa, levels of serum TG3 antibodies, or the duration of the gluten-free diet.

This dissertation provides new information regarding the IgA class TG2 and TG3 autoantibody responses in coeliac disease and dermatitis herpetiformis: Firstly, it showed that in addition to TG2 antibodies, TG3 antibodies are also secreted at the small-bowel mucosal level, particularly in active dermatitis herpetiformis patients. In addition, while TG2 antibody-secreting cells were detected in both the coeliac disease and dermatitis herpetiformis patients, TG3 antibody-secreting plasma cells were shown to be characteristic of dermatitis herpetiformis. The presence of both cell populations was shown to be gluten-dependent, but it did not always parallel with the corresponding serum or deposited tissue-bound antibodies, suggesting that autoantibody production also outside of the small-bowel mucosa occurs in both manifestations. Finally, the disappearance of cutaneous IgA and TG3 in dermatitis herpetiformis patients was shown to occur slowly but in parallel during a strict, long-

term gluten-free diet, further supporting the existence of IgA and TG3 in the skin as immune complexes.

TIIVISTELMÄ

Keliakia on yleinen autoimmuunivälitteinen sairaus, jossa ravinnon gluteeni aiheuttaa perimältään alttiilla henkilöillä ohutsuolen limakalvon kroonisen tulehduksen ja vähittäisen vaurioitumisen sekä lukuisia suolioireita. Keliakia ei kuitenkaan ole pelkästään suoliston sairaus vaan se voi ilmetä myös erilaisina suoliston ulkopuolisina oireina. Yksi keliakian tunnetuimmista suoliston ulkopuolisista ilmentymismuodoista on dermatitis herpetiformis eli ihokeliakia, joka ilmenee rakkulaisena ja kutisevana ihottumana erityisesti kyynärpäissä, polvissa ja pakaroissa. Iho-oireiden lisäksi ihokeliakassa nähdään usein myös keliakialle tyypillisiä rakenteellisia ja tulehduksellisia muutoksia ohutsuolen limakalvolla. Sekä keliakian että ihokeliakian ainoa hoitomuoto on elinikäinen gluteeniton ruokavalio, jonka aikana ohutsuoli ja iho paranevat ja kliiniset oireet helpottuvat.

Sekä keliakiassa että ihokeliakiassa gluteeni aiheuttaa IgA-luokan vasta-ainereaktion elimistön omaa transglutaminaasi (TG) 2 -entsyymiä kohtaan. Näitä TG2-autovasta-aineita esiintyy hoitamattomien potilaiden seerumissa sekä kertyminä ohutsuolen limakalvolla, missä niiden tiedetään osin myös muodostuvan. Ihokeliakiapotilailla nähdään TG2-vasta-ainereaktion lisäksi myös IgA-luokan vasta-ainereaktio epidermaalista transglutaminaasia, TG3:a, kohtaan. TG3-vasta-aineita esiintyy kertyminä ihokeliakiapotilaiden iholla ja niitä havaitaan ihokeliakiapotilailla ja pienellä osalla keliakiapotilaista myös seerumissa. Sekä seerumin että suolen ja ihon vasta-aineet häviävät gluteenittoman ruokavaliohoidon aikana.

Tämän väitöskirjan tarkoituksena oli lisätä tutkimustietoa IgA-luokan TG2- ja TG3-vasta-ainereaktioista keliakiassa ja ihokeliakiassa. Osatyössä **I** määritettiin ohutsuolen limakalvon TG2-vasta-aineita tuottavien plasmasolujen esiintyvyys keliakiapotilailla taudin eri vaiheissa. Tulokset osoittivat, että TG2-vasta-aineita tuottavia plasmasoluja esiintyy jo taudin alkuvaiheessa ennen suolen limakalvovaurion syntyä ja että niiden osuus lisääntyy suolivaurion kehittyessä. Gluteenittoman ruokavaliohoidon aloittamisen jälkeen solujen esiintyvyys laski huomattavasti vuoden aikana ja pitkän hoidon jälkeen soluja ei enää juurikaan havainnoitu, mikäli ruokavaliohoito oli tiukka. Solujen suhteellinen määrä korreloi seerumin vasta-aineiden ja ohutsuolen limakalvon vasta-ainekertymien kanssa, joskaan yksittäisten potilaiden kohdalla ne eivät aina kulkeneet käsi kädessä.

Osatyössä **II** TG2- ja TG3-vasta-ainereaktioita tutkittiin ohutsuolen kudosviljelymallia hyödyntäen. Tulokset osoittivat, että aktiivisten keliakiapotilaiden ohutsuolen koepaloista erittyi kudosviljelyliuokseen pääosin TG2-vasta-aineita, kun taas ihokeliakiapotilaiden koepaloista erittyi pääosin TG3-vasta-aineita. Niiltä ihokeliakiapotilailta, jotka erittivät TG3-vasta-aineita kudosviljelyliuokseen, havainnoitiin TG3-vasta-aineita tuottavia soluja ohutsuolen limakalvolta.

Osatyössä **III** tutkittiin ohutsuolen limakalvon TG2- ja TG3-vasta-aineita tuottavien plasmasolujen esiintyvyyttä ja gluteenireaktiivisuutta ihokeliakiapotilailla gluteenialtistuksen aikana. Kumpaakaan solutyyppiä ei esiintynyt pitkään gluteenitonta ruokavaliota noudattaneilla potilailla, kun taas gluteenialtistuksen jälkeen sekä TG2- että TG3-vasta-aineita tuottavia plasmasoluja havaittiin yli puolella potilaista. Solujen suhteellinen määrä altistuksen jälkeen korreloi seerumin vasta-ainepitoisuuksien kanssa, mutta yksittäisten potilaiden kohdalla yhteyttä ei aina havaittu. Verrokkeina käytetyiltä keliakiapotilailta TG3-vasta-aineita tuottavia plasmasoluja ei pääsääntöisesti löytynyt.

Osatyössä **IV** selvitettiin ihokeliakiapotilaiden iholla esiintyvien IgA- ja TG3-kertymien häviämistä pitkän gluteenittoman ruokavaliohoidon aikana ja tutkittiin, onko häviäminen yhteydessä seerumin TG3-vasta-ainepitoisuuteen, ruokavaliohoidon pituuteen ja ohutsuolen limakalvon paranemiseen. Tulokset osoittivat, että TG3-kertymät häviävät iholta samanaikaisesti IgA-vasta-ainekertymien kanssa pitkän gluteenittoman ruokavaliohoidon aikana. Kertymien häviäminen ei ollut yhteydessä seerumin TG3-vasta-aineisiin, ohutsuolen limakalvovaurioon eikä hoidon kestoon.

Tämä väitöskirjatutkimus toi uutta tietoa IgA-luokan TG2- ja TG3-vasta-ainereaktioista keliakiassa ja ihokeliakiassa: Tutkimus osoitti ensimmäistä kertaa, että TG2-vasta-aineiden ohella myös TG3-vasta-aineita tuotetaan ohutsuolen limakalvolla, erityisesti aktiivisessa ihokeliakiassa. Lisäksi tutkimus näytti, että TG2-vasta-aineita tuottavia plasmasoluja esiintyy ohutsuolen limakalvolla niin keliakia- kuin ihokeliakiapotilaillakin, kun taas tässä tutkimuksessa havainnoidut TG3-vasta-aineita tuottavat plasmasolut näyttäisivät olevan tunnusomaisia ihokeliakialle. Vaikka molemmat solupopulaatiot olivat gluteenireaktiivisia, niiden yhteys seerumin sekä ohutsuolen ja ihon vasta-aineisiin ei aina ollut suoraviivainen, mikä viittaa siihen, että sekä keliakiassa että ihokeliakiassa vasta-aineita tuotetaan paitsi ohutsuolessa, myös sen ulkopuolella. Tutkimus osoitti myös, että ihokeliakiapotilaiden iholla esiintyvät IgA- ja TG3-kertymät häviävät hitaasti ja samanaikaisesti gluteenittoman ruokavaliohoidon aikana, mikä tukee vallitsevaa teoriaa siitä, että IgA ja TG3 esiintyvät ihon rakenteisssa komplekseina.

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ABBREVIATIONS

AGA	anti-gliadin antibodies
APC	antigen-presenting cell
ATI	α-amylase/trypsin inhibitors
BSA	bovine serum albumin
CD	coeliac disease
DH	dermatitis herpetiformis
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibody
FBS	foetal bovine serum
FITC	fluorescein-isothiocyanate
GFD	gluten-free diet
GDP	guanosine-5´-diphosphate
GTP	guanosine-5´-triphosphate
HLA	human leucocyte antigen
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MICA	major histocompatibility complex class I molecule A
PBS	phosphate-buffered saline
PT-gliadin	pepsin-trypsin digested gliadin
RT	room temperature
TCR	T cell receptor
TG	transglutaminase
TLR	toll-like receptor
TRITC	tetramethylrhodamine-isothiocyanate
Vh/CrD	villous height:crypt depth ratio

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (**I–IV**).

I Hietikko M, Koskinen O, Kurppa K, Laurila K, Saavalainen P, Salmi T, Ilus T, Huhtala T, Kaukinen K, Lindfors K (2018). Small-intestinal TG2-specific plasma cells at different stages of coeliac disease. BMC Immunol. 6;19(1):36.

II Hietikko M, Hervonen K, Ilus T, Salmi T, Huhtala H, Laurila K, Rauhavirta T, Reunala T, Kaukinen K, Lindfors K (2018). Ex vivo culture of duodenal biopsies from dermatitis herpetiformis patients indicates that transglutaminase 3 antibody production occurs in the gut. Acta Derm Veneorol. 98(3):366-372.

III Sankari H, Hietikko M, Kurppa K, Kaukinen K, Mansikka E, Huhtala H, Laurila K, Reunala T, Hervonen K, Salmi T, Lindfors K. Transglutaminase 2 and transglutaminase 3-specific plasma cell responses in dermatitis herpetiformis patients undergoing a gluten challenge. Submitted.

IV Hietikko M, Hervonen K, Salmi T, Ilus T, Zone JJ, Kaukinen K, Reunala T, Lindfors K (2018). Disappearance of epidermal transglutaminase and IgA deposits from the papillary dermis of dermatitis herpetiformis patients after a long-term gluten-free diet. Br J Dermatol. 178(3):e198-e201.

INTRODUCTION

Coeliac disease is an immune-mediated systemic disorder classically manifesting in the small intestine. The disease develops in genetically predisposed individuals in response to dietary gluten and proceeds gradually from the early-stage disease with small-bowel mucosal inflammatory changes in normal villi to the full-blown disease characterised by villous atrophy and crypt hyperplasia (Marsh et al. 1992). In addition, during the disease course, highly specific immunoglobulin (Ig) A-class antibodies targeting transglutaminase (TG) 2, the main autoantigen, are produced (Dieterich et al. 1997). Coeliac disease typically presents with gastrointestinal symptoms, but a wide variety of extraintestinal manifestations are also prevalent (Laurikka et al. 2018). These extraintestinal manifestations can affect almost any site of the body, and present with or without coeliac-type small-intestinal symptoms or mucosal alterations. Furthermore, in the context of the extraintestinal manifestations, the autoantibody response may be different (Yu et al. 2018).

Dermatitis herpetiformis specifically refers to the cutaneous manifestation of coeliac disease. It is the most common extraintestinal manifestation, occurring in approximately 13% of coeliac disease patients in Finland (Salmi et al. 2011). A diagnostic feature of dermatitis herpetiformis is an itchy, blistering rash and the deposition of granular IgA in the papillary dermis of the skin (Zone et al. 1996). These IgA deposits have been shown to target another member of the transglutaminase family, epidermal transglutaminase, TG3, which is currently regarded as the main autoantigen of dermatitis herpetiformis (Sardy et al. 2002). Gastrointestinal symptoms in dermatitis herpetiformis are rare (Pasternack et al. 2017). However, parallel to coeliac disease, the majority of dermatitis herpetiformis patients have villous atrophy and crypt hyperplasia in the small intestine (Gawkrodger et al. 1984; Reunala et al. 1978; Mansikka et al. 2017 and 2018), and the remainder evince coeliac-type inflammation (Fry et al. 1972; Savilahti et al. 1992; Järvinen et al. 2003; Salmi et al. 2014).

A hallmark of both coeliac disease and dermatitis herpetiformis is the presence of TG2-targeting autoantibodies in the serum (Dieterich et al. 1997; Dieterich et al. 1999). In addition, such antibodies can be found as deposits in the small-bowel

mucosa (Korponay-Szabo et al. 2004; Salmi et al. 2014) where also a high frequency of plasma cells specific for TG2 has been described in untreated coeliac disease (Di Niro et al. 2012 and 2016). In addition to the TG2 antibody response, the majority of dermatitis herpetiformis patients – and also a small subset of coeliac disease patients (Salmi et al. 2016) – have IgA class TG3 antibodies in the serum (Sardy et al. 2002; Hull et al. 2008). In contrast to TG2 antibody response in coeliac disease, the TG3 antibody response in terms of small-bowel mucosal plasma cell responses is not as well characterised.

During a strict, life-long gluten-free diet, the treatment of choice for both manifestations, the small-bowel mucosa and skin heal and the clinical symptoms disappear. Likewise, the serum and deposited small-bowel mucosal and cutaneous antibodies disappear. While serum antibodies disappear rapidly, within months, the small-bowel mucosal TG2-targeting IgA deposits may persist longer, even for years (Koskinen et al. 2010). Similarly, the IgA deposits in dermatitis herpetiformis patient skin have been shown to disappear slowly (Garioch et al. 1994). Whether their target, TG3, disappears simultaneously with IgA has not been previously investigated.

The aim of the present study was to investigate the IgA-class autoantibody responses towards TG2 and TG3 in coeliac disease and dermatitis herpetiformis. Specifically, the study focused on the presence, frequency, and gluten-dependence of small-bowel mucosal TG2 and TG3 autoantibody-secreting cells and their connection to the corresponding serum and deposited small-bowel mucosal and cutaneous antibodies. Furthermore, the disappearance of cutaneous IgA and TG3 in dermatitis herpetiformis patients after a long-term gluten-free diet was studied.

REVIEW OF THE LITERATURE

1 COELIAC DISEASE

1.1 Overview of coeliac disease

Coeliac disease is a systemic autoimmune-mediated disorder triggered by dietary gluten present in wheat, rye, and barley in genetically susceptible individuals. Once considered a rare malabsorptive disease of childhood, it is currently one of the most frequent autoimmune conditions; it is estimated to affect around 1% of the population worldwide (Singh et al. 2018) and as many as 2% in Finland (Lohi et al. 2007). The prevalence is further increasing, not only due to increased awareness and effective diagnostics, but also because of a true rise in prevalence (Lohi et al. 2007; Rubio-Tapia et al. 2009; Kang et al. 2013; Singh et al. 2018). Today, coeliac disease can be diagnosed at any age, and its prevalence has been shown to increase with age (Mäki et al. 2003; Lohi et al. 2007; Vilppula et al. 2008). The disease is more common in females than in males (Singh et al. 2018).

In coeliac disease patients, ingestion of gluten leads to small-bowel mucosal damage, which develops gradually from minor inflammatory changes to overt lesions characterised by villous atrophy and crypt hyperplasia (Marsh 1992). In addition, antibodies mainly targeting gluten-derived peptides as well as the self-antigen TG2 are generated (Dieterich et al. 1997). The clinical presentation is wide and includes numerous gastrointestinal and extraintestinal manifestations (Laurikka et al. 2018). Furthermore, the symptoms vary from mild to severe, and some patients may even be completely asymptomatic (Kivelä et al. 2015). Due to its heterogeneous nature, diagnosing coeliac disease can be challenging and a large proportion of patients remains unrecognised (Singh et al. 2018). In Finland, for example, it is estimated that less than half of coeliac disease patients have been found (Ilus et al. 2014).

1.2 Clinical presentations of coeliac disease

The typical presentation of coeliac disease comprises gastrointestinal symptoms such as diarrhoea, indigestion, and abdominal pain as well as signs of malabsorption, which were long regarded as the dominant features of the disease. However, as

awareness of the disease has increased, it has become evident that the symptoms are highly variable and not restricted solely to the small intestine (Mäki et al. 1988; Volta et al. 2014; Kivelä et al. 2015). In addition, over the decades, the clinical presentation has shifted towards milder forms, and patients may present with only minor gastrointestinal symptoms, be completely asymptomatic (Ukkola et al. 2011; Kivelä et al. 2015), or present with one of the many extraintestinal manifestations associated with the disease (Mäki et al. 1988; Kivelä et al. 2015; Laurikka et al. 2018).

Extraintestinal manifestations of coeliac disease can affect almost any site of the body, appear at any age, and be the sole presentation of the disease (Laurikka et al. 2018). One of the best-characterised manifestations is dermatitis herpetiformis, coeliac disease of the skin, which presents with an itching and blistering rash typically on the elbows, knees, and buttocks (Collin et al. 2017; Chapter 2.2). In addition to dermatitis herpetiformis, a range of neurological signs and symptoms have been connected to coeliac disease, the most commonly associated conditions being gluten ataxia and peripheral neuropathy (Hadjivassilious et al. 2006 and 2008). Coeliac disease has also been associated with elevated liver enzymes, and even severe liver failure has been described (Kaukinen et al. 2002; Äärelä et al. 2016). Anaemia resulting from deficiency of iron, B_{12} vitamin, and folate is also common (Repo et al. 2017; Saukkonen et al. 2017). Dental enamel defects (Aine et al. 1990), loss of bone mineral density leading to osteoporosis, and an increased risk for fractures (Di Stefano et al. 2013; Heikkilä et al. 2015) as well as infertility and other reproductive health problems (Tersigni et al. 2014) are also observed. While some of the manifestations are age-related, appearing primarily in childhood or adulthood (Jericho et al. 2017), some have been associated with a more severe clinical and histological presentation of coeliac disease (Nurminen et al. 2018; Laurikka et al. 2018). Generally, they are effectively treated with a gluten-free diet, but some of the manifestations might be irreversible and lead to complications if not recognised early enough (Jericho et al. 2017; Laurikka et al. 2018).

Coeliac disease frequently occurs in parallel with other autoimmune diseases such as type 1 diabetes, autoimmune thyroiditis, autoimmune hepatitis, and Sjögren syndrome (Collin et al. 2002). In addition, the disease is associated with severe complications such as gastrointestinal malignancies and non-Hodgkin's lymphoma, but the risk reduces during a gluten-free diet (Holmes et al. 1989; Grainge et al. 2012).

1.3 Small-bowel mucosa in coeliac disease

1.3.1 Small-bowel mucosal morphology

In coeliac disease, the ingestion of gluten leads to gradual changes in the small-bowel mucosa (Figure 1), which develop over years or even decades during continuous gluten consumption (Mäki et al. 1990; Lähdeaho et al. 2005). The degree of damage can be evaluated using different methodologies (Adelman et al. 2018). One way is to categorise the damage subjectively using the Marsh classification (Marsh 1992): in the healthy small-bowel mucosa, the villi are long and finger-like and the crypts are short (Marsh 0) (Figure 1). In the early stage of coeliac disease, the villous morphology remains normal but increased infiltration of inflammatory cells in the epithelium and the lamina propria can be detected (Marsh I). Thereafter, elongation of the crypts occurs (Marsh II) and, finally, overt villous atrophy develops (Marsh III) (Marsh 1992). The level of villous atrophy can be further classified into partial, subtotal, and total villous atrophy (Marsh 1992). Another, more objective way to evaluate the degree of small-bowel mucosal damage is by quantifying the villous height:crypt depth ratio (Vh/CrD) (Taavela et al. 2013), with a ratio above two being considered a cut-off for the normal value (Kuitunen et al. 1982; Taavela et al. 2013). Regardless of the method, the small-bowel mucosal morphology should always be assessed in well-oriented and high-quality biopsies for reliable interpretation (Taavela et al. 2013). In addition, it should be noted that the mucosal damage can be patchy (Ravelli et al. 2010) and that it is not a specific finding to coeliac disease, as it occurs also in other conditions as well as upon treatment with certain medications (DeGaetani et al. 2013; Aziz et al. 2017).

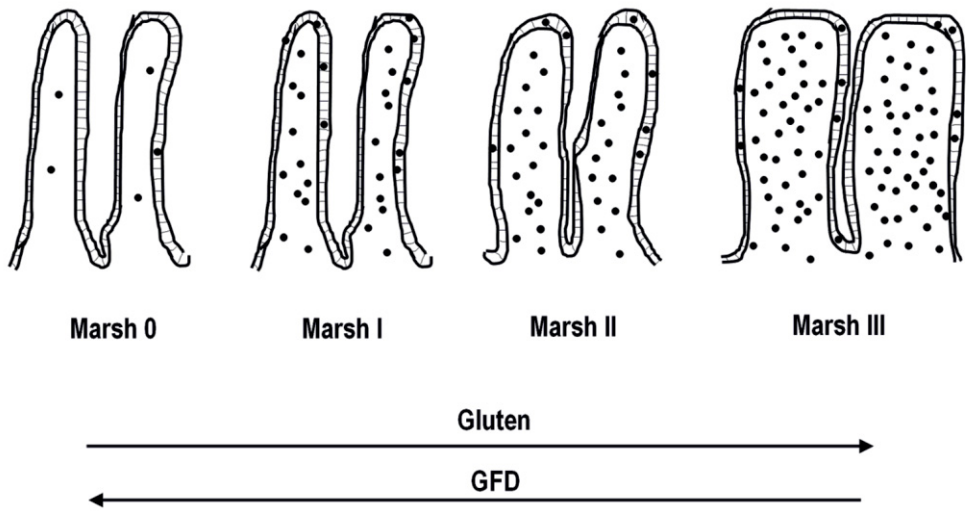

Figure 1. Gradual development of small-bowel mucosal inflammation and damage in an untreated coeliac disease patient upon ingestion of gluten as graded according to the Marsh classification. In the healthy small-bowel mucosa, the villi are long and the crypts are short (Marsh 0). In the early stage of coeliac disease, increased numbers of inflammatory cells can be detected in the epithelium and lamina propria in the otherwise normal mucosa (Marsh I). During continuous gluten exposure, the crypts become elongated (Marsh II) and eventually, overt villous atrophy with crypt hyperplasia develops (Marsh III). All these changes are reversed during treatment with a gluten-free diet (GFD). Figure adapted from Marsh (1992).

1.3.2 Small-bowel mucosal inflammation

In addition to the structural changes to the small-bowel mucosa, the coeliac lesion is characterised by the infiltration of immune cells into the epithelium and the lamina propria. In the epithelium, increased numbers of intra-epithelial lymphocytes (IEL) are typically detected, which, as described above, is regarded as the first sign of the small-intestinal lesion in coeliac disease (Ferguson and Murray, 1971; Kuitunen et al. 1982; Marsh et al. 1992; Ferguson et al. 1993; Järvinen et al. 2003). The majority of the IELs are characterised by the expression of the CD3 surface molecule and can be further classified into αβ+ and γδ+ IELs according to the T cell receptor that they express (Savilahti et al. 1992). Even though intraepithelial lymphocytosis is indicative for coeliac disease, it is not entirely disease-specific and can also be found in other conditions, such as milk allergy, other autoimmune diseases, and parasitic

infections (Kuitunen et al. 1982). Of the IELs, the γδ+ subtype is regarded as the most specific for predicting forthcoming coeliac disease (Savilahti et al. 1992; Järvinen et al. 2003 and 2004). During a gluten-free diet, the density of the IELs decreases (Ferguson and Murray 1971) but may remain elevated for years despite the recovery of the mucosal morphology (Iltanen et al. 1999; Ilus et al. 2012).

In addition to IELs, infiltration of other inflammatory cells – such as plasma cells, T cells, and antigen-presenting cells – can be detected in the lamina propria (Savilahti et al. 1992; Baklien et al. 1977). Plasma cells in particular are extensively expanded in untreated coeliac disease (Baklien et al. 1977). These plasma cells produce mainly antibodies of the IgA isotype, as well as antibodies of the IgM and IgG isotype to a lesser extent (Sollid and Jabri 2013). It has been shown that of all the IgA-secreting plasma cells in the lamina propria, on average 10% are specific for TG2, the main autoantigen of coeliac disease (Dieterich et al. 1997), whereas only 1% produce antibodies recognising gluten-derived peptides (Di Niro et al. 2012 and 2016; Steinsbø et al. 2014).

1.4 Coeliac disease antibodies

1.4.1 Serum antibodies

Antibodies against various antigens have been described in the context of coeliac disease and its different manifestations. The earliest antibodies described were those targeting the native gluten-derived gliadin (Du Pré and Sollid 2015). These anti-gliadin antibodies (AGA) were commonly used in case finding and detected using an enzyme-linked immunosorbent assay (ELISA) technique before the development of other detection methods. However, today they are known to be unspecific for coeliac disease, being found in healthy individuals as well as in other conditions, so they are no longer in clinical use. By contrast, IgA and IgG class antibodies against deamidated gliadin peptides (DGP) generated during the pathogenesis of coeliac disease have been shown to have better accuracy in detecting coeliac disease compared to the AGA tests (Kurppa et al. 2011; Kaukinen et al. 2007), and they perform almost as well as the primarily used TG2 autoantibodies described below (Lewis et al. 2010). Their usage in clinical practice varies, but they may be helpful in identifying patients with early-stage coeliac disease (Kurppa et al. 2011) as well as

those with IgA deficiency or patients seronegative for TG2-targeting autoantibodies (Sugai et al. 2006; Dahle et al. 2010; Kurppa et al. 2011).

