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# CHARACTERIZING THE AUTOIMMUNE RESPONSES IN DERMATITIS HERPETIFORMIS

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# ABSTRACT

Henna Lehtola: Characterizing the Autoimmune Responses in Dermatitis Herpetiformis
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Dermatitis herpetiformis (DH) is a cutaneous manifestation of celiac disease (CD) associated with an itchy blistering rash typically on the elbows, knees and buttocks. The disease is driven by the response to dietary gluten and characterized by granular deposits of immunoglobulin A (IgA) targeting transglutaminase (TG) 3 in the papillary dermis. The skin symptoms disappear by following a strict gluten-free diet (GFD), but the anti-TG3 IgA deposits may persist in the skin for years after starting the diet. Although DH is considered a T cell mediated disease, the T cell responses are still poorly understood in DH. Plenty of cytokines, *e.g.* interleukin (IL)-4, IL-8 and IL-17, have been linked to DH. To date, no conclusive evidence has been presented, however, for their exact role in the DH pathogenesis. Therefore, comprehensive systemic cytokine profiling studies are needed. While CD is considered a T helper ($T_H$) 1-mediated autoimmune disorder, $T_H$2-type cytokines seem to dominate the molecular findings linked to DH. The primary aim of this thesis was to characterize the systemic cytokine profile of DH patients and to evaluate whether it involves different effector T cell population than seen in CD, further explaining the cutaneous manifestation of DH. The second aim was to investigate the tissue origin of the circulating cytokines in DH patients.

First, we used multiplex cytokine assay to characterize the serum cytokine pattern of untreated DH patients. Second, we investigated the serum cytokine response of treated DH patients to gluten re-exposure using enzyme-linked immunosorbent assay (ELISA). Lastly, we used indirect immunofluorescence microscopy to study the cytokine expression in the two primary disease associated tissues - the skin and the small intestinal epithelium. We compared the cytokine expression to CD patients without skin lesions. As controls, we included patients with other skin diseases without CD or DH, and healthy subjects.

To our knowledge, this study presents the first comprehensive T cell cytokine profiling of DH patients. The results showed significant variation in the cytokine levels between untreated DH patients. IL-23 was the most elevated T cell cytokine in the serum of DH patients with higher average concentration compared to CD patients and controls. Treated DH patients did not respond to the gluten re-exposure through serum IL-4, IL-6, IL-8 or IL-23. Finally, we found perilesional and intestinal expression of IL-4, IL-6 and IL-23 in DH patients concurrent with the cytokine profiling. These findings suggest that the response to gluten in DH patients might be mainly mediated by $T_H$2- and $T_H$17 T cell subsets. The cutaneous expression of IL-4 as well as the intestinal expression of IL-23 could be characteristic for DH. Moreover, the elevated serum IL-8 potentially reflects increased secretion from the small intestine in DH patients.

In view of these results, this study suggests that the differences in the cytokine pattern of CD and DH patients could lead to these two separate manifestations of systemic gluten-induced autoimmune response. This supports the hypothesis of a switch occurring in the $T_H$-profile during the development of gluten-induced autoimmune response in DH, and potentially explains the two manifestations of CD. However, the exact role of the characterized cytokines in the pathogenesis of DH remains to be established.

Keywords: dermatitis herpetiformis, TG3, effector T cell, cytokine response, celiac disease

# TIIVISTELMÄ

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Dermatitis herpetiformis (DH) eli ihokeliakia on iholla esiintyvä keliakian (CD) ilmenemismuoto, johon liittyy kutiava rakkulainen ihottuma, tyypillisesti kyynärpäissä, polvissa ja pakaroissa. Ravinnon sisältämä gluteeni aiheuttaa sairaudelle tunnusomaisen immuunivasteen, joka todetaan verinahan päällimmäisestä kerroksesta löytyvien anti-transglutaminaasi (TG) 3 immunoglobuliini (IgA) -kertymien perusteella. Iho-oireet katoavat ehdotonta gluteenitonta ruokavaliota noudattamalla, mutta anti-TG3 IgA -kertymät voivat sen sijaan säilyä ihossa vielä vuosia ruokavalion aloittamisen jälkeen. Vaikka ihokeliakian ajatellaan olevan T-soluvälitteinen sairaus, sille ominaiset T-soluvasteet tunnetaan huonosti. Useita sytokiineja, kuten interleukiini (IL)-4, IL-8 ja IL-17, on liitetty ihokeliakiaan, mutta niiden tarkkaa roolia taudin synnyssä ja kehityksessä ei ole toistaiseksi esitetty. Tämän vuoksi tarvitaan entistä kattavampia systeemisiä sytokiinien profilointitutkimuksia. Vaikka keliakia luokitellaan T-auttaja ($T_H$) 1-välitteiseksi autoimmuunisairaudeksi, $T_H$2-tyypin sytokiinit näyttävät hallitsevan ihokeliakiaan liitettyjä molekulaarisia löydöksiä. Tämän tutkielman tavoitteena oli karakterisoida ihokeliakian systeemistä sytokiiniprofiilia ja arvioida, liittyykö siihen keliakiasta poikkeava efektori-T-solupopulaatio, mikä selittäisi DH:n ilmentymisen iholla. Lisäksi tutkielma pyrki selvittämään DH-potilaiden verenkierrossa esiintyvien sytokiinien kudosalkuperää.

Käytimme hoitamattomien DH-potilaiden seerumisytokiinien määrittämiseen multiplex -sytokiinimääritystä. Tutkimme, miten hoidettujen DH-potilaiden uudelleenaltistus gluteenille vaikuttaa potilaiden seerumin sytokiinitasoihin käyttämällä entsyymivälitteistä immunosorbenttimääritystä (ELISA). Lisäksi käytimme epäsuoraa immunofluoresenssimikroskopiaa tutkiaksemme sytokiinien ilmentymistä kahdessa ihokeliakiaan ensisijaisesti liitetyssä kudoksessa – ihossa ja ohutsuolen epiteelikudoksessa. Vertasimme sytokiinien ilmentymistä CD-potilaisiin, joilla ei ole DH:lle ominaisia ihovaurioita. Kontrolleina käytimme terveitä henkilöitä sekä potilaita, joilla on todettu jokin muu ihosairaus ilman keliakiaa.

Tietääksemme, tämä tutkimus on ensimmäisen kattava DH-potilaiden T-solusytokiinitutkimus. Tulokset osoittivat huomattavaa sytokiinitasojen vaihtelua hoitamattomien DH-potilaiden välillä. IL-23 oli kohonnein T-solusytokiini DH-potilaiden seerumissa ja sen keskimääräinen seerumikonsentraatio oli DH-potilailla korkeampi verrattuna CD-potilaisiin ja kontrolleihin. Hoidettujen DH-potilaiden IL-4, IL-6, IL-8 ja IL-23-seerumitasot eivät reagoineet gluteenialtistukselle. Osoitimme, että IL-4:n, IL-6:n ja IL-23:n ilmentyminen ihovaurioiden ympärillä sekä ohutsuolessa on yhteneväistä esitetyn DH-potilaiden sytokiiniprofiilin kanssa. Nämä löydökset esittävät, että DH-potilaiden vaste gluteenille saattaa olla pääasiallisesti $T_H$2- ja $T_H$17-välitteinen. IL-4:n ilmentyminen iholla, kuin myös IL-23:n ilmentyminen suolessa, voisi olla ihokeliakialle ominaista. Lisäksi koholla oleva seerumin IL-8-pitoisuus kuvastaa mahdollisesti sen lisääntynyttä eritystä DH-potilaiden ohutsuolesta.

Näiden löydösten valossa tämä tutkimus esittää, että DH:n ja CD:n sytokiiniprofiilien erot voisivat johtaa näiden kahden toisistaan poikkeavan gluteeniriippuvaisen systeemisen autoimmuunivasteen ilmentymiseen. Tämä tukee hypoteesia siitä, että gluteenin aiheuttaman autoimmuunivasteen kehittymisen aikana ihokeliakiassa tapahtuu $T_H$-profiilin vaihdos, mikä mahdollisesti selittää keliakian kaksi erilaista ilmenemismuotoa. Edellä kuvattujen sytokiinien tarkka rooli ihokeliakian synnyssä ja kehittymisessä on kuitenkin vielä selvittämättä.

Avainsanat: dermatitis herpetiformis, ihokeliakia, TG3, efektori-T-solu, sytokiinivaste, keliakia

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*Henna Lehtola*

# ABBREVIATIONS

| | |
|---|---|
| APC | Antigen-presenting cell |
| BCR | B cell receptor |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium ion |
| CD | Celiac disease |
| DAPI | 4',6-diamidino-2-phenylindole |
| DGP | Deamidated gliadin peptide |
| DH | Dermatitis herpetiformis |
| DIF | Direct immunofluorescence |
| ELISA | Enzyme-linked immunosorbent assay |
| GFD | Gluten-free diet |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GSE | Gluten sensitive enteropathy |
| HLA | Human leukocyte antigen |
| HRP | Horseradish peroxidase |
| IBS | Irritable bowel syndrome |
| IEL | Intraepithelial lymphocyte |
| IF | Immunofluorescence |
| IFN | Interferon |
| IgA | Immunoglobulin A |
| IL | Interleukin |
| ITAC | Interferon-inducible T-cell alpha chemoattractant |
| MIP | Macrophage inflammatory protein |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate-buffered saline |
| PSF | Point spread function |
| RCD | Refractory celiac disease |
| RT | Room temperature |
| TCR | T cell receptor |
| TGF | Transforming growth factor |
| TG2 | Tissue transglutaminase |
| TG3 | Epidermal transglutaminase |
| $T_H$ cell | T helper cell |
| TNF-α | Tumor necrosis factor alpha |
| $T_{reg}$ | Regulatory T cell |

# CONTENTS


1. INTRODUCTION ..... 1
2. LITERATURE REVIEW ..... 2
   2.1 Overview to Dermatitis Herpetiformis ..... 2
      2.1.1 Historical Background ..... 3
      2.1.2 Clinical Presentation ..... 3
      2.1.3 Epidemiology ..... 4
      2.1.4 Diagnosis ..... 5
      2.1.5 Treatment ..... 6
   2.2 Pathogenesis of Dermatitis Herpetiformis ..... 7
      2.2.1 Genetic and Environmental Factors ..... 8
      2.2.2 Intestinal Manifestations ..... 9
      2.2.3 Cutaneous Manifestations ..... 10
   2.3 Immunopathogenic Aspects of Dermatitis Herpetiformis ..... 11
      2.3.1 Neutrophil Infiltration ..... 13
      2.3.2 Effector T Cells ..... 14
      2.3.3 Development of B Cell and Plasma Cell Responses ..... 15
      2.3.4 T Cell Cytokine Signaling ..... 17
3. AIMS OF THE STUDY ..... 21
4. MATERIALS AND METHODS ..... 22
   4.1 Characterization of Serum Cytokines ..... 23
      4.1.1 Milliplex Immunoassay ..... 23
      4.1.2 Enzyme-Linked Immunosorbent Assay (ELISA) ..... 24
      4.1.3 Statistics ..... 25
   4.2 Characterization of Tissue Origin of Cytokines ..... 26
      4.2.1 Immunofluorescence Staining and Imaging ..... 26
5. RESULTS ..... 28
   5.1 Serum Cytokines in Dermatitis Herpetiformis ..... 28
   5.2 Tissue Origin of Cytokines in Dermatitis Herpetiformis ..... 31
6. DISCUSSION ..... 39
   6.1 Possible Limitations of the Study ..... 40
   6.2 Future Aspects ..... 41
7. CONCLUSIONS ..... 42
REFERENCES ..... 43

# 1. INTRODUCTION

Dermatitis herpetiformis (DH) is a cutaneous manifestation of celiac disease (CD) associated with an itchy blistering rash typically on the elbows, knees and buttocks, and like CD, is driven by T cell mediated autoimmune response initiated by dietary gluten in wheat, barley and rye. DH is characterized by granular deposits of immunoglobulin A (IgA) targeting transglutaminase (TG) 3 in the papillary dermis (Sárdy et al., 2002). These autoantibodies are also found from the circulation of most DH patients (Sankari et al., 2020; Sárdy et al., 2002), and usually disappear with the skin symptoms on a strict gluten-free diet (GFD). The anti-TG3 IgA deposits, instead, may persist in the skin for years despite dietary adherence (Bardella et al., 2003; Hietikko et al., 2018). Varying degree of small intestinal damage typical in CD, *i.e.* villous atrophy and crypt hyperplasia, are also exhibited in DH patients (Alakoski et al., 2012; Marks et al., 1966).

In recent decades, considerable progress has been made in understanding the immunopathogenesis of DH, but it has still remained undiscovered, how the autoimmune responses in the small intestine progress into the skin. This is mainly suggested to be a result of epitope spreading from another member of the TG family, TG2, to TG3 or cross-reactivity between these two proteins (Kárpáti et al., 2018). Secondly, although DH is considered a T cell mediated disease, the early T cell responses are still poorly understood in DH, including the gluten-induced T cell responses. Plenty of cytokines, *e.g.* IL-4, IL-8 and IL-17 have been sporadically linked to DH (Caproni et al., 2021; Hall et al., 2007; Żebrowska et al., 2013), primarily based on the positive correlation between serum levels and disease status or gluten exposure. Thereby, there is no conclusive evidence for their exact role in the DH pathogenesis, and comprehensive systemic cytokine profiling studies are needed (Kemppainen et al., 2021). It is intriguing that while CD is considered a $T_H1$-mediated autoimmune disorder (Nilsen et al., 1995), $T_H2$-type cytokines seem to dominate the molecular findings linked to DH (Kemppainen et al., 2021). In this Thesis, the aim was to define the T cell cytokine responses regulating the pathogenesis of DH and to assess whether such a profound difference between these two manifestations of the same disease could truly exist.

# 2. LITERATURE REVIEW

## 2.1 Overview to Dermatitis Herpetiformis

Dermatitis herpetiformis (DH) is a cutaneous manifestation of celiac disease (CD), an autoimmune-mediated condition affecting the small intestine. DH manifests as an itchy blistering rash typically on the elbows, knees and buttocks. CD like enteropathy, including villous atrophy, crypt hyperplasia and increased number of intraepithelial lymphocytes, also occur in DH (Brow et al., 1971; Järvinen et al., 2003). DH arises from intolerance toward dietary gluten found in wheat, barley and rye. Despite having different primary manifestations, DH and CD share the human leukocyte antigen (HLA)-DQ2 and -DQ8 mediated susceptibility (Balas et al., 1997; Karell et al., 2002). This is supported by the common occurrence of DH and CD in families (Hervonen et al., 2002; Reunala, 1996).

DH is characterized by granular deposits of immunoglobulin A (IgA) in the papillary dermis targeting transglutaminase (TG) 3, which is considered as the primary autoantigen in DH (Sárdy et al., 2002). These IgA autoantibodies likely originating from the small intestine are also found from the circulation of most DH patients (Sankari et al., 2020; Sárdy et al., 2002). In addition to TG3 autoantibodies, most DH patients are seropositive for antibodies against TG2, another member of the TG family and the main autoantigen in CD (Dieterich et al., 1999). The circulating autoantibodies and the skin symptoms disappear as a result of strict gluten-free diet (GFD), the treatment of choice. However, the anti-TG3 IgA deposits may persist in the skin for years (Bardella et al., 2003; Hietikko et al., 2018). Moreover, the anti-TG3 IgA antibodies are able to form immunocomplexes with TG3 (Sárdy et al., 2002), but the role of these immunocomplexes in the pathogenesis of DH is unclear.

In recent decades, considerable progress has been made in understanding the immunopathogenesis of DH, but it is still a riddle, how the autoimmune responses in the small intestine progress into the skin. This is suggested to be a result of epitope spreading from TG2 to TG3 or cross-reactivity between them (Kárpáti et al., 2018). There is also a possibility that the epitope spreading occurs between gliadin and TG3, mediated by anti-TG3 B cells interacting with anti-gliadin T cells, as Kaunisto et al. (2022) have suggested. The early T cell responses are poorly understood in DH, including the gluten-induced T cell responses. Recently, by identifying phenotypic markers of gluten-reactive T cells in DH patients, Risnes et al. (2022) suggested that the gluten-induced T cell responses in DH have similar characteristics to CD. While CD is considered a $T_H1$-dominant disorder (Nilsen et al., 1995), the expression of $T_H2$-type and $T_H17$-type cytokines have been linked to DH (Caproni et al., 1998; Smith et al., 1999; Żebrowska et al., 2013). However, there are no solid evidence for their exact role in the immunopathogenesis of DH.

