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INSIGHTS INTO THE MOLECULAR MECHANISMS OF SKIN PATHOLOGY IN DERMATITIS HERPETIFORMIS

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ABSTRACT

Paula Rantala: Insights into the molecular mechanisms of skin pathology in Dermatitis herpetiformis

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Dermatitis herpetiformis (DH) is a skin-related manifestation of celiac disease, in which dietary gluten causes itching and blistering rash, especially in the elbows, knees and buttocks. Therefore, the treatment is a strict gluten-free diet. DH is characterized by granular immunoglobulin A (IgA) deposits found in the upper dermis of the skin. The main antigen of these IgA autoantibodies is epidermal transglutaminase (TG3), which is normally present in the keratinocyte layer of epidermis, but not in the dermal-epidermal junction where it accumulates complexed with the IgA in the case of DH. Although many factors, including strong genetic background involved in the pathogenesis of DH has been identified, the details of the molecular mechanisms by which these factors end up affecting the skin, are unknown. The aim of this thesis is to identify if certain structural proteins in the skin attract IgA and TG3 into the dermal papillae and further cause the development of the symptoms. In addition, the mechanism of blister formation, *i.e.,* skin layer separation, particularly the potential involvement of neutrophils is assessed.

Indirect immunofluorescence method was used for observing colocalization of the TG3-IgA complexes and selected structural proteins of the skin. Frozen skin biopsy sections of DH patients were stained with antibodies against TG3, IgA and structural proteins: type IV collagen, type VII collagen and fibrinogen and the colocalization between TG3 and others were visualized with labelled secondary antibodies and fluorescence microscopy. Further, in order to confirm if circulating TG3-IgA immunocomplexes in DH patient's serum interacts with fibrinogen, fibrinogen protein was western blotted separately in native and denatured forms. Isolated fibrinogen membranes were treated with DH patient's serum and IgA was observed by immunofluorescence. For neutrophil treatment of the skin biopsies, samples were activated with DH patient's serum and incubated with isolated granulocyte solution.

First, the immunofluorescence results showed that TG3 colocalizes with IgA and fibrinogen. To the contrary collagen IV and collagen VII were seen to localize separate from TG3. Secondly, fibrinogen protein immunoblots did not show binding between isolated fibrinogen and DH patient serum derived IgA. Finally, no visible difference in dermal-epidermal junction was seen between control and granulocyte treated skin section samples.

To conclude, this study supports the evidence that fibrinogen might play a part in pathogenesis of DH with TG3. Also based on the findings of this study, two subtypes of collagen proteins, type IV and type VII can be convincingly excluded as potential interaction partners for TG3 in the dermal-epidermal junction. Lastly, at least the methods used in this thesis were not able to demonstrate IgA and fibrinogen interaction nor neutrophil driven skin layer separation *in vitro*.

Keywords: dermatitis herpetiformis, transglutaminase, TG3, celiac disease, IgA, fibrinogen, collagen

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TIIVISTELMÄ

Paula Rantala: Ihokeliakian patogeneesin molekulaarisista mekanismeista iholla

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Dermatitis herpetiformis (DH) eli ihokeliakia on iholla esiintyvä keliakian ilmentymä, jossa ruuasta saatu gluteeni aiheuttaa kutisevaa ja rakkulaista ihottumaa, erityisesti kyynärpäissä, polvissa ja pakaroissa. Hoitona on gluteenittoman ruokavalion tiukka noudattaminen lopun elämää. Ihokeliakia tunnistetaan immunofluoresenssitutkimuksella, jolla voidaan havaita taudille tyypilliset immunoglobuliini A (IgA) esiintymät ihon dermiksen eli verinahan ja epidermiksen eli orvaskesin yhdyskohdassa. Ihokeliakiassa IgA-autovasta-aineiden antigeeninä on epidermaalinen transglutaminaasi (TG3). TG3:a esiintyy normaalisti terveessä ihossa epidermiksen keratinokyyttikerroksessa, mutta ei dermiksen ja epidermiksen liitoskohdassa, mihin se ihokeliakiassa kerääntyy immunokompleksina IgA:n kanssa. Vaikka useita ihokeliakian kehittymiseen osallistuvia tekijöitä, kuten vahva geneettinen tausta, on tunnistettu, sen molekulaarisia mekanismeja, jotka saavat ihon oirehtimaan gluteenista, ei vielä tunneta yksityiskohtaisesti. Tämän tutkimuksen tavoitteena on tunnistaa vuorovaikuttavatko tietyt ihon rakenneproteiinit IgA:n ja TG3:n kanssa, sitoen ne aiheuttamaan paikallisen immuunireaktion iholla. Lisäksi tutkimus pyrkii havainnollistamaan ihokerrosten erkaantumisen mekanismia ja erityisesti neutrofiilien potentiaalista osallisuutta.