The first autoantibodies described in coeliac disease were shown to target the reticular fibres in the endomysium, the connective tissue surrounding smooth muscle cells, and they were initially known as reticulin antibodies (ARA) (Seah et al. 1971a). Later, a new autoantibody targeting monkey oesophagal endomysium was discovered (Chorzelski et al. 1983). These antibodies, called endomysial antibodies (EmA), were detected using an indirect immunofluorescence method on monkey oesophagus sections (Chorzelski et al. 1983). The method was subsequently improved and monkey oesophagus was replaced by human umbilical cord as the substrate (Ladinser et al. 1994). The target of both ARA and EmA, TG2, was identified only later (Dieterich et al. 1997; Korponay-Szabo et al. 2000 and 2003b). Thereafter, an ELISA-based method for the detection of TG2 antibodies was developed using either human or guinea pig TG2 as the antigen (Sulkanen et al. 1998a; Sblattero et al. 2000). Today, the levels of IgA class TG2-targeting antibodies in patient serum are measured using both the EmA and the TG2 ELISA method. In the case of IgA deficiency, TG2 antibodies in IgG class can be measured (Korponay-Szabo et al. 2003a). In addition to the traditional EmA and TG2 antibody tests, rapid point-of-care tests have been developed for the detection of TG2-targeting autoantibodies in a whole blood sample from the fingertip (Korponay-Szabo et al. 2005; Raivio et al. 2006), and they are also available for the detection of DGP antibodies (Lau et al. 2018).

In addition to TG2-targeting autoantibodies, autoantibodies targeting other transglutaminase family members can also be detected in the serum of coeliac disease patients, particularly in the context of extraintestinal manifestations (Yu et al. 2018). Serum IgA class autoantibodies towards epidermal transglutaminase, TG3, are a typical feature of dermatitis herpetiformis, but they are also found in the serum of coeliac disease patients, albeit with less frequency (Sardy et al. 2002; Heil et al. 2005; Marietta et al. 2008; Salmi et al. 2016). Furthermore, antibodies targeting a neuronal TG isoform, TG6, are associated with the neurological manifestations of coeliac disease, especially gluten ataxia (Hadjivassiliou et al. 2006 and 2008) and gluten neuropathy (Hadjivassiliou et al. 2013). In contrast to TG2 antibodies, however, the gluten dependency of TG3 and TG6 antibodies in coeliac disease is not as evident (Lindfors et al. 2011; Salmi et al. 2016; De Leo et al. 2018).

1.4.2 Small-bowel mucosal antibodies

Besides being present in the serum, TG2-targeting IgA antibodies can also be found as deposits in the small-bowel mucosa (Shiner and Ballard 1972; Korponay-Szabo et al. 2004), where they can be detected using a direct immunofluorescence staining method (Korponay-Szabo et al. 2004). In coeliac disease patients, these antibody deposits have been shown to target extracellular TG2 in the basement membrane below the epithelial layer and around mucosal blood vessels (Korponay-Szabo et al. 2004) whereas in non-coeliac subjects, IgA is detected only inside the plasma cells and epithelial cells (Korponay-Szabo et al. 2004). The TG2-targeting IgA deposits are specific for coeliac disease and found in practically all patients with villous atrophy (Salmi et al. 2006a; Koskinen et al. 2010). In addition, they can be present in the small-bowel mucosa already before the development of mucosal alterations (Salmi et al. 2006a; Koskinen et al. 2010; Kaukinen et al. 2005; Tosco et al. 2008) and before the appearance of TG2-targeting autoantibodies in the serum (Salmi et al. 2006b). Therefore, detection of small-intestinal IgA deposits may be helpful in identifying early-stage coeliac disease in the absence of villous atrophy as well as patients with negative and borderline positive serology. In IgA-deficient patients, IgG and IgM class deposits can be detected (Korponay-Szabo et al. 2004; Borrelli et al. 2010).

In addition to the small intestine, TG2-targeting IgA deposits have also been found in various extraintestinal locations in untreated coeliac disease patients, such as the kidneys, muscles, and lymph nodes (Korponay-Szabo et al. 2004), and around vessel walls in the brain of gluten ataxia patients (Hadjivassiliou et al. 2006).

1.5 Diagnosis of coeliac disease

The diagnosis of coeliac disease is based on a combination of serological testing of disease-specific TG2-targeting autoantibodies and the determination of small-bowel mucosal morphology in biopsies taken during invasive gastroscopy during a gluten-containing diet (Lindfors et al. 2019). Previously, the demonstration of small-intestinal villous atrophy and crypt hyperplasia was considered mandatory for the final diagnosis, whereas serological tests had more of a supportive role (Walker-Smith et al. 1990). However, due to the high specificity and sensitivity of both EmA (95–100% and 83–100%, respectively) and TG2 antibodies (77.8–100% and 89–100%, respectively) (Lewis and Scott 2006; Giersepien et al. 2012), diagnostics is

now moving towards non-invasive methods, and the assessment of serum autoantibodies has become increasingly important. According to the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), the diagnosis in symptomatic children with the coeliac genotype can be made without the assessment of small-bowel mucosal biopsies (Husby et al. 2012). Similar criteria have also been shown to be applicable to adult patients (Fuchs et al. 2019). In Finland, the national current care guidelines have recently been revised, and small-intestinal mucosal biopsy is no longer required in individuals showing highly elevated levels of serum TG2-targeting autoantibodies (10x the upper limit of normal value of TG2 antibodies and positive EmA) (Coeliac disease, Current Care Guidelines 2018).

1.5.1 Differential diagnosis

In addition to coeliac disease, the consumption of gluten-containing cereals has been associated with a condition called non-coeliac gluten sensitivity (NCGS) (Biesiekierski et al. 2011; Pinto-Sanchez and Verdu 2018). Individuals with gluten sensitivity experience a range of coeliac-type gastrointestinal and/or extraintestinal symptoms in response to the ingestion of gluten-containing products, but lack the genetic predisposition and the histological and serological markers characteristic for coeliac disease (Pinto-Sanchez and Verdu 2018). Nevertheless, the symptoms improve during dietary treatment and reoccur upon the reintroduction of gluten to the diet. The underlying mechanisms and true prevalence of gluten sensitivity are not as yet known, and there are no specific biomarkers available (Pinto-Sanchez and Verdu 2018). In addition, it remains unclear whether other non-gluten components of wheat, such as poorly absorbed short-chain carbohydrates (FODMAPs) or α-amylase/trypsin inhibitors (ATIs), are also involved in the development of the symptoms (Biesiekierski et al. 2013; Zevallos et al. 2017; Dale et al. 2018; Skodje et al. 2018). The diagnosis of NCGS is currently based on the exclusion of coeliac disease and wheat allergy during a gluten-containing diet, the disappearance of symptoms during a gluten-free diet, and the recurrence of symptoms upon gluten reintroduction (Al-Toma et al. 2019).

1.6 Treatment of coeliac disease

Currently, the only available treatment for coeliac disease is a lifelong gluten-free diet, i.e. the strict avoidance of wheat, rye, and barley (See et al. 2015). During the diet, the small-intestinal mucosal morphology recovers, serum antibodies disappear, and the clinical symptoms resolve. In addition, the risk for developing disease-associated complications decreases. Upon re-introduction of gluten to the diet, the signs and symptoms rapidly reappear.

While the dietary treatment is successful in the majority of patients, in some cases the small-intestinal mucosa does not heal (Leffler et al. 2007), and/or the symptoms persist despite a strict diet (Laurikka et al. 2016). The most common reason for persistent symptoms and villous atrophy is poor, inadvertent, or advertent adherence to the diet. However, a minority of patients are truly non-responsive even to a strict gluten-free diet. These patients have a condition called refractory coeliac disease, which in Finland, for example, affects approximately 0.3% of the patients (Ilus et al. 2014). Due to these challenges, alternative or additional non-dietary therapies for the gluten-free diet are being developed, ranging from the modification of dietary gluten to targeting one of the steps involved in the pathogenesis of coeliac disease (Lähdeaho et al. 2014; Leffler et al. 2015). However, none of the new treatment options has yet reached clinical practice.

1.7 Pathogenesis of coeliac disease

The development of coeliac disease is a result of genetic and environmental factors as well as innate and adaptive immune mechanisms (Stamnaes and Sollid 2015a; Lindfors et al. 2019).

1.7.1 Genetic factors

The development of coeliac disease requires genetic susceptibility. The major predisposing genetic factors are the genes encoding for the human leucocyte antigen (HLA)-DQ molecules HLA-DQ2 and HLA-DQ8, which are expressed on the surface of antigen-presenting cells (Stokes et al. 1972; Sollid et al. 1989; Sollid 2017). These molecules are found in practically all coeliac disease patients and are considered a prerequisite for disease development. The majority of the patients carry

a variant of HLA-DQ2 called HLA-DQ2.5 (90%) (Sollid et al. 1989; Sollid 2017) and the rest carry either HLA-DQ8 or another HLA-DQ2 variant, DQ2.2 (Spurkland et al. 1992; Karell et al. 2003). However, as 30–40% of the general population carries these predisposing HLA molecules, so they alone are not sufficient for disease development; other susceptibility genes and/or environmental factors are also required. To date, over 40 non-HLA regions have been identified, many of which relate to immunity and are also associated with other autoimmune diseases, such as rheumatoid arthritis and type I diabetes (van Heel et al. 2007; Dubois et al. 2010; Trynka et al. 2011; Sollid 2017). However, while the HLA-DQ variants contribute as much as 40% of the genetic risk of coeliac disease, the effect of the non-HLA regions is thought to be more modest, at approximately 14% (Trynka et al. 2011).

1.7.2 Environmental factors

The main external factor of coeliac disease is dietary gluten (Dicke et al. 1953). The term gluten is commonly used to describe a group of storage proteins – prolamins – found in wheat, rye, and barley. In wheat, these storage proteins are subdivided based on their alcohol-solubility into insoluble glutenins and soluble gliadins (Wieser et al. 2007), whereas in rye and barley, they are termed secalins and hordeins, respectively. The gluten proteins have a particularly high content of glutamine- and proline-rich areas (Vader et al. 2002; Wieser et al. 2007), and especially the high proline content makes them extremely resistant to degradation by gastrointestinal proteases (Shan et al. 2002). Consequently, long polypeptides remain in the intestine, some of which, in the context of coeliac disease, are either immunogenic or toxic (Shan et al. 2002). One of the most immunogenic gluten-derived peptides characterised is the cleavage-resistant 33-mer, which contains six T cell epitopes and is regarded as a particularly strong activator of the adaptive immune response in coeliac disease (Shan et al. 2002).

While gluten remains the major environmental factor of coeliac disease, the role of other predisposing factors has also been studied. Breastfeeding and infant feeding habits (Ivarsson et al. 2000; Ivarsson et al. 2013), viral infections during both childhood and adulthood (Kagnoff et al. 1987; Kemppainen et al. 2017; Bouziat et al. 2017; Karsh et al. 2019), and alterations in the intestinal microbiota (Kalliomäki et al. 2012; Wacklin et al. 2013; Verdu et al. 2015; Caminero et al. 2016), among others, have all been proposed to be associated with the development and

progression of coeliac disease. However, data on many of the factors and their causality, such as breastfeeding habits (Szajewska et al. 2015), are currently controversial.

1.7.3 The autoantigen TG2

TG2, discovered as the coeliac disease autoantigen in 1997 (Dieterich et al. 1997), is a multifunctional enzyme belonging to a structurally and functionally related transglutaminase family of nine distinct proteins (Lorand and Graham 2003). TG2 is the most widely expressed member of the family and constitutively present in most tissues of the body, including the small intestine (Villanacci et al. 2009). Similarly to other transglutaminases, the main function of TG2 is the posttranslational modification of proteins: TG2 can create isopeptide bonds between two target proteins by cross-linking protein-bound residues sequence-specifically in a reaction called transamidation or, alternatively, convert glutamine residues in the proteins into glutamate in a reaction termed deamidation (Lorand and Graham 2003). The enzymatic activity of TG2 is allosterically regulated by the availability of Ca^{2+} and guanosine nucleotides, guanosine-5´-diphosphate (GDP) and guanosine-5´-triphosphate (GTP) (Achyuthan et al. 1987; Liu et al. 2002). The Ca^{2+}-bound TG2 has an open, catalytically active conformation, whereas in the GTP/GDP-bound state, it adopts a closed conformation and is catalytically inactive (Pinkas et al. 2007). In addition, an oxidative environment has been shown to inactivate the enzyme (Siegel et al. 2008; Stamnaes et al. 2010; Iversen et al. 2014).

The functions of TG2 typically depend on its cellular location (Park et al. 2010) and enzymatic activity. Intracellularly, TG2 is inactive and mainly located in the cytosol, where it participates in signal transduction as a G-protein (Nakaoka et al. 1994). TG2 is also actively externalised from the cells (Zemskov et al. 2011; Adamczyk et al. 2015): on the cell surface, TG2 is bound to the plasma membrane, where it associates with integrins and functions as a co-receptor for fibronectin binding, mediating cell adhesion and spreading (Akimov et al. 2000; Akimov et al. 2011). Extracellularily, TG2 interacts with its various substrate proteins, particularly fibronectin (Cardoso et al. 2015), and participates in the stabilisation of the cytoskeleton and extracellular matrix, as well as the regulation of cell adhesion, matrix assembly, and cell motility. Extracellular TG2 has been shown to be mostly inactive due to prevailing oxidative conditions, but it is activated upon inflammation, tissue injury, and a reducing environment (Siegel et al. 2008; Stamnaes et al. 2010).

In coeliac disease, TG2 plays a central role. Firstly, it is the main target of the autoantibody reaction, and secondly, it modifies gluten-derived peptides to be more immunogenic. Due to their high content of glutamine residues, gluten-derived peptides are excellent substrates for TG2 (Vader et al. 2002; Fleckenstein et al. 2002; Piper et al. 2002). TG2 introduces negative charges into the peptides by converting specific glutamine residues into glutamic acid in a deamidation reaction (Fleckenstein et al. 2002). As a result, the binding affinity of the gluten-derived peptides to HLA-DQ molecules on antigen-presenting cells increases and stable gluten-HLA-DQ complexes capable of eliciting an immune response are formed (Molberg et al. 1998; Van de Wal et al. 1998; Kim et al. 2004). In addition to deamidation, TG2 also crosslinks gluten-derived peptides to itself, which has significance in the production of TG2-targeting antibodies, as described below (Fleckenstein et al. 2004).

1.7.4 Adaptive and innate immune mechanisms

Under normal conditions, the small-intestinal epithelium is highly resistant and impermeable to macromolecules. In coeliac disease, however, epithelial integrity is impaired and thus the gluten-derived peptides resulting from incomplete digestion can cross the epithelial barrier via the transcellular or paracellular route (Schumann et al. 2017). After entering the lamina propria, the peptides can initiate the adaptive and innate immune responses characteristic of coeliac disease (Figure 2).

1.7.4.1 Adaptive immune mechanisms

Adaptive immune response is initiated when gluten-derived peptides are presented to gluten-specific CD4+ T cells through coeliac disease-associated HLA-DQ molecules on antigen-presenting cells. While native gluten-derived peptides bind only poorly to HLA-DQ2 and -DQ8 molecules (van de Wal et al. 1996), their deamidated counterparts have an increased binding affinity towards these molecules, as described above. Upon presentation of the deamidated, HLA-DQ-bound gluten peptides to gluten-specific CD4+ T cells, which are found only in the small intestine of patients with coeliac disease and preferentially recognise the deamidated gluten peptides through their T cell receptors (TCR) (Lundin et al. 1993; Lundin et al. 1994; Molberg et al. 1998; Dorum et al. 2009; Sollid 2017), the CD4+ T cells in the lamina propria become activated. This results in the secretion of various proinflammatory cytokines, such as interferon (IFN)-γ and interleukin (IL)-21 (Nilsen et al. 1995;

Bodd et al. 2010), which, in turn, promote the activation of cytotoxic IELs (Zeng et al. 2005) and thereby contribute to the subsequent epithelial destruction and the development of villous atrophy, as described below. Interestingly, while the antigen-presenting cells in coeliac disease have been previously thought to comprise mainly macrophages and dendritic cells (Ráki et al. 2006; Beitnes et al. 2011; Beitnes et al. 2012), it was recently demonstrated that the majority of the cells presenting gluten-derived peptides to CD4+ T cells in the lamina propria of coeliac disease patients are actually B cells and plasma cells (Høydal et al. 2018).

In addition to the proinflammatory response, CD4+ T cells are thought to play an important role in the generation of the antibody responses characteristic of coeliac disease: they activate disease-specific B cells and promote their differentiation into plasma cells that secrete antibodies towards gluten-derived peptides and TG2. In parallel with the activation of the B cells, the CD4+ T cells themselves become activated and start proliferating and clonally expanding (Du Pre and Sollid 2015). As TG2-specific CD4+ T cells have not been detected in the small-intestinal mucosa, the generation of TG2-targeting autoantibodies has been explained to occur with the help of gluten-specific CD4+ T cells (Mäki et al. 1994; Sollid et al. 1997). According to this so-called hapten-carrier model, gluten-derived peptides are taken up by B cell receptors on TG2-specific B cells as covalent complexes with TG2, and subsequently presented to gluten-specific CD4+ T cells in the context of HLA-DQ molecules (Mäki et al. 1994; Sollid et al. 1997; Fleckenstein et al. 2004; Di Niro et al. 2012; Stamnaes et al. 2015b). As a result, the TG2-specific B cells become activated and capable of differentiating into TG2 antibody-producing plasma cells. It has also been shown that TG2 can effectively multimerise with itself, resulting in TG2-multimers into which gluten peptides can be incorporated (Stamnaes et al. 2015b). These structures can induce an even more efficient activation of gluten-specific T cells than the TG2 monomers (Stamnaes et al. 2015b).

Both the serum and small-bowel mucosal TG2-targeting autoantibodies were initially thought to originate from plasma cells residing in the small-intestinal mucosa; antibodies produced locally in the small intestine were suggested to first deposit in the small-intestinal mucosa and then spill over from the small intestine into the circulation (Marzari et al. 2001). However, recent evidence suggests that while the serum and small-intestinal antibodies are clonally related, they have a different molecular composition, and serum antibodies might actually be produced in the lymphoid tissues outside the small intestine (Iversen et al. 2017).

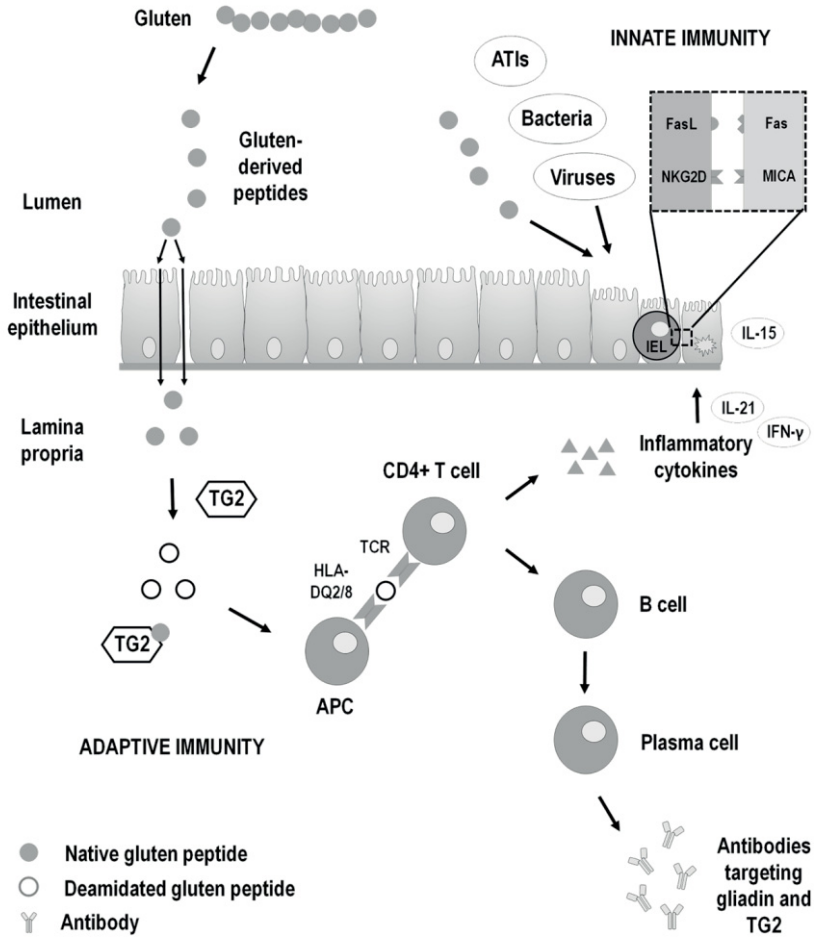

Figure 2. Simplified illustration of the pathogenetic mechanisms underlying coeliac disease. Insufficiently degraded gluten-derived peptides can cross the small-intestinal epithelium via the transcellular or the paracellular pathway. Upon reaching the lamina propria, the peptides are modified by TG2, resulting in the formation of either deamidated gluten peptides or peptides cross-linked to TG2. The modified peptides are subsequently presented to CD4+ T cells in the context of coeliac disease-associated HLA-DQ molecules on antigen-presenting cells. As a result, the CD4+ T cells are activated and various inflammatory cytokines are produced. In addition, T cells provide help to disease-specific B cells, which differentiate into plasma cells secreting antibodies towards gluten and TG2. In parallel, stressed epithelial cells produce IL-15 in response to different stimuli, which leads to apoptosis of the epitheal cells through different pathways, such as the Fas/FasL pathway. Figure adapted and modified from Sollid and Jabri 2013. APC, antigen presenting cell; ATI, α-amylase/trypsin inhibitors; HLA, human leucocyte antigen; IEL, intraepithelial lymphocyte; IFN, interferon; IL, interleukin; MICA, major histocompatibility complex class I molecule A; TCR, T cell receptor; TG2, transglutaminase 2

1.7.4.2 Innate immune mechanisms

Adaptive immune response in itself is not sufficient to cause the characteristic small-intestinal mucosal alterations in coeliac disease, and innate immune mechanisms also play a role (Sollid and Jabri 2013). Activation of the innate immune mechanisms occurs along with the activation of lamina propria CD4+ T cells and is thought to be mediated by IL-15, which is secreted particularly by stressed epithelial cells in response to various triggers (Figure 2). Such triggers include, for example, certain gluten-derived peptides, viruses and bacteria, and other non-gluten components of wheat such as ATIs (Mention et al. 2003; Maiuri et al. 2003; Hüe et al. 2004; Sollid and Jabri 2013; Setty et al. 2015; Zevallos et al. 2017; Brown et al. 2018; Bouziat et al. 2017). IL-15 induces the upregulation of activating receptors, such as NKG2D and CD94-NKG2C, on CD8+ IELs, turning them into CD8+ CTLs killer cells (Hüe et al. 2004; Meresse et al. 2004; Meresse et al. 2006). Simultaneously, the upregulation of stress molecules such as major histocompatibility complex class I molecule A (MICA) and HLA-E on the surface of intestinal epithelial cells occurs. Interaction of the receptors with their ligands drives epithelial cells to apoptosis through different pathways, which in part promotes small-intestinal damage and increased epithelial permeability (Hüe et al. 2004).

1.7.5 Role of antibodies in the pathogenesis

Besides being valuable diagnostic tools, numerous studies have addressed the possible role of TG2 antibodies in the pathogenesis of coeliac disease (Halttunen and Mäki 1999; Barone et al. 2007; Myrsky et al. 2008; Boscolo et al. 2010). Nevertheless, their exact contribution remains controversial. As the activity of TG2 is crucial for the modification and the subsequent increased immunogenicity of the gluten-derived peptides, several studies have addressed the capability of the antibodies to interfere with the enzymatic activity of TG2. However, the results have been contradictory: the antibodies have been reported to have both inhibitory (Byrne et al. 2010; Dieterich et al. 2003; Esposito et al. 2002; Anjum et al. 2009) and stimulatory (Kiraly et al. 2006; Myrsky et al. 2009) effects, as well as no effect at all (Di Niro et al. 2012). On the other hand, TG2 antibodies have been shown to target at least four different epitopes on TG2 (Simon-Vecsei et al. 2012; Iversen et al. 2013; Iversen et al. 2014), and recently, epitope-dependent functional effects for the antibodies were described: antibodies targeting certain epitopes were shown to protect TG2 from oxidative inactivation and increase its Ca^{2+} sensitivity, thus

keeping the enzyme active, whereas others were shown to reduce the rate of TG2-mediated deamidation (Hnida et al. 2016). Therefore, the contradictory results could depend on the experimental settings as well as the type of antibodies used (Hnida et al. 2016).