### 2.1.1 Historical Background

The history of DH begins in 1884, when American dermatologist Louis Duhring (1884) described DH for the first time and named it after its appearance similar to herpes. The second notable step in the early history of DH took place in 1952, when Esteves and Brandao (1952) discovered dapsone as an effective treatment for the skin symptoms of DH. In the late 1960s, the association between DH and CD started to reveal. Structural abnormality of the small intestine typical in CD was detected in DH patients, first by Marks et al. (1966), and after a year from that by Fry et al. (1967) with a suggestion that the intestinal abnormality occurs due to gluten sensitivity. The suggestion by Fry et al. (1967) proved to be true when it was discovered that GFD has an improving effect on intestinal lesions, as well as on skin lesions (Fry et al., 1968; Marks & Whittle, 1969; Reunala et al., 1977). It was further confirmed by gluten challenge studies, first by Fry et al. (1969), with the demonstration of the relapse of skin and intestinal symptoms as a result of gluten reintroduction, proving that DH is indeed gluten dependent disease and requires continual GFD (Kósnai et al., 1986; Leonard et al., 1983).

After the increased incidence of symptoms inside families of DH patients was discovered and high frequency of small intestinal abnormalities among DH patients was proved, scientists came to a conclusion that the connection between DH and CD could be genetic (Brow et al., 1971; Marks & Shuster, 1971; May & Roberts, 1971). Soon after that, Katz et al. (1972) discovered the genetic link between DH and CD, although knowing already then that the genetic factor cannot be the only factor involved in their pathogenesis. In the late 1990s, Balas et al. (1997) confirmed that DH and CD patients share the HLA-mediated susceptibility.

In 1969, a major advance in understanding DH was made, when Van der Meer (1969) revealed the granular IgA deposits in the papillary dermis of the uninvolved skin of DH patients by immunofluorescence. Since then, IgA deposits in the skin have been considered as the hallmark of DH and thus used as the diagnostic finding of DH. The latest significant step in the study of DH was taken in the 21$^{st}$ century, when Sárdy et al. (2002) discovered that the primary autoantigen in DH is TG3 instead of TG2, the autoantigen in CD. In the same paper they also showed the colocalization of TG3 and IgA deposits in the papillary dermis of DH patients.

### 2.1.2 Clinical Presentation

The clinical picture of DH consists of small blisters, papules and erythema on the skin typically distributed symmetrically on elbows, knees and buttocks (Bolotin & Petronic-Rosic, 2011). The skin lesions can also appear on the scalp, face, upper back, abdomen and groin (Collin et al., 2017;

Reunala et al., 2021). The lesions induce an intense itch, which compels patients to scratch the affected area. As a result, the primary lesions are replaced by erosions, crusts and post-inflammatory hyperpigmentation that often dominate the clinical picture (Reunala et al., 2021).

Although intestinal biopsies have revealed a varying degree of villous atrophy and crypt hyperplasia in three-fourths of DH patients, obvious gastrointestinal symptoms are rarely experienced in DH (Alakoski et al., 2012; Reunala et al., 2021). Mainly occasional loose stools and minor gastrointestinal complaints have been described in up to one-third of patients (Collin & Reunala, 2003; Pasternack et al., 2017). The severity of mucosal lesions in the small intestine varies between patients, but all DH patients irrespective of the small intestinal morphology have increased densities of γδ+ T cells, a subset of intraepithelial lymphocytes (IEL), as has also been found to be the case with CD patients (Savilahti et al., 1992).

Dental abnormalities have been as well described in DH patients. In the study by Aine et al. (1992), mild celiac-type dental enamel defects were detected in over half of the patients. The defects included horizontal grooves, shallow pits and colour defects on the surface of enamel (Aine et al., 1992). In addition, DH may affect the oral mucosa. The mucosal findings consist of vesicles and erosions with possible soreness and burning sensation (Bolotin & Petronic-Rosic, 2011). Variable data on the prevalence of oral lesions has been reported without evidence that they are caused by DH alone (Bolotin & Petronic-Rosic, 2011; Lähteenoja et al., 1998).

### 2.1.3 Epidemiology

DH is a common extraintestinal manifestation of CD. While the prevalence of CD in Tampere University Hospital district has been reported to be 661 per 100 000, the prevalence of DH has been reported to be 75,3 per 100 000 at its highest (Salmi et al., 2011; Virta et al., 2009). Thus, the ratio between DH and CD is 1:8. Furthermore, the prevalence of DH has been estimated to be even 84 per 100 000 due to great migration into the hospital district (Salmi et al., 2011). The same prevalence ratio between DH and CD was calculated in the UK, where the national prevalence of CD and DH was 240 per 100 000 and 30 per 100 000, respectively (West et al., 2014). The prevalence of DH varies geographically, which has been mainly explained by genetic predisposition and differing wheat consumption habits (Bolotin & Petronic-Rosic, 2011; Kang et al., 2013). DH is most common in Europe and the US, where the prevalence has ranged between 10,4 and 75,3 per 100 000, Finland holding the highest reported prevalence to date (Salmi et al., 2011). In turn, the occurrence of DH is thought to be rare in Asian population due to prevalence of 10-34 per 150 000 000 in Japan, and even rarer among Africans (Bolotin & Petronic-Rosic, 2011; Ohata et al., 2012; Shibahara et al., 2002).

Studies conducted in Finland and the UK showed that the incidence of DH has decreased over the past decades (Salmi et al., 2011; West et al., 2014). In the Finnish study, the incidence showed significant decrease from 5.2 to 2.7 per 100 000 (Salmi et al., 2011), and in the UK study from 1.8 to 0.8 per 100 000 (West et al., 2014). Simultaneously, an increasing trend has been shown in the incidence rate of CD in European studies (Kang et al., 2013), and even a fourfold increase was observed in the UK between 1990 and 2011 (West et al., 2014). Exceptionally, also a declining trend has been reported in the incidence of CD in the 21st century in Finland, which is thought to be a result of high general awareness of CD and active case determination for more than two decades inside the country (Virta et al., 2017). The study also suggests that exposure to yet unidentified triggering factors for CD has plateaued among the Finnish adult population (Virta et al., 2017).

In theory, DH can occur at any age, but the mean age at diagnosis was 49 years in the early 2000s in Finland (Salmi et al., 2011). Since 1970, the mean age at diagnosis has increased significantly from 35 to 46 years in women and from 35 to 51 years in men (Salmi et al., 2011). Based on the Statistics Database of Natural Resources Institute Finland (online, referred: 10.12.2022), the annual average consumption of wheat, barley and rye per capita has decreased from 116 kg to 60 kg in a time frame from 1950 to 2021, which is suggested to explain the increase in the mean age at diagnosis (Reunala et al., 2018; Salmi et al., 2011). In two recent large studies (Salmi et al., 2011; West et al., 2014), the male to female ratio of DH has been found to be close to 1:1, suggesting that gender has less effect to DH than earlier studies have presented (Bolotin & Petronic-Rosic, 2011; Salmi et al., 2011).

### 2.1.4 Diagnosis

The diagnosis of DH is based on the presence of granular IgA deposits in the papillary dermis at the dermal-epidermal junctions (Bolotin & Petronic-Rosic, 2011; Caproni et al., 2009). Together with a compatible clinical picture, the detection of granular IgA deposits with direct immunofluorescence (DIF) is considered to be sufficient for DH diagnosis (Caproni et al., 2009). Since greater amounts of IgA deposits occur near the active lesions, the skin biopsy should preferably be taken from healthy-appearing area adjacent to the rash, called perilesional skin (Caproni et al., 2009; Reunala et al., 2021). Other itchy and blistering skin diseases, such as linear IgA disease and bullous pemphigoid, may resemble the clinical picture of DH, but are nevertheless distinguishable from DH by DIF examination (Collin et al., 2017; Reunala et al., 2021).

Beyond DIF, serological measurements can be used to support the diagnosis. DH patients often produce IgA autoantibodies against TG2, which can be detected by enzyme-linked immunosorbent

assay (ELISA) with over 90% specificity and sensitivity for the diagnosis of DH. Anti-TG2 autoantibodies are mostly confined to patients with small intestinal villous atrophy, and therefore the diagnosis of DH cannot be excluded by negative result (Collin & Reunala, 2003; Mansikka et al., 2017). However, with a compatible clinical picture, TG2 and EmA antibody levels can be used as a suggestive of DH and, in a case of positive diagnosis, as an indication of small bowel mucosal damage. If elevated, TG2 antibody measurement can further be utilized to monitor patient's adherence to GFD (Collin & Reunala, 2003; Salmi & Hervonen, 2020). Although circulating anti-TG3 autoantibodies are suggested to be highly specific marker of DH, their exact role and value are still somewhat unclear and therefore anti-TG3 autoantibody testing is not currently in clinical use (Borroni et al., 2013; Reunala et al., 2021; Salmi & Hervonen, 2020). Even though the mucosal changes in the small intestine vary from inflammatory changes to severe villous atrophy in DH, it has been shown that the severity of mucosal damage at diagnosis does not have effects on the long-term prognosis of DH (Mansikka et al., 2018). Moreover, serological tests and skin biopsies are sufficient to monitor GFD adherence after diagnosis. Thus, it has been rationalized that there is no need for routine small intestine biopsies at diagnosis (Caproni et al., 2009; Reunala et al., 2021; Salmi & Hervonen, 2020).

Despite the relatively specific clinical picture of DH, there are challenges with correct diagnosis in primary care. Clinical presentation of DH can be difficult to diagnose correctly by general practitioners, easily leading to misdiagnosis and delay in diagnosis of DH. In Finland, where the prevalence of DH has been reported to be the highest, a diagnostic delay of at least 2 years occurred in one-third of patients between 1970 and 2014 (Mansikka et al., 2018). During recent decades, the diagnostic delay has shortened possibly due to better guidelines available for physicians in Finland (Mansikka et al., 2018; Reunala et al., 2021).

### 2.1.5 Treatment

The treatment of choice for all DH patients is a strict, lifelong gluten-free diet (GFD) (Ludvigsson et al., 2014). On a GFD, gluten-containing wheat, barley and rye are excluded from the daily diet. Successful adherence to the GFD leads to relief of possible gastrointestinal symptoms within a few weeks and recovery of small intestinal mucosa within 1-3 years (Reunala et al., 2015). The rash and related pruritus starts to alleviate slowly (Garioch et al., 1994), in several weeks to months, and therefore a majority of the DH patients benefit from dapsone (4,4-diaminodiphenylsulfone) medication along with the GFD (Reunala et al., 2018). Dapsone is a sulfure drug with anti-inflammatory properties (Zhu & Stiller, 2001). By suppressing the neutrophil recruitment and activation on the lesional area, dapsone relieves the itchy rash within a few days, but does not affect the IgA deposits or enteropathy (Reunala et al., 2021). Thus, the idea is to slowly reduce the dapsone dose and

eventually end it when the GFD alone clears the rash, which usually takes 2 to 3 years (Garioch et al., 1994; Mansikka et al., 2018). Among large series of DH patients, only very few of them (1.7%) had a rash non-responsive to GFD and needed continuous dapsone treatment (Hervonen et al., 2016). The IgA-TG3 deposits can persist in the skin much longer than the rash itself, even after patients have been asymptomatic and on a strict GFD for several years (Hietikko et al., 2018).

It has been suggested that during GFD, a proportion of DH patients can achieve a tolerance to gluten (Bardella et al., 2003; Garioch et al., 1994; Paek et al., 2011), and consequently they can be re-introduced back to gluten-containing diet without relapsing. However, it seems that the development of gluten tolerance in DH is rare (Mansikka et al., 2019). In addition, DH patients especially with villous atrophy have been shown to have an increased risk of developing lymphomas, which seems to decline with time on a strict GFD (Hervonen et al., 2005; Lewis et al., 1996). Thus, to treat the symptoms of DH as well as to prevent complications, lifelong strict adherence to GFD is justified in all patients with DH (Mansikka et al., 2019).

## 2.2 Pathogenesis of Dermatitis Herpetiformis

The pathogenesis of DH is likely characterized by complex interactions between genetic and environmental factors and active immune system. As an extraintestinal manifestation of CD, it likely develops in a similar T cell mediated manner as CD. Gluten-intolerance induces the production of antibodies against gluten peptides and TG2 in the small intestine. The mechanism for the development of antibody response against TG3, the primary autoantigen in DH, is however unknown. Very little research has been performed to establish the origin of TG3 antibodies, but different models, *e.g.* epitope spreading in the small intestine, have been suggested (Kaunisto et al., 2022). Skin lesions accompanied by perilesional deposits of IgA and TG3 are the primary diagnostic criteria that distinguish DH from CD. Although the TG3-IgA immunocomplexes in the dermal-epidermal junction seem not to be pathogenic by themselves, they are still likely mediating local immunoreactions leading to blister formation in the skin (Hall et al., 2007; Smith et al., 2002). However, it is still unknown how the TG3 and IgA end up co-localizing in the skin. It is currently assumed that either TG3–IgA complexes found from the skin originate from the circulation (Görög et al., 2016) and bind to structural proteins of the skin, or IgA autoantibodies from the circulation bind and form complexes with TG3 in the papillary dermis (Zone et al., 2011).

### 2.2.1 Genetic and Environmental Factors

DH has been shown to be a familial disease. Reunala (1996) and colleagues showed that 10.5% of 999 patients with DH had one or several affected first-degree relatives who can be affected both with DH and CD. Similarly, in the study of Hervonen et al. (2002), DH seemed to cluster in the families of CD patients and vice versa, indicating the same genetic background for DH and CD. Indeed, patients with DH and CD have the same genetic susceptibility. Virtually every patient with DH and CD carries the HLA-DQ2 and/or HLA-DQ8 haplotype. HLA-DQ2 and HLA-DQ8 are class II human leukocyte antigen molecules expressed on the surface of antigen-presenting cells (APCs). Most of the patients express the HLA-DQ2 molecule encoded by DQA1*05/DQB1*02 genes, and almost all of the remaining patients express HLA-DQ8 molecule encoded by DQA1*03/DQB1*0302 genes (Balas et al., 1997; Karell et al., 2003; Spurkland et al., 1997). Approximately 40% of North American and European populations have inherited the risk alleles, but still only a proportion of them eventually develop the disease (Lindfors et al., 2019). This indicates that HLA-DQ2 or HLA-DQ8 is necessary but not sufficient for DH and CD to develop, and thus other factors beyond HLA-DQ2/8 are required for the disease onset.

The only unquestionable environmental factor linked to the development of DH is the consumption of gluten, which is commonly used when referred to the prolamins. Grains, including wheat, barley and rye, contain gluten. Wheat gluten is a complex mixture of gliadin and glutenin peptides, which are particularly rich in proline and glutamine amino acids. Due to the high proline content, gluten polypeptides are protected from proteolytic enzymes and end up being undigested. The long structure of non-degraded gluten polypeptide increases its immunogenicity, meaning that it can provoke the immune response seen in DH and CD patients. The great majority of people with the genetic risk consume gluten, but only a subset of them will eventually develop the disease. Therefore, other yet unknown environmental factors have been suspected to be involved in the disease development (Lindfors et al., 2019).

The impact of environmental stressors such as infections or environmental toxins has not been thoroughly studied in DH, although iodine, for example, is known to exacerbate the skin lesions, potentially by activating the immunocomplex associated TG3 in the skin (Taylor & Zone, 2018). Dysbiotic microbiota has been linked to dysregulated immune response, and the imbalance of gut microbiota has been suspected to be one of the risk factors of CD (Akobeng et al., 2020). However, there have been no studies on how the maintenance of the microbial homeostasis throughout life might impact the development of DH. Lastly, ageing is considered one of the factors that promote autoimmunity due to its impact on the decline or dysregulation of the immunological functions. Although B and T cells have been shown to become gradually less responsive to antigens with age, the memory cells tend to accumulate and the $T_H1$ responses to shift towards $T_H2$-type of responses during ageing. As

the immune responses become $T_H2$-like, tolerance mechanisms might fail leading to increased autoreactivity (Boren & Gershwin, 2004). Such age-related phenomena are particularly interesting since DH has a notably higher mean age of onset in comparison with CD (Reunala et al., 2021). CD patients have also been shown to present with age-dependent titres of TG3-antibodies at diagnosis (Salmi et al., 2016).