Coeliac disease patient-derived antibodies, either total serum IgA or isolated TG2 antibodies, have also been shown to have various effects in different in vitro and in vivo settings that could have functional significance in the pathogenesis of coeliac disease. Firstly, their possible effect on the epithelial cells has been studied: they have been reported to interfere with the differentiation (Halttunen and Mäki 1999) and proliferation (Barone et al. 2007) of epithelial cells as well as to affect their permeability (Zanoni et al. 2006) and adhesion (Teesalu et al. 2012). Furthermore, it has been suggested that the antibodies could modulate the transportation of gluten-derived peptides through the small-intestinal epithelium (Matysiak-Budnik et al. 2008; Caputo et al. 2010; Rauhavirta et al. 2011; Lebreton et al. 2012). They have also been shown to disturb angiogenesis (Myrsky et al. 2008; Caja et al. 2010; Kalliokoski et al. 2013) and increase vascular permeability (Myrsky et al. 2009). In vivo studies have shown, for example, that the injection of antibodies in mice induces a condition mimicking early-stage coeliac disease (Kalliokoski et al. 2015; Kalliokoski et al. 2017).

TG2-targeting antibodies have also been thought to contribute to the development of extraintestinal manifestations, in particular those associated with the deposition of IgA antibodies as described above (Korponay-Szabo et al. 2004; Hadjivassiliou et al. 2006). The injection of mice with TG2-targeting antibodies has led to the development of gluten ataxia-like symptoms (Boscolo et al. 2010). Furthermore, a role for TG2 autoantibodies in reproductive disorders associated with coeliac disease has been suggested (Anjum et al. 2009; Di Simone et al. 2010; Simon-Vecsei et al. 2012; Sóñora et al. 2014). In addition, as antibodies targeting TG6 are frequently found in connection with neurological manifestations of coeliac disease (Hadjivassiliou et al. 2006, 2008 and 2013), and the presence of TG3 antibodies is a characteristic feature of dermatitis herpetiformis (Sardy et al. 2002), it has been proposed that the different autoantibody responses could have a role in the development of these specific extraintestinal manifestations of coeliac disease.

2 DERMATITIS HERPETIFORMIS: COELIAC DISEASE OF THE SKIN

2.1 Overview of dermatitis herpetiformis

The most common extraintestinal manifestation of coeliac disease is the cutaneous manifestation, dermatitis herpetiformis, first described by Louis Duhring in 1884 (Duhring 1983). The association with coeliac disease was recognised when it was shown that, in addition to skin symptoms, patients with dermatitis herpetiformis also present with coeliac-type small bowel mucosal changes (Marks et al. 1966; Fry et al. 1973) and that both the skin and small-intestinal symptoms resolve upon commencement of a gluten-free diet (Fry et al. 1973; Reunala et al. 1977). Currently, dermatitis herpetiformis affects approximately one in eight coeliac disease patients in Finland (Salmi et al. 2011). Both conditions share the same genetic HLA-DQ background (Katz et al. 1972; Spurkland et al. 1997), they occur frequently in the same families (Reunala 1996; Hervonen et al. 2002), and the different phenotypes can even be encountered in identical twins (Bardella et al. 2000; Hervonen et al. 2000). Despite the similarities, differences between the two manifestations also exist: in contrast to coeliac disease, the incidence of dermatitis herpetiformis has been shown to be decreasing (Salmi et al. 2011; West et al. 2014). Furthermore, while coeliac disease is found in all age groups with a female predominance (Singh et al. 2018), dermatitis herpetiformis is rarely diagnosed in childhood (Hervonen et al. 2014) and is slightly more common in males (Salmi et al. 2011; West et al. 2014). A comparison of the features of dermatitis herpetiformis and coeliac disease is presented in Table 1.

Table 1. Comparison of the features of dermatitis herpetiformis and coeliac disease. Table modified from Reunala et al. (2015a).

	Dermatitis herpetiformis	Coeliac disease	References
Gender predominance	Male	Female	Salmi et al. 2011; West et al. 2014; Singh et al. 2018
Age	Mainly adults	Children and adults	Hervonen et al. 2014
HLA-DQ2 or -DQ8	95–100%	95–100%	Katz et al. 1972; Spurkland et al. 1997
Prevalence in Finland	0.08%	2%	Lohi et al. 2007; Salmi et al. 2011
Incidence	Decreasing	Increasing	Lohi et al. 2007; Rubio-Tapia et al. 2009; Salmi et al. 2011; Kang et al. 2013; West et al. 2014
Dominant autoantigen	TG3	TG2	Dieterich et al. 1997; Sardy et al. 2002
Small-intestinal mucosal villous damage present	75%	100%	Gawkrodger et al. 1984; Reunala et al. 1984; Mansikka et al. 2017 and 2018
Small-intestinal TG2-IgA deposits present	79%	100%	Koskinen et al. 2010; Salmi et al. 2011
Cutaneous IgA deposits present	100%	0%*	Zone et al. 1996
Serum EmA antibodies	89%	95%	Reunala et al. 2015b
Serum TG2 antibodies	70–86%	92%	Dieterich et al. 1999; Reunala et al. 2015b
Serum TG3 antibodies	86%	24–28%	Reunala et al. 2015b; Salmi et al. 2016
Treatment	GFD and dapsone medication	GFD	Reunala et al. 2015a

HLA, human leucocyte antigen; TG, transglutaminase; IgA, immunoglobulin A; EmA, endomysial antibodies; GFD, gluten-free diet

* Presence of cutaneous IgA deposits in the healthy skin of coeliac disease patients has been reported in two studies by Cannistraci et al. (2007) and Bonciolini et al. (2019), but not verified on a larger scale.

2.2 Characteristics of dermatitis herpetiformis

2.2.1 Clinical findings and diagnosis

Dermatitis herpetiformis is characterised by an itchy, blistering rash particularly on the elbows, knees, and buttocks. A pathognomonic feature is the deposition of granular IgA in the dermis along the dermo-epidermal junction, the papillary dermis (Van der Meer et al. 1969). The basis for the diagnosis of dermatitis herpetiformis is the detection of these IgA deposits in a skin biopsy next to the active lesion by direct immunofluorescence staining together with the typical clinical picture (Zone et al. 1996). Interestingly, despite being a diagnostic feature of dermatitis herpetiformis, two studies have reported the presence of cutaneous IgA deposits also in the healthy skin of coeliac disease patients (Table 1) (Cannistraci et al. 2007; Bonciolini et al. 2019).

Besides the skin symptoms, patients with dermatitis herpetiformis may present with gastrointestinal symptoms, but they are often mild and the majority of patients are asymptomatic and show no signs of malabsorption (Pasternack et al. 2017). Parallel to coeliac disease, dermatitis herpetiformis is associated with complications such as an increased risk for developing non-Hodgkin's lymphoma (Leonard et al. 1983a; Hervonen et al. 2005; Grainge et al. 2012). Notably, the mortality rate in dermatitis herpetiformis is not increased as seen in coeliac disease (Hervonen et al. 2012).

2.2.2 Serum antibodies and small-bowel mucosa

The majority of untreated dermatitis herpetiformis patients have IgA class antibodies targeting the epidermal transglutaminase, TG3, in the serum (Table 1) (Sardy et al. 2002; Marietta et al. 2008; Hull et al. 2008; Rose et al. 2009; Reunala et al. 2015b). In addition, approximately 70–90% of patients present with serum EmA and TG2-targeting autoantibodies (Dieterich et al. 1999; Reunala et al. 2015b) and also antibodies against deamidated gliadin peptides (Reunala et al. 2015b). In contrast to coeliac disease, however, detection of neither serum TG3- nor TG2-targeting autoantibodies is used in the diagnostics of dermatitis herpetiformis, even though TG2 antibodies can be considered supportive of the diagnosis.

Approximately 75% of dermatitis herpetiformis patients have small-intestinal villous atrophy and crypt hyperplasia (Gawkrodger et al. 1984; Reunala et al. 1984; Mansikka et al. 2017 and 2018) and the rest show at least inflammatory changes characteristic of coeliac disease, such as an increased density of γδ+ IELs (Table 1) (Järvinen et al. 2003; Savilahti et al. 1992; Salmi et al. 2014). In addition, similar to coeliac disease, TG2-targeting IgA deposits can be detected in the small-bowel mucosa of dermatitis herpetiformis patients, although with less frequency (Kárpáti et al. 1988; Korponay-Szabo et al. 2004; Salmi et al. 2014).

2.2.3 The autoantigen TG3

While TG2 is the main autoantigen target in the small-bowel mucosa in both coeliac disease and dermatitis herpetiformis, the target of the cutaneous IgA deposits in dermatitis herpetiformis has been shown to be TG3 (Sardy et al. 2002). TG3 belongs to the same transglutaminase family as TG2: it shares a similar structure, particularly in the catalytically active site, and the ability to deamidate and form thioester-linked complexes with gluten-derived peptides, albeit with less efficiency than TG2 (Stamnaes et al. 2010). In contrast to the ubiquitously expressed TG2, the tissue distribution of TG3 is not as wide, and it has thus far been found in the kidneys, lungs, and skin (Sardy et al. 2002). In normal skin, TG3 is expressed in the upper layers of the epidermis where it contributes to epidermal keratinisation and to the formation of the cornified envelope (Eckert et al. 2005). In dermatitis herpetiformis, however, TG3 is also located in the papillary dermis (Sardy et al. 2002; Donaldson et al. 2007). Whether TG3 in this unusual location originates from epidermal keratinocytes by diffusing through the basement membrane in regions of trauma (Donaldson et al. 2007; Zone et al. 2011) or aggregates in the papillary dermis as complexes with IgA from the circulation (Sardy et al. 2002; Preisz et al. 2005; Reunala et al. 2015a; Görög et al. 2016) remains unclear.

2.3 Treatment of dermatitis herpetiformis

Similarly to coeliac disease, dermatitis herpetiformis is treated with a strict, life-long gluten-free diet (Reunala et al. 2015a). During the diet, both the skin and small-bowel mucosa heal and the circulating TG2- and TG3-targeting autoantibodies disappear (Reunala et al. 2015a). By contrast, the rash has been shown to persist longer after

commencement of a gluten-free diet (Fry et al. 1973; Reunala et al. 1977), and treatment with dapsone (4,4-diaminodiphenylsulfone) is commonly used initially, generally for a period of two years (Fry et al. 1973; Garioch et al. 1994). The cutaneous IgA deposits have likewise been reported to disappear slowly during the adoption of the diet (Reunala 1978; Fry et al. 1978; Frödin et al. 1981; Fry et al. 1982; Leonard et al. 1983b; Garioch et al. 1994; Hardman et al. 1997; Reunala et al. 1998; Bardella et al. 2003). Whether the disappearance of their target in the papillary dermis, TG3, parallels that of the IgA deposits is not known. As in coeliac disease, it has been shown that in rare cases patients with dermatitis herpetiformis are non-responsive to a strict dietary treatment. In these patients, the small-bowel mucosa recovers, but dapsone medication is needed to control the persistent rash (Garioch et al. 1994; Hervonen et al. 2016).

2.4 Pathogenesis of dermatitis herpetiformis

The pathogenetic mechanisms leading to the development of dermatitis herpetiformis, as well as the reason why it occurs only in a subset of coeliac disease patients, are obscure. Currently, dermatitis herpetiformis is thought to arise from the small intestine as a result of hidden, undiagnosed, or neglected coeliac disease which, upon long-lasting gluten exposure, develops into a cutaneous manifestation (Sardy et al. 2002; Salmi et el. 2016; Mansikka et al. 2019). This hypothesis has been supported by several findings. Firstly, studies have shown that coeliac disease can evolve into dermatitis herpetiformis in patients during a normal gluten-containing diet (Kurppa et al. 2008; Salmi et al. 2015). Secondly, it has been shown that dermatitis herpetiformis affects mainly adults (Salmi et al. 2011). Thirdly, TG3 antibodies characteristic of dermatitis herpetiformis are rarely found in children with coeliac disease (Hull et al. 2008; Jaskowski et al. 2009; Salmi et al. 2016). Finally, the decreasing incidence of dermatitis herpetiformis has been explained by the increased diagnosis and treatment of coeliac disease (Salmi et al. 2011).

The existence of TG3 antibodies may also result from the initial antibody response towards TG2 in coeliac disease (Sardy et al. 2002). TG3 antibodies can be found in both dermatitis herpetiformis and coeliac disease patients, but those in dermatitis herpetiformis patients are present at a higher frequency and have also been reported to have higher avidity and specificity towards TG3 (Sardy et al. 2002). Thus, it has been suggested that a prolonged gluten consumption could result in epitope spreading due to the structural homology between the two TG isoforms,

further maturation of the antibodies, and, eventually, the production of high-affinity antibodies towards TG3 (Sardy et al. 2002; Reunala et al. 2015a; Salmi et al. 2016). Furthermore, it has been speculated that those coeliac disease patients presenting with elevated levels of TG3 antibodies could be those who develop dermatitis herpetiformis over time (Sardy et al. 2002; Hull et al. 2008). On the other hand, as both TG3 and TG6 have been shown to be capable of deamidating gluten-derived peptides and forming covalent complexes with them (Stamnaes et al. 2010), it has also been suggested that the corresponding antibodies could arise independently from those targeting TG2 (Hadjivassiliou et al. 2008; Stamnaes et al. 2010; De Leo et al. 2018).

The deposition of IgA antibodies in the skin is thought to lead to an immunological cascade with T cells, neutrophils, and macrophages accumulating in the papillary dermis. As a result, various cytokines, chemokines, and proteases are produced, and the formation of basement membrane zone vesicles eventually occurs (Reitamo et al. 1981; Graeber et al. 1993; Airola et al. 1995; Smith et al. 2002). Whether the IgA deposits participate directly in blister formation is not clear, as they can be found in sites away from the skin lesion (Donaldson et al. 2007), and two patients have been reported to develop a rash without the appearance of IgA deposits during a gluten challenge study (Mansikka et al. 2019). On the other hand, it has been shown that the IgA-bound TG3 in the skin of dermatitis herpetiformis patients is enzymatically active and capable of binding fibrinogen, a protein with proinflammatory properties and the capability of attracting inflammatory proteins (Taylor et al. 2015). Thus, it has been suggested that this activity could relate to the development of the skin lesion (Reunala et al. 2015a; Taylor et al. 2018).

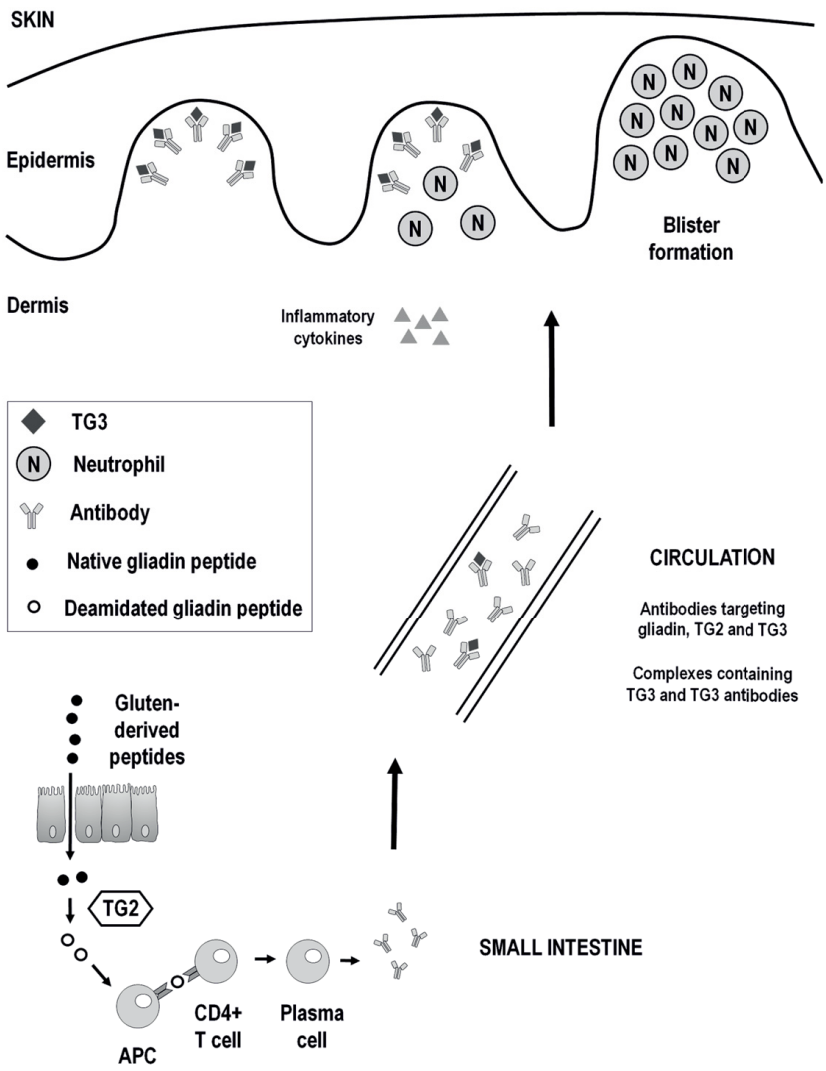

Figure 3. Simplified illustration of the pathogenesis of dermatitis herpetiformis. According to the current hypothesis, dermatitis herpetiformis arises from the small intestine as a consequence of untreated coeliac disease. Continuous gluten exposure results in the production of high affinity TG3 antibodies, which can be found in the circulation as such or as complexes with TG3, in parallel with antibodies targeting TG2 and gluten-derived gliadin peptides. In the skin, TG3 antibodies are deposited in the dermo-epidermal junction, the papillary dermis. Subsequently, inflammatory cells, predominantly neutrophils, are accumulated in the skin and various cytokines and proteases are produced. Eventually, the formation of subepidermal blisters and the typical dermatitis herpetiformis rash occurs. APC, antigen presenting cell, N, neutrophil, TG, transglutaminase. Figure adapted and modified from Reunala et al. (2015a).

THE PRESENT STUDY

3 AIMS OF THE STUDY

The aim of this study was to characterise the IgA autoantibody responses towards TG2 and TG3 in coeliac disease and dermatitis herpetiformis.

The specific aims were:

1. To investigate the serum and small-bowel mucosal TG2 and TG3 antibody responses in untreated and treated coeliac disease patients with various phenotypes (**I–III**).

2. To study the TG2 and TG3 antibody responses in coeliac disease and dermatitis herpetiformis exploiting the organ culture method of patient-derived small-bowel mucosal biopsies (**II**).

3. To study the TG2 and TG3 antibody responses in dermatitis herpetiformis patients undergoing a gluten challenge (**III**).

4. To investigate the disappearance of cutaneous IgA and TG3 from dermatitis herpetiformis patients after a long-term gluten-free diet (**IV**).

4 MATERIALS AND METHODS

4.1 Patients (**I–IV**)

4.1.1 Coeliac disease patients (**I–III**)

Altogether 90 coeliac disease patients undergoing upper gastrointestinal endoscopy at the Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, were included in Studies **I–III**. These patients comprised all coeliac disease phenotypes except dermatitis herpetiformis.

In Study **I**, the coeliac disease cohort consisted of 46 patients. Of them, 15 were clinically suspected of having coeliac disease but had a normal small-intestinal mucosal morphology (Vh/CrD $\geq$ 2) (defined as early-stage coeliac disease). These 15 patients were prospectively followed up for one year on a gluten-containing diet, during which their mucosal morphology deteriorated and the levels of serum EmA and/or TG2 antibodies increased (defined as overt coeliac disease). Thereafter, the patients started a gluten-free diet and after one year, their mucosal morphology had improved and the levels of serum autoantibodies decreased in all patients. Of the prospectively followed-up patients, serum and small-bowel mucosal samples were available from 14 of the patients with early-stage coeliac disease, all 15 patients with the overt disease, and 11 of the patients after one year on the gluten-free diet. In addition, 31 long-term treated coeliac disease patients were included in the study (median duration of gluten-free diet 9 years, range 3–34). Of them, 15 were in clinical, serological (EmA and TG2 antibodies), and small-bowel mucosal remission, 11 patients had non-responsive coeliac disease with persistent villous atrophy despite a strict gluten-free diet, and the remaining five showed poor adherence to the diet. Serum samples and small-intestinal mucosal biopsies were available from all the long-term treated patients.

The coeliac disease cohort in Study **II** comprised 20 patients. Ten of the patients were untreated and recruited at the time of diagnosis, and the remaining ten had adhered to a gluten-free diet for one year and were in clinical remission. Serum and

small-bowel mucosal biopsies were available from all the patients. Moreover, two additional small-bowel mucosal biopsies were taken for organ culture studies.

The coeliac disease cohort in Study **III** comprised 24 patients. Eighteen of the patients were on a gluten-containing diet. Nine of these 18 patients and six additional patients were also investigated after adhering to a gluten-free diet for one year and achieving clinical remission. Serum and small-bowel mucosal biopsies were available from all the patients.

4.1.2 Dermatitis herpetiformis patients (**II–IV**)

Altogether 69 patients with dermatitis herpetiformis were included in Studies **II–IV**. All the patients had been diagnosed at the Department of Dermatology, Tampere University Hospital, based on the typical clinical picture and the demonstration of granular IgA deposits in the papillary dermis in cutaneous biopsy samples by direct immunofluorescence staining.

The dermatitis herpetiformis cohort in Study **II** consisted of 17 patients. Five of the patients had the active disease in terms of cutaneous IgA deposits and a rash, and they were on dapsone medication and following either a strict (n=3) or partial (n=2) gluten-free diet (median duration 25 years, range 4–40). The remaining twelve patients were in clinical remission and following a strict gluten-free diet (median duration 25 years, range 8–38). Serum and small-bowel mucosal samples were available from all the patients. For organ culture studies, two additional small-bowel mucosal biopsies were obtained.

The dermatitis herpetiformis cohort in Study **III** consisted of 19 patients undergoing a gluten challenge. The inclusion criteria of the challenge were a confirmed diagnosis of dermatitis herpetiformis, adherence to a gluten-free diet for at least five years, and the absence of skin symptoms for at least three years (Mansikka et al. 2019). At pre-challenge, the patients had been on a gluten-free diet for a median of 22 years (range 5–40) and were in clinical, serological (EmA and TG2 antibodies), and small-bowel mucosal remission. At the beginning of the challenge, all patients received 200 g of commercially available wheat bread for a three-day period, followed by a normal gluten-containing diet with a minimum of 10 g of wheat per day. Post-challenge examinations were performed at the time of the appearance of a rash or serological relapse (positive EmA or TG2 antibodies), or after 12 months of gluten challenge. The median duration of the challenge was 3.5 months (range 1–12 months). At pre- and post-challenge, serum EmA, TG2, and

TG3 antibodies were measured and cutaneous and small-bowel mucosal biopsies were taken.

The dermatitis herpetiformis cohort in Study **IV** consisted of 33 patients from whom cutaneous and small-bowel mucosal biopsies and serum samples were available. Seven of the patients had the untreated disease and presented with an active rash. Twenty-one patients were following a strict gluten-free diet (mean duration 22 years, range 5–38), were in clinical remission, and had no rash or dapsone medication. The remaining five patients were following either a strict (n=3) or partial (n=2) gluten-free diet (mean duration 28 years, range 9–40); they presented with an active rash and used dapsone (12.5–100 mg/day).

4.1.3 Non-coeliac control subjects (**I, III**)

Altogether 30 subjects, all excluded for coeliac disease based on negative serology for EmA and TG2 antibodies and a normal small-intestinal mucosal morphology, were used as controls in Studies **I** and **III**. Of these 30 subjects, 16 had self-reported gluten sensitivity and experienced abdominal symptoms after the consumption of gluten-containing products, and 14 had dyspepsia.

4.2 Small-bowel mucosal morphology (**I–IV**)

In Studies **I–III**, the small-bowel mucosal morphology was determined based on Vh/CrD on haematoxylin-eosin stained sections cut from well-oriented paraffin-embedded biopsies as described earlier (Taavela et al. 2013). A ratio of ≥2 was considered normal (Kuitunen et al. 1982). In Study **IV**, the small-bowel mucosal findings were graded as normal villous architecture, partial villous atrophy (PVA), or subtotal villous atrophy (SVA).

4.3 Serology (**I–IV**)

IgA-class EmA in patient serum was determined by an indirect immunofluorescence method using human umbilical cord as the substrate (Sulkanen et al. 1998b). A serum dilution of 1≥5 was considered positive.

IgA-class TG2 and TG3 antibody levels in patient serum were determined by commercial enzyme-linked immunosorbent assays (ELISA) according to the

manufacturer's instructions in samples diluted 1:100 (Celikey®, Phadia, Freiburg, Germany and anti-heTG IgA ELISA, Immunodiagnostik, Bensheim, Germany, respectively). The optimal cut-off value for serum sample positivity was ≥5 U/ml for TG2 and >22 AU/ml for TG3 antibodies, as suggested by the manufacturer.

4.4 HLA genotype (**I–III**)

Determination of the coeliac disease-associated HLA-DQ2 and HLA-DQ8 genotypes was performed using a DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland), SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden), or HLA-tagging single-nucleotide peptides (Koskinen et al. 2009).

4.5 Organ culture (**II**)

Two small-bowel mucosal biopsies from each patient were obtained for organ culture studies in Study **II**. Biopsies were cultured in RPMI-1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) supplemented with 15% foetal bovine serum (Invitrogen-Gibco), 100 U/ml penicillin (Invitrogen-Gibco), 4 mM L-glutamine (Invitrogen-Gibco), 50 ug/ml insulin (Sigma Aldrich Co, St. Louis, Missouri, USA), 2 mg/ml glucose (Sigma Aldrich), and 10 mM HEPES buffer (Invitrogen-Gibco) for 24 h at 37°C. One biopsy was cultured in the presence of medium only and one was subjected to peptic-tryptic (PT) digest of gliadin (1 mg/ml). After culture, the culture medium was collected and stored at -20ºC until analysed. EmA, TG2, and TG3 antibody levels were determined in undiluted culture medium samples as described above.

4.6 Immunofluorescence stainings (**I–IV**)

Immunofluorescence stainings were performed on sections cut from frozen small-bowel mucosal and skin biopsies embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek Europe, Holland). Before staining, the sections were air-dried for 20 min at room temperature and washed in phosphate-buffered saline (PBS). All incubations were carried out at room

temperature unless otherwise indicated. The antibodies used in the stainings are presented in Table 2.

4.6.1 Small-intestinal IgA deposits (**I–III**)

Small-intestinal TG2-targeting IgA deposits in Studies **I–III** were detected using an established staining method (Korponay-Szabo et al. 2004; Salmi et al. 2006a): TG2 was detected using mouse monoclonal anti-TG2 antibody (CUB7402; Neomarkers, Fremont, California, USA) and IgA using fluorescein-isothiocyanate (FITC)-labelled rabbit anti-human IgA antibody (Dako A/S, Glostrup, Denmark) (Table 2). In coeliac disease patients, IgA is known to deposit on extracellular TG2 under the small-intestinal epithelium and around mucosal blood vessels, whereas in non-coeliac subjects, it is localised only inside plasma cells and epithelial cells (Korponay-Szabo et al. 2004; Salmi et al. 2006a). The IgA deposits were graded as negative, weak, moderate, or strong based on their intensity along the basement membrane in the villous-crypt area and around mucosal vessels (Salmi et al. 2006a).

The possible presence of small-intestinal IgA targeting TG3 was investigated by double staining IgA and TG3 in Study **II**. TG3 was detected using rabbit polyclonal anti-TG3 antibody (1:100; A015, Zedira, Darmstadt, Germany) for 1 h, followed by Alexa Fluor 568-conjugated goat anti-rabbit antibody (1:2000; Invitrogen) for 1 h (Table 2). Co-localisation of TG3 with IgA was evaluated using FITC-labelled rabbit anti-human IgA antibody (1:40; Dako A/S) for 15 min.

4.6.2 Small-intestinal TG2 and TG3 antibody-secreting cells (**I–III**)

The detection of small-intestinal TG2 and TG3 antibody-secreting cells was based on a previously established method using soluble biotinylated TG2 and the plasma cell marker CD138 for the visualisation of TG2-specific plasma cells (Di Niro et al. 2012 and 2016). In the present study, the method was also applied to TG3 antibodies, and either plasma cells (**I**, **III**) or IgA-positive cells (**II**) were considered antibody secreting cells. TG2 and TG3 antibodies were detected using biotinylated human recombinant TG2 (2 µg/ml; T002, Zedira) or TG3 (5 µg/ml; T024, Zedira), respectively, followed by tetramethylrhodamine-isothiocyanate (TRITC)-labelled streptavidin (1:1000; KPL, Gaithersburg, MD, USA) in BSA-PBS for 30 min.

Plasma cells were identified using a mouse monoclonal CD138 antibody (1:25; B-A38, Bio-Rad), followed by goat anti-mouse IgG Alexa Fluor 488 (1:2000; A-

11001, Thermo-Fisher Scientific). IgA-positive cells were detected using FITC-conjugated anti-human IgA antibody (1:40; Dako A/S) for 15 min.

The stainings were analysed at 20x or 40x magnification (Olympus BX60F5, Olympus Optical co. LTD, Japan) and the percentage of TG2 and TG3 antibody-secreting cells out of all lamina propria plasma cells was determined on two consecutive small-intestinal biopsy sections.

4.6.3 Cutaneous IgA and TG3 deposits (**IV**)

Cutaneous IgA and TG3 deposits were detected using TRITC-conjugated goat anti-human IgA (1:50) (A18786, Life Technologies, Frederick, Maryland, USA) for 1 h and FITC-conjugated rabbit polyclonal TG3 antibody (1:100) (A030, Zedira, Darmstadt, Germany) overnight at 4 °C, respectively. Alternatively, TG3 was detected using a goat polyclonal TG3 antibody (1:25) (Zone et al. 2011) for 45 min, followed by Alexa Fluor 488-conjugated AffiniPure $F(ab')_2$ Fragment donkey anti-goat antibody (1:500) (705-546-147, Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. The presence of IgA and TG3 was evaluated separately using single stainings and their co-localisation using double stainings.

Stainings were analysed at 20x magnification (Olympus BX60F5, Olympus Optical Co. LTD, Japan) and graded according to staining intensity as negative, weak (+), moderate (++), or strong (+++).

4.7 Ethical considerations (**I–IV**)

The study protocols were approved by the Ethics Committee of Tampere University Hospital (permission numbers R12267, R17042, R03041, R04097, R04105, R00204, E95025), and written informed consent was obtained from all participating subjects.

4.8 Statistics (**I–IV**)

Data are expressed as the number of subjects (n) and percentages, and as means or medians and ranges. Statistical analyses were performed using the non-parametric Wilcoxon test, Mann–Whitney test, and Fisher's test, as appropriate. Spearman's correlation test was used to analyse correlations. Statistical testing was performed

using statistical analysis software (IBM SPSS Statistics, SPSS Inc., IBM Corp., Armonk, NY, USA). A p-value <0.05 was considered statistically significant.

Table 2. Summary of the reagents used in immunofluorescence stainings in Studies **I–IV**.

Staining	Reagent	Primary antibody	Clone and manufacturer	Dilution or concentration	Study
Small-intestinal IgA deposits targeting TG2 or TG3 (Chapter 4.6.1)	anti-human IgA	FITC-conjugated	Dako A/S, Glostrup, Denmark	1:40	**I–III**
	anti-TG2 antibody	mouse monoclonal	Clone CUB7402, Neomarkers	1:200	
	anti-TG3 antibody	rabbit polyclonal	A015, Zedira, Darmstadt, Germany	1:100	
Small-intestinal TG2 and TG3 antibody-secreting cells (Chapter 4.6.2)	recombinant TG2*		T002, Zedira, Darmstadt, Germany	2 ug/ml	**I–III**
	recombinant TG3*		T024, Zedira, Darmstadt, Germany	5 ug/ml	
	anti-human IgA	FITC-conjugated	Dako A/S, Glostrup, Denmark	1:40	
	anti-CD138 antibody	mouse monoclonal	Clone B-A38, Bio-Rad Antibodies, Oxford, UK	1:25	
Cutaneous IgA and TG3 deposits (Chapter 4.6.3)	anti-human IgA	TRITC-conjugated	A18786, Life Technologies, Frederick, Maryland, USA	1:50	**IV**
	anti-TG3 antibody	FITC-conjugated	A030, Zedira, Darmstadt, Germany	1:100	
		goat polyclonal	Zone et al. 2011	1:25	

* recombinant TG2 and TG3 were used for the detection of the corresponding antibodies in the tissue

5 RESULTS

5.1 TG2 antibody response in coeliac disease (**I, III**)

In order to elucidate the TG2 antibody responses in the serum and small-bowel mucosa, samples from altogether 70 coeliac disease patients at different disease stages were analysed and compared to those from 30 non-coeliac control subjects in Studies **I** and **III** (Table 3).

In patients with early-stage coeliac disease and a normal small-bowel mucosal morphology (median Vh/CrD 2.8, range 2.2–3.6), serum EmA levels were elevated in 93% of the patients, the median titre being 1:50 (Table 3). Serum TG2 antibody levels, in turn, were elevated in 64% of the patients (median 8.4 U/ml). In patients with overt coeliac disease and a deteriorated mucosal morphology (median Vh/CrD 0.6, range 0.04–2.2), the serum EmA and TG2 antibody levels were both elevated in 87% of the patients, the median values being 1:200 and 41.7 U/ml, respectively. After one year of a gluten-free diet and the improvement of the mucosal morphology (median Vh/CrD 2.7, range 0.8–4.5), the serum EmA and serum TG2 antibody levels had normalised in 72% (median 0) and 78% (median 2.1 U/ml) of the patients, respectively. In long-term dietary-treated patients, the median Vh/CrD was 1.5 (range 0.1–4.2). It is noteworthy within this group that the recovery of the small-bowel mucosa was evident only in all the patients in clinical remission but in none of the non-responsive patients or patients with dietary lapses. Furthermore, within the long-term treated patients, the normalisation of serum EmA and the serum TG2 antibodies was seen in 84% (median 0) and 71% (median 0.9 U/ml) of the patients, respectively. The normalisation of serum antibody levels was most evident in patients in clinical remission and those with non-responsive coeliac disease. No serum autoantibodies were detected in any of the non-coeliac control subjects with a normal mucosal morphology (median Vh/CrD 3.4, range 2.3–4.6).

Small-intestinal TG2-targeting IgA deposits were present in the majority of patients with early stage (100%) and overt (97%) coeliac disease, but they were found with less frequency in patients after a one-year adherence to a gluten-free diet (71%) (Table 3). In long-term treated patients, IgA deposits were present in 66% of the patients, being detectable mostly in non-responsive patients and those with dietary

lapses. With the exception of three (13%) patients with gluten sensitivity, all non-coeliac control subjects were negative for mucosal IgA deposits.

Small-intestinal TG2 antibody-secreting cells were present in 12 (80%) out of the 15 patients with early-stage coeliac disease, the median cell percentage out of all lamina propria plasma cells being 2.3% (range 0–12.7%) (Table 3; Figure 4). Upon the development of the overt disease on a gluten-containing diet, the percentage of the cells increased to a median of 4.5% (range 0–20.0%) (Figure 3); at this point, the cells were present in 29 (97%) out of the 30 patients with available samples. During a one-year strict gluten-free diet, the percentage of the cells declined to a median of 0.9% (range 0–4.6%), the cells being found in 20 (77%) out of the 26 patients. In long-term treated patients, the cells were mostly absent (median percentage 0%, range 0–7%), and found in seven (23%) out of the 31 patients: of them, three were patients in clinical remission, one had non-responding coeliac disease, and three presented with dietary lapses (Figure 3). With the exception of one patient with dyspepsia, no TG2 antibody-secreting cells were found in non-coeliac control subjects.

When comparing the percentage of TG2 antibody-secreting cells in all coeliac disease patients regardless of disease activity with other disease parameters, a positive correlation was found with serum EmA (R_s=0.640, *p*-value<0.001) and TG2 antibody levels (R_s=0.653, *p*-value<0.001) and the intensity of small-intestinal TG2-targeting IgA deposits (R_s=0.419, *p*-value<0.001), and a negative correlation was found for small-intestinal morphology (R_s=-0.307, *p*-value=0.002) (Table 4).

Table 3. Serum and small-bowel mucosal findings of coeliac disease patients and non-coeliac control subjects. Statistically significant ($p<0.05$) p-values as compared to overt coeliac disease or non-coeliac controls are shown, as appropriate.

	Early stage CD n=15	Overt CD n=37	1-year GFD n=34	Long-term GFD n=31	Non-CD controls n=30
Vh/CrD					
<2.0, n (%)	0/15 (0)	34/37 (92)	6/29 (21)	16/31 (52)	0/30 (0)
median (range)	2.8 (2.2–3.6) *†	0.6 (0.04–2.2) †	2.7 (0.8–4.5) *†	1.5 (0.1–4.2) †	3.4 (2.3–4.6) *
EmA					
positive cases, n (%)	14/15 (93)	32/37 (87)	8/29 (26)	5/31 (16)	0/30 (0)
median (range), titre	1:50 (0–1:2000) †	1:200 (0–1:4000) †	0 (0–1:200) *†	0 (0–1:2000) *†	0 (0–0) *
Serum TG2 antibodies					
positive cases, n (%)	7/11 (64)	31/36 (86)	7/32 (22)	5/30 (17)	0/30 (0)
median (range), U/ml	8.4 (3.3–101) †	41.7 (2.9–101) †	2.1 (0–26.4) *†	0.9 (0–101) *	0.85 (0–3.9) *
Mucosal TG2-IgA deposits					
positive cases, n (%)	15/15 (100)	29/30 (97)	15/21 (71)	19/29 (66)	3/23 (13)
median (range), intensity	2 (0.5–2) *†	2.75 (0–3)†	1 (0–3) *†	1 (0–3) *†	0 (0–0.5) *
Mucosal TG2 antibody-secreting cells					
positive cases, n (%)	12/15 (80)	29/30 (97)	20/26 (77)	7/31 (23)	1/30 (3)
median (range), % ^	2.3 (0–12.7) *†	4.5 (0–20) †	0.9 (0–4.6) *†	0 (0–7) *†	0 (0–0.4) *

* $p<0.05$ compared to overt coeliac disease group

† $p<0.05$ compared to non-coeliac control group

^ out of all lamina propria plasma cells

CD, coeliac disease; EmA, endomysial antibodies; GFD, gluten-free diet; IgA, immunoglobulin A; TG2, transglutaminase 2; Vh/CrD, villous height:crypt depth ratio

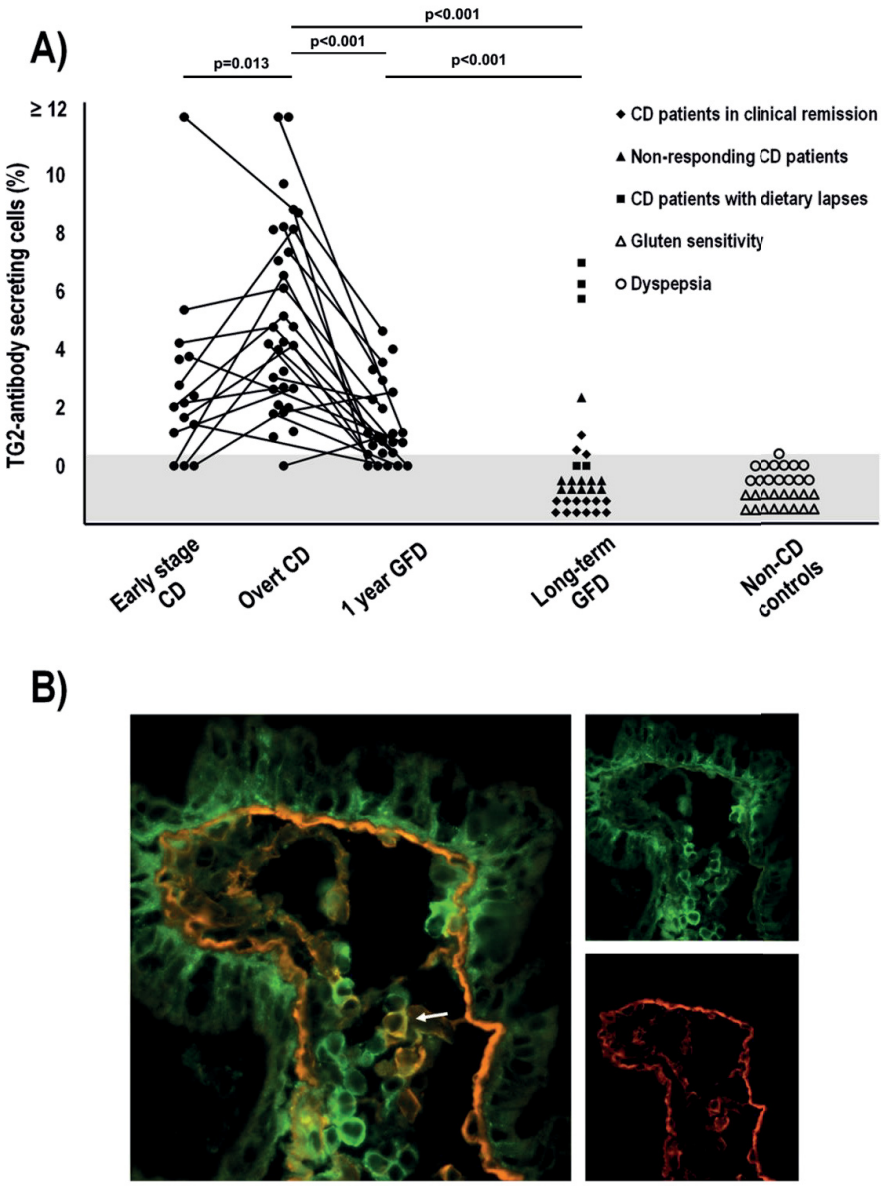

Figure 4. A) The percentage of transglutaminase (TG) 2-antibody secreting cells in coeliac disease patients at different disease stages and in non-coeliac control subjects. The grey area indicates negative values. Statistically significant ($p<0.05$) p-values are indicated. All groups differed statistically significantly from the non-coeliac controls ($p<0.05$). B) A representative immunofluorescence picture of TG2 antibody-secreting cells in an early-stage coeliac patient. Recombinant TG2 is shown in red, plasma cell marker CD138 in green, and their colocalisation in yellow (arrow). CD, coeliac disease; GFD, gluten-free diet

Table 4. Correlations between the percentage of TG2 antibody-secreting cells out of all lamina propria plasma cells and the levels of serum EmA and TG2 antibodies, the intensity of small-intestinal TG2-targeting IgA deposits and villous height:crypt depth ratio (Vh/CrD) in the entire coeliac disease (CD) patient cohort as well as in untreated coeliac disease patients. R_s, Spearman's correlation coefficient

	Serum EmA		Serum TG2 antibodies		Mucosal TG2-IgA deposits		Vh/CrD	
	R_s	*p*-value	R_s	*p*-value	R_s	*p*-value	R_s	*p*-value
All CD patients	0.640	>0.001	0.653	>0.001	0.419	>0.001	-0.307	0.002
Untreated CD patients	0.210	0.257	0.133	0.483	-0.109	0.612	0.04	0.83

5.2 Organ culture of small-bowel mucosal biopsies (II)

In order to further elaborate the serum and small-intestinal TG2 antibody responses in coeliac disease and to extend the findings to dermatitis herpetiformis, 20 coeliac disease patients and 17 dermatitis herpetiformis patients were investigated in Study **II**. In addition to studying the TG2 antibody response, antibody response towards TG3 was also investigated. Furthermore, in both coeliac disease and dermatitis herpetiformis patients, an organ culture of the small-bowel mucosal biopsies was used for evaluating autoantibody secretion at the small-intestinal level.

In coeliac disease, serum EmA and TG2 antibody levels were positive in nine (90%) and all 10 (100%) of the patients with the active disease, respectively (median values 1:200 and 16.5 U/ml) (Table 5; Figure 1 in original publication **II**). By contrast, such antibodies were found in three (30%) and none (0%) of the 10 patients in remission, respectively (median values 0 and 0.5 U/ml). In the organ culture of small-bowel mucosal biopsies in the presence of medium only, EmA secretion was observed in eight (80%) of the patients with the active disease and in two (20%) of those in remission (Table 5). Similarly, TG2 antibody secretion above 20 U/ml was observed in seven (70%) of the patients with the active disease (median 74.8 U/ml) whereas only low secretion or none at all (<20 U/ml) was detected in patients in remission (median 4.6 U/ml). Treatment of the biopsies with PT-gliadin had no effect on the secretion of either EmA or TG2 antibodies. When investigating the presence of TG2 antibody-secreting cells in the small-bowel mucosal biopsies, such cells were found in eight (80%) of the patients with the active disease and in four (40%) of those in remission (Table 5).

In contrast to the coeliac disease patients, all the dermatitis herpetiformis patients regardless of disease activity were negative for serum EmA and TG2 antibodies

(Table 5). Similarly, in the organ culture of small-intestinal biopsies, EmA secretion was observed in only one (20%) of the patients with the active disease and none of those in remission. TG2 antibody secretion was below <20 AU/ml in all patients with the active disease and those in remission. Furthermore, small-intestinal TG2 antibody-secreting cells were detected in only one (8%) of the patients in remission.

As TG3 is the dominant autoantigen of dermatitis herpetiformis (Sardy et al. 2002), and circulating TG3 antibodies can also be detected in a subset of coeliac disease patients (Sardy et al. 2002, Marietta et al. 2008, Salmi et al. 2016), TG3 antibody responses in the serum and small-intestinal biopsies of both patient groups were investigated in parallel with TG2. In coeliac disease patients, serum TG3 antibody levels were above the cut-off value in three (30%) patients with the active disease (median 21.5 AU/ml) and in one (10%) of those in remission (median 15 AU/ml) (Table 5). Nevertheless, apart from one patient with the active disease, TG3 antibody levels in the organ culture medium were either negative or fairly low (<20 U/ml) in the majority of patients with the active disease (median 8.5 AU/ml) and in all of those in remission (median 1.4 AU/ml). Treatment of the biopsies with PT-gliadin had no effect on the TG3 antibody secretion. When investigating the presence of mucosal TG3 antibody-secreting cells, such cells were found in only one (10%) patient with the active disease; this patient also presented with high levels of TG3 antibodies in the serum (122 AU/ml) and organ culture medium (<190 AU/ml).

As compared to coeliac disease patients, serum TG3 antibody levels in dermatitis herpetiformis patients were in general lower. Serum TG3 antibody levels were positive in only one (20%) of the patients with the active disease (median 9 AU/ml) and one (8%) of those in remission (median 7.5 AU/ml) (Table 5). On the contrary, TG3 antibody secretion into the organ culture medium was observed in all patients with the active disease (median 108.6 AU/ml) and one patient in remission (median 1.7 AU/ml). Mucosal TG3 antibody-secreting cells were present in three (60%) of the patients with the active disease but in none of the patients in remission; the patients presenting with TG3 antibody-secreting cells were the ones presenting with the highest levels of TG3 antibodies in the organ culture medium.

Table 5. Serum, organ culture medium, and small-intestinal biopsy findings in coeliac disease and dermatitis herpetiformis patients. Statistically significant ($p<0.05$) p-values are shown, as appropriate.

		Active CD n=10	CD in remission n=10	Active DH n=5	DH in remission n=12
Serum	EmA				
	positive cases, n (%)	9/10 (90)	3/10 (30)	0/5 (0)	0/12 (0)
	median (range), titre	1:200 (0–4000)	0 (0–0) †	0 (0–0) †	0 (0–0)
	TG2 antibodies				
	positive cases, n (%)	10/10 (100)	0/10 (0)	0/5 (0)	0/12 (0)
	median (range), U/ml	16.5 (4.9–130.5)	0.5 (0–2.5) †	0 (0–0) †	0 (0–0)
	TG3 antibodies				
	positive cases, n (%)	3/10 (30)	1/10 (10)	1/5 (20)	1/12 (8)
	median (range), AU/ml	21.5 (5–122)	15 (5–24)	9 (7–28)	7.5 (0–35)
Organ culture medium	EmA				
	present; n (%)	8/10 (80)	2/10 (20) †	1/5 (20) †	0/12 (0)
	TG2 antibodies				
	median (range), U/ml	74.8 (0–111.4)	4.6 (0–17.4) †	0 (0–11.4) †	0 (0–3.7)
	TG3 antibodies				
	median (range), AU/ml	8.5 (0–190*)	1.4 (0–7.2) †	108.6 (39.5–146.7) †	1.7 (0–76.7) ‡
Small-intestinal biopsy	TG2 antibody-secreting cells				
	positive cases, n (%)	8/10 (80)	4/10 (40)	0/5 (0)	1/12 (8)
	TG3 antibody-secreting cells				
	positive cases, n (%)	1/10 (10)	0/10 (0)	3/5 (60)	0/12 (0)

* Upper limit of detection range

† $p<0.05$ compared to active coeliac disease group

‡ $p<0.01$ compared to active dermatitis herpetiformis group

CD, coeliac disease; DH, dermatitis herpetiformis; EmA, endomysial antibodies; TG, transglutaminase

5.3 TG2 and TG3 antibody responses in dermatitis herpetiformis patients undergoing gluten challenge (**III**)

To extend the findings made in Study **II** and to clarify the TG2 and TG3 antibody responses during a gluten challenge, altogether 19 dermatitis herpetiformis patients were investigated in Study **III**. According to the inclusion criteria of the challenge, at the time of the recruitment at pre-challenge, all the patients were in clinical, serological (EmA and TG2 antibodies), and small-bowel mucosal remission (Table 6; Table 1 in original publication **III**). At this point, mucosal TG2 antibody-secreting cells were found in two (12%) out of the 17 patients with available biopsies, the percentage of the cells out of all lamina propria plasma cells being 0.18% and 0.93%, respectively, with a median of 0% (range 0–0.93) for the whole group (Table 6; Figure 1 in original publication **III**). After a gluten challenge lasting a median of 3.5 months (range 1–12), 15 (79%) out of 19 patients evinced small-intestinal villous atrophy (median Vh/CrD 2.7). Small-intestinal TG2-targeting IgA deposits were present in 10 (56%) out of 18 patients, and 11 (61%) out of 18 patients showed elevated levels of serum TG2 antibodies (median 32 U/ml). At this point, mucosal TG2 antibody-secreting cells were found in 13 (72%) out of 18 patients, and the median cell percentage increased statistically significantly ($p=0.002$) from pre-challenge to 1.15% (range 0–12.6%).