### 2.2.2 Intestinal Manifestations

The development of DH is believed to initiate from the gluten intolerant intestine, like CD. Underlying the gluten intolerance is tissue transglutaminase (TG2), which is one of the nine enzymes of the transglutaminase family. These enzymes crosslink (transamidate) proteins covalently in a calcium ($Ca^{2+}$)-dependent manner. TG2, present in large quantities in the intestine, also catalyzes the deamidation of gliadin where glutamine is converted to glutamate (Odii & Coussons, 2014). After the deamidation, the gliadin peptide binds better to the HLA-DQ2/DQ8 molecule on the surface of antigen presenting cell (APC), resulting in an efficient presentation to T cells (Sollid, 2017). Eventually, the overactive immune response leads to anti-deamidated gliadin peptide (DGP) IgA/IgG and anti-TG2 IgA autoantibody production. The anti-TG2 autoantibodies are a highly specific marker of CD but also widely detected in DH patients (Lewis & Scott, 2006; Salmi et al., 2014). Instead, anti-TG3 autoantibodies have been predominantly linked to DH patients. Although the anti-TG3 autoantibodies can also be produced in CD patients, the anti-TG3 autoantibodies produced in DH patients seem to bind TG3 more selectively and with higher affinity than the ones produced in CD patients (Sárdy et al., 2002). However, the mechanism for anti-TG3 autoantibody production as well as the role of these autoantibodies in DH is still unclear. TG3, which is best known for its role in the formation of the cornified envelope (Candi et al., 2005), has also been shown to deamidate gliadin peptides *in vitro* (Stamnaes et al., 2010). Compared to TG2, it can form enzyme–peptide thioester complexes less efficiently and lacks the ability to form iso-peptide-linked complexes with gliadin (Stamnaes et al., 2010).

The activated immune system in DH can evoke inflammation in the small intestine, which is indicated by the increased density of intraepithelial lymphocytes, more specifically γδ+ T cells (Salmi et al., 2014; Savilahti et al., 1992). Also, the density of αβ+ T cells in the small intestinal epithelium seems to increase on a gluten-dependent manner (Savilahti et al., 1992). With varying severity, the inflammatory environment in the small intestine causes villous atrophy and crypt hyperplasia in most of the DH patients (Alakoski et al., 2012; Marks et al., 1966). The abovementioned mucosal lesions in the small intestine can cause intestinal symptoms, such as loose stools and abdominal discomfort. However, obvious gastrointestinal symptoms or signs of malabsorption are rarely experienced in DH (Alakoski et al., 2012; Reunala et al., 2021).

### 2.2.3 Cutaneous Manifestations

In the skin, DH manifests as an itchy blistering rash showing as small blisters, papules and erythema. The affected areas on the skin are typically distributed symmetrically on elbows, knees and buttocks (Bolotin & Petronic-Rosic, 2011). The skin lesions can also appear on the scalp, face, upper back, abdomen and groin (Collin et al., 2017; Reunala et al., 2021). The blisters usually tend to replace with erosions, crusts and post-inflammatory hyperpigmentation due to intense itchiness of the lesions (Reunala et al., 2021).

The mechanisms leading to the development of skin lesions in DH are only superficially known. Granular IgA deposits can be found in the papillary dermis predominantly at the dermal-epidermal junctions together with TG3, and they are specifically co-localized in the perilesional areas of the skin (Bolotin & Petronic-Rosic, 2011; Caproni et al., 2009). Papillary dermis is an untypical location for TG3 since it is endogenously produced in the skin only by the cells in the uppermost layer of epidermis called the cornified envelope (Hitomi et al., 2003). The reason for this puzzling protein pattern could be simply spontaneous diffusion of TG3 towards dermis, where it would attract the circulating anti-TG3 autoantibodies to bound to itself (Zone et al., 2011). Alternatively, circulating immunocomplexes formed of TG3 and anti-TG3 IgA autoantibodies in DH patients (Görög et al., 2016) may potentially adhere to structural proteins found in the dermis that are substrates for TG3, such as fibrinogen (Taylor et al., 2015).

The role of the IgA-TG3 immunocomplexes in the pathogenesis of DH is unclear since they can also be found in the asymptomatic areas of the skin in DH patients as well as from the skin of DH patients in clinical remission (Hietikko et al., 2018; Mansikka et al., 2019). Thus, the presence of immunocomplexes alone in the skin seems not to be pathogenic. In fact, the IgA-TG3 immunocomplexes may persist in the skin for years despite strict adherence to GFD and drastically decreased level of circulating anti-TG3 autoantibodies (Bardella et al., 2003; Hietikko et al., 2018). The reason for this occurrence has however remained unknown to date, possibly due to the weak understanding of the antigen specificity. The clearance of skin deposits might simply be prolonged, or antibodies might be actively produced (at a level below detection) by *e.g.* long-lived plasma cell or memory T cell populations that have not been discovered yet in DH patients. Persistent IgA-TG3 immunocomplexes have also been detected from the circulation of asymptomatic DH patients following the GFD (Görög et al., 2016). Similar long-lasting circulating immunocomplexes have been detected among patients in remission with other diseases, such as in the study of Ferrari et al. (2014), but the reason however remains elusive.

## 2.3 Immunopathogenic Aspects of Dermatitis Herpetiformis

In the early 2000s, major progress was made in the field of DH research by Sárdy and his colleagues (2002), who identified TG3 as the main autoantigen of DH and its pivotal role in the development of skin lesions. However, the immunopathogenesis of DH, manifesting along the gut-skin axis, remains only partly understood. Our understanding of several key aspects of the DH pathogenesis, *e.g.* the development of anti-TG3 antibodies and their role in the development of skin lesions, has remained ambiguous. The putative pathogenesis of DH is summarized in Figure 1.

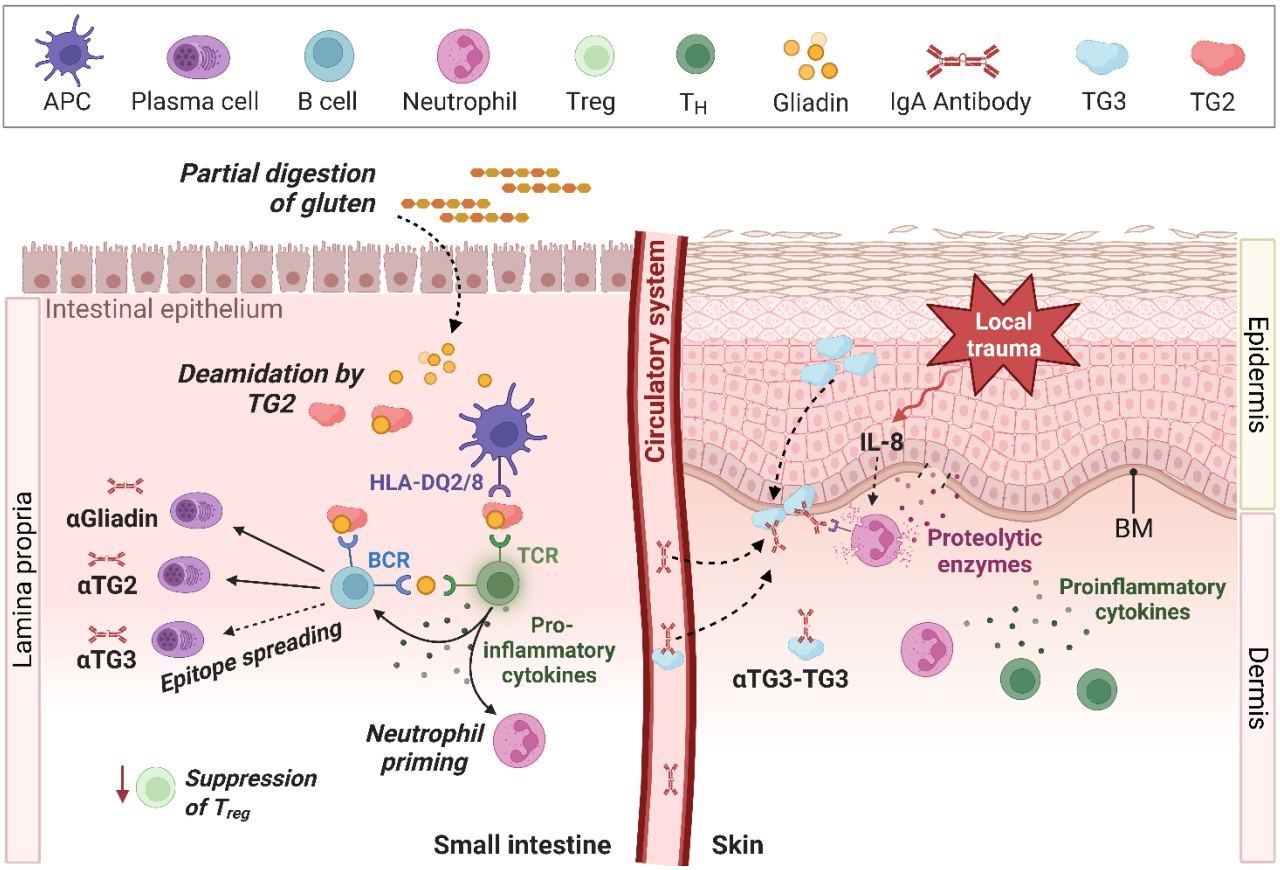


***Figure 1.*** *Simplified depiction of the putative pathogenesis of dermatitis herpetiformis. The early gluten-induced autoimmune response in the small intestine results in the production of autoantibodies targeting gluten-derived gliadin peptides, TG2, and eventually TG3. The autoimmune responses are mediated by effector $T_H$ cells. In the skin, IgA-TG3 immunocomplexes accumulate into dermal papillae. These deposits might be a result of circulating IgA-TG3 immunocomplexes binding to these sites, or spontaneous diffusion of TG3 towards dermis. Tissue bound IgA attract neutrophils to dermal-epidermal junctions leading to the cleavage of the basement membrane and eventually to blister formation, presumably catalyzed by neutrophil secreted proteolytic enzymes. Abbreviations: αGliadin: anti-gliadin antibody, αTG2/TG3: anti-TG2/TG3 autoantibody, APC: Antigen presenting cell, BCR: B cell receptor, BM: Basement membrane, HLA: Human leukocyte antigen, IgA: Immunoglobulin A, IL-8: Interleukin 8, TCR: T cell receptor, TG: Transglutaminase, $T_H$: T helper cell, Treg: Regulatory T cell. Redrawn from Antiga et al. (2019) and Kemppainen et al. (2021). Created with BioRender.com.*

Even though the cascade of T and B cell driven events induced by the ingestion of gluten is far less well characterized in DH than in the case of CD, the early events occurring in the small intestine of DH patients are thought to follow the same path as in CD. In the small intestinal mucosa, the partially digested peptides from dietary gluten end up being deamidated by TG2. The deamidation increases the binding affinity of gluten derived gliadin peptides to HLA-DQ2/DQ8 molecules that are expressed on the surface of APCs. The gliadin peptides bound to HLA are then presented to gluten-specific $CD4^+$ T helper ($T_H$) cells via T cell receptor (TCR) (Sollid, 2017). Through this presentation, $T_H$ cells become reactive to gliadin and secrete proinflammatory cytokines, thereby creating an inflammatory environment in the small intestine (Lindfors et al., 2019). Although the gliadin-reactive T cells from the gut of DH patients have not been studied, gliadin-induced T cell response has been established *ex vivo* (Kalliokoski et al., 2020). Furthermore, the antigen-specificity of T cells in DH seems to at least partially overlap with that seen in CD patients. Since TG2 is able to form covalent complexes with gliadin peptides, which however has been shown only *in vitro* and in a mouse model (Fleckenstein et al., 2004; Lindstad et al., 2021), TG2 is also presented to gluten-specific $CD4^+$ $T_H$ cells. This mechanism is generally known as the hapten-carrier effect, where the carrier (gliadin peptide) elicits an immune response against the bound hapten (TG2), which would not be immunogenic alone (Sollid et al., 1997). TG3, the primary autoantigen in DH, has also been found to create complexes with gliadin peptides (Monneaux & Muller, 2002).

Besides the contribution of T cells, the autoimmunity against gliadin and TG2 is a result of the pivotal participation of B cells. Gliadin and TG2-gliadin complexes are recognized by B cell receptors (BCRs) and end up being internalized by B cells. The processed gliadin peptides are then presented to gluten-specific $T_H$ cells, which can provide help signals, including accessory molecules and T cell-derived cytokines, to both gluten-specific and TG2-specific B cells once activated. These signals promote the differentiation of B cells into plasma cells secreting DGP- and TG2-antibodies (Lindfors et al., 2019). To date, TG2-reactive T cells have not been found either from DH or CD patients, which has made the T cell dependency of TG2-specific B cells questionable. However, several observations suggest that the generation of antibodies is more likely T cell dependent. First, the possibility of TG2 functioning as a thymus-independent antigen have been theoretically excluded, since the molecular structure of TG2 does not fit the classic profile of a thymus-independent antigen. Second, multimers of several TG2 molecules complexed with gliadins could possibly crosslink BCRs of autoreactive B cells, but this type of recognition normally leads to clonal deletion of B cells (Kaunisto et al., 2022). In addition, the proliferation of TG2-specific B cells is markedly reduced *in vitro*, in the absence of T cell help (Lindstad et al., 2021). The TG2-reactive T cells may not be necessary in the T cell dependent pathogenesis however, since TG2-specific B cells are shown to present gliadin to gluten-reactive T cells *in vitro* when TG2–gliadin complexes are available (Di Niro et al., 2012). The

interaction of TG3-specific B cells and gliadin-specific $CD4^+$ T cells has not been established (Kaunisto et al., 2022).

While dendritic cells and macrophages are generally considered the predominant APCs, gliadin- and TG2-specific B cells and plasma cells have been identified as the most abundant cells presenting gliadin to T cells in the gut of CD patients, thus suggesting them to be the predominant APCs in CD (Høydahl et al., 2019). Assuming similar initial development of the immune responses in CD and DH, B cells and plasma cells could also be the predominant APCs in DH. The existence of memory cell populations has not been documented in DH, but it is possible that populations of long lived memory T cells or B cells develop in response to gluten in DH patients, as has been observed in CD patients (Sollid, 2022).

### 2.3.1 Neutrophil Infiltration

In the lesional skin of DH patients, neutrophils are found to accumulate into the papillary dermis (Caproni et al., 2009; Katz et al., 1980). The neutrophil infiltration is linked to the subepidermal blister formation, since the blister fluid from DH lesions has been shown to contain collagenases and elastases that may be derived from neutrophils (Oikarinen et al., 1986). These proteolytic enzymes could cause the destruction of basement membrane and the subsequent blister formation seen in the skin of DH patients (Oikarinen et al., 1986). Also, co-localization of another protease, granzyme B, and neutrophils in the dermal-epidermal junction indicates the neutrophil involvement in the enzyme secretion (Russo et al., 2018). Accompanied by the increased level of granzyme B, Russo et al. (2018) demonstrated the disruption of the key components of basement membrane, *i.e.* α6/β4 integrin, collagen VII, and collagen XVII, in the skin of DH patients.

In order for neutrophils to accumulate at sites of inflammation, they must be activated. Indeed, circulating neutrophils from patients with active DH have shown signs of activation with increased expression of CD11b and decreased expression of L-selectin (Smith et al., 2002). The study of Smith et al. (2002) also showed that neutrophils from DH patients have an increased ability to bind IgA through Fc IgA receptors, enabling their co-localization with IgA skin deposits. The expression of CD11b would then allow primed neutrophils to bind to endothelial cells, move into the dermis and co-localize with the IgA deposits, leading to the development of skin lesions (Smith et al., 2002). To support this, Hall et al. (2006) showed increased expression of E-selectin, a mediator for tethering neutrophils to epithelium, in the upper dermis of DH patients. From DH patients, Hall and his colleagues (2006) found elevated serum IL-8, which has been demonstrated earlier to increase with E-selectin as a result of minor local trauma in the skin of healthy subjects (Takeuchi et al., 2003). IL-8 is a chemokine that is shown to increase expression of CD11b and induce shedding of L-selectin on

the surface of neutrophils (Huber et al., 1991). Thus, after the neutrophils have been primed in pro-inflammatory environment, possibly in the gut, they could move towards IgA deposits in the papillary dermis by chemotaxis created by chemokines, such as IL-8, induced by minor local trauma in the skin (Hall et al., 2007).

### 2.3.2 Effector T Cells

The interaction between APCs and naïve $CD4^+$ T cells activates $CD4^+$ T cells and enables them to differentiate into a certain type of effector cells depending on the cytokine signals they encounter from the environment (Figure 2). Once activated, $T_H$ cells are able to regulate the response of other immune cells. So far, three major, classical $T_H$ subclasses have been characterized based on the way they stimulate the immune response with different pattern of cytokines: $T_H1$, $T_H2$ and $T_H17$. The main subclass of effector $T_H$ cells in DH patients, however, has not been characterized. Thus, the exact nature of the gluten-induced T cell response in DH is not known (Kemppainen et al., 2021).

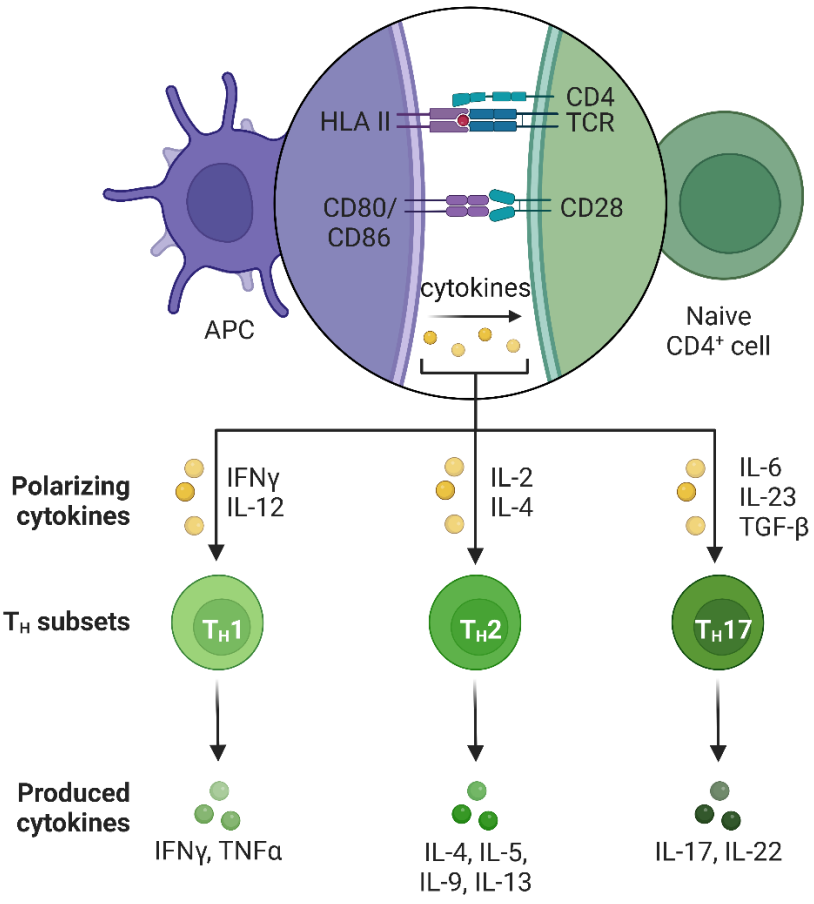


***Figure 2.*** *Caption on the next page.*

***Figure 2. (Caption).*** *T cell activation and differentiation by APC. By displaying co-stimulatory molecule and presenting antigen on the surface via HLA II -molecule, APC can influence the differentiation of naïve CD4$^+$ T cell into an effector T cell subtype. Simultaneous cytokine secretion of APC determines the outcome of T cell differentiation. $T_H1$, $T_H2$ and $T_H17$ are the major, classical $T_H$ subclasses. Different subtypes of $T_H$ cells produce different cytokine patterns directing immune responses. Abbreviations: APC: Antigen presenting cell, HLA: Human leukocyte antigen, IFN: Interferon, IL: Interleukin, TCR: T cell receptor, TGF: Transforming growth factor, $T_H$: T helper cell, TNF: Tumor necrosis factor. Modified from Lazaratos (2020) BioRender. Created with BioRender.com.*

The loss of tolerance to gluten and self-antigens may also be caused by the impaired function of another effector T cell group, regulatory T (Treg) cells. Inadequate activity of Tregs has been linked to CD (Granzotto et al., 2009; Hmida et al., 2012) and the same phenomenon could affect the Treg population in DH. The pathological role of Tregs in DH is supported by reduced levels of FOXP3$^+$ Tregs in the skin of DH patients (Antiga et al., 2015). The reduction of Tregs may lead to insufficient suppressive function and subsequently to the development of skin lesions.

### 2.3.3 Development of B Cell and Plasma Cell Responses

DH patients produce autoantibodies against TG3, which is considered the primary autoantigen in DH. However, comprehensive knowledge about the development of TG3 responses in DH is still lacking, and therefore TG2-targeted autoimmune responses in CD has been used to hypothesize how TG3 antibody responses might presumably develop. Like TG2 antibodies in CD, TG3 antibodies have been suggested to result from target antigen switch from gliadin to TG3 due to B cell epitope spreading in DH (Figure 3). This theory is supported by the ability of TG2-specific B cells to present gliadin to gluten-reactive T cells *in vitro* (Di Niro et al., 2012) and could explain the absence of TG2- and TG3-reactive T cells in DH patients. The epitope spreading might arise from TG2 as well, meaning that the original autoimmunity against TG2 would later expand to cover other members of the transglutaminase family (Kárpáti et al., 2018; Kaunisto et al., 2022). Alternatively, TG3 antibodies could originate from the cross-reactivity between these two transglutaminases due to their high degree of homology (Kárpáti et al., 2018; Kaunisto et al., 2022).

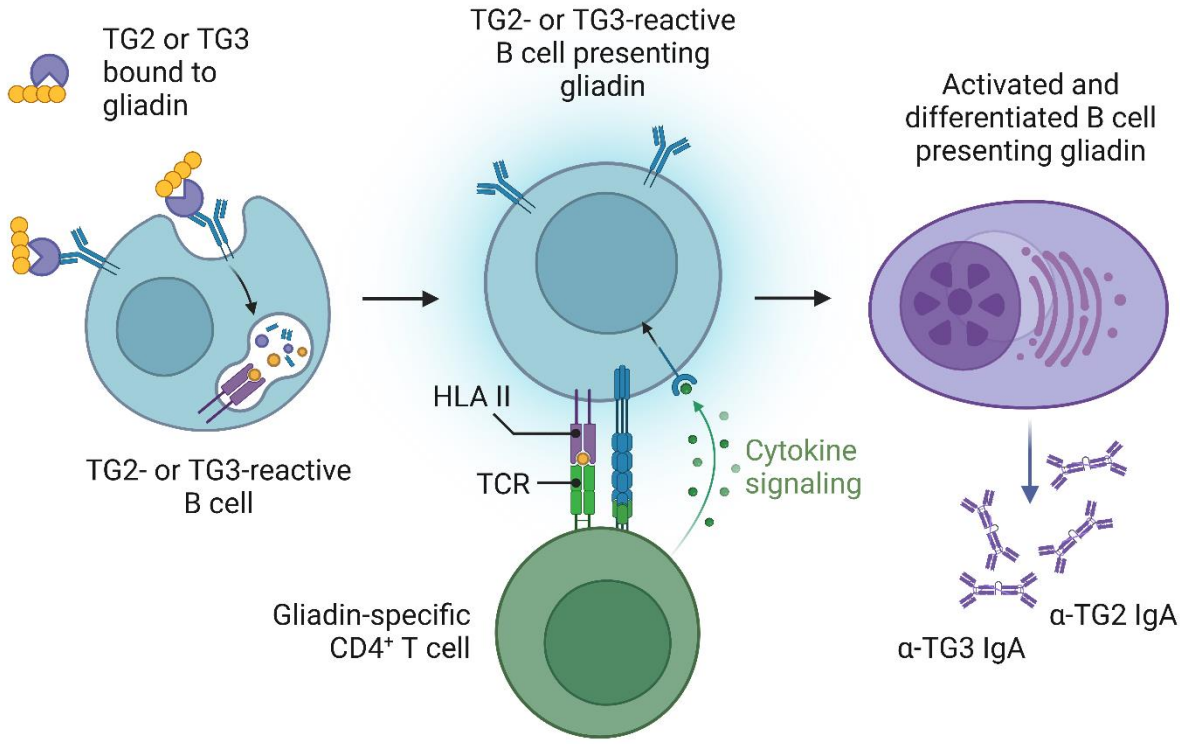


***Figure 3.*** *Epitope spreading from gliadin to TG2 and TG3. Simplified depiction of the suggested mechanism for epitope spreading in DH. TG2- or TG3-reactive B cell internalize gliadin-TG2 or -TG3 complexes and presents gliadin peptide via HLA II -molecule. Gliadin-specific CD4⁺ T cell recognizes presented gliadin by TCR and stimulates gliadin-presenting B cell by releasing cytokines. Activated and differentiated B cell produces IgA class αTG2 or αTG3 antibodies. Abbreviations: αTG2/TG3: anti-TG2/TG3 autoantibody, HLA: Human leukocyte antigen, IgA: Immunoglobulin A, TCR: T cell receptor TG: Transglutaminase. Redrawn from Kaunisto et al. (2022). Created with BioRender.com.*

Intestinal TG2- and TG3 antibody producing plasma cells have been found from DH patients (Sankari et al., 2020), and they are presumably responsive to gluten as their number have been shown to increase during gluten challenge (Sankari et al., 2020). However, B cells reactive for TG3 have not been modelled in animal studies (Kaunisto et al., 2022). The presence of intestinal anti-TG3 plasma cells and serum TG3 autoantibodies seems not to coincide in all cases (Sankari et al., 2020), referring to more than one autoantibody subset originating from different plasma cells (Kemppainen et al., 2021), as has also been suggested to be the case with TG2 autoantibody producing plasma cells in CD (Iversen et al., 2017). Furthermore, the anti-TG3 plasma cells seem to be strictly DH-specific since these cells have only rarely been detected in CD patients (Hietikko et al., 2018; Sankari et al., 2020). This is supported by the results of Sankari and colleagues (2020), suggesting that the TG3-specific autoantibodies secreted by intestinal plasma cells are not cross-reactive with TG2. These findings indicate strict epitope specificities and would thus not support the much-proposed epitope spreading theory.

The skin of DH patients seems to be devoid of gliadin-reactive T cells (Baker et al., 1995), but it however carries αβ+ and γδ+ T cells (Garioch et al., 1997; Kell et al., 1994) that are also present in the patients' small intestine (Salmi et al., 2014; Savilahti et al., 1992). It still remains open whether the cell populations in skin and small intestine are linked, but the discovery of Holtmeier et al. (2002) about γδ+ T cells suggests that the cutaneous T cell population would not originate from the inflamed intestine. On the contrary, the limited literature implies that the IgA deposits in the skin would be dimeric, thereby suggesting a connection with the gut (Unsworth et al., 1982). However, at this point, the immunological processes underlying the development of gluten driven TG3 autoimmunity can only be speculated.

### 2.3.4 T Cell Cytokine Signaling

Cytokines are a broad group of cell signaling molecules controlling the immune system. Along with many other cell types, $T_H$ cells are able to secrete cytokines, and based on their cytokine profile they can be divided into three major, classical subclasses: $T_H1$, $T_H2$ and $T_H17$. Defined by IFN-γ secretion, $T_H1$ response activates the immune defence against intracellular pathogens (Mosmann & Coffman, 1989). $T_H2$ cells instead act in response to allergens and invading parasites mainly through IL-4 and IL-5 secretion (Zhu & Zhu, 2020). Both subsets have been linked to immune pathogenicity, $T_H1$ cells specifically to many chronic autoinflammatory diseases and $T_H2$ cells to atopy and asthma (Harrington et al., 2005). $T_H17$ is one of the more recent $T_H$ subsets to be discovered, playing a role in the immune response against certain extracellular pathogens (Zhu & Zhu, 2020). $T_H17$ cells are characterized by the production of IL-17 and strongly linked to the disease progression of *e.g.* rheumatoid arthritis and psoriasis, but might have a significant role in other human autoimmune diseases as well (Steinman, 2007).

Much like that of CD, the pathogenesis of DH is very likely modulated by the action of gluten-reactive $T_H$ cells. The cytokines, as well as the main subclass of $T_H$ cells, that promote the development of DH, have however remained uncharacterized. To date, cytokines have been sporadically linked to DH primarily based on the correlation between serum levels and disease status or gluten exposure. Interestingly, while CD is considered primarily a $T_H1$-mediated disease (Nilsen et al., 1995), a few studies suggest that the predominant effector T cell population in DH might be $T_H2$-type or $T_H17$-type based on the T cell cytokines linked to DH patients (Table 1).

***Table 1.*** *T cell cytokines linked to DH. Abbreviations: GFD: Gluten-free diet, IL: Interleukin, NA: No published data available, TNF: Tumor necrosis factor.*

| Cytokine | Serum | Skin | Small intestine |
|---|---|---|---|
| IL-4 | Increased concentrations (Makino et al., 2017) | I.) Strong expression (Caproni et al., 1998)<br>II.) Increased frequency of IL-4 producing $CD4^+$ T cells (Caproni et al., 2021) | Increased expression (Hall et al., 2000; Smith et al., 1999) |
| IL-5 | Increased concentrations (Makino et al., 2017) | N/A | Specific expression (Desreumaux et al., 1998) |
| IL-6 | N/A | Weak expression (Gornowicz-Porowska et al., 2014). | N/A |
| IL-8 | I.) Increased concentrations (Hall et al., 2006)<br>II.) Decreased level on GFD (Hall et al., 2007) | Increased expression (Hall et al., 2007) | Increased expression on gluten containing diet (Hall et al., 2007). |
| IL-10 | N/A | Low expression (Antiga et al., 2015) | N/A |
| IL-13 | Increased concentrations (Makino et al., 2017) | Increased expression (Amerio et al., 2000). | N/A |
| IL-17 | I.) Increased concentrations (Velikova et al., 2019; Żebrowska et al., 2013, 2017)<br>II.) Correlation between the frequency of IL-$17^+$ cells and the disease activity (Caproni et al., 2021)<br>III.) Reduced frequency of IL-$17^+$ cells on a GFD (Caproni et al., 2021) | I.) Increased frequency of IL-$17^+$ lymphocytes (Zebrowska et al., 2013)<br>II.) Increased frequency of IL-17 producing $CD4^+$ T cells (Caproni et al., 2021) | N/A |
| IL-31 | I.) Reduced concentrations (Kulczycka-Siennicka et al., 2017).<br>II.) Increased concentrations (Bonciani et al., 2017). | Increased frequency of IL-31+ cells (Bonciani et al., 2017). | N/A |
| IL-36 | Increased concentrations (Żebrowska et al., 2017). | N/A | N/A |
| TNF-α | I.) Increased concentrations (Hall et al., 2006)<br>II.) Correlation between the frequency of TNF-$\alpha^+$ cells and the disease activity (Caproni et al., 2021)<br>III.) Reduced frequency of TNF-$\alpha^+$ cells on a GFD (Caproni et al., 2021) | I.) Increased frequency of TNF-$\alpha^+$ cells (Caproni et al., 2021)<br>II.) Increased expression (Amerio et al., 2000; Żebrowska et al., 2014) | Enriched TNF-α producing cells (Caproni et al., 2021) |

Significant increase in one of the $T_H2$ cytokines, IL-4, has been reported in the serum of DH patients compared to healthy subjects (Makino et al., 2017). IL-4 has also shown strong expression in both perilesional and lesional skin of DH patients (Caproni et al., 1998), as well as higher expression in the small intestine of DH compared to patients with gluten sensitive enteropathy (GSE) (Hall et al., 2000; Smith et al., 1999). Caproni et al. (2021) found a nearly significant ($p = 0.054$) increase in the frequency of skin derived $CD4^+$ T cells producing IL-4 in DH compared to CD patients. Another $T_H2$ cytokine, IL-5, has been reported to be increased in the serum of DH patients by Makino and colleagues (2017). Before that, specific expression of IL-5 has been detected in the small intestine of DH patients (Desreumaux et al., 1998). In addition, elevated expression of IL-13 in the lesional skin of DH patients has been reported (Amerio et al., 2000), contributing the hypothesis that $T_H2$ cytokines might be important in the pathogenesis of DH. Makino et al. (2017) have also reported about significant increase in the serum IL-13 levels in DH patients compared to healthy subjects.