Serum TG3 antibodies were positive in four (21%) out of 19 dermatitis herpetiformis patients at pre-challenge (median 4 AU/ml), and mucosal TG3 antibody-secreting cells were found in two (12%) of those 17 patients with available biopsies (median cell percentage in the whole group 0%, range 0–1.24%) (Table 6; Table 1 and Figure 1 in original publication **III**). Cutaneous IgA deposits were present in three (16%) patients at pre-challenge. At post-challenge, 17 (94%) patients had elevated levels of TG3 antibodies in the serum (median 89 AU/ml), and 10 (56%) had mucosal TG3 antibody-secreting cells; as compared to pre-challenge, the median cell percentage increased statistically significantly ($p=0.008$) to a median of 0.98% (range 0–10.93%). At the end of the challenge, 13 (68%) of the patients presented with cutaneous IgA deposits.

Table 6. Serum antibodies and small-bowel and cutaneous findings in 19 dermatitis herpetiformis patients at pre- and post-challenge.

		Pre-challenge	Post-challenge
Serum	EmA		
	positive cases, n (%)	0 (0)	12 (56)*
	median (range), titre	0 (0–0)	1:200 (0–1:4000)*
	TG2 antibodies		
	positive cases, n (%)	0 (0)	11 (61)*
	median (range), U/ml	0 (0–0)	32 (0–101)*
	TG3 antibodies		
	positive cases, n (%)	4 (21)	17 (89)
	median (range), AU/ml	4 (0–41)	89 (5–190^)
Small-intestinal biopsy	Vh/CrD		
	<2.0, n (%)	0 (0)	15 (79)
	median (range)	2.7 (2.1–4.5)	0.8 (0.1–3.1)
	TG2-targeting IgA deposits		
	positive cases, n (%)	0 (0)	10 (56)*
	TG2 antibody-secreting cells		
	positive cases, n (%)	2 (12)**	13 (72)*
	median (range), % ^^	0 (0–0.93)**	1.15 (0–12.6)*
	TG3 antibody-secreting cells		
	positive cases, n (%)	2 (12)**	10 (56)*
	median (range), % ^^	0 (0–1.2)**	0.98 (0–10.9)*
Skin biopsy	IgA deposits		
	positive cases, n (%)	3 (16)	13 (68)

* data available for 18 patients

** data available for 17 patients

^ Upper limit of detection range

^^ out of all lamina propria plasma cells

EmA, endomysial antibodies; IgA, immunoglobulin A; TG, transglutaminase; Vh/CrD, villous height:crypt depth ratio

When comparing the percentage of TG2 antibody-secreting cells in all dermatitis herpetiformis patients at post-challenge with other disease parameters, a positive correlation with serum EmA and TG2 antibody levels (R_S=0.670, p=0.002 and R_S=0.685, p=0.002, respectively) and the intensity of small-intestinal mucosal TG2-targeting IgA deposits (R_S=0.673, p=0.002) was detected. Similarly, the percentage of TG3 antibody-secreting cells in all dermatitis herpetiformis patients at post-challenge was positively correlated with serum TG3 antibody levels (R_S=0.547, p=0.019) (Table 7).

Table 7. Correlations between the percentage of TG2 and TG3 antibody-secreting cells out of all lamina propria plasma cells and the serum, small-bowel mucosal, and cutaneous findings in dermatitis herpetiformis patients at post-challenge. R_s, Spearman's correlation coefficient

	R_s	*p*-value
Percentage of TG2 antibody-secreting cells		
Serum EmA	0.670	0.002
Serum TG2 antibodies	0.685	0.002
Intensity of mucosal TG2-IgA deposits	0.673	0.002
Vh/CrD	-0.188	0.455
Percentage of TG3 antibody-secreting cells		
Serum TG3 antibodies	0.547	0.019
Intensity of cutaneous IgA deposits	0.063	0.803
Vh/CrD	-0.350	0.155

EmA, endomysial antibodies; TG, transglutaminase; IgA: immunoglobulin A; Vh/CrD: villous height:crypt depth ratio

In the 24 coeliac disease patients and 7 non-coeliac control subjects used as controls in Study **III**, no TG3 antibody-secreting cells were found, with the exception of one coeliac disease patient on a gluten-free diet.

5.4 Cutaneous IgA and TG3 deposits in dermatitis herpetiformis (**IV**)

In order to investigate the disappearance of cutaneous IgA and TG3 deposits after a long-term gluten-free diet, samples from a total of 33 dermatitis herpetiformis patients were analysed. Of these patients, serum TG3 antibody levels were available for all 26 treated patients; serum TG3 antibodies were positive in two (40%) of the five patients with an active rash and two (10%) of the 21 patients in remission (Table 8). Three (60%) of five untreated patients evinced either subtotal or total villous atrophy, whereas the remaining two had a normal small-bowel morphology (Table 8). With the exception of one (7%) patient presenting with partial villous atrophy, all patients with available biopsies in the dietary-treated group in remission showed normal morphology. In the group of dietary-treated patients with an active rash, two patients with strict dietary compliance had normal villous architecture and the other two with dietary lapses had partial villous atrophy.

Table 8. Serological and small-bowel mucosal findings in dermatitis herpetiformis patients at the time of the study.

	Untreated DH patients n=7	Treated DH patients in remission n=21	Treated DH patients with an active rash n=5
Serum TG3 antibodies; median (range) AU/ml	na	6 (0–36)	17 (9–28)
Small-bowel histology; n (%)			
Normal	2/5 (40)	13/14 (93)	2/4 (50)
SVA	2/5 (40)	-	-
PVA	1/5 (20)	1/14 (7)	2/4 (50)

na, data not available

DH, dermatitis herpetiformis; GFD, gluten-free diet; TG, transglutaminase; SVA, subtotal villous atrophy; PVA, partial villous atrophy

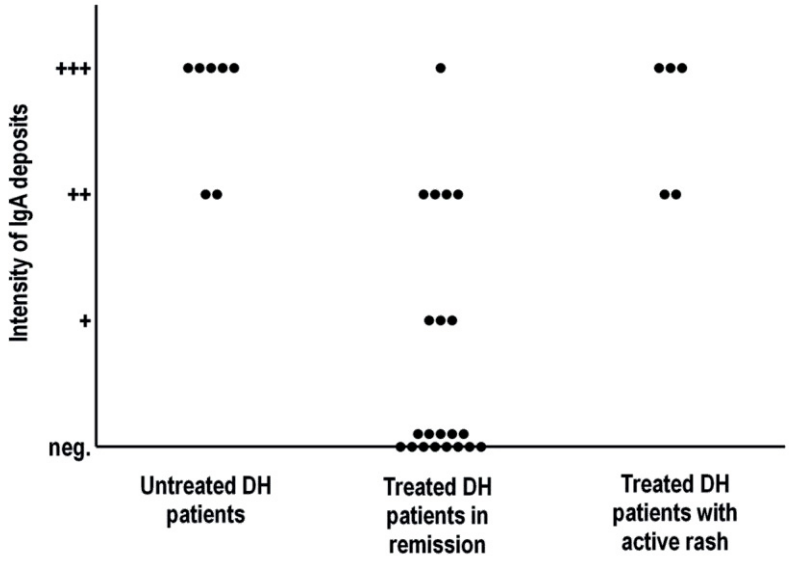

Figure 5. The intensity of cutaneous immunoglobulin A (IgA) deposits in dermatitis herpetiformis patients. Intensity graded as negative (neg.), weak (+), moderate (++), or strong (+++). The intensity of TG3 staining paralleled the IgA staining in all but three cases. DH, dermatitis herpetiformis; GFD, gluten-free diet

When investigating the presence of cutaneous IgA and TG3 separately using single stainings, all seven untreated patients were shown to present with strong or moderate IgA deposits in the papillary dermis (Figure 5; Table I and Figure 1 in original publication **IV**). Stainings for TG3 paralleled those for IgA. Similar findings were observed in all five gluten-free diet-treated patients with an active rash. By contrast, of the 21 treated patients in clinical remission, eight (38%) had IgA and TG3 in the papillary dermis. In double stainings investigating the presence of both IgA and TG3 in cutaneous samples, IgA and TG3 were always shown to colocalise, and, with the exception of three patients, their staining intensities paralleled each other (Table 1 in original publication **IV**). The presence of the IgA and TG3 deposits was not associated with the duration of the dietary treatment, the levels of serum TG3 antibodies, or the small-intestinal morphology.

6 DISCUSSION

6.1 TG2 and TG3 antibody responses in coeliac disease and dermatitis herpetiformis

6.1.1 Small-bowel mucosal TG2 and TG3 antibody-secreting cells

The first study providing evidence for TG2 antibody production by small-bowel mucosal lymphocytes came in 2001 (Marzari et al. 2001). Thereafter, plasma cells specific for TG2 were visualised in the small-intestinal lamina propria of coeliac disease patients using immunofluorescence staining: during the active disease, their frequency is high (Di Niro et al. 2012 and 2016), but they decline considerably within 6–12 months of gluten withdrawal (Di Niro et al. 2016). Such observations were also made in the present study. Small-intestinal mucosal TG2 antibody-secreting cells were detected in the great majority (97%) of patients with overt coeliac disease and their percentage of all lamina propria plasma cells decreased sharply within one year of a gluten-free diet. After long-term treatment of a median of 9 years, the cells were mostly absent in patients with strict dietary adherence. The current study further expands on the previous findings by showing that mucosal TG2 antibody-secreting cells could be detected already in 81% of the patients with early-stage coeliac disease. In addition, they were present in the majority of treated patients having dietary lapses but not in patients with non-responsive coeliac disease on a strict diet. Thus, supporting the earlier findings (Di Niro et al. 2012 and 2016), the results of the current study suggest that the mucosal TG2 antibody-secreting cells in coeliac disease are gluten-dependent; once gluten is removed from the diet, the presentation of gluten-TG2-containing complexes to disease-specific T cells ceases, which could subsequently affect the survival of the TG2 autoantibody-secreting plasma cells and eventually lead to the observed drop in their frequency (Di Niro et al. 2016).

Parallel with coeliac disease, patients with dermatitis herpetiformis show an autoimmune response against TG2 (Salmi et al. 2014; Reunala et al. 2015b) but also against TG3 (Sardy et al. 2002). However, no studies regarding the presence of small-bowel mucosal TG2 or TG3 antibody-secreting cells – or their frequency or gluten-

responsiveness – in dermatitis herpetiformis are available. The current study demonstrates that both TG2 and TG3 antibody-secreting cells can be detected in the small-intestinal mucosa of dermatitis herpetiformis patients. Both cell populations were mostly absent in long-term treated patients in clinical remission, but they appeared in 56% and 72% of the patients during a gluten challenge of 1–12 months, respectively. Regarding the presence and frequency of the cells, the long-term treated dermatitis herpetiformis patients thus equalled the long-term treated coeliac disease patients, whereas after the gluten challenge, they were more comparable to patients with early-stage rather than overt coeliac disease. This finding could reflect the observation that mucosal alterations in dermatitis herpetiformis are often less severe than in coeliac disease and therefore more characteristic of early-stage coeliac disease (Collin et al. 2017). On the other hand, as the patients ingested gluten for a period of 1 to 12 months only, longer gluten exposure might have been required for the cell frequency to be higher and for the cells to appear in all patients. Nevertheless, similarly to TG2 antibody-secreting cells in coeliac disease, the presence of TG2 and TG3 antibody-secreting cells in dermatitis herpetiformis appears to be gluten-dependent.

Interestingly, even though a subset of untreated coeliac disease patients without apparent skin symptoms present with circulating antibodies towards TG3 (Sardy et al. 2002; Heil et al. 2005; Marietta et al. 2008; Salmi et al. 2016), in the present study, small-intestinal mucosal TG3 antibody-secreting cells were mostly absent in coeliac disease patients irrespective of disease activity and the presence or absence of serum TG3 antibodies. It thus appears that the TG3 antibody-secreting cells are specific to dermatitis herpetiformis. Earlier studies have shown serum TG3 antibodies to be different in coeliac disease and dermatitis herpetiformis. While high-affinity TG3 antibodies recognising TG3 selectively can be detected only in dermatitis herpetiformis patients, antibodies with less affinity and the ability to cross-react with TG2 are found in both manifestations (Sardy et al. 2002). Furthermore, serum TG3 antibodies in dermatitis herpetiformis have been shown to be gluten-dependent and to decrease in parallel with EmA and TG2 antibodies during a gluten-free diet (Reunala et al. 2015b), whereas in coeliac disease, their levels do not decrease as rapidly (Salmi et al. 2016). The results of the current study could thus possibly reflect these previously reported differences between the TG3 antibody populations in coeliac disease and dermatitis herpetiformis.

6.1.2 Association of TG2 and TG3 antibody-secreting cells with serum and deposited small-bowel mucosal and cutaneous antibodies

Despite the gluten-dependency of both TG2 and TG3 antibody-secreting cells, their presence and frequency did not always parallel with the presence or levels of the corresponding serum antibodies either in coeliac disease or in dermatitis herpetiformis patients. Such a lack of association has also been noted in previous studies on TG2 antibodies in both untreated and treated coeliac disease patients (Di Niro et al. 2012 and 2016; Iversen et al. 2017; Høydal et al. 2019). The presence of antibody secreting cells in treated patients with negative serology could be explained by the local production of antibodies in the small-intestinal mucosa due to minor lapses in the gluten-free diet, for example (Di Niro et al. 2016). However, the absence of such cells in seropositive patients, as well as the lack of correlation in general, is likely due to other factors. Recently, Iversen et al. (2017) showed that unlike previously suggested (Picarelli et al. 1996; Marzari et al. 2001), serum TG2 antibodies in coeliac disease do not seem to originate from plasma cells residing in the small-intestinal mucosa but instead derive mostly from lymphoid tissues outside the small intestine. The discrepancies between the serum antibodies and small-bowel mucosal plasma cell findings of the current study could therefore at least in part be explained by the extraintestinal production of antibodies both in coeliac disease and dermatitis herpetiformis.

Regardless of the origin of serum antibodies, small-intestinal TG2 antibody-secreting cells could contribute to the formation of the intestinal TG2-targeting IgA deposits, which have been shown to appear already early on in the disease process and to precede the appearance of antibodies in the serum (Korponay-Szabo et al. 2004; Kaukinen et al. 2005; Salmi et al. 2006a and b; Koskinen et al. 2008). In this study, the frequency of TG2 antibody-secreting cells was shown to correlate with the intensity of the mucosal TG2-targeting IgA deposits within both the coeliac disease and dermatitis herpetiformis patients. However, also in this case, the presence of TG2 antibody-secreting cells did not always coincide with the presence of mucosal antibody deposits at the level of individual patients. In non-responsive coeliac disease patients, for example, TG2 antibody-secreting cells were detected in only one patient, despite the presence of mucosal TG2-targeting IgA deposits in all of them. Previously, the long persistence of IgA deposits in non-responsive coeliac disease patients has been suggested to be explained by high-avidity antibodies that are generated during the progression of the disease and then strongly bound to small-bowel mucosa (Salmi et al. 2006b; Westerlund et al. 2007; Koskinen et al. 2010).

However, in addition to non-responsive patients, three early-stage coeliac disease patients and three non-coeliac control patients with self-reported gluten-sensitivity presented with TG2-targeting IgA deposits in the small-intestinal mucosa in the absence of TG2 antibody-secreting cells. Whether the absence of TG2 antibody-secreting cells in these cases is due to the extraintestinal production of antibodies as discussed in the context of serum antibodies remains to be established. However, methodological issues cannot be ruled out either.

The current study also investigated the association between TG3 antibody-secreting cells and the cutaneous TG3-targeting IgA deposits in dermatitis herpetiformis patients. After the gluten challenge, 13 patients presented with cutaneous IgA deposits, but four of them had no TG3 antibody-secreting cells in the small-bowel mucosa, suggesting that antibody production outside the small-bowel mucosa could also contribute to the development of cutaneous deposits. Previous studies on the origin of the cutaneous IgA deposits have been contradictory, and both intestinal and extraintestinal origins have been suggested (Seah et al. 1971b; Unsworth et al. 1982; Hall et al. 1985; Olbricht et al. 1986). Even though the current results imply that TG3 antibodies are secreted in the small-intestinal level, no conclusions as to the origin of the cutaneous IgA deposits can be made based on them; future studies addressing this topic are needed.

6.2 Antibody responses in the organ culture system

The ex vivo culture of patient-derived small-bowel mucosal biopsies has been previously used for studying the various pathogenetic processes of coeliac disease, including the intestinal secretion of TG2 antibodies at different stages of the disease (Picarelli et al. 1996; Vogelsang et al. 1999; Carroccio et al. 2002; Stenman et al. 2008; Rauhavirta et al. 2013; Tosco et al. 2013 and 2014). In the present study, TG2-targeting antibodies were detected in intestinal secretions in the majority of patients with active coeliac disease, whereas in treated patients, the level of secretion was lower, as also reported previously (Stenman et al. 2008; Rauhavirta et al. 2013). Besides coeliac disease, no previous studies have investigated TG2 antibody secretion in small-bowel mucosal biopsies obtained from patients with dermatitis herpetiformis. In the present study, TG2 antibody secretion was not observed in any of the patients with dermatitis herpetiformis, irrespective of disease activity.

The present study also demonstrates that in addition to TG2-targeting antibodies, also TG3 antibodies can be studied exploiting the organ culture method. While the

levels of TG3 antibodies in intestinal secretions were generally either negative or rather low in both untreated and treated coeliac disease patients, high-level TG3 antibody secretion was observed in all patients with active dermatitis herpetiformis.

In the current setting, autoantibody secretion from the cultured biopsies was not affected by supplementation of the culture medium with PT-gliadin in any of the patient groups. In accordance with numerous previous studies, this lack of stimulatory effect is most likely dependent on the short culture period (Picarelli et al. 2001; Stenman et al. 2008; Rauhavirta et al. 2013; Tosco et al. 2013 and 2014).

The current study also sought to investigate the origin of the antibodies in the organ culture medium, suggested to be due to the detachment of antibodies from local antibody deposits in the tissue (Stenman et al. 2008) or secretion by plasma cells. Patients secreting TG2-targeting antibodies into the culture medium had small-bowel mucosal TG2-targeting IgA deposits, and the majority of them also had detectable TG2 antibody-secreting cells in the small-bowel mucosa, making both scenarios possible. However, no extracellular TG3-targeting IgA deposits were found in any of the patients secreting TG3 antibodies into the culture medium – instead the majority of them had detectable TG3 antibody-secreting cells in the small-bowel mucosa – so the TG3 antibodies in the culture medium most likely derived from such cells.

6.3 Cutaneous IgA and TG3 deposits in dermatitis herpetiformis

The cutaneous IgA deposits in dermatitis herpetiformis patients have been previously shown to colocalise with TG3 (Sardy et al. 2002). In the present study, the disappearance of IgA and TG3 was investigated and both of them were shown to disappear in parallel in two thirds of dermatitis herpetiformis patients in clinical remission during long-lasting dietary treatment, but to persist in all patients with the active disease regardless of the diet. While the IgA deposits seem to reappear quickly, within 1–12 months, in treated dermatitis herpetiformis patient skin upon gluten challenge (Leonard et al. 1983b; Mansikka et al. 2019), their long persistence after the commencement of a gluten-free diet has been well documented in numerous previous studies (Table 9). In the earliest studies with the maximum duration of the gluten-free diet being seven years, the cutaneous IgA deposits were shown to persist in all patients (Table 9) (Fry et al. 1978; Reunala, 1978; Frödin et al. 1981). Thereafter, longer treatment periods (mean duration of a gluten-free diet more than 5.5 years) were studied and the disappearance of IgA was observed in 14–42% of the patients

(Fry et al. 1982; Leonard et al. 1983b; Garioch et al. 1994; Hardman et al. 1997; Reunala et al. 1998; Bardella et al. 2003). The duration of the dietary treatment in the present study exceeds that in the previous investigations as the patients in clinical remission had been on the diet for a mean of 22 years. After such a long treatment, 62% of the patients no longer had IgA deposits (Table 9). Surprisingly, the IgA-negative patients had more or less the same mean duration of a gluten-free diet as those with persisting IgA deposits (22 vs 19 years). Moreover, the absence or presence of IgA deposits was not associated with small-bowel mucosal damage at diagnosis and, importantly, after gluten-free diet, the small-bowel mucosal morphology was normal also in the patients with persisting IgA deposits. The response to treatment in terms of the rash and small-bowel mucosal damage was therefore equally good regardless of the presence of cutaneous IgA deposits.

Table 9. Studies reporting the disappearance of immunoglobulin A (IgA) deposits from the skin of dermatitis herpetiformis patients after adherence to a gluten-free diet (GFD).

Study	Number of patients	Duration of GFD, mean years (range)	Loss of IgA from the skin, n (%)
Original Study IV	21	22 (5–38)	13 (62)
Bardella et al. 2003	38*	8 (na)	16 (42)
Reunala et al. 1998	10	6 (0.5–19)	4 (40)
Hardman et al. 1997	10	16 (6–26)	3 (30)
Garioch et al. 1994	41	13 (5–24) **	10 (24)
Leonard et al. 1983b	12	10 (3–20)	3 (24)
Fry et al. 1982	22	10, 14,14**	3 (14)
Frödin et al. 1981	22	2 (1–4)	0 (0)
Fry et al. 1978	19	na (1–7)	0 (0)
Reunala et al. 1978	12	3 (1–7)	0 (0)

na, data not available

* 26 patients on a strict, six on a moderate, and six on a poor gluten-free diet.

** Duration of gluten-free diet in patients showing loss of IgA from the skin.

While the persistence of IgA deposits in the skin of dermatitis herpetiformis patients is well documented, less is known about the persistence of their target, TG3: only one previous study has reported the presence of TG3 in dietary-treated dermatitis herpetiformis patients (Donaldson et al. 2007). The study in question included nine dermatitis herpetiformis patients who, regardless of the diet, had the active disease and IgA deposits in the skin. TG3 was found in all but one patient on a strict gluten-free diet, this subject having sparse IgA in perilesional skin in the absence of TG3. Moreover, the authors reported that the intensity of TG3 staining roughly correlated with that of IgA, which is in line with the present findings. In the present study, the

staining intensities of IgA and TG3 were of the same magnitude in all but three cases. The current study further documents that after a long-term gluten-free diet, TG3 is not found in the papillary dermis without IgA, thus strongly suggesting that the disappearance of TG3 parallels that of IgA. Consistent with this finding, during a gluten challenge, the IgA and TG3 deposits have been shown to re-appear simultaneously in dermatitis herpetiformis skin (Mansikka et al. 2019).

The parallel disappearance of IgA and TG3 supports the prevailing conception that IgA and TG3 are deposited in the papillary dermis as immune complexes (Sardy et al. 2002; Görög et al. 2016; Mansikka et al. 2019). The existence of such complexes has long been speculated over but supported by the unusual localisation of TG3 in the papillary dermis (Sardy et al. 2002). However, opposing views as to their origin have been reported: the presence of both IgA and TG3 in the vessel walls of dermatitis herpetiformis patient skin (Preisz et al. 2005) and the presence of IgA- and TG3-containing complexes in the circulation of dermatitis herpetiformis patients (Görög et al. 2016) could imply that the deposited complexes derive from the circulation. On the other hand, a study by Zone et al. (2011) proposed an alternative theory. Using a passive transfer mouse model with normal human skin grafts, they were able to produce IgA-TG3 deposits in mouse skin. As the only source of TG3 was the human skin graft, they suggested that TG3 could derive from the epidermis and be deposited in the papillary dermis together with IgA antibodies from the circulation. Regardless of its origin, the dermal TG3 in dermatitis herpetiformis patient skin has been shown to be enzymatically active (Taylor et al. 2015). It is therefore possible that the TG3-IgA aggregates are tightly crosslinked to the extracellular matrix of the papillary dermis, which could provide an explanation for the long persistence of the cutaneous immune complexes during a gluten-free diet (Taylor et al. 2015).

6.4 Strengths and limitations of the study

In the present study, the IgA-specific antibody responses towards TG2 and TG3 in coeliac disease and dermatitis herpetiformis were studied and compared comprehensively in different settings: the responses were analysed in the serum and organ culture medium, as well as in small-bowel mucosal and cutaneous biopsies. Both the coeliac disease and dermatitis herpetiformis patient cohorts were well-defined and covered different stages of the diseases, which allowed investigating the fluctuation of the responses according to gluten intake.

Studies **I–III** provided new aspects on TG2 and TG3 antibody responses in coeliac disease, especially regarding the small-bowel mucosal plasma cell responses and their connection to serum and deposited small-bowel mucosal antibodies. The immunofluorescence staining method for detecting small-intestinal mucosal TG2 antibody-secreting cells had been established earlier (Di Niro et al. 2012) and was here further modified and applied to identify mucosal TG3 antibody-secreting cells. Regarding the detection of TG2 antibody-secreting cells, a limitation of the study method is the possible binding of recombinant TG2 to endogenous fibronectin, the abundantly available substrate protein of TG2, in small-bowel mucosal tissue sections (Di Niro et al. 2016). This makes interpretation of the results more challenging than when using recombinant TG3, which does not bind in a similar way. In addition, the patchiness of the small-intestinal lesion could affect the results in that the frequency of autoantibody secreting cells might not be consistent throughout the intestine (Di Niro et al. 2016). On the other hand, a previous study addressed this issue, and preliminarily results from one coeliac disease patient showed the density of TG2-specific plasma cells to be equal in all individual small-bowel mucosal biopsies taken from different locations (Di Niro et al. 2016). A further limitation of the study was the small number of coeliac disease patients with elevated levels of serum TG3 antibodies. Although representative, more studies are needed in order to confirm the present findings regarding this patient group.

Study **II** was the first to exploit the organ culture method of small-bowel mucosal biopsies in investigating both the TG2 and TG3 antibody responses in coeliac disease and dermatitis herpetiformis. The method is well established and has been used in coeliac disease research for decades (Browning and Trier 1969). However, it comes with certain limitations: in the current study design, a limitation was the short culture period, during which no effect of gluten on autoantibody secretion was observed. A prolonged culture period might have therefore been needed to achieve the desired effects (Picarelli et al. 2001; Stenman et al. 2008). Regarding the patients, a major limitation was the active dermatitis herpetiformis patient group, which consisted of patients having the active disease in terms of the rash and cutaneous IgA deposits but who had been following a gluten-free diet for a relatively long time, were seronegative, and had normal small-bowel mucosal structure. Therefore, the group was not directly comparable to the active coeliac disease patient group consisting of untreated, newly diagnosed patients. Similarly, the treated dermatitis herpetiformis patients had been on a gluten-free diet for a much longer time than their coeliac disease counterparts, who had been dietary-treated for one year only.

The gluten challenge in Study **III** provided an opportunity to investigate the different aspects of the TG2 and TG3 antibody responses in dermatitis herpetiformis patients in a prospective manner. The study shed new light especially on the small-intestinal mucosal TG2- and TG3-specific plasma cells responses, which had not been previously investigated in dermatitis herpetiformis. However, as the patients ingested gluten for a rather short time during the study, they are not directly comparable to patients at diagnosis, nor to coeliac disease patients of the present study consisting mainly of newly diagnosed and one-year treated patients.

Study **IV** was the first one to address the disappearance of both IgA and TG3 from the skin of dermatitis herpetiformis patients after a long-term gluten-free diet. As compared to previous studies addressing the persistence of cutaneous IgA deposits in dermatitis herpetiformis patients, the patients in the current study had adhered to a gluten-free diet for a particularly long time. This, along with the strict dietary compliance and inclusion of gluten-free diet-treated patients with the active disease, enabled the reliable evaluation of the disappearance of the IgA and TG3 deposits during the diet. A limitation was the relatively small size of the patient groups and the cross-sectional nature of the study.

7 SUMMARY AND CONCLUSIONS

The present study revealed several new aspects of IgA-specific TG2 and TG3 autoantibody responses in coeliac disease and dermatitis herpetiformis.

Firstly, the study provided new information regarding the presence and frequency of small-bowel mucosal TG2 antibody-secreting cells at different stages of coeliac disease. TG2 antibody-secreting cells were found already in the early stage of coeliac disease, and their frequency was shown to increase towards the development of the overt disease with small-bowel mucosal damage and to decline upon commencement of a gluten-free diet. After long-term treatment, the cells were mostly absent if dietary adherence was strict. The presence of TG2 antibody-secreting cells in coeliac disease at different stages was thus shown to reflect the presence of gluten in the diet.

Secondly, exploiting the ex vivo organ culture method of patient-derived small-bowel mucosal biopsies, the current study demonstrated that in addition to TG2 antibodies, TG3 antibodies are also secreted at the small-bowel mucosal level. TG2 antibody secretion was shown to be most prevalent in active coeliac disease patients, whereas TG3 antibody secretion was observed primarily in patients with active dermatitis herpetiformis. In patients secreting the highest levels of TG3 antibodies into the organ culture medium, TG3 antibody-secreting cells were identified in the small-bowel mucosa.

Thirdly, the present study showed that the small-bowel mucosal TG2 and TG3 antibody-secreting cells in dermatitis herpetiformis are gluten-dependent. Both cell populations were mostly absent in long-term-treated dermatitis herpetiformis patients in clinical remission, but they were shown to appear in more than half of the patients during a gluten challenge lasting up to one year. As TG3 antibody-secreting cells were generally absent in coeliac disease patients, the present findings also indicate that such cells are specific to dermatitis herpetiformis.

Regardless of the observed gluten-dependency of both TG2 and TG3 antibody-secreting cells, the present study further showed that their presence did not always parallel the corresponding serum or the deposited small-bowel mucosal and cutaneous antibodies in coeliac disease or dermatitis herpetiformis. This finding may suggest that antibody production occurs also outside of the small-bowel mucosa in both manifestations, as was recently shown in coeliac disease.

Finally, the current study established that the disappearance of TG3 from the papillary dermis of the skin of dermatitis herpetiformis patients during a long-term gluten-free diet is slow and parallels that of IgA. This finding provides further support for the prevailing theory that TG3 and IgA are deposited in the skin in the form of immune complexes.

In the future, more studies are needed in order to reveal the mechanisms leading from TG2 antibody production in coeliac disease to the formation of TG3 antibodies and IgA-TG3 complexes in dermatitis herpetiformis, and to establish the possible contribution of these different autoantibody responses to the development of the two manifestations of coeliac disease.

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PUBLICATION I

Small-intestinal TG2-specific plasma cells at different stages of coeliac disease

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Small-intestinal TG2-specific plasma cells at different stages of coeliac disease

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Abstract

Background: In coeliac disease, ingestion of gluten induces the production of transglutaminase 2 (TG2)-targeted autoantibodies by TG2-specific plasma cells present at high frequency in the small intestinal mucosa in untreated disease. During treatment with a gluten-free diet (GFD), the number of these cells decreases considerably. It has not been previously investigated whether the cells are also present prior to development of villous atrophy, or in non-responsive patients and those with dietary lapses. We aimed to define the frequency of small bowel mucosal TG2-specific plasma cells in coeliac disease patients with varying disease activity, and to investigate whether the frequency correlates with serum and small intestinal TG2-targeting antibodies as well as mucosal morphology and the number of intraepithelial lymphocytes.

Results: Mucosal TG2-specific plasma cells were found in 79% of patients prior to development of mucosal damage, in all patients with villous atrophy, and in 63% of the patients after 1 year on GFD. In these disease stages, TG2-specific plasma cells accounted for median of 2.3, 4.3, and 0.7% of all mucosal plasma cells, respectively. After long-term treatment, the cells were present in 20% of the patients in clinical remission (median 0%) and in 60% of the patients with poor dietary adherence (median 5.8%). In patients with non-responsive coeliac disease despite strict GFD, the cells were found in only one (9%) subject; the cells accounted for 2.4% of all plasma cells. A positive correlation between the percentage of TG2-specific plasma cells and serum TG2 antibody levels ($r_S = 0.69$, $P < 0.001$) and the intensity of mucosal TG2-targeting IgA deposits ($r_S = 0.43$, $P < 0.001$) was observed.

Conclusions: Our results show that TG2-specific plasma cells are already detectable prior to villous atrophy, and that generally their frequency increases during overt disease. By contrast, on GFD, the percentage of these cells decreases. Overall, the presence of TG2-specific plasma cells in the small bowel mucosa mirrors the presence of gluten in the diet, but the frequency is not always parallel to the level of serum or intestinal TG2 antibodies. These findings increase the knowledge about the development of the TG2 plasma cell responses especially in the early phases of coeliac disease.

Keywords: Coeliac disease, Gluten, Transglutaminase 2, Autoantibody, Small intestine

Background

In coeliac disease, dietary gluten in wheat, rye, and barley functions as a driving antigen for an abnormal immune response that develops in genetically susceptible individuals carrying the human leukocyte antigen (HLA)-DQ2 or -DQ8 haplotypes. The disease is characterised by small-bowel mucosal damage which develops gradually from normal villous morphology to inflammation and finally to villous atrophy with crypt hyperplasia diagnostic of coeliac disease. The intestinal damage is often coupled with numerous gastrointestinal symptoms, although various extraintestinal manifestations are also prevalent. A specific characteristic of coeliac disease is the generation of immunoglobulin class A (IgA) antibodies towards the main autoantigen, transglutaminase 2 (TG2) [1]. These autoantibodies are generally found in the circulation of coeliac disease patients [2] and as deposits in the small intestinal mucosa below the subepithelial basement membrane and around blood vessels [3]. Interestingly, intestinal TG2-targeted deposits can be detected even prior to manifest mucosal damage and in the absence of serum antibodies [4–6].

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Upon removal of gluten from the diet, the only currently available treatment, the clinical symptoms and histopathological changes in the small intestine resolve, and both the circulating and intestinal antibodies disappear within 1 year in most patients [7]. However, a subset of patients fails to respond to the dietary treatment and the villous atrophy persists despite a strict gluten-free diet (GFD). The most common reason for persistent villous atrophy is either advertent or inadvertent gluten intake or, in rare cases, refractory coeliac disease [8].

TG2-targeting antibodies were long thought to be generated by intestinal plasma cells [9–11], but recent data suggests that they might also be produced in lymphoid tissues outside the gut [12]. In the small intestine, the TG2-specific plasma cells are present at high frequency during the active disease [10, 11], and they decrease considerably within 6–12 months after commencement of a strict GFD [11]. However, no data exist regarding the presence of these cells in the early phases of coeliac disease when the mucosal morphology is still normal. In addition, their existence in non-responding coeliac disease patients or those with dietary lapses has not been previously investigated. With this in mind, we enumerated the TG2-specific plasma cells in untreated and treated coeliac patients with varying degrees of disease activity, and investigated whether the number of these cells correlates with serum TG2 antibody levels, the intensity of mucosal TG2-targeting IgA deposits, and small intestinal mucosal morphology and inflammation.

Methods

Patients and study design

The study cohort comprised 46 coeliac disease patients who underwent upper gastrointestinal endoscopy at the Department of Gastroenterology and Alimentary Tract Surgery of Tampere University Hospital (Table 1). Fifteen of the patients were clinically suspected of having coeliac disease based on gastrointestinal symptoms and positive coeliac disease-specific autoantibodies (endomysial and/or TG2 antibodies) despite having normal small bowel mucosa (villous height crypt depth ratio (Vh/CrD) ≥ 2). These patients were prospectively followed up while they continued on a normal gluten-containing diet

Table 1 Demographic data and the small-bowel mucosal and serological findings of patients participating in the study

	Prospectively studied coeliac patients (*n* = 15)			Long-term treated coeliac patients (*n* = 31)			Disease controls (*n* = 25)	
	CD prior to atrophy *n* = 14	Overt CD *n* = 15	1 year GFD *n* = 11	Patients in remission *n* = 15	Non-responding CD *n* = 11	Patients with dietary lapses *n* = 5	Gluten sensitivity *n* = 18	Dyspepsia *n* = 7
Female; n (%)	10 (71)	10 (67)	7 (64)	10 (67)	7 (64)	5 (100)	16 (89)	1 (14)
Age; median (range), years	55 (16–70)	55 (17–71)	57 (28–72)	59 (24–66)	49 (40–76)	51 (31–77)	49 (24–65)	47 (24–76)
Duration of GFD; median (range), years	0	0	1 (1–1)	8 (3–34)	7 (3–24)	10 (9–17)	0	0
HLA-DQ2 or -DQ8-positive; n (%)	14 (100)	15 (100)	11 (100)	15 (100)	11 (100)	4[a] (100)	9 (50)	1 (14)
EmA; median (range), titer	1:100 (0–1:2000)	1:100 (0–1:4000)	0 (0–1:50)	0 (0)	0 (0–1:5)	1:200 (0–1:2000)	0 (0)	0 (0)
TG2 abs; median (range), U/ml	9.4 (3.3- > 100)	11.9 (4.2- > 100)	3.9 (0–8.6)	0.5 (0–2.8)	1.3 (0–9.9)	56.9 (12.9- > 100)	1.4 (0–4.1)	0.6 (0–2.8)
Mucosal TG2-IgA deposits present; n (%)	14 (100)	14[a] (100)	7[b] (88)	5 (33)	10[a] (100)	3[c] (100)	4 (22)	0 (100)
Vh/CrD; mean (95% CI), ratio	2.9 (2.6–3.1)	1.4 (1.1–1.7)	3.4 (2.4–4.5)	3.4 (3.2–3.6)	0.2 (0.0–0.4)	0.5 (−0.3–1.3)	3.5 (3.0–4.1)	3.5 (3.0–4.1)
$CD3^+$ IELs; median (range), cells/mm	54 (12–79)	67 (38–116)	39 (23–80)	42 (25–77)	60 (30–109)	50 (38–69)	21 (7–59)	26 (16–40)
$\alpha\beta^+$ IELs; median (range), cells/mm	30 (12–50)	43 (21–75)	22 (14–43)	31 (20–46)	44 (26–105)	38 (35–56)	16 (5–26)	22 (17–31)
$\gamma\delta^+$ IELs; median (range), cells/mm	18.8 (0–38.5)	23.7 (14–58.7)	v13.6 (1.4–56.3)	14.0 (7.3–27.8)	12.1 (0–37.8)	13.0 (4.4–20.5)	2.7 (0–10.2)	1.6 (0.7–16.1)

abs antibodies, *CD* coeliac disease, *CI* confidence interval, *EmA* endomysial antibodies, *GFD* gluten-free diet, *GS* gluten sensitive; *IgA* immunoglobulin A; *IELs* intraepithelial lymphocytes; TG2-abs, transglutaminase 2 antibodies; *Vh/CrD* villous height crypt depth ratio

Reference values set at 2.0 for Vh/CrD, 37 cells/mm for $CD3^+$ IELs, 25 cells/mm for $\alpha\beta^+$ IELs, and 4.3 cell/mm for γδ+ IELs (Järvinen et al., [15])

Cut-off value for TG2 antibodies ≥ 5 AU/ml

[a]Data missing from one patient

[b]Data missing from three patients

[c]Data missing from two patients

for 1 year, during which villous atrophy developed (Vh/CrD < 2) in all patients. Thereafter, the patients started a GFD, and after 1 year on the diet, their mucosal morphology had recovered. Small bowel samples from 14 of the patients at the time of the normal mucosal morphology, all 15 patients at the time of the villous atrophy, and 11 of the patients after 1 year on a GFD were available for the current study (Table 1).

Furthermore, 15 coeliac disease patients on a long-term GFD without symptoms and evincing full histological recovery, 11 non-responsive coeliac disease patients with persistent villous atrophy despite a strict GFD, and five patients with poor dietary adherence were investigated. Twenty patients with self-reported gluten sensitivity experiencing abdominal symptoms after consumption of gluten-containing products [13] and seven patients with dyspepsia served as the non-coeliac controls in the study. All controls had been excluded for coeliac disease, as demonstrated by negative serology and normal small bowel mucosal morphology. The demographic data and small-bowel mucosal and serological findings of all subjects are reported in Table 1.

The study protocol was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland, and written informed consent was obtained from all participating subjects.

Small-intestinal mucosal morphology and inflammation

Small-intestinal mucosal biopsies were obtained upon upper gastrointestinal endoscopy. For morphological studies, one formalin-fixed biopsy sample was stained with haematoxylin and eosin to determine the villous height-crypt depth ratios (Vh/CrD) according to a previously published procedure [14]. A ratio of ≥2 was considered normal. One of the biopsies was submerged in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek Europe, Holland), followed by snap-freezing in liquid nitrogen. Thereafter, 5-μm-thick sections were cut. According to an established protocol [15], the sections were stained for $CD3^+$, $\alpha\beta^+$, and $\gamma\delta^+$ intraepithelial lymphocyte (IEL) subsets. The reference values were 37 cells/mm, 25 cells/mm, and 4.3 cells/mm for $CD3^+$ IELs, $\gamma\delta^+$ IELs and $\alpha\beta^+$ IELs, respectively [15].

Serological measurements and HLA genotyping

Serum endomysial antibodies (EmA) in IgA class were determined by an indirect immunofluorescence method exploiting human umbilical cord as substrate. A dilution of 1:≥5 was considered positive [16]. Serum IgA-class TG2 antibodies were measured by a commercially available enzyme-linked immunosorbent assay (Celikey®, Phadia, Freiburg, Germany) in samples diluted 1:100. A titre of ≥ 5 AU/ml was set as the cut-off for positivity.

SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden), DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or HLA-tagging single-nucleotide peptides [17] were used for HLA genotyping.

Small-intestinal TG2-specific IgA deposits

For the determination of mucosal TG2-targeting IgA deposits, frozen sections were stained with mouse monoclonal anti-TG2 antibody (CUB7402; NeoMarkers, Fremont, California, USA), followed by detection with fluorescein isothiocyanate (FITC) -labelled rabbit anti-human IgA antibody (Dako A/S, Glostrup, Denmark) [3]. Based on their intensity along the basement membrane in the villous-crypt area, the deposits were graded blinded as a negative, or a weak, moderate, or strong positive, as described previously [6].

Small-intestinal TG2-specific plasma cells

An earlier described technique was used to detect mucosal TG2-specific plasma cells [10]. Initially, 5-μm-thick frozen sections were air-dried for 20 min at room temperature (RT). After washing in PBS, the sections were incubated with biotinylated human recombinant TG2 (2 μg/ml; T002, Zedira) for 45 min at RT. Biotinylation was performed using EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Scientific, Waltham, MA, USA) according to the instructions provided by the manufacturer. Thereafter, the sections were incubated with rhodamine-labelled streptavidin (1:1000; KPL, Gaithersburg, MD, USA) for 30 min at RT. Plasma cells were identified using a mouse monoclonal CD138 antibody (1:25; B-A38, Bio-Rad), followed by goat anti-mouse IgG Alexa Fluor 488 (1:2000; A-11001, Thermo-Fisher Scientific). Stainings were analysed at 20x and 40x magnification (Olympus BX60F5, Olympus Optical Co. LTD, Japan) on two consecutive small intestinal biopsy sections and the percentage of TG2-specific cells out of all *lamina propria* plasma cells in the entire section was determined.

Statistical analyses

Data are expressed as the number of subjects (n) and percentages, or as medians and ranges. Statistical analyses were performed using the Wilcoxon test or Mann–Whitney test as appropriate. Correlation was evaluated using Spearman's correlation. Statistical testing was performed using statistical analysis software (IBM SPSS Statistics, SPSS Inc., Chicago, IL, USA). A *P*-value < 0.05 was considered statistically significant.

Results

Of the 15 prospectively studied coeliac disease patients, TG2-specific plasma cells were already present in 11 out

of the 14 available small bowel samples (79%) from the patients before the development of villous atrophy. The median percentage of the cells was 2.3% (range 0–12.7%) of all *lamina propria* plasma cells (Fig. 1a and b). After continuing on a gluten-containing diet for 1 year and developing overt small bowel mucosal damage, all fifteen patients had intestinal TG2-specific plasma cells, and the median percentage of the cells was 4.3% (range 1.8–8.8%) ($P = 0.055$ when compared to patients with early-stage coeliac disease). By contrast, after 1 year on a GFD, the cells were found in 7 out of 11 (64%) patients with available samples, and the median percentage of the cells significantly decreased to 0.7% (range 0–2.9%, $P = 0.003$) when compared to the overt disease.

In long-term GFD-treated patients in clinical remission responding well to dietary treatment, only a few remaining TG2-specific plasma cells (median 0.0%, range 0–1.1%) were detected in 3 out of 15 (20%) of the patients (Fig. 1a and b). In non-responding coeliac disease patients on a strict GFD, the cells were mostly absent, being present in only one patient (9%) who had 2.4% of TG2-specific cells out of all *lamina propria* plasma cells. Of the coeliac disease patients with dietary lapses, three out of five (60%) had TG2-specific plasma cells, the median being 5.8% (range 0–7.0%). No TG2-specific plasma cells were found in any of the non-coeliac control patients with either gluten sensitivity or dyspepsia.

A positive correlation between the percentage of TG2-specific plasma cells and serum TG2 antibody levels ($r_S = 0.690$, $P < 0.001$, Fig. 2a) as well as EmA ($r_S = 0.712$, $P < 0.001$) was observed when data from all coeliac disease patient groups were included in the analysis. Similarly, the percentage of the TG2-specific plasma cells correlated with the intensity of the small intestinal IgA deposits in all coeliac disease patients ($r_S = 0.430$, $P < 0.001$) (Fig. 2b). However, the percentage of TG2-specific plasma cells did not correlate with serum

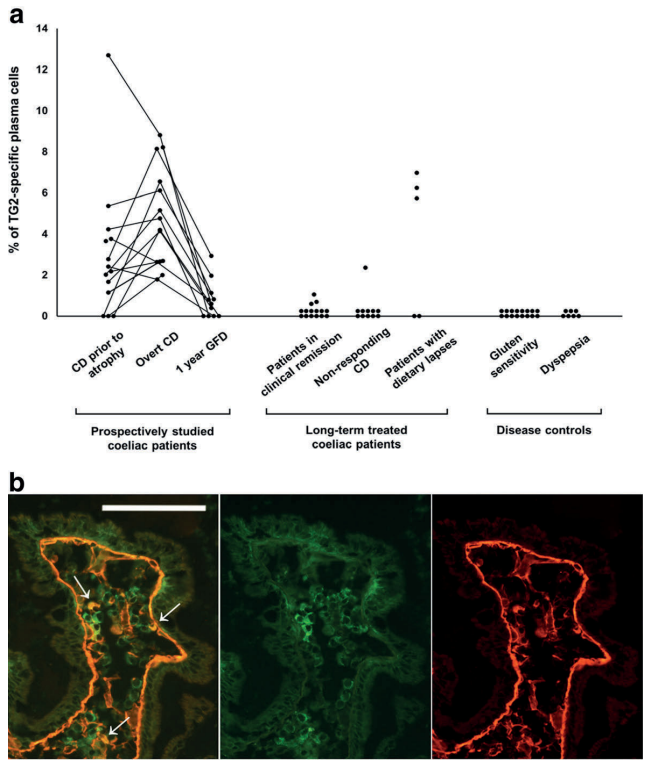

Fig. 1 a. The percentage of small-bowel mucosal transglutaminase 2 (TG2) -specific plasma cells out of all *lamina propria* plasma cells in the different patient groups. **b.** Immunofluorescence staining for transglutaminase 2 (TG2) antibodies and plasma cells in small-bowel mucosal sections. Representative picture of a coeliac disease patient prior to villous atrophy showing positive staining for TG2-specific plasma cells (arrows). Recombinant TG2 (red), plasma cell marker CD138 (green), and their colocalisation (yellow) at 20x magnification. Scale bar = 100 μm. Abbreviations: CD, coeliac disease; GFD, gluten-free diet

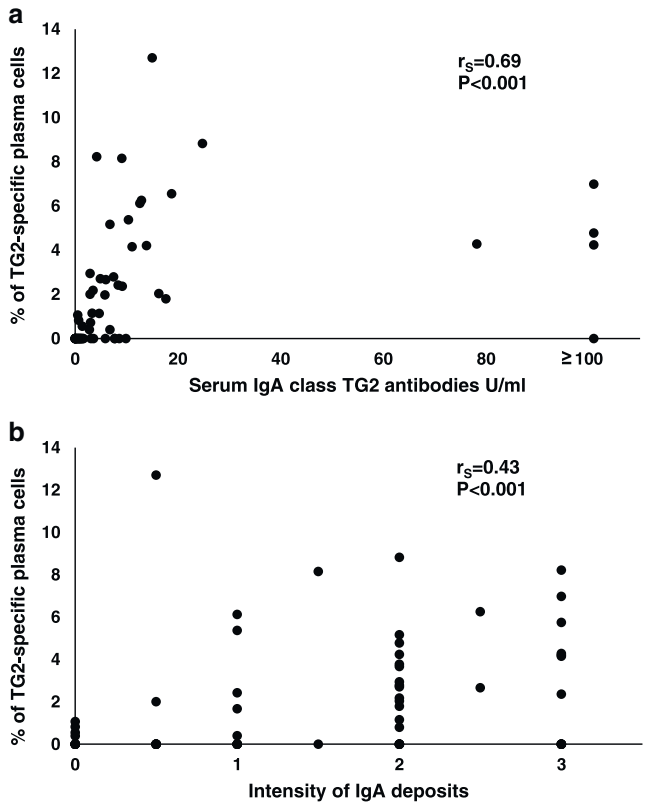

Fig. 2 Correlation between the percentage of small intestinal transglutaminase 2 (TG2) -specific plasma cells and serum IgA class TG2 antibody levels (**a**) and the intensity of small intestinal TG2-targeting immunoglobulin a (IgA) deposits (**b**) in all coeliac disease patients. Grading of IgA deposits as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = strong

or mucosal TG2 antibodies in any of the individual coeliac disease patient groups (Additional file 1 Table S1). Considering all coeliac disease patients, there was no correlation between the percentage of the plasma cells and Vh/CrD. Of the IELs, there was a modest correlation between the percentage of TG2-specific plasma cells and $\gamma\delta^+$ IELs. Detailed information about the correlations is presented in Additional file 1 Table S1.