Predominantly $T_H2$ associated IL-31 has been linked to DH, although the findings are inconsistent. In the study by Kulczycka-Siennicka et al. (2017), lower serum IL-31 levels were detected in DH patients compared with healthy subjects, whereas Bonciani et al. (2017) reported significantly higher serum levels in DH patients and high numbers of $IL\text{-}31^+$ cells infiltrated in the skin lesions. The enhanced expression of IL-31 is associated with the induction and persistence of pruritus in chronic inflammatory skin diseases (Bonciani et al., 2017). Low level of Treg produced IL-10, which is known to suppress $T_H2$ responses, has been detected in the lesional skin of DH patients (Antiga et al., 2015). This suggests that insufficient immunosuppressive action of Tregs in DH might result in an uncontrolled local immune response, and critically, to the development of skin lesions.

$T_H17$-type cytokine IL-17 has been linked to DH as well. Serum IL-17 levels were significantly higher in DH patients compared to healthy subjects (Velikova et al., 2019) and patients with other autoimmune bullous skin conditions (Żebrowska et al., 2013, 2017). The quantity of IL-17 producing $CD4^+$ T cells have also been reported to significantly reduce during the GFD (Caproni et al., 2021). In addition, Zebrowska et al. (2013) detected increased amount of $IL\text{-}17^+$ cells from lesional DH skin compared to healthy subjects' skin. Żebrowska et al. (2017) have also studied the expression of IL-36, a proinflammatory cytokine linked to the development of psoriasis, and showed significantly higher serum IL-36 levels in DH patients than in other autoimmune bullous skin conditions and healthy subjects. In addition, weak expression of IL-6 in the lesional skin of DH patients has been detected (Gornowicz-Porowska et al., 2014). IL-6 is a pleiotropic cytokine which is postulated to play a major role in neutrophil recruitment (Gornowicz-Porowska et al., 2014).

The expression of IL-8, a chemokine responsible for T cell and neutrophil migration, was shown to be significantly increased in the serum of DH patients compared to healthy subjects and patients

with other immune mediated skin diseases (Hall et al., 2006). After that, Hall et al. (2007) demonstrated the decreasing serum IL-8 level in response to the GFD. In the same study, Hall et al. (2007) detected significantly increased expression of IL-8 in the small intestine and in the lesional skin of DH patients when compared to healthy subjects. Another $T_H1$-type cytokine, tumor necrosis factor alpha (TNF-α), has also been linked to DH. Zebrowska and colleagues (2014) discovered that TNF-α expression was significantly higher in DH lesions than in the perilesional skin or healthy skin. Furthermore, Caproni et al. (2021) reported that TNF-α producing cells were significantly increased only in the skin samples from DH patients and enriched in $CD4^+$ lymphocytes from duodenal mucosa. Moreover, they discovered that the frequency of circulating TNF-α producing $CD4^+$ T cells correlated with the disease activity and decreased on the GFD. Hall et al. (2016) have as well detected significantly increased serum concentration of TNF-α in patients with DH compared to normal subjects.

The aforementioned studies indicate the levels of singular cytokines in DH patients and thus broader cytokine pattern studies are still lacking. In general, the current knowledge (summarized in Table 1) about DH-linked T cell cytokines is very limited and there is no solid evidence for the exact role of any of these cytokines in the immunopathogenesis of DH. Therefore, more comprehensive studies are needed to establish the cytokine profile of DH patients and to better understand the autoimmune responses in DH.

# 3. AIMS OF THE STUDY

The primary aim of this study was to characterize the systemic cytokines driving DH pathophysiology, as identified using DH patient sera. The second aim was to investigate the tissue origin of the cytokines in DH patients, using the two primary disease associated tissues - the skin and the small intestinal epithelium.

# 4. MATERIALS AND METHODS

The work was implemented as a pilot study prior to full-scale research projects. The work was divided into two parts: investigating the systemic serum cytokine responses in DH and characterizing the tissue origin of potentially DH-linked cytokines. The project workflow is outlined in Figure 4. The characterization of tissue origin of cytokines comprised of immunofluorescence (IF) staining of frozen patient tissue and IF microscopy, whereas the characterization of serum cytokines was performed using luminex-based multiplex (Milliplex) immunoassay and ELISA assays. Since the role of cytokines in DH immunopathology is so poorly understood and DH is hypothesized to develop as a result of undiagnosed CD (Reunala et al., 2018), we investigated the cytokine expression also in CD patients.

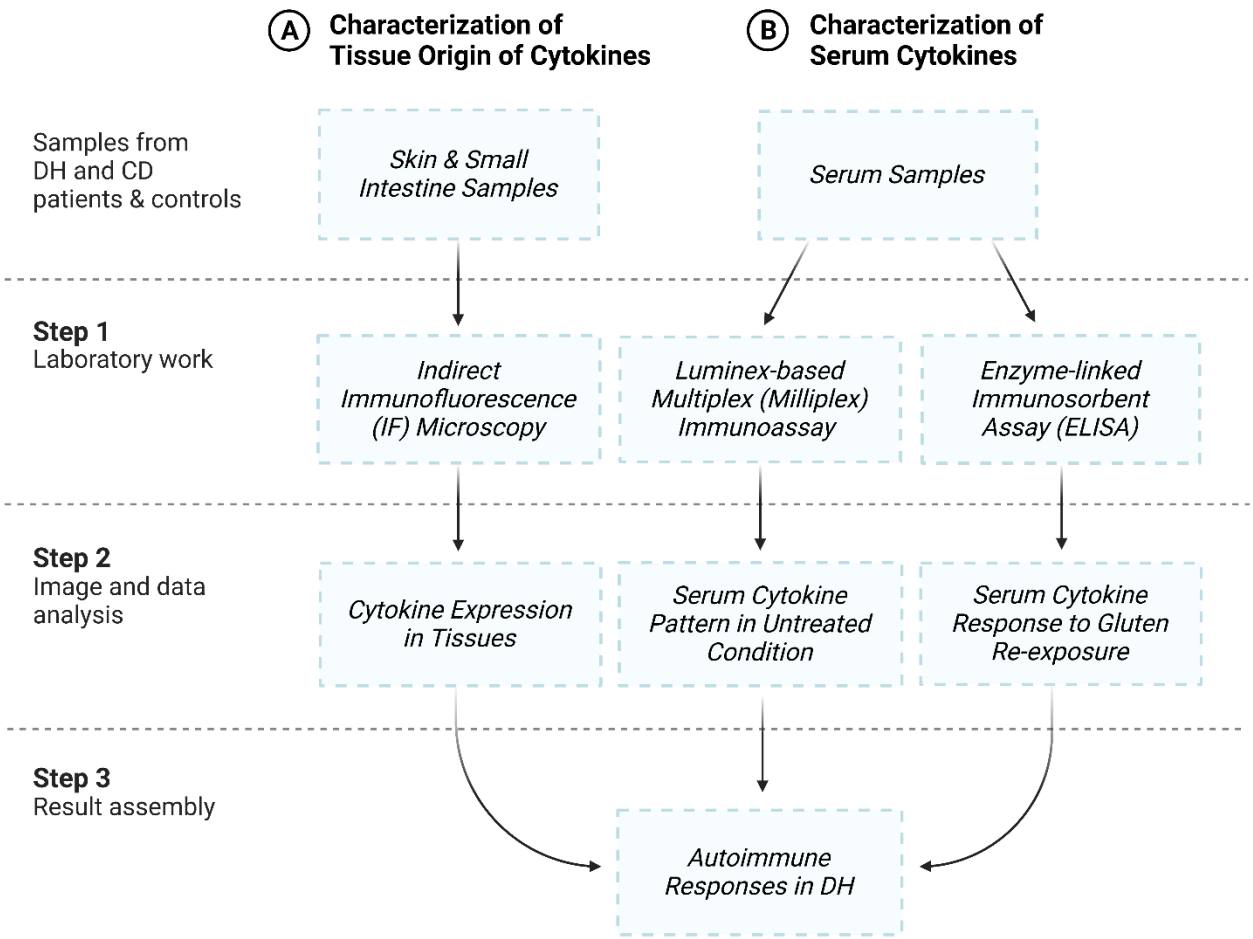


***Figure 4.*** *Workflow of the thesis. Abbreviations: CD: Celiac Disease, DH: Dermatitis Herpetiformis. Created with BioRender.com.*

**Patients**

The clinical samples used at this study were collected at Tampere University Hospital with the consent of the patients and with the approval (R16309, R07122, R98012, R03041, R17042) of the regional ethical committee. Serum, skin and small intestinal biopsies were collected from adult individuals. Samples were collected from untreated DH and CD patients upon diagnosis and after one year on GFD. Samples were also collected from GFD treated DH and CD patients before, during and after a gluten challenge (see below).

## 4.1 Characterization of Serum Cytokines

In this work, the serum cytokines of DH and CD patients were analyzed using Milliplex immunoassay and ELISA assays. In all DH patients, the diagnosis had been based on the typical clinical picture and the presence of granular IgA deposits in the papillary dermis demonstrated with a DIF examination. CD had been excluded from the control patients used in the assays.

**Gluten challenge**

The gluten challenged DH patients were re-exposed to wheat after being on a GFD for a median of 22 (range 5-40) years. The gluten challenge comprised an initial three-day challenge with 200 g of commercially available wheat-based bread (equivalent to 10 slices) daily followed by a gluten-containing diet with a minimum of 10 g of wheat per day. The CD patients had been adhered to a GFD for one year, and the control samples were from the challenged patients with unspecific abdominal symptoms (Sankari et al., 2020).

### 4.1.1 Milliplex Immunoassay

The Milliplex immunoassay was used to detect the concentration of 21 different T cell associated cytokines from serum samples. The assay contained serum samples from 6 untreated DH and CD patients. As controls, we had serum samples from 6 non-celiac individuals who have been diagnosed with a skin disease other than DH. MilliPlex® Human High Sensitivity T Cell Panel Premixed 21-plex - Immunology Multiplex Assay (HSTCMAG28SPMX13, Sigma-Aldrich) was used. All steps were done as instructed by the manufacturer. Serum matrix, wash buffer, standard solutions and quality controls were prepared as per instructions. 200 µl of wash buffer was added into each well and the plate was mixed on a plate shaker for 10 minutes at room temperature (RT). The wash buffer was

decanted, and the residual amount was removed by inverting the plate and tapping it into towels. 50 µl of each standard solution and quality control was added into wells. 25 µl of assay buffer was added into sample wells, followed by 25 µl of each serum sample. Finally, 25 µl of mixed magnetic beads were added into each well. The samples were incubated on a plate shaker overnight at +4°C covered from light. After incubation, the well contents were emptied, and the plate was washed two times. 50µl of detection antibodies were added into each well. The wells were covered from light and the samples were incubated on a plate shaker for 1 hour at RT. 50 µl of Streptavidin-Phycoerythrin was added into each well and the samples were incubated for 30 minutes. After incubation, the washing procedure was done as previously. 150 µl of sheath fluid was added into each well and the beads were resuspended on a plate shaker for 5 minutes at RT. The plate was analyzed immediately using Bio-Plex 200 Reader (Bio-Rad).

### 4.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assays (ELISA) were used to detect the changes in IL-4, IL-6, IL-8 and IL-23 serum concentrations after gluten challenge. Commercial ELISA kits from Sigma-Aldrich were used (Table 2). With the assays, we studied serum samples from DH and CD gluten challenge patients before re-exposure to gluten (day 0) and after being re-exposed to gluten for 6 days. As controls, we had serum samples from non-celiac individuals exposed to wheat. The samples used in this work are listed in Table 3. For IL-4, IL-6 and IL-23 assays, the serum samples were diluted 1:1 with water. For IL-6 assay, samples (day 0 and 6) of two CD challenge patients were diluted again 1:1 with water. The dilutions were taken into account in the absorbance values of samples.

***Table 2.*** *ELISA kits used in the study. Abbreviations: IL: Interleukin.*

| Target | Catalog | Kit |
|---|---|---|
| IL-4 | RAB0298 | Human IL-4 ELISA Kit |
| IL-6 | RAB0306 | Human IL-6 ELISA Kit |
| IL-8 | RAB0319 | Human IL-8 / CXCL8 ELISA Kit |
| IL-23 | RAB0697 | Human IL23A / Interleukin-23 Subunit Alpha ELISA Kit |

***Table 3.*** *The number of samples from different patient groups used in ELISA Assays. Abbreviations: CD: Celiac Disease, DH: Dermatitis herpetiformis, 0d: day 0, 6d: day 6.*

| Sample type | | IL-4 | IL-6 | IL-8 | IL-23 |
|---|---|---|---|---|---|
| **CD challenge** | *0d* | 3 | 3 | 5 | 2 |
| | *6d* | 3 | 3 | 5 | 2 |
| **DH challenge** | *0d* | 6 | 6 | 5 | 6 |
| | *6d* | 6 | 6 | 5 | 6 |
| **Non-celiac control** | | 5 | 5 | 3 | 4 |

All the assays were based on a standard sandwich ELISA principle and performed according to manufacturer's protocol. Standard solutions were prepared for each assay. Detection antibody was diluted with 11 ml of 1x assay diluent buffer B. 100 µl of each sample and standard were added into wells. The plate was sealed, and the samples were incubated on a plate shaker overnight at +4°C. After incubation, the wells were washed three times with wash buffer. 100 µl of detection antibody was added into the wells and the samples were incubated on a plate shaker for 1 hour at RT. The wells were washed twice with wash buffer. 100 µl of Horseradish peroxidase (HRP)-Streptavidin was added into each well and the samples were incubated on a plate shaker for 45 minutes at RT. The wells were washed twice with wash buffer. 100 µl of TMB One-Step Substrate Reagent was added into each well and the wells were covered from light. The samples were incubated on a plate shaker for 30 minutes at RT. 50 µl of stop solution was added into each well and absorbance was immediately read at 450 nm by Multiskan FC Microplate Photometer (Thermo Scientific).

### 4.1.3 Statistics

T-test was used to determine the statistical significance of the differences in average serum levels between patient groups. A p-value $< 0.05$ was considered statistically significant. Serum samples with a cytokine level below the Milliplex assay range (< 0.11 pg/ml) were considered to have a cytokine concentration 0.21 pg/ml, according to the lowest detected cytokine concentration in the assay read.

## 4.2 Characterization of Tissue Origin of Cytokines

In this work, DH patients' skin and small intestine biopsies were stained. Healthy skin and small intestine biopsies and CD patients' small intestine biopsies were stained for comparison. We stained the biopsies with antibodies against five potential cytokines or chemokines: IL-4, IL-6, IL-8, IL-23 and IL-31, and against IL-8 receptor, IL-8RA.

### 4.2.1 Immunofluorescence Staining and Imaging

The study included a total of 3 DH patients with skin biopsies taken from perilesional skin, 3 DH patients with small intestine biopsies and 3 CD patients with small intestine biopsies. All patients were untreated, except one of the DH patients with a skin biopsy (Skin I) was a refractory celiac disease (RCD) patient, thus unresponsive to GFD. Skin biopsies from two control patients were used as skin controls. The control skin biopsies were diagnosed as IgA negative, and they were from HLA-DQ2 and HLA-DQ8 negative individuals. As a small intestine control, one healthy small intestine biopsy was used.

For staining, unconjugated primary antibodies (Table 4) and Alexa Fluor™ 488-conjugated secondary antibodies (Table 5) were used. All antibodies were diluted with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Frozen sections were air-dried for 20 minutes at RT and washed 2 x 3 minutes with PBS on a plate shaker. After air-drying, the sections were blocked with 4% BSA-PBS. The sections were incubated with primary antibody overnight at +4°C and with appropriate secondary antibody for 1 hour at RT. The sections were washed 2 x 3 minutes with PBS between every step before antibody staining, and 3 x 3 minutes between every step during antibody staining. After the last washing step, the stained sections were air-dried and mounted under coverslips (Menzel-Gläser, Thermo Fisher Scientific) by using Vectashield Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Applying the aforementioned staining method, secondary antibody control staining was performed with 1% BSA-PBS. Skin and small intestine samples from DH patients were used for the control staining.

The stained sections were imaged using Olympus IX-51 epifluorescence microscope (Olympus Corporation, Tokyo, Japan). Acquired images were processed with Fiji Image J (Schindelin et al., 2012) and Biorender (Toronto, Canada). The images were deconvoluted with Fiji Image J and its plugins PSF Generator and DeconvolutionLab2. For each image, theoretical point spread function (PSF) was generated and deconvolution was done with following parameters shown in Table 6.

***Table 4.*** *Primary antibodies used for immunofluorescence staining. Abbreviations: IL: Interleukin, RA: Receptor.*

**Primary antibodies**

| Target | Manufacturer | Catalog | Host | Dilution |
|---|---|---|---|---|
| IL-4 | Sigma-Aldrich | MABF3181-25UG | Mouse | 1:100 |
| IL-6 | Novus Biologicals | AF-206-NA | Goat | 1:100 |
| IL-8 | Novus Biologicals | MAB-208 | Mouse | 1:100 |
| IL-8RA | Novus Biologicals | MAB-330 | Mouse | 1:100 |
| IL-23 | Novus Biologicals | NBP1-77257-0.025mg | Rabbit | 1:100 |
| IL-31 | Novus Biologicals | NBP1-76419-0.025mg | Rabbit | 1:100 |

***Table 5.*** *Secondary antibodies used for immunofluorescence staining. Abbreviations: IgG: Immunoglobulin G.*

**Secondary antibodies**

| Target | Manufacturer | Catalog | Host | Wavelength | Dilution |
|---|---|---|---|---|---|
| Mouse IgG | Invitrogen | A11001 | Goat | 488 nm | 1:500 |
| Goat IgG | Invitrogen | A21468 | Chicken | 488 nm | 1:500 |
| Rabbit IgG | Invitrogen | A11034 | Goat | 488 nm | 1:500 |

***Table 6.*** *Parameters for PSF Generator and DeconvolutionLab2 for images taken at 20x magnification. Abbreviations: NA: Numerical aperture, PSF: Point spread function, RI: Refractive index.*

| Theoretical PSF | | | | | Deconvolution | |
|---|---|---|---|---|---|---|
| **Optical Model** | **RI** | **Wavelength** | **Pixel Size** | **NA** | **Algorithm** | **Iterations** |
| Born and Wolf | 1,0 | 350/488 | 330 nm | 0,45 | Richardson-Lucy | 10 |

# 5. RESULTS

## 5.1 Serum Cytokines in Dermatitis Herpetiformis

The T cell cytokine profile of untreated adult DH patients was characterized with Milliplex immunoassay (Figure 5). In addition, the cytokine levels of untreated adult CD patients and controls diagnosed with other skin diseases without CD were measured. In all DH patients, the serum levels of the investigated cytokines were inside the detectable range. The IL-4 level was below the detectable range in two control samples and in one CD sample. The IL-6 level was below the detectable range in one control sample. IL-23 was the most elevated T cell cytokine in the serum of DH patients based on the average concentration, although there was a significant variation in the concentrations between DH patients. DH patients had higher average concentration of IL-23 compared to CD patients and controls, although the difference was not statistically significant. The average ± standard deviation (SD) of IL-23 concentration was 1903 ± 1014 pg/ml in the DH group ($p = 0.51$, compared with controls; $p = 0.22$, compared with CD group), 1440 ± 958 pg/ml in the CD group ($p = 0.99$, compared with controls), and 1427 ± 1370 pg/ml in the control group.

Fractalkine (also known as CX3CL1) and IL-4 had the next highest average concentration in the serum of DH patients. However, the level was significantly lower compared to the level of IL-23. The same fashion in the cytokine levels of CD patients and controls was observed, but the average concentration of fractalkine and IL-4 was higher in CD patients compared to DH patients. DH patients had even lower IL-4 level in average than the controls. The average (± SD) IL-4 concentration was 164 ± 242 pg/ml in the DH group ($p = 0.71$, compared with controls; $p = 0.21$, compared with CD group), 405 ± 361 pg/ml in the CD group ($p = 0.59$, compared with controls), and 257 ± 530 pg/ml in the control group. The average (± SD) concentration of fractalkine was 246 ± 59 pg/ml in the DH group ($p = 0.71$, compared with controls; $p = 0.35$, compared with CD group), 384 ± 325 pg/ml in the CD group ($p = 0.31$, compared with controls), and 231 ± 69 pg/ml in the control group. Other cytokines analyzed in the T cell cytokine panel had average serum concentration ranging from 5 pg/ml to 99 pg/ml in DH patients, and similar serum levels were detected from CD and control patients as well.

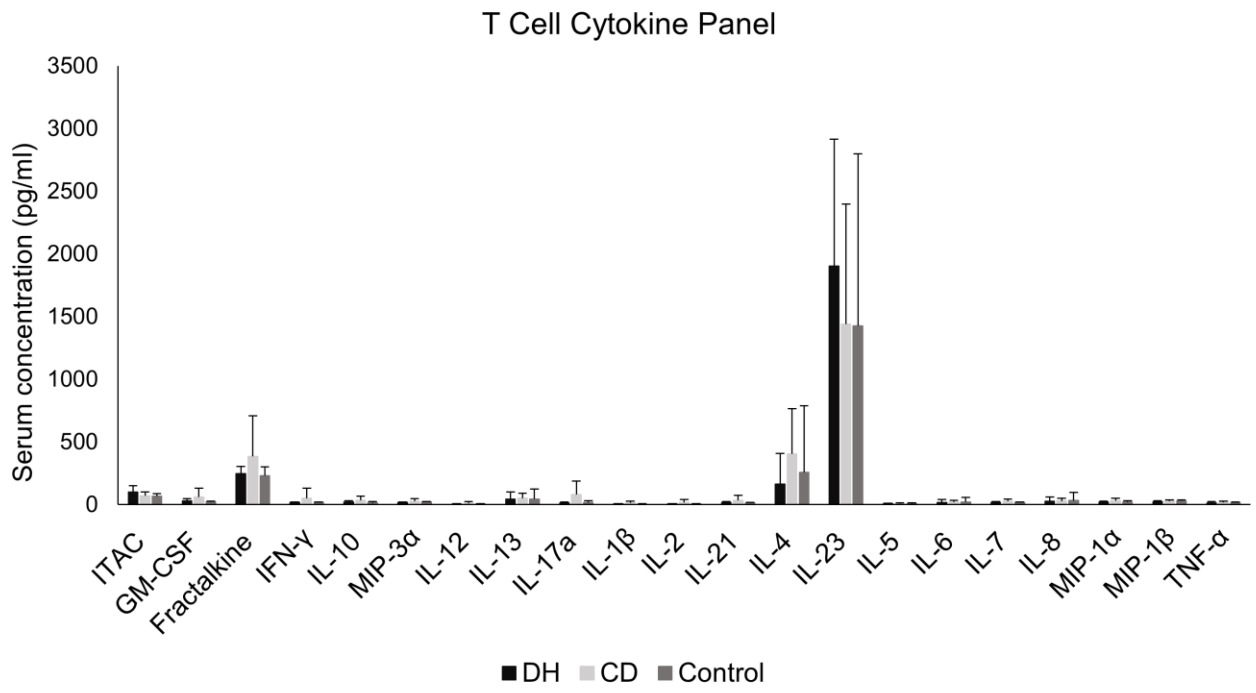


***Figure 5.*** *T cell cytokine serum concentrations in DH, CD and control patient groups. Abbreviations: CD: Celiac Disease, DH: Dermatitis Herpetiformis, GM-CSF: Granulocyte-macrophage colony-stimulating factor, IL: Interleukin, INF: Interferon, ITAC: Interferon-inducible T-cell alpha chemoattractant, MIP: Macrophage inflammatory protein, TNF: Tumor necrosis factor.*

The response of serum IL-4, IL-6, IL-8 and IL-23 to a 6-day gluten re-exposure in challenge patients was characterized using ELISA assays. The responses are presented as fold change (Figure 6), based on the serum concentration changes in patients between day 0 and 6. Before the gluten challenge (day 0), the average serum concentration of IL-4 and IL-23 in DH and CD patients was shown to be already higher than in the control group despite the GFD (Table 7). Changes in the serum cytokine levels were not detected in DH patients after the gluten challenge. The average fold change in serum IL-4, IL-6 and IL-23 level was higher in the CD group compared to the DH and control group, whereas the control group had higher average fold change in serum IL-8 level compared to the DH and CD group. However, the differences in the changes did not reach statistical significance between the patient groups.

The average (± SD) fold change of IL-4 levels between day 0 and 6 was 0.98 ± 0.22 in the DH group ($p = 0.32$, compared with healthy controls; $p = 0.44$, compared with CD group), 13.00 ± 23.57 in the CD group ($p = 0.46$, compared with healthy controls), and 1.66 ± 1.35 in the healthy control group. The average (± SD) fold change of IL-6 levels between day 0 and 6 was 0.97 ± 0.16 in the DH group ($p = 0.18$, compared with healthy controls; $p = 0.43$, compared with CD group), 7.45 ± 11.50 in the CD group ($p = 0.42$, compared with healthy controls), and 0.79 ± 0.23 in the healthy control group. The average (± SD) fold change of IL-8 levels between day 0 and 6 was 0.99 ± 0.06 in the DH group

(p = 0.47, compared with healthy controls; p = 0.22, compared with CD group), 0.86 ± 0.20 in the CD group (p = 0.42, compared with healthy controls), and 1.97 ± 1.93 in the healthy control group. The average (± SD) fold change of IL-23 levels between day 0 and 6 was 1.00 ± 0.04 in the DH group (p = 0.32, compared with healthy controls; p = 0.54, compared with CD group), 7.21 ± 10.11 in the CD group (p = 0.54, compared with healthy controls), and 0.89 ± 0.19 in the healthy control group.

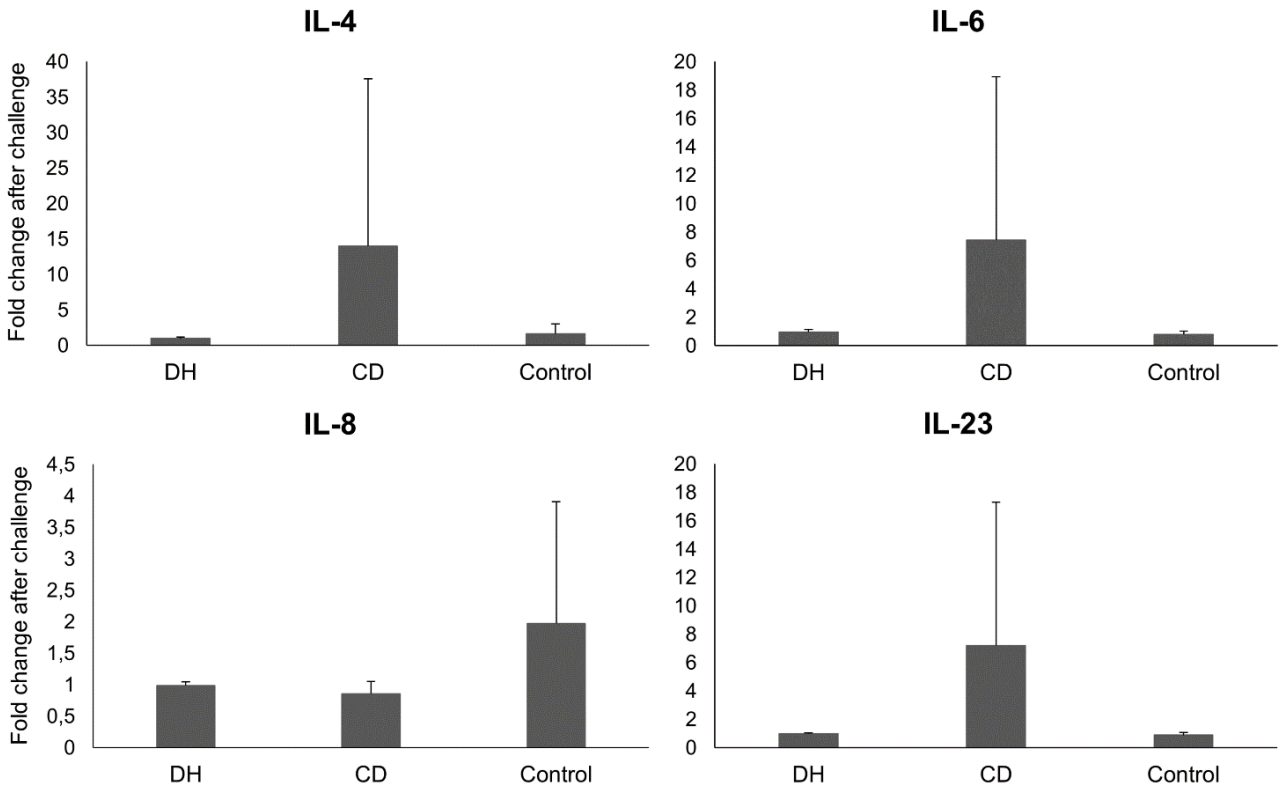


***Figure 6.*** *Changes in the serum concentrations of IL-4, IL-6, IL-8 and IL-23 in CD and DH patients and healthy controls between day 0 and 6. The changes are presented as fold change. Abbreviations: CD: Celiac Disease, DH: Dermatitis Herpetiformis, IL: Interleukin.*

***Table 7.*** *Average concentration of IL4, IL-6, IL-8 and IL-23 in the patient groups on day 0 and 6. Abbreviations: IL: Interleukin.*

| Sample type | | IL-4 (pg/ml) | IL-6 (pg/ml) | IL-8 (pg/ml) | IL-23 (pg/ml) |
|---|---|---|---|---|---|
| **CD challenge** | *0d* | 395 | 2672 | 321 | 7344 |
| | *6d* | 331 | 5163 | 299 | 6796 |
| **DH challenge** | *0d* | 138 | 2213 | 346 | 6303 |
| | *6d* | 136 | 2147 | 472 | 6415 |
| **Non-celiac control** | *0d* | 39 | 2307 | 418 | 3175 |
| | *6d* | 43 | 1884 | 411 | 3274 |

## 5.2 Tissue Origin of Cytokines in Dermatitis Herpetiformis

To investigate the original production site of serum cytokines in DH patients, skin and small intestine samples from adult DH patients were stained using indirect IF. The samples were stained to detect IL-4, IL-6, IL-8, IL-23, IL-31 and IL8-RA (IL-8 receptor). For comparison, small intestine samples from adult CD patients as well as skin and small intestine samples from healthy adult patients were stained.

The staining results are summarized in Table 8. The results were mostly consistent inside the patient groups (DH, CD & control). IL-4 was detected from all the DH skin and small intestine samples, as well as from the CD small intestine samples. The skin controls for IL-4 were negative, whereas the small intestine control was positive. IL-6 was detected in all of the DH skin samples, but only in one DH small intestinal sample. IL-6 was also detected from two of the three small intestine samples of CD patients. All the control samples were positive for IL-6. No IL-8 or IL-8RA was found from any of the DH skin samples, but instead they were detected from all the DH and CD small intestine samples. For IL-8 and IL-8RA, the skin controls were negative and the small intestine control positive. Lastly, IL-23 and IL-31 were detected from all stained samples. Refractory type of disease did not reveal any notable differences in the skin staining results compared to the GFD-responsive form of the disease.

***Table 8.*** *Summary of the tissue staining results. Abbreviations: CD: Celiac Disease, DH: Dermatitis Herpetiformis, IL: Interleukin, RCD: Refractory Celiac Disease ++: Clearly present, +: Faintly present, —: Not visible, N/A: No result available.*

| Sample type (n=14) | | IL-4 | IL-6 | IL-8 | IL-8RA | IL-23 | IL-31 |
|---|---|---|---|---|---|---|---|
| DH | skin I (RCD) | ++ | ++ | — | — | ++ | ++ |
| | skin II | + | ++ | — | — | ++ | ++ |
| | skin III | ++ | ++ | — | — | + | + |
| | intestine I | ++ | — | ++ | ++ | ++ | ++ |
| | intestine II | ++ | — | ++ | ++ | ++ | ++ |
| | intestine III | + | + | + | ++ | ++ | ++ |
| CD | intestine I | ++ | — | ++ | ++ | + | + |
| | intestine II | ++ | ++ | ++ | ++ | ++ | + |
| | intestine I | ++ | ++ | ++ | ++ | ++ | + |
| Control | skin I | — | + | — | — | ++ | + |
| | skin II | — | + | — | — | ++ | ++ |
| | intestine I | N/A | N/A | ++ | ++ | N/A | N/A |
| | intestine II | N/A | N/A | N/A | N/A | + | + |
| | intestine III | + | + | N/A | N/A | N/A | N/A |

Immunofluorescence staining of intestinal samples revealed that IL-4 was expressed in the small intestine of all DH patients (Figure 7A) and CD patients (Figure 7C). The expression of IL-4 was significantly lower in the healthy small intestine sample (Figure 7B) compared to DH and CD samples. The IL-8 staining showed that IL-8 was expressed in the intestinal crypts of all three DH patients (Figure 7D). Healthy small intestine seemed to contain a few infiltrated IL-8$^+$ cells (Figure 7E), but IL-8 was not that specifically located inside the crypts as in the small intestine of DH patients. The expression of IL-8 in the small intestine samples of all three CD patients (Figure 7F) was similar to observed in DH patients. All three DH patients also expressed IL-8RA in their small intestine (Figure 7G), and some IL-8RA$^+$ cells were found from healthy small intestine as well (Figure 7H). The expression of IL-8RA was higher in the crypts of CD patients (Figure 7I) compared to DH patients.