Discussion

In the current study, we have discovered that TG2-specific plasma cells are already present in most patients prior to detectable mucosal damage. Moreover, we showed that in the majority of cases the percentage of these cells increases upon continuous gluten intake and the subsequent development of villous atrophy. On a strict GFD, the percentage of these cells declines. After long-term treatment, the cells are mostly absent both in patients in clinical remission and in non-responding patients with persistent villous atrophy. By contrast, TG2-specific plasma cells can be detected in patients with dietary lapses. In controls, even those with gluten-related symptoms, no cells were detected.

Our results from untreated and treated coeliac disease patients are in line with previous studies [10, 11], showing that the amount of TG2-specific plasma cells is elevated in the small intestinal mucosa at the time of diagnosis and this amount decreases on a GFD. It has earlier been shown that the percentage of these cells out of all *lamina propria* plasma cells accounts for up to 24% in overt disease, being on average 10% [10, 11]. However, in the current study, the corresponding percentages were lower. Our patients had been recruited to the study while still having normal mucosal morphology, and they developed overt villous atrophy within a one-year follow-up on a gluten-containing diet. Thus, it is conceivable that the patients had had flat mucosal lesion for a reasonably shorter time than in the previous

studies, which might explain the lower percentages of TG2-specific plasma cells in our study.

TG2-specific plasma cells correlated positively with serum TG2 antibody levels when the data of all coeliac disease patients were analysed together. This correlation most likely mirrors the responsiveness of the plasma cells to gluten exposure which is not surprising in the light that TG2 antibodies have been suggested to arise by a hapten-carrier-like mechanism involving TG2-catalysed generation of gluten-TG2 complexes [18, 19]. On the other hand, correlations between the percentages of the plasma cells and serum antibody levels were not detected when different coeliac disease groups were analysed separately; this is in agreement with previous findings in untreated coeliac disease patients [11]. It has been proposed that the lack of correlation could be explained by the production of the antibodies also outside the gut [11]. This concept has recently been further supported by the finding that coeliac patient serum and intestinal TG2 antibodies are clonally related but have different molecular compositions, pointing to different sites of origin [12]. Such extraintestinal production of TG2 antibodies could also explain why some patients in our study had serum TG2 antibodies in the absence of intestinal TG2-specific plasma cells.

Although small intestinal TG2-specific plasma cells are not likely to be the major source of serum TG2 antibodies [12], it would be logical to assume that the TG2 antibodies bound to their antigen in the small intestinal mucosa and predicting forthcoming mucosal damage are produced locally by *lamina propria* TG2-specific plasma cells. In this study, we observed a correlation between the percentage of the plasma cells and the intensity of mucosal TG2-specific IgA deposits, which supports this hypothesis. Interestingly, however, TG2-specific plasma cells were mostly absent in non-responding coeliac patients, even though they all presented with strong TG2-targeting IgA deposits in the small intestinal mucosa despite a strict GFD. It has been proposed that the long persistence of small intestinal IgA deposits in non-responsive patients on a strict diet may be explained, for instance, by the high avidity binding of the IgA antibodies to small intestinal TG2 [6, 7]. This could explain the presence of IgA deposits in the absence of TG2 antibody-secreting plasma cells in our non-responsive patients. However, it does not provide an explanation for the presence of IgA deposits in the absence of plasma cells in the small subset of patients prior to development of villous atrophy. Similarly, it does not explain the presence of weak IgA deposits without TG2-antibody secreting plasma cells in a few gluten-sensitive control patients. Whether extraintestinal production of TG2 antibodies occurring for instance in the bone marrow, spleen and lymph nodes contributes to the appearance of mucosal IgA deposits remains to be addressed in future studies.

Conclusions

We conclude that the TG2-specific plasma cells are already present in the early phases of coeliac disease when the mucosal morphology is still normal, their percentage increases upon the development of villous atrophy and decreases on a GFD. Overall, the frequency of TG2 antibody-secreting plasma cells in the different phases of coeliac disease reflects the presence of gluten in the diet, but the frequency of these cells is not always parallel with serum TG2 antibody levels or the intensity of small intestinal TG2-targeting IgA deposits. Our findings widen the understanding of small-bowel mucosal TG2-specific plasma cells in coeliac disease and thus provide further insight into the generation of TG2 antibody responses.

Additional file

Additional file 1: Table S1. Correlations between the percentage of TG2-specific plasma cells and other study parameters. (DOCX 15 kb)

Abbreviations

EmA: Endomysial antibody; GFD: Gluten-free diet; HLA: Human leukocyte antigen; IEL: Intraepithelial lymphocyte; IgA: Immunoglobulin A; RT: Room temperature; TG2: Transglutaminase 2; Vh/CrD: Villous height-crypt depth ratio

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Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to restrictions defined by the Ethics Committee.

Authors' contributions

Conceived and designed the study: KL, KaKa. Conceived, designed, and performed the experiments: MH, OK, KL, PS. Analysed the data: MH. Performed the statistical analysis: MH, HH. Participated in patient recruitment and material sampling: OK, KuKa, TS, TI, KaKa. All authors have read, revised, and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland, and written informed consent was obtained from all participating subjects.

Consent for publication

Not applicable.

Competing interests

All authors have read the journal's policy on the disclosure of potential conflicts of interest and have none to declare.

Publisher's Note

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PUBLICATION II

Ex vivo culture of duodenal biopsies from dermatitis herpetiformis patients indicates that transglutaminase 3 antibody production occurs in the gut

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INVESTIGATIVE REPORT

Ex vivo Culture of Duodenal Biopsies from Patients with Dermatitis Herpetiformis Indicates that Transglutaminase 3 Antibody Production Occurs in the Gut

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Coeliac disease and dermatitis herpetiformis (DH) are characterized by autoantibodies targeting transglutaminase (TG)2 and TG3, respectively. Previous studies show that TG2 antibodies are produced in the gut and can be assessed in organ culture of small-intestinal biopsies from patients with coeliac disease. Thus far, no studies have investigated TG3 antibodies in organ culture of biopsies from patients with DH, or exploited the method in DH. The aim of this study was to investigate TG3 and TG2 antibody responses in serum and small-intestinal biopsies from patients with DH with active disease, and from those in remission. The majority of patients with DH were negative for both serum and organ culture medium TG2-targeting antibodies. Surprisingly, patients with active DH secreted TG3 antibodies into the culture medium despite seronegativity. In patients secreting high levels of TG3 antibodies into the culture medium, we also detected TG3-antibody-positive cells in the small-intestinal mucosa. These findings suggest that TG3 antibodies can be investigated in the organ culture system and that their secretion occurs in the small intestine, especially in active DH.

Key words: coeliac disease; dermatitis herpetiformis; transglutaminase; autoantibody.

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Malabsorption, diarrhoea and other gastrointestinal complaints are classical symptoms of coeliac disease, a systemic autoimmune-mediated condition occurring in a subset of individuals carrying the susceptibility genotype, human leucocyte antigen (HLA)-DQ2 or -DQ8. Typically, in patients with coeliac disease the ingestion of gluten, which is present in wheat, rye and barley, induces gradual destruction of the small-intestinal mucosal architecture, leading eventually to villous atrophy and crypt hyperplasia, as well as chronic inflammation within the intestinal epithelium and in the lamina propria. Active coeliac disease is further characterized by circulating gluten-dependent IgA endomysial autoantibodies (EMA) known to target transglutaminase 2 (TG2) (1). In addition to being present in serum, the autoantibodies are bound to TG2 in various tissues, including the small intestine (2), which is where they are produced (3–6).

Although classically associated with gastrointestinal symptoms, coeliac disease has a wide variety of extraintestinal manifestations. One of the best characterized of these is the cutaneous manifestation dermatitis herpetiformis (DH), a blistering autoimmune disorder characterized by granular IgA deposits in the papillary dermis (7). Belonging to the same spectrum of gluten sensitivity disorders, DH and coeliac disease share similar genetic predisposition, HLA-DQ2/8, and these different disease phenotypes can occur in the same families (8) and even in monozygous twins (9). Moreover, it has been reported that coeliac disease with classic enteropathy may evolve over time into DH on a gluten-containing diet (10, 11). The majority of patients with DH also show villous atrophy and crypt hyperplasia in the small intestine (12), and the remainder at least have signs of inflammatory changes characteristic of coeliac disease (13, 14). In addition, 70–80% of patients with DH have TG2-targeting autoantibodies in the serum (15) and small bowel mucosa (16). However, instead of TG2, the pathognomonic dermal IgA targets transglutaminase 3 (TG3), which is currently regarded as the main autoantigen in DH (17). The majority of patients with DH also have specific TG3 antibodies in the circulation (17, 18), which are also occasionally detected in the serum of patients with coeliac disease without skin symptoms (19). In contrast to TG2 antibodies, however, the site of TG3 antibody formation is, thus far, unknown.

Coeliac disease and DH are both treated with a gluten-free diet, which results in alleviation of symptoms and recovery of the small intestinal mucosa. Dietary treatment also alleviates DH rash, albeit often relatively slowly, and hence patients with severe skin symptoms are also treated with dapsone medication at the beginning of the gluten-free diet. During treatment, TG2-specific antibodies disappear from the serum and, although more slowly, from the small-intestinal mucosa in both coeliac disease and DH (20, 21). In DH, TG3-targeting antibodies also disappear from the serum concomitant with a gluten-free diet (22).

Previous studies have demonstrated that intestinal TG2-targeting antibodies at different stages of coeliac disease can be assessed in the organ culture system of small-intestinal biopsies (23–28). However, no studies are available for the TG3 antibody response and DH. The aim

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of the current study was therefore to better characterize the antibody responses targeting different TG isoforms in sera of patients with DH and those with coeliac disease and in organ culture of small-intestinal biopsies, both during active disease and in remission.

METHODS

Patients and small-intestinal mucosal biopsy samples

The study included a total of 17 patients with DH who had been diagnosed based on typical clinical picture and demonstration of granular IgA deposits in the papillary dermis (**Table I**) (29). Five of the patients had active DH in terms of rash and dermal IgA deposits despite being on a gluten-free diet (median duration 25 years, range 4–40 years) and needed dapsone medication to control the rash (defined as the active DH group). Two of these reported regular dietary lapses. The remaining 12 were following a strict diet (median duration 25 years, range 8–38 years) and were in clinical remission and not using dapsone (defined as the DH in remission group). Four of these 12 still had dermal IgA deposits. The DH patient cohort has been described in greater detail by Hervonen et al. (30). In addition, the study included a total of 20 patients with coeliac disease who had been diagnosed based on the demonstration of villous atrophy and crypt hyperplasia in small-bowel mucosal samples (31). Ten of these patients were enrolled at the time of diagnosis and were thus untreated (defined as the active coeliac disease group) and 10 had been on a gluten-free diet for one year (defined as the coeliac disease in remission group).

In the current study, all participants with active disease and in remission were invited to undergo upper gastrointestinal endoscopy, upon which small-bowel mucosal biopsies were taken. A total of 4 biopsy samples were obtained from each patient; 2 to assess small-intestinal mucosal findings and 2 for organ culture studies. In addition, serum samples taken at the time of the endoscopy were available. The study protocol was approved by the ethics committee of Tampere University Hospital, Tampere, Finland, and written informed consent was obtained from all participating subjects.

Small-intestinal mucosal morphology and inflammation

For morphological studies, one small-intestinal biopsy sample was fixed in formalin and processed for haematoxylin and eosin staining. The villous height-crypt depth ratios (Vh/CrD) were determined as described previously (32) and a ratio of ≥2 was considered normal. One small-intestinal biopsy was freshly embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek Europe, Holland), snap-frozen in liquid nitrogen and cut into 5-µm-thick sections. $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ intraepithelial lymphocytes (IELs) in the frozen sections were detected by immunohistochemistry, as described previously. The reference values were set at 37 cells/mm for $CD3^+$ IELs, 25 cells/mm for $\alpha\beta^+$ IELs, and 4.3 cell/mm for $\gamma\delta^+$ IELs (14).

Organ culture studies

Organ culture of small-intestinal mucosal biopsies was performed as described previously (33). Biopsies were cultured in RPMI-1640 medium (Invitrogen-Gibco, Paisley, UK) supplemented with 15% foetal bovine serum (Invitrogen-Gibco), 100 U/ml penicillin (Invitrogen-Gibco), 4 mM L-glutamine (Invitrogen-Gibco), 50 µg/ml insulin (Sigma-Aldrich Co, St Louis, MO, USA), 2 mg/ml glucose (Sigma-Aldrich) and 10 mM HEPES buffer (Invitrogen-Gibco) for 24 h at 37°C. From each patient one biopsy was cultured in the presence of medium only and one was subjected to a peptic-tryptic (PT) digest of gliadin (1 mg/ml), prepared, as described previously (34). Thereafter, culture supernatants were harvested and preserved at –20°C for further analysis.

IgA-class TG2 and TG3 autoantibodies in patient serum and organ culture medium

IgA-class endomysial antibodies (EMA) in serum and organ culture medium were determined by an indirect immunofluoresence method using human umbilical cord as substrate (35). In the case of serum samples, a dilution of 1:≥5 was considered positive. For organ culture medium, undiluted samples were used and EMA titres were graded, according to staining intensity, as negative (–), weak positive (+), or strong positive (++).

TG2 and TG3 antibodies in serum and organ culture medium were measured by commercially available enzyme-linked immunosorbent assay kits (ELISA) (Celikey®, Phadia, Freiburg, Germany, and anti-heTG IgA ELISA, Immunodiagnostik AG, Bensheim, Germany, respectively). Measurements were performed according to the manufacturers' instructions in serum samples diluted 1:100 and in centrifuged, undiluted organ culture supernatants. The optimal cut-off values for serum sample positivity were ≥5 and >22 AU/ml for TG2 and TG3 antibodies, respectively.

Small-intestinal IgA

Small-intestinal mucosal IgA deposits and their co-localization with TG2 and TG3 was investigated on cryosections of small-intestinal mucosal biopsies. TG2-specific IgA deposits were stained by a direct immunofluorescence method using mouse monoclonal anti-TG2 antibody (CUB7402; NeoMarkers, Fremont, CA, USA) and fluorescein isothiocyanate (FITC)-labelled rabbit anti-human IgA antibody (Dako A/S, Glostrup, Denmark), as previously described (2). In coeliac disease, subepithelial IgA deposition can be found below the basement membrane along the villous and crypt epithelium and around mucosal blood vessels, whereas in normal small-bowel mucosa IgA is detected inside the plasma and epithelial cells (2). To determine the presence of TG3 in the small-bowel mucosa, sections were incubated with rabbit polyclonal anti-TG3 antibody (1:100; A015, Zedira, Darmstadt, Germany) for 1 h at room temperature (RT), followed by incubation with Alexa Fluor 568-conjugated goat anti-rabbit antibody (1:2000; Invitrogen) for 1 h at RT. Co-localization of TG3 with IgA was assessed by further incubating the sections with FITC-labelled rabbit anti-human IgA antibody (1:40; Dako A/S) for 15 min at RT.

Table I. Characteristics and small-intestinal mucosal findings in patients participating in the study

	Active DH, $n=5$	DH in remission, $n=12$	Active CD, $n=10$	CD in remission, $n=10$
Female; *n* (%)	4 (80)	8 (67)	4 (40)	6 (60)
Age, years, median (range)	56 (22–69)	64 (26–75)	48.5 (31–63)	53 (31–75)
Vh/CrD; mean (95% CI), ratio	2.0 (0.9–3.1)	3.1 (2.7–3.4)[a]	0.6 (0.2–1.0)[a]	2.7 (1.7–3.6)[b]
$CD3^+$ IELs; mean (95% CI), cells/mm	38 (27–50)[c]	47 (29–65)	79 (61–97)	64 (33–94)
$\alpha\beta^+$ IELs; mean (95% CI), cells/mm	28 (2–54)[c]	31 (21–42)	56 (42–70)	40 (20–59)
$\gamma\delta^+$ IELs; mean (95% CI), cells/mm	13.5 (8.7–18.3)[c]	14.4 (6.1–22.6)	29.1 (18.8–39.4)	20.7 (14.9–26.5)

[a]Data available for 9 patients. [b]Data available for 8 patients. [c]Data available for 3 patients.
Reference values set at 2.0 for villous height crypt depth ratio (Vh/CrD), 37 cells/mm for CD3+ IELs, 25 cells/mm for $\alpha\beta^+$ IELs and 4.3 cell/mm for $\gamma\delta^+$ IELs (14).
CD: coeliac disease; CI: confidence interval; DH: dermatitis herpetiformis; IELs: intraepithelial lymphocytes.

Immunofluorescence staining of recombinant TG2- and TG3-binding cells in the small intestine

Immunofluorescence staining of small-intestinal recombinant TG2- and TG3-binding cells was performed using a technique formerly established by Di Niro et al. (5) with minor modifications. Cryosections of small-intestinal biopsies 5-µm-thick were air-dried for 20 min at RT, washed with phosphate-buffered saline (PBS) and incubated with either biotinylated human recombinant TG2 (2 µg/ml; T002, Zedira) or TG3 (5 µg/ml; T024, Zedira) in 1% bovine serum albulin – phosphate-buffered saline (BSA-PBS) for 45 min RT. Biotinylation of both recombinant proteins was performed using EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Scientific, Waltham, MA, USA) according to manufacturer's instructions. After washing, rhodamine-labelled streptavidin (1:1000; KPL, Gaithersburg, MD, USA) in 10% BSA-PBS was applied for 30 min at RT. For double stainings, an additional incubation step with FITC-conjugated anti-human IgA antibody (1:40; Dako A/S) for 15 min at RT was applied.

Statistical analyses

Statistical analyses were performed using the non-parametric Wilcoxon test, Mann–Whitney test and Fisher's test, as appropriate. Correlation was evaluated using Spearman's correlation. A p-value <0.05 was considered statistically significant.

RESULTS

Two patients with active DH reporting dietary failures had abnormal Vh/CrD representing partial villous atrophy, and the remaining patients with DH had normal villous architecture (Table I). Vh/CrD was below normal in all patients with active coeliac disease; 4 had total, 2 had subtotal, and 3 had partial villous atrophy. In contrast, all patients with coeliac disease in remission evinced normal mucosal architecture. The mean levels of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ T cells were above the reference level in all 4 study groups. The levels of all IEL subsets were comparable between the active DH and DH in remission groups, but lower than in both of the coeliac disease patient groups (Table I).

Serum EMA was negative in all patients with DH irrespective of disease activity. In contrast, 90% of patients with coeliac disease with active disease and 30% of patients in remission had positive serum EMA (**Fig. 1**A). When assessing the secretion of EMA into the organ culture medium, only one patient with active DH was observed to secrete EMA, in contrast to 80% and 20% of patients with active coeliac disease and coeliac patients in remission, respectively (Fig. 1B). TG2 antibody results in patient serum samples paralleled the EMA findings, being negative in both DH patient groups, positive in all patients with active coeliac disease and negative in patients with coeliac disease in remission (Fig. 1C). Similarly, TG2 antibody levels in the organ culture medium were either negative or very low (<20 AU/ml) in both DH patient groups and in patients with coeliac disease in remission (Fig. 1D). In contrast, 70% of patients with active coeliac disease secreted noticeable amounts of TG2 antibodies into the organ culture medium (>50 AU/ml). The levels of TG2 antibodies in the organ culture medium correlated with those in serum ($R=0.394$, $p=0.017$). PT-gliadin had

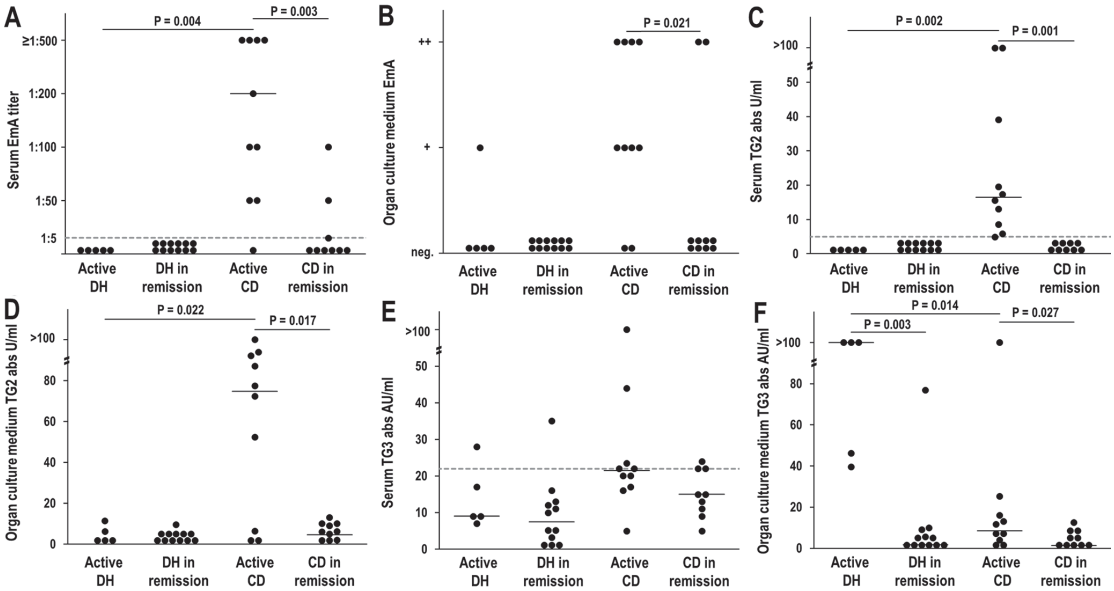

Fig. 1. Levels of antibodies in the serum and organ culture medium of dermatitis herpetiformis (DH) and coeliac disease (CD) patients. (A and B) Endomysial (EMA), (C and D) transglutaminase 2 (TG2) and (E and F) TG3 antibodies (abs) in DH and coeliac disease patient serum and organ culture medium as assessed by immunofluorescence staining or enzyme-linked immunoassay (ELISA). Horizontal lines represent median values, dashed lines cut-off values for positivity provided by the ELISA kit manufacturer for serum samples. Only p-values considered statistically significant (<0.05) are indicated.

no effect on the secretion of EMA or TG2 antibodies into the organ culture medium (data not shown).

When TG2 autoantibodies were assessed in the small-intestinal mucosa, none of the patients with DH in either group was found to have TG2-bound IgA in the basement membrane or around blood vessels (**Fig. 2**). This was in contrast to patients with coeliac disease, of whom all patients with active coeliac disease and 60% of DH patients in remission presented with such small bowel mucosal TG2-targeting IgA deposits.

Applying an approach previously used to identify TG2-specific plasma cells in the small-intestinal mucosa of patients with coeliac disease (5, 6), we further investigated the possible presence of such IgA-positive recombinant TG2-binding cells in the small intestine of our patients with DH and those with coeliac disease. They were not found in patients with DH of either group, with the exception of one patient with DH in remission. This patient had a detectable, but low, number of recombinant TG2-binding cells. In contrast, such cells were found in 8 out of 10 patients with active coeliac disease and in 4 out of 10 patients with coeliac disease in remission (Fig. 2).

Similarly, we next addressed the autoantibody response against the other TG isoform, TG3, at serum and small-intestinal level. Serum TG3 antibody levels were below the cut-off value (>22 AU/ml) in the majority of patients in all groups; only 1 patient with active DH and 1 with DH in remission had positive serum TG3 antibodies in addition to 3 patients with active coeliac patients and 1 in remission (Fig. 1E). Interestingly, however, TG3 antibody titres of ≥40 AU/ml were detected in the organ culture medium of all patients with active DH, one patient with DH in remission and one patient with active coeliac disease (Fig. 1F). There was no correlation between the TG3 antibody levels in the organ culture medium and serum ($R=0.241$, $p=0.157$). Moreover, PT-gliadin had no effect on the secretion of TG3 antibodies into the organ culture medium in any of the patient groups (data not shown).