In the IL-6 staining, one of the three DH patients had few IL-6$^+$ cells in the small intestine (Figure 8A). Similar expression was observed in the healthy small intestine sample (Figure 8B). Two of the three CD patients had IL-6$^+$ cells infiltrated in the small intestine (Figure 8C). The staining for IL-23 showed that IL-23 was expressed in the small intestine of all three DH patients (Figure 8D). Healthy small intestine (Figure 8E) had lower expression of IL-23 with weaker localization inside the crypts compared to the small intestines of DH patients. All the small intestine samples of CD patients expressed

IL-23 mainly inside the crypts (Figure 8F). In the IL-31 staining, all three DH patients had IL-31$^+$ cells infiltrated in the small intestine (Figure 8G). The healthy small intestine sample (Figure 8H) contained couple of weakly fluorescent IL-31$^+$ cells. The small intestines of CD patients (Figure 8I) expressed more IL-31 inside the crypts but had fewer IL-31$^+$ cells compared to the DH patients.

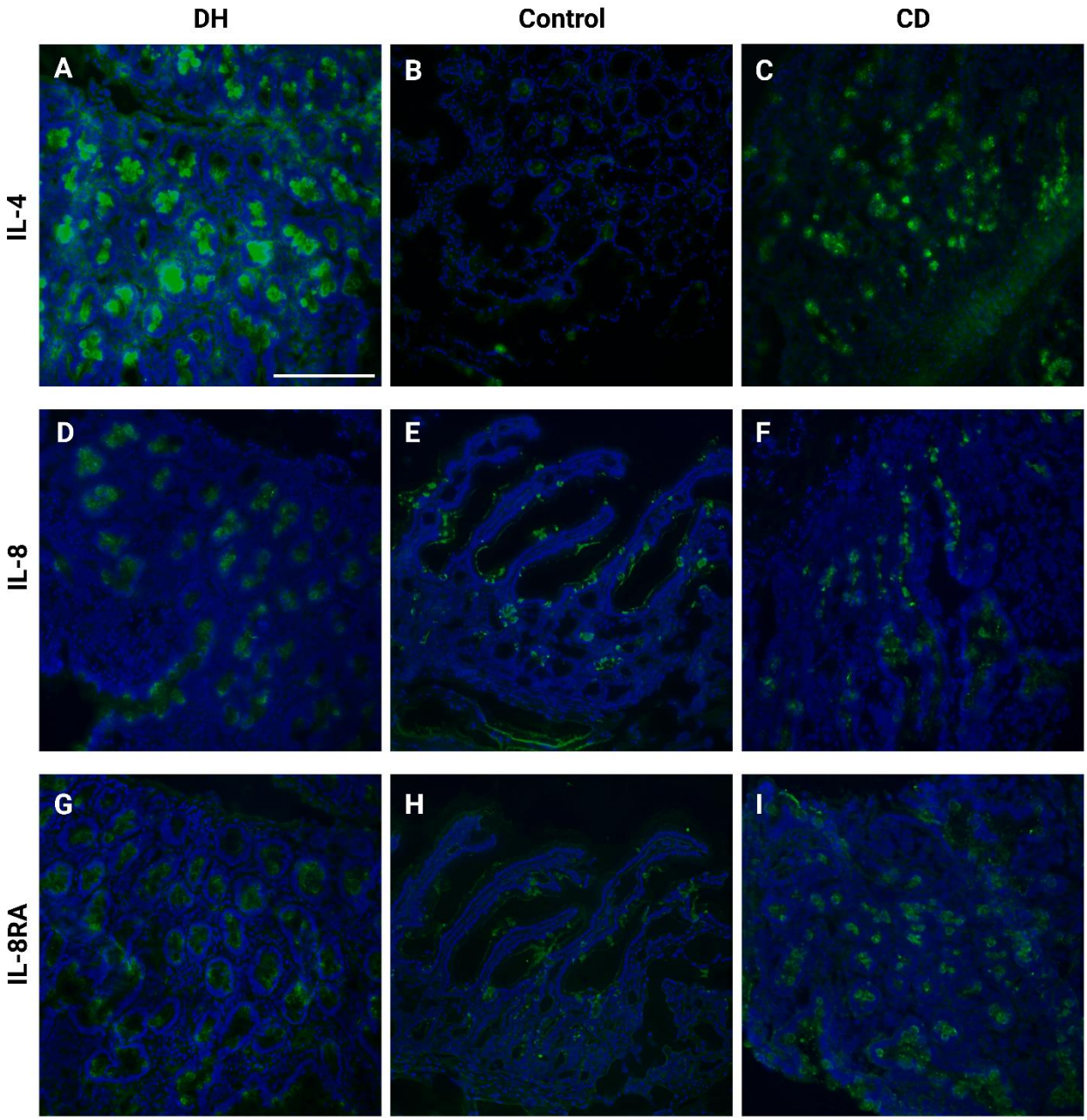


***Figure 7.*** *Expression of IL-4, IL-8 and IL-8RA (green) with DAPI (blue) staining in the small intestine of DH and CD patients and healthy controls. Images taken at 20x magnification. Scale bar 200 µm. Abbreviations: CD: Celiac Disease, DAPI: 4',6-diamidino-2-phenylindole, DH: Dermatitis Herpetiformis, IL: Interleukin.*

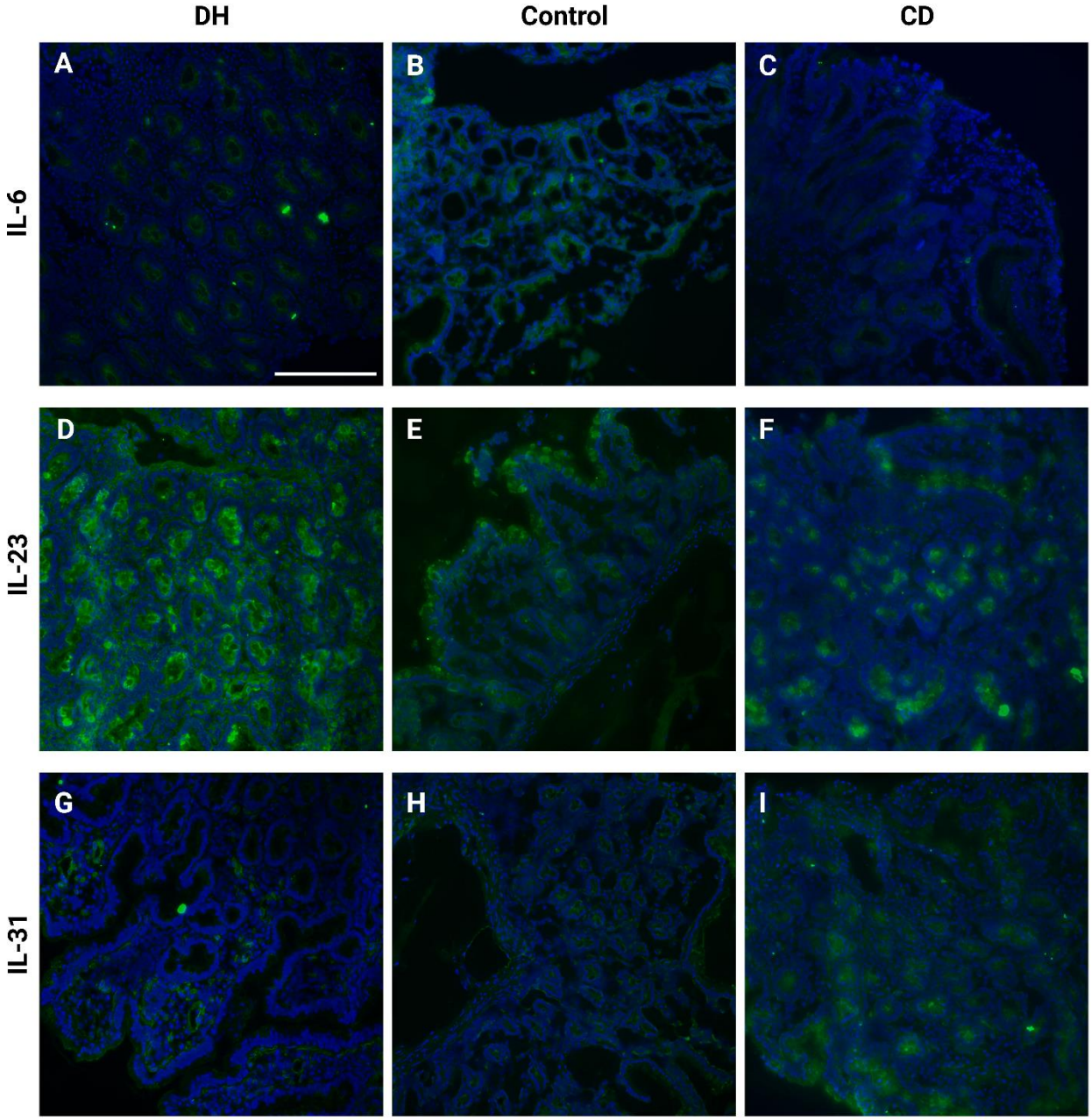


***Figure 8.*** *Expression of IL-6, IL-23 and IL-31 (green) with DAPI (blue) staining in the small intestine of DH and CD patients and healthy controls. Images taken at 20x magnification. Scale bar 200 µm. Abbreviations: CD: Celiac Disease, DAPI: 4',6-diamidino-2-phenylindole, DH: Dermatitis Herpetiformis.*

Immunofluorescence staining of skin samples revealed that in all three DH patients IL-4$^{+}$ cells were infiltrated in the upper dermis and epidermis of perilesional skin (Figure 9A). Possibly, secretory IL-4 was also diffusely expressed throughout the tissue. No expression of IL-4 was observed in the healthy skin samples (Figure 9B). The IL-8 staining did not show any IL-8$^{+}$ cells infiltrated in the perilesional skin of DH patients (Figure 9C) nor in the healthy skin samples (Figure 9D). The staining for IL-8RA showed that IL-8RA$^{+}$ cells were not infiltrated in the perilesional skin of DH patients (Figure 9E). No expression of IL-8RA was observed in the healthy skin samples either (Figure 9F).

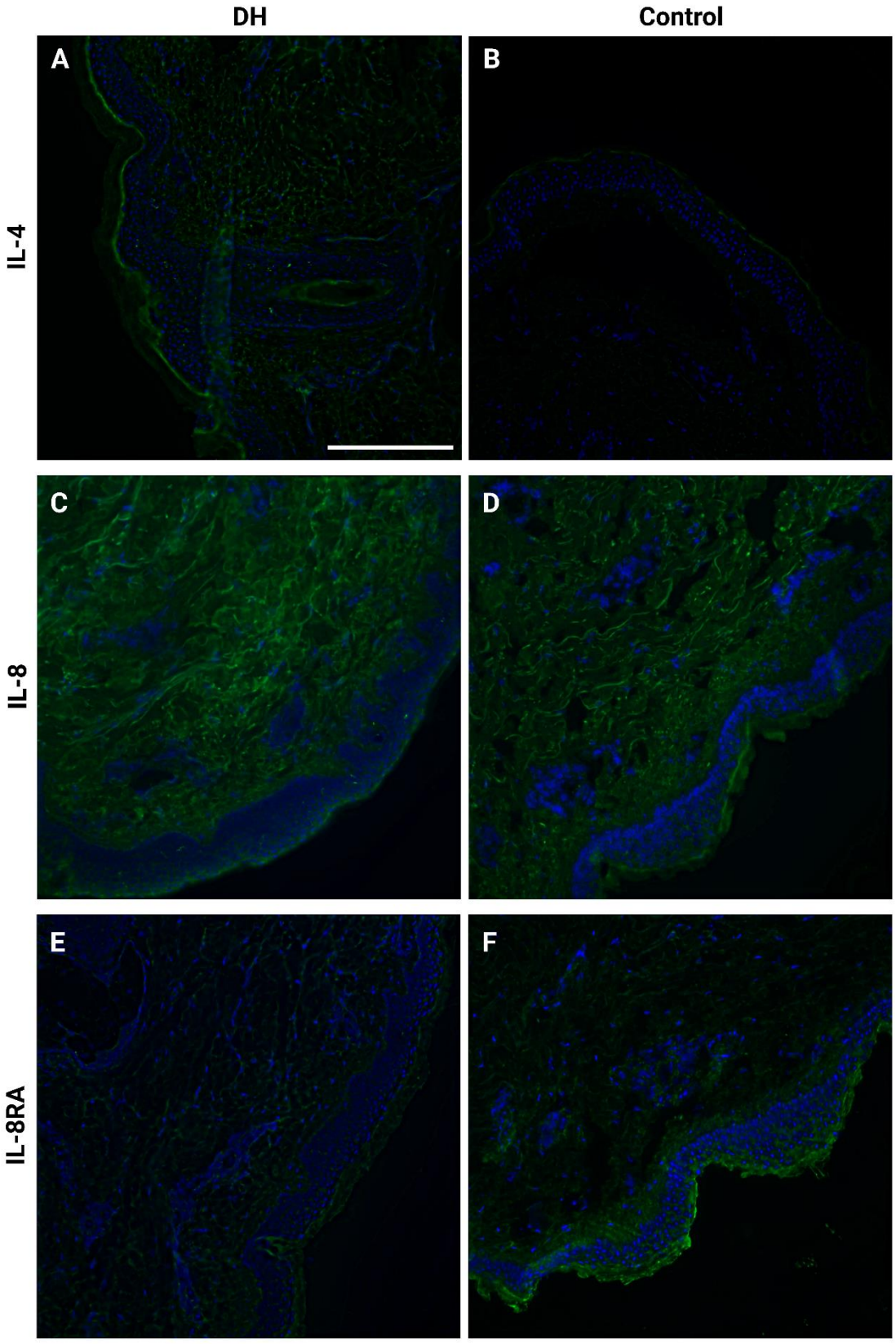


***Figure 9.*** *Expression of IL-4, IL-8 and IL-8RA (green) with DAPI (blue) staining in the skin of DH patients and healthy controls. Images taken at 20x magnification. Scale bar 200 µm. Abbreviations: DAPI: 4',6-diamidino-2-phenylindole, DH: Dermatitis Herpetiformis, IL: Interleukin.*

The IL-6 staining showed that all DH patients had IL-6$^+$ cells infiltrated in the epidermis, dermal-epidermal junction and dermis of perilesional skin (Figure 10A). In the healthy skin, the signal for IL-6 was weaker, although present (Figure 10B). In the IL-23 staining, IL-23$^+$ cells were infiltrated in the upper dermis and epidermis of perilesional skin in all DH patients (Figure 10C). IL-23$^+$ cells were also found from dermis and dermal-epidermal junction in both healthy skin samples (Figure 10D). The staining for IL-31 revealed that IL-31$^+$ cells were infiltrated in the dermis of perilesional skin of all DH patients (Figure 10E). IL-31$^+$ cells were also found from the dermis of the healthy skin (Figure 10F).

In order to demonstrate the specific binding of the secondary antibodies used in this work, control staining was performed. All the secondary antibodies had tendency to stain some structures in the dermis of the skin samples (Figure 11A, C, E). In the small intestine samples, the secondary antibodies caused insignificant amount of background (Figure 11B, D, F). The background staining pattern of the secondary antibody was considered when interpreting the results.

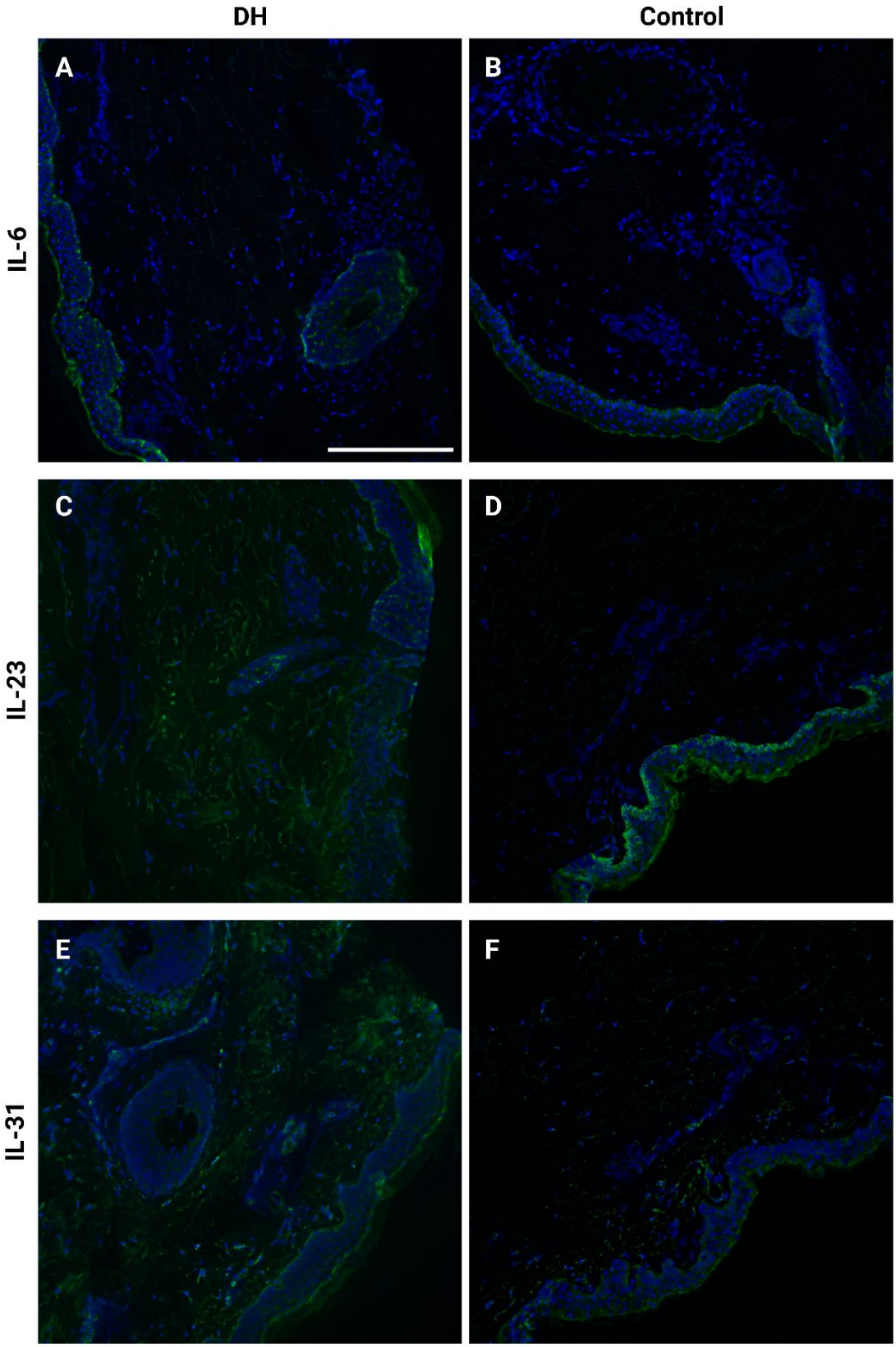


***Figure 10.*** *Expression of IL-6, IL-23 and IL-31 (green) with DAPI (blue) staining in the skin of DH patients and healthy controls. Images taken at 20x magnification. Scale bar 200 µm. Abbreviations: DAPI: 4',6-diamidino-2-phenylindole, DH: Dermatitis Herpetiformis.*

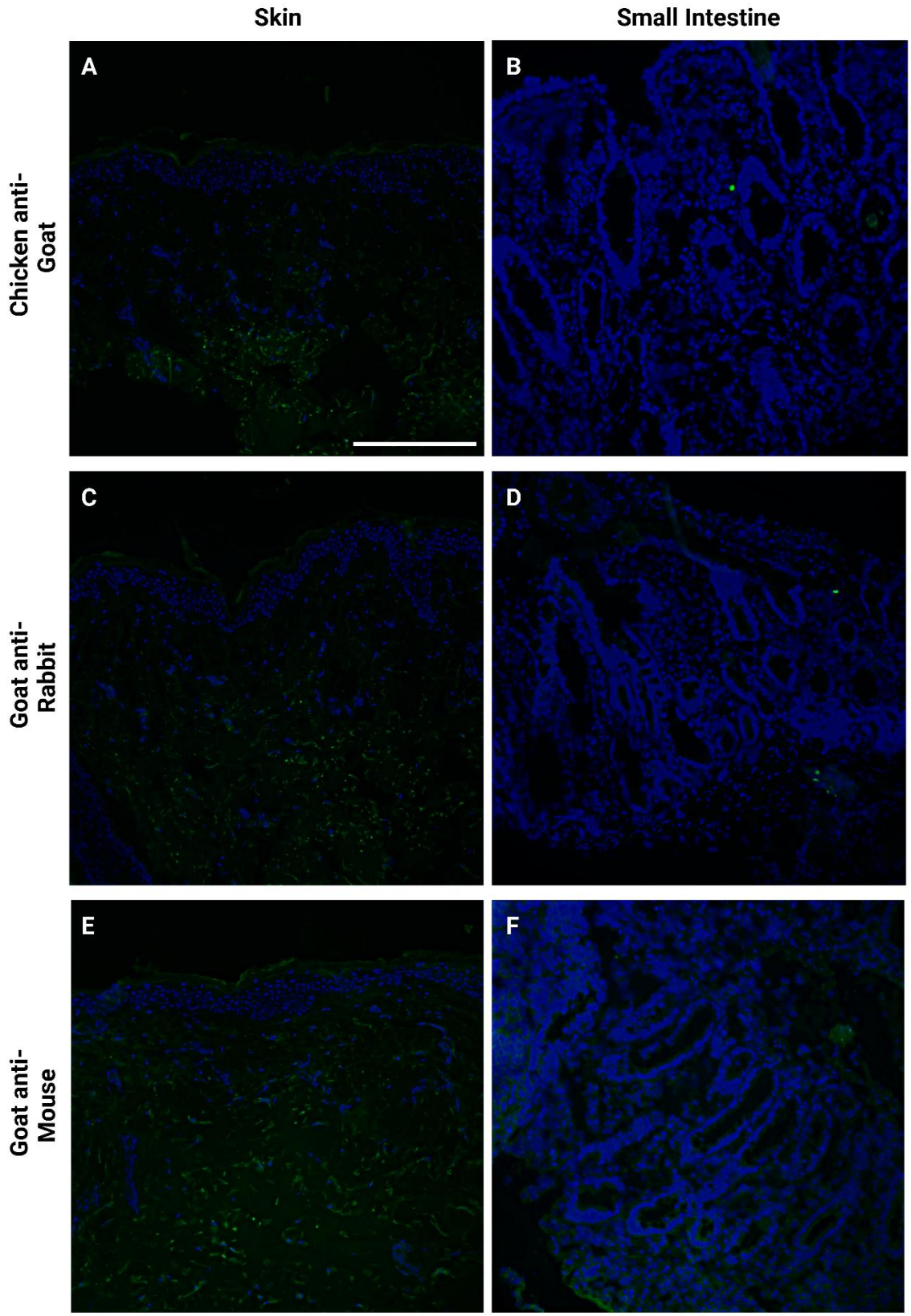


***Figure 11.*** *Control staining for the secondary antibody expression (green) with DAPI staining (blue). Stained sections from DH patients. Images taken at 20x magnification. Scale bar 200 µm. Abbreviations: DAPI: 4',6-diamidino-2-phenylindole, DH: Dermatitis Herpetiformis.*

# 6. DISCUSSION

In this work we aimed to characterize the systemic cytokine profile of DH patients and evaluate whether the autoimmune response involves different effector T cell populations than are known to be involved in CD pathogenesis, further explaining the cutaneous manifestation of DH. DH patients seem to have $T_H17$-type (IL-23) and $T_H2$-type (IL-4) activity, as previous studies have suggested (Caproni et al., 1998, 2021; Żebrowska et al., 2013). We were not able to identify DH-specific cytokines, but instead, a similar systemic serum cytokine pattern was seen in DH and CD patients as well as in patients diagnosed with other skin diseases. These results indicate that the elevated cytokines, *i.e.* IL-4 and IL-23, are generally produced during chronic, systemic inflammatory response. Considering the heterogenetic nature of DH, the variability of results between the DH patients was somewhat inevitable. After following the GFD, the DH patients seemed not to respond to gluten re-exposure by up-regulating IL-4, IL-6, IL-8 or IL-23 production, while CD patients did respond with increased serum levels of IL-4, IL-6 and IL-23. Similar studies have been previously conducted only with CD patients, using implementations different from this study (the duration of GFD, the amount of ingested gluten and the duration of gluten exposure) (Goel et al., 2019; Tye-Din et al., 2020). The unresponsiveness could indicate that the response of DH patients to gluten is simply mediated by other cytokines, or that the cytokine levels remain increased in patients even after becoming asymptomatic, concurrent with the antibody deposits persisting in the skin of DH patients after years on GFD (Hietikko et al., 2018). It is noteworthy, that the duration of GFD before the gluten challenge varied significantly between DH and CD patients from a median of 22 years to one year, respectively. Considering the long-term GFD of DH patients, longer than six days might be required for the peripheral gliadin-specific T cells of DH patients to produce detectable response, as Kalliokoski et al. (2020) have suggested in their *ex vivo* study with DH patient derived peripheral blood mononuclear cells (PBMCs), further proposing a reason for a low percentage of gluten-responsive DH patients compared to CD patients.

The tissue stainings showed cytokine expression concurrent with the serum cytokine profiling. Based on our results, the expression of IL-4 in the skin could be characteristic for DH. This is supported by Caproni and colleagues (1998), who have earlier detected strong expression of IL-4 in the skin of DH patients. In addition, the small intestine could function as the site of production of IL-4. Since the signal for IL-4 expression was stronger in the small intestine of DH patients than in the healthy small intestine, IL-4 mediated immune response could play a role in the clinical presentation of DH, like Smith et al. (1999) have suggested. The measured IL-4 serum level in DH patients was low compared to CD patients, even though IL-4 was shown to be produced in the skin as well. We suggest couple of reasons for that. First, the detection window of cytokines is narrow since the majority of

cytokines are known to have a short half-life *in vivo* and to be targeted by rapid degradation (Liu et al., 2021). Second, the low expression level of cytokines could partly explain why DH patients typically undergo milder intestinal symptoms than CD patients. Lastly, the production level of cytokines in the skin might be relatively low, and therefore may not be enough to increase the serum concentration after all.

Gornowicz-Porowska and colleagues (2014) have studied the role of IL-6 in DH skin and suggested that a weak IL-6 expression might reflect defective IL-6 signaling in the acute DH. Our results support the low production of IL-6, albeit in the perilesional skin. The expression of IL-6 was more apparent in the skin, indicating the prominent role of epidermal keratinocytes in the production of IL-6 (Gornowicz-Porowska et al., 2014). In DH patients, IL-8 and IL-8RA were detected only from the small intestine. This result supports the suggestion of Hall et al. (2007), in which the gastrointestinal mucosa may be the source of the elevated serum IL-8 instead of inflamed epidermis and further contribute to the development of skin lesions in DH. The scarce perilesional expression of IL-31 detected in this work show consistent with the study of Bonciani et al. (2017), but the specificity of IL-23 and IL-31 in the DH skin remained unclear. The frequency of IL-31$^+$ cells could be increased in the small intestinal epithelium of DH patients, but there is no previous data to support this, although increased serum IL-31 has been detected in DH patients (Bonciani et al., 2017). The expression of IL-23 was significantly greater in the intestine of DH patients, suggesting its specific role in the pathogenesis of DH. To our knowledge, the IL-23 expression has not been studied earlier in DH, but IL-23 has been implicated in several other autoimmune diseases including psoriasis, rheumatoid arthritis and inflammatory bowel disease, where it has a central role due to its ability to create continuous inflammatory loops through the positive feedback of IL-22 and IL-17 arms (Abdo & Tye, 2020). From these results we could draw the conclusion that the secretion of IL-4 and IL-23 take part in the pathogenesis of DH, but their specificity and possible role in the development of skin symptoms remains unclear. This work suggests that the differences in the cytokine pattern of DH and CD patients could lead to these two different manifestations of systemic gluten-induced autoimmune response.

## 6.1 Possible Limitations of the Study

Although this work was meant to serve as a pilot study for further larger studies, the relatively small sample size in this work should be emphasized. In addition, relatively long storage time of the healthy tissue samples might have impacted their quality and thus the antibody staining patterns have to be interpreted with caution. Indirect IF microscopy has high sensitivity, and it provides amplified signal since several conjugated secondary antibodies can bind to each primary antibody. However, the challenge of fluorescence microscopy is autofluorescence, the intrinsic fluorescence of tissues,

which can distort the signal originating from specific antibody staining. For example, structural proteins collagen and elastin, as well as lipofuscin pigment, are identified as endogenous fluorophores in the skin (Giovannacci et al., 2019). The same endogenous fluorophores have been detected also in the intestinal tissues, where the amount of autofluorescence signals seems to increase during inflammation (Wizenty et al., 2018).

## 6.2 Future Aspects

In this work, skin and small intestine samples were from different patients, but studying tissue pairs from the same patient should be considered in further studies. It would enable to study the personal cytokine expression ratio between skin and intestine and furthermore how it is related to the patient's disease phenotype. Adding this information to the serum cytokine study would allow us to see how the tissue expression is linked to the level of circulating cytokines in a patient level. Thus, using a sample set containing skin, small intestine and serum sample from each patient combined with clinical data could be one idea to fulfill in future studies. Considering the variable nature of DH, this could give extensive data about the cytokine profile of DH patients, further enabling their comparison and mirroring to different levels of the disease. Other future perspective would be to define other cytokine responses that could mediate the autoimmune response against gluten in DH patients by gluten re-exposure, and ideally reveal cytokines that are involved in the initial stage of DH.

The impact of factors other than gluten on DH has not been thoroughly studied, including ageing, which is supported by the high mean age at diagnosis (Salmi et al., 2011). With age, the $T_H1$ cytokine profile tend to shift towards $T_H2$, increasing autoreactivity of the immune system (Boren & Gershwin, 2004). As the $T_H2$-mediated cytokines seem to dominate the findings of DH studies instead of $T_H1$-mediated cytokines linked to CD, it would be an intriguing idea that the same phenomenon seen in ageing could underlie the progression of DH. It remains to be determined whether the switch from $T_H1$ cytokine profile to $T_H2$ in the development of DH occurs through the mechanism of connected with ageing or a separate pathway.

# 7. CONCLUSIONS

The object of this study was to characterize the systemic cytokine profile of DH and to evaluate whether different $T_H$-subpopulations are involved in DH and CD pathogenesis, potentially explaining the cutaneous manifestation of DH. We were able to perform the first comprehensive T cell cytokine profiling of a small panel of DH patients. We elucidated that untreated DH patients present with varying cytokine patterns, qualitatively indicative of mainly $T_H17$- and $T_H2$-type activity. We also demonstrated that DH patients do not respond to gluten with increased IL-4, IL-6, IL-8 or IL-23 production after GFD. Lastly, we presented tissue cytokine expression concurrent with the cytokine profiling, and also supported previous findings of other authors. In conclusion, these results suggest that a switch in the $T_H$-profile occurs during the development of DH, which could explain the two separate manifestations of CD.

This study provides preliminary insight into the effector T cell function in DH. The cytokine profile of DH patient immune responses should be studied further in order to establish which cytokines are involved in the skin lesion development, distinguishing the manifestation of DH from CD.

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