When double stainings for TG3 and IgA were performed to establish the presence of IgA antibodies bound to TG3 in the small-bowel mucosa (similarly to coeliac-type TG2-bound IgA deposits), no extracellularly located TG3, and thus no TG3-targeting IgA deposits, were detected in the small-intestinal sections. Instead, we were able to detect TG3-positive cells, the majority of which were also positive for IgA (**Fig. 3**). A few such cells were present in 3 and 4 patients with active DH and DH patients in remission, respectively. In patients with active coeliac disease,

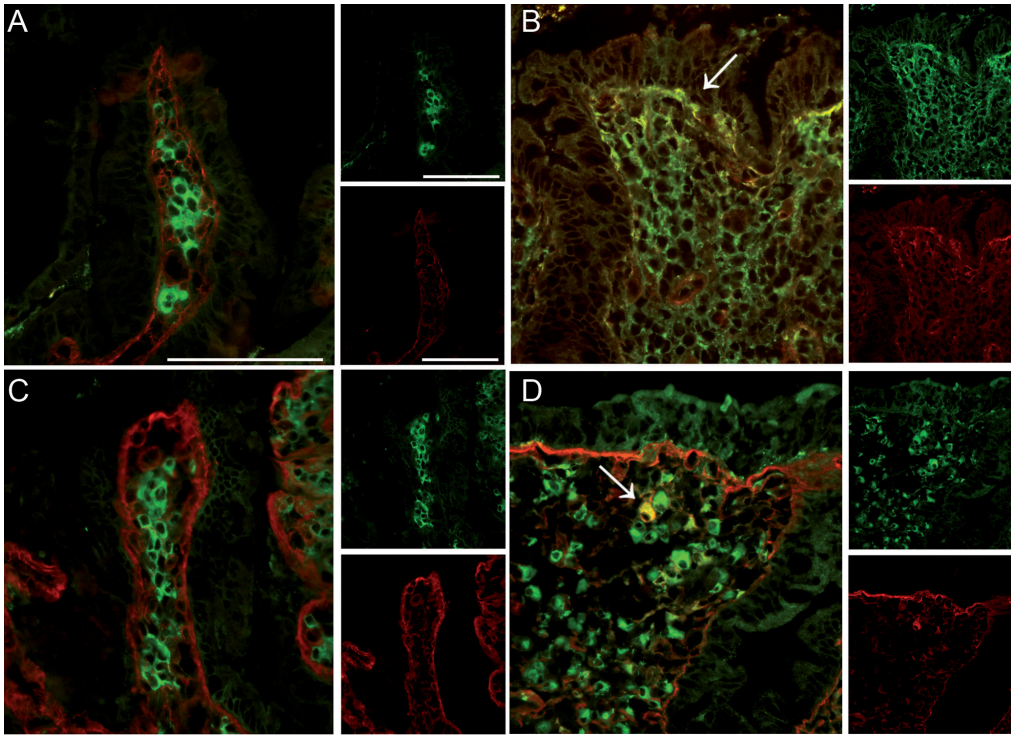

Fig. 2. Transglutaminase 2 (TG2)-targeting IgA deposits as well as recombinant TG2-binding, IgA-positive cells in the small intestine of patients with active dermatitis herpetiformis (DH) and those with coeliac disease. Small-intestinal TG2-targeting IgA deposits (A) were not present in patients with DH, but (B) were evident in patients with coeliac disease (*arrow*). (C) Recombinant TG2-binding IgA-positive cells were generally not detected in active patients with DH, but (D) were found in the majority of patients with active coeliac disease (*arrow*). TG2 or recombinant TG2 (*red*), IgA (*green*) and their co-localization (*yellow*) at 20× magnification. Scale bar =100 µm in all panels.

such cells were found in 4 out of 10 patients, whereas in patients with coeliac disease in remission no such cells were detected (Fig. 3). The presence and number of the cells was not associated with TG3 antibodies in the serum or the organ culture medium.

Finally, when the presence of IgA-positive cells binding recombinant human TG3 in the small-intestinal sections was investigated, such cells were found at low frequency in 3 out of 5 patients with active DH (Fig. 3). These 3 patients were those secreting the highest levels of TG3 antibodies into the organ culture medium (> 100 AU/ml). In addition, such cells were also detected in the one patient with active coeliac disease secreting TG3 antibodies into the organ culture medium, whereas no such cells were visualized in patients with DH in remission or the rest of the patients with coeliac disease.

DISCUSSION

The present study showed that, even though the majority of patients with active DH were negative for serum TG3 antibodies, they all secreted these antibodies into the organ culture medium. In contrast, patients with active coeliac disease had higher levels of TG3 antibodies in the serum, but low levels of secretion into the organ culture medium. Thus, the levels of TG3 autoantibodies in serum and organ culture medium did not parallel each other, in contrast to EMA and TG2 antibodies, which were mostly absent in all patients with DH, but present in patients with active coeliac disease in both type of samples.

The presence of TG2-targeting autoantibodies in the organ culture medium of coeliac disease patient-derived small-intestinal biopsies has been held to be explicable by the detachment of the autoantibodies from tissue-bound deposits and their release into the culture supernatant (28). This hypothesis seemed plausible, as all of our patients with considerable amounts of organ culture antibodies also had intestinal TG2-targeting deposits. We therefore tested whether this could also explain the presence of TG3 antibodies in the organ culture medium. We observed, however, that TG3 was not found extracellularly in the small intestine of any of our patients, which is in line with one previous study (17), and thus, the TG3 autoantibodies in the organ culture medium are not derived from detached mucosal TG3-targeted IgA deposits. Interestingly, however, a subset of patients with DH and those with coeliac disease were found to have occasional TG3-positive cells, the majority of them also being positive for IgA, but the

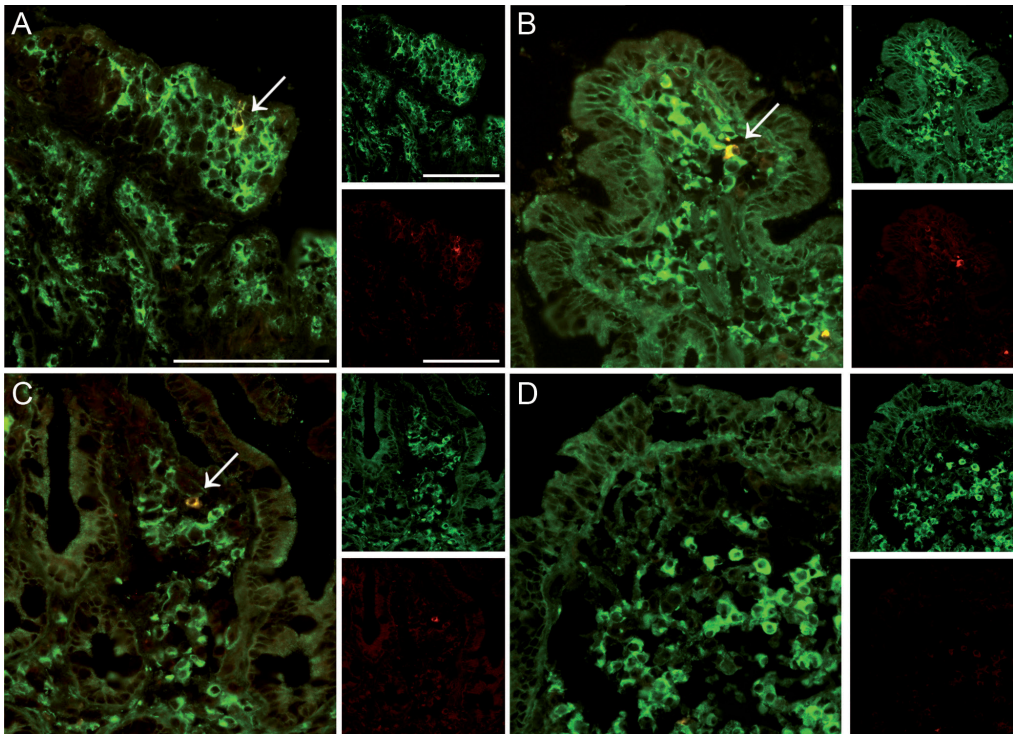

Fig. 3. Transglutaminase 3 (TG3)-positive cells, as well as recombinant TG3-binding, IgA-positive cells in the small intestine of patients with active dermatitis herpetiformis (DH) and coeliac disease. TG3- and IgA-positive cells were present in a few patients with (A) DH (*arrow*), and (B) coeliac disease (*arrow*). Recombinant TG3-binding IgA-positive cells (*arrow*) were detected in (C) patients with DH, but (D) were generally absent in patients with coeliac disease. TG3 or recombinant TG3 (*red*), IgA (*green*) and their co-localization (*yellow*) at 20× magnification. *Scale bar* = 100 µm in all panels.

presence and amount of these cells did not parallel TG3 autoantibodies in the culture medium. Whether these cells are antigen-presenting cells which have been suggested to express TG3 (36) remains to be determined in future studies, together with their possible involvement in DH and coeliac disease.

In addition, TG2 antibodies in the organ culture medium have been suggested to be secreted by TG2-specific plasma cells (3, 26, 37), which have recently been described in the small-intestinal mucosa of patients with coeliac disease (5, 6). In our study, those patients secreting TG2 antibodies into organ culture medium had, with the exception of one individual, recombinant TG2-binding cells in the lamina propria. Investigating the presence of TG3-antibody secreting cells in a similar manner, we detected IgA-positive recombinant TG3-binding cells in those 3 patients with DH and one patient with coeliac disease secreting the highest levels of TG3 antibodies into the organ culture medium. Even though the presence of such cells did not entirely coincide with the secretion of TG3 antibodies into the organ culture medium, our findings would indicate the TG3-binding IgA-positive cells as the source of the antibodies in the medium. It would therefore appear that, similarly to TG2-targeting antibodies (3–6), TG3 antibodies are also secreted at the small intestinal level, particularly in active DH. However, our results do not exclude the possibility that TG3 antibodies are also produced in other tissues. In fact, extraintestinal TG3 antibody secretion occurring, for instance, in bone marrow, spleen and lymph nodes could possibly explain the presence of these antibodies in the serum of those patients with coeliac disease who are negative for organ culture medium antibodies and intestinal TG3-binding IgA-positive cells.

On the other hand, the inconsistency between the general TG3 seronegativity of the patients with active DH and the presence of these antibodies in the organ culture medium along with TG3-binding intestinal cells can probably be explained by other factors. As 2 of our patients with active DH admitted regular dietary transgressions, this continued gluten intake might be sufficient to maintain antibody production locally only in the small intestine, as suggested previously (6). Moreover, it has been shown recently that, in DH, circulating TG3 antibodies can exist as immune complexes with TG3 even without free TG3 antibodies (18), which might provide another explanation for the seronegativity of the patients.

The pathognomonic IgA in DH has been suggested to be deposited in the dermis as immune complexes with TG3, which is found at this location only in DH (17). All our active DH patients and 33% of those in remission had cutaneous IgA deposits. Moreover, in active DH IgA always co-localizes with TG3 (38) and we recently showed that they disappear in parallel during a gluten-free diet, supporting their dermal deposition as immune complexes (39). Although the results of the current study point towards an intestinal origin of TG3 autoantibodies, the site of immune complex formation remains unknown.

The major strength of the present study is the well-defined cohorts of patients with DH and coeliac disease at different stages of disease. Moreover, the antibody responses against the 2 TG isoforms were studied in multiple settings, namely serum, organ culture medium and small bowel-mucosal biopsies, addressing both the presence of the antibodies as well as their possible origin. A major weakness is the particular nature of the DH patient groups. In contrast to patients with active coeliac disease, the patients with active DH were not newly diagnosed, but had active disease despite a gluten-free diet. This very likely explains many of the inconsistencies in antibody response between these patient groups, including the TG2 and TG3 seronegativity of the active DH patient group. Moreover, the patients with DH in remission had been on a gluten-free diet for a considerably longer time than the patients with coeliac disease in remission. Another limitation is the 24-h duration of the organ culture, which, even though most commonly used, may explain the unresponsiveness of autoantibody secretion to gluten in all patient groups, as also noted elsewhere (24, 25, 28, 37, 40).

Despite these limitations, our study provides important information on the autoantibody responses against TG2 and TG3 in DH and coeliac disease. Firstly, at least in our patient cohort the TG2 and TG3 autoantibody responses in serum and small-bowel mucosa were clearly different between patients with DH and those with coeliac disease. Secondly, our study revealed that, similarly to TG2 antibodies, TG3 antibodies can be detected not only in the serum, but also in the organ culture medium of small-intestinal mucosal biopsies. Most of all, our results strongly suggest that TG3 autoantibody secretion occurs in the small intestine, especially in active DH. Moreover, as the cells secreting these antibodies were found in patients with DH on a long-term gluten-free diet of up to 33 years, they must belong to an exceptionally long-lived plasma cell population (41). Even though the results of this study are not directly applicable to patients with DH at diagnosis or after short-term treatment, they provide a platform for future studies addressing this issue.

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The authors have no conflicts of interest to declare.

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PUBLICATION III

Intestinal transglutaminase 3- and transglutaminase 2-specific plasma cell responses in dermatitis herpetiformis patients undergoing a gluten challenge

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Submitted

PUBLICATION IV

Disappearance of epidermal transglutaminase and IgA deposits from the papillary dermis of dermatitis herpetiformis patients after a long-term gluten-free diet

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Research letter

Disappearance of epidermal transglutaminase and IgA deposits from the papillary dermis of patients with dermatitis herpetiformis after a long-term gluten-free diet

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Dear Editor, Dermatitis herpetiformis (DH) is an itchy, blistering skin disease characterized by the deposition of granular IgA in the papillary dermis. It is regarded as the cutaneous manifestation of coeliac disease, an autoimmune-mediated condition affecting the small intestine. In addition to skin symptoms, patients with DH have mostly subclinical small-intestinal villous atrophy and crypt hyperplasia or at least coeliac-type inflammatory changes. Moreover, patients with DH have specific antibodies targeting epidermal transglutaminase 3 (TG3), the dominant autoantigen in DH.[1] These IgA class TG3 antibodies can be found in the serum of the majority of

Table 1 Characteristics, duration of gluten-free diet (GFD), and cutaneous and serological findings in 33 patients with dermatitis herpetiformis at the time of the study and small-bowel mucosal histology at diagnosis and at the time of the study[a]

Sex and age (years) at diagnosis	Rash	GFD, years	IgA staining	TG3 staining	TG3 + IgA staining	Serum TG3 antibodies (AU mL^{-1})[b]	Small-bowel histology at diagnosis/present
Untreated group							
F/56	+	0	+++	+++	+++	na	SVA
M/57	+	0	+++	+++	+++	na	Normal
M/64	+	0	+++	++	+++	na	SVA
M/45	+	0	++	+++	+++	na	–
M/78	+	0	++	++	+++	na	PVA
F/20	+	0	++	++	++	na	–
M/60	+	0	++	++	++	na	Normal
Remission group							
M/23	–	33	+++	+++	+++	16	SVA/normal
M/21	–	19	++	++	++	6	PVA/na
M/54	–	11	++	++	++	2	PVA/na
M/60	–	5	++	++	++	21	SVA/normal
M/40	–[c]	29	+	+	++	13	PVA/normal
M/37	–	22	+	+	+	4	PVA/na
M/28	–	22	+	+	+	36	PVA/na
F/56	–	10	+	+	+	5	PVA/normal
M/12	–	38	–	–	–	10	PVA/normal
M/7	–	38	–	–	–	< 2	PVA/normal
M/53	–[c]	31	–	–	–	< 2	Normal/normal
M/32	–	31	–	–	–	< 2	PVA/normal
M/24	–	29	–	–	–	8	SVA/na
M/35	–	27	–	–	–	4	PVA/na
F/40	–	25	–	–	–	2	PVA/normal
M/42	–	24	–	–	–	12	SVA/PVA
F/43	–	22	–	–	–	< 2	PVA/normal
F/53	–	10	–	–	–	5	SVA/normal
M/66	–[c]	9	–	–	–	11	PVA/normal
M/21	–	8	–	–	–	35	PVA/normal
M/36	–	8	–	–	–	6	PVA/na
Active disease group							
M/28	+[d]	40[e]	+++	+++	+++	9	SVA/PVA
M/20	+[d]	36	+++	+++	+++	23	SVA/na

(continued)

Table 1 (continued)

Sex and age (years) at diagnosis	Rash	GFD, years	IgA staining	TG3 staining	TG3 + IgA staining	Serum TG3 antibodies (AU mL^{-1})[b]	Small-bowel histology at diagnosis/present
F/44	+[d]	25	++	+	++	17	SVA/normal
M/25	+[d]	28[c]	+	+	++	28	PVA/PVA
M/12	+[d]	9	+	+	++	9	SVA/normal

TG3, transglutaminase; F, female; M, male; na, not available; SVA, subtotal villous atrophy; PVA, partial villous atrophy. [a]Seven patients were untreated (untreated group), 21 were in clinical remission and had no rash on a GFD (remission group) and five were nonresponsive, i.e. had active rash and needed dapsone during long-lasting GFD treatment (active disease group). Presence of IgA and TG3 in the papillary dermis was examined with single and double stainings and the results interpreted as strong (+++), moderate (++) and weak (+) positive or negative (−). The small-bowel mucosal findings were graded as normal villous architecture, PVA or SVA. [b]Cut-off value for serum TG3 antibody positivity > 22 AU mL^{-1}; [c]dapsone (7·5–25 mg daily) stopped at the time of the skin biopsy; [d]dapsone (12·5–100 mg daily) needed to control the rash; [e]GFD not strict.

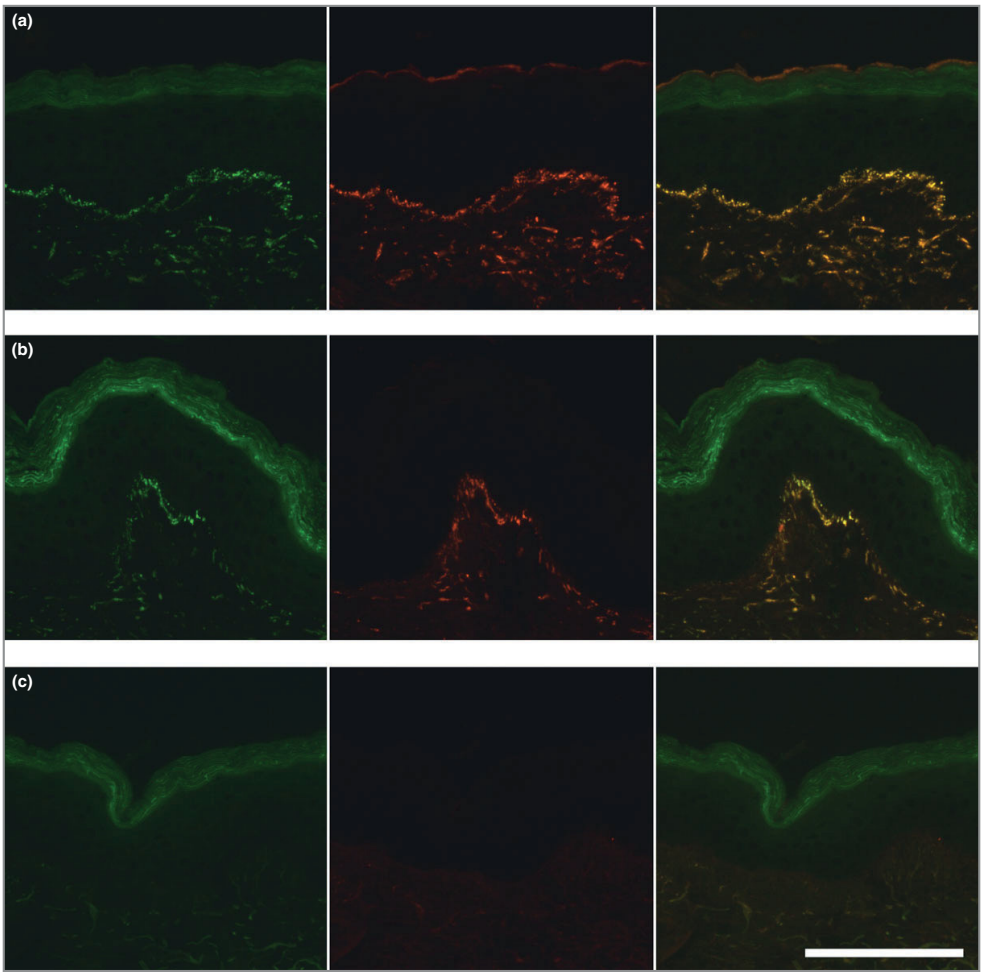

Fig 1. Immunofluorescence staining for transglutaminase 3 (TG3) and IgA in skin sections of patients with dermatitis herpetiformis (DH). Representative pictures of sections from (a) a patient with untreated DH showing strong (+++) staining; (b) a gluten-free diet (GFD)-treated patient showing moderate (++) staining and (c) a GFD-treated patient showing negative (–) staining. TG3 is shown in green, IgA in red and their colocalization in yellow. Scale bar = 100 μm in all panels.

untreated patients.[2] Moreover, the pathognomonic IgA at dermoepidermal junctions also targets TG3 that is localized at this site only in DH.[1]

The treatment of choice for DH is a lifelong strict gluten-free diet (GFD), during which the dermal IgA disappears slowly. However, after several years on a GFD, when the rash has healed, IgA is still present in the majority of patients.[3–5] Whether the disappearance of TG3 from the papillary dermis follows a similar or longer time span is not known. With this in mind, we investigated 33 patients with DH: seven in which the DH was untreated with active rash at the time of diagnosis (untreated group); 21 with DH in clinical remission adhering to a strict GFD (mean duration 22 years, range 5–38) (remission group); and five with active disease following either a strict (n = 3) or partial (n = 2) GFD (mean duration 28 years, range 9–40) (active disease group) for the presence of dermal TG3 and IgA (Table 1).

Skin biopsies were taken from perilesional skin or, when there was no rash, routinely from normal-appearing forearm skin. Sections cut from the biopsies were stained using FITC-conjugated rabbit polyclonal TG3 antibody (1 : 100) (A030, Zedira, Darmstadt, Germany) or, alternatively, a goat polyclonal TG3 antibody (1 : 25)[6] as the primary antibody, followed by Alexa Fluor 488-conjugated antigoat antibody (1 : 500) (705–546–147, Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) as the secondary antibody. For double stainings, the sections were further incubated with TRITC-conjugated goat antihuman IgA (1 : 50) (A18786, Thermo Fisher Scientific, Waltham, MA, U.S.A.). IgA class TG3 antibody levels in patient serum were determined using an antihuman-epidermal-transglutaminase IgA enzyme-linked immunosorbent assay kit (Immundiagnostik, Bensheim, Germany). Moreover, data on small-bowel mucosal morphology at diagnosis and at the time of the study was available for a subset of patients. The study protocol was approved by the ethical committee of Tampere University Hospital, Tampere, Finland.

All patients in the untreated and active disease groups with active rash showed strong or moderate IgA deposits in the papillary dermis, which colocalized with TG3 (Table 1, Fig. 1). In contrast, of the 21 patients in the remission group, only eight had IgA and TG3 in the papillary dermis whereas the rest were negative for both. Apart from three individuals, the staining intensities of IgA and TG3 were of the same magnitude (Table 1). Corresponding results were obtained with a noncommercial TG3 antibody[6] used previously in addressing TG3 deposition in the skin of patients with DH (data not shown).[7]

The eight patients in remission but with IgA and TG3 deposits had been on the strict GFD diet for a mean of 19 years (range 5–33) and the 13 without any deposits a mean of 22 (range 8–38) years (Table 1). The presence or absence of IgA and TG3 deposits did not thus show any correlation with the duration of GFD treatment ($P = 0{\cdot}078$).

Serum TG3 antibodies were positive in only two individuals in the active disease group and in two in the remission group (Table 1), and there was no association with the antibody levels and the presence of cutaneous IgA and TG3. Moreover, the absence or presence of IgA and TG3 deposits was not associated with small-bowel mucosal damage at diagnosis or at the time of the study or dapsone medication.

To conclude, we have demonstrated that IgA and TG3 disappear in parallel from the papillary dermis of patients with DH, as seen in a previous study showing that TG3 is always found in conjunction with IgA in active DH.[7] The disappearance is particularly slow and occurs only in a subset of patients despite a long and strict GFD. In addition, it appears that the response to GFD treatment in terms of rash and small-bowel mucosal damage was equally good regardless of the presence of dermal IgA deposits. Altogether, the present findings provide further evidence for the prevailing view that IgA and TG3 exist as immune complexes in the skin of patients with DH.[1,8,9]

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