Kolokalisaation tarkasteluun käytettiin epäsuoraa immunofluoresenssimenetelmää. DH-potilaiden iholeikkeet värjättiin TG3:n, IgA:n ja tyypin IV kollageenin, tyypin VII kollageenin ja fibrinogeenin vasta-aineilla, jonka jälkeen TG3:n vuorovaikutusta muiden kanssa tarkasteltiin fluoresoivilla sekundaarisilla vasta-aineilla ja fluoresenssimikroskopialla. Sen tutkimiseksi, vuorovaikuttavatko DH-potilaan verenkierrossa kiertävät TG3-IgA-immunokompleksit fibrinogeenin kanssa, fibrinogeeniproteiini ajettiin *western blot* -menetelmällä sekä natiivissa että denaturoidussa muodossa. Proteiinikalvot käsiteltiin DH-potilaan seerumilla ja IgA havaittiin immunofluoresenssimenetelmin. Iholeikkeiden neutrofiilikäsittelyä varten näytteet aktivoitiin DH-potilaan seerumilla ja inkuboitiin kokoverinäytteestä eristetyn granulosyyttiliuoksen kanssa.

Immunofluoresenssitulokset osoittivat, että TG3 kolokalisoituu IgA:n ja fibrinogeenin kanssa. Päinvastoin kollageeni IV ja kollageeni VII nähtiin selvästi kolokalisoituvan erillään TG3:sta. Fibrinogeeniproteiinin immunoblottauksessa fibrinogeenin ja TG3-IgA immunokompleksien välistä vuorovaikutusta ei pystytty kuitenkaan todentamaan. Myöskään näkyvää eroa ei ollut havaittavissa dermiksen ja epidermiksen liitoksessa kontrollin ja granulosyytti-käsiteltyjen ihonäytteiden välillä.

Yhteenvetona tämä tutkimus tukee näyttöä siitä, että fibrinogeeni saattaa olla osana ihokeliakian patogeneesiä TG3:n kanssa. Tämän tutkimuksen tulosten perusteella kaksi kollageeniproteiinien alatyyppiä, tyyppi IV ja tyyppi VII voidaan sulkea pois TG3: n mahdollisina sitoutumispartnereina ihossa. Tässä tutkimuksessa käytetyillä menetelmillä ei pystytty osoittamaan IgA:n ja fibrinogeenin yhteisvaikutusta eikä neutrofiileistä johtuvaa ihokerroksien erkaantumista *in vitro*.

Avainsanat: dermatitis herpetiformis, ihokeliakia, keliakia, transglutaminaasi, TG3, IgA, fibrinogeeni, kollageeni

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

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LIST OF SYMBOLS AND ABBREVIATIONS

APCs	Antigen presenting cells
BSA	Bovine serum albumin
CD	Celiac disease
DAPI	4′,6-diamidino-2-phenylindole
DEJ	Dermal-epidermal junction
DH	Dermatitis Herpetiformis
DIF	Direct immunofluorescence
EBA	Epidermolysis bullosa acquisita
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GFD	Gluten-free diet
GzmB	Granzyme B
HCl	Hydrochloride
HE	Hematoxylin and eosin
HLA	Human leukocyte antigen
IgA	Immunoglobulin A
IL	Interleukin
INF- γ	Interferon γ
kDa	Kilodalton
MMPs	Matrix metalloproteinases
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
RBC	Red blood cell
RT	Room temperature
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TBST	Tris buffered saline Tween 20
TG2	Tissue transglutaminase
TG3	Epidermal transglutaminase
Th	T helper cells
TRITC	Tetramethylrhodamine

INTRODUCTION

Skin, supported by the immune system, protects us from external pathogens and physical trauma. Still, disturbances in the immune system can turn it against the same tissues it is protecting causing interference in the normal physiological functions. One of these disorders is dermatitis herpetiformis (DH), a chronic cutaneous manifestation of gluten-sensitive enteropathy called celiac disease (CD). Along with many other autoimmune diseases, the incidence of celiac disease is increasing (Lerner et al. 2015, Bolotin and Petronic-Rosic 2011), potentially due to gradual changes in dietary habits and the living environment. The incidence of DH on the other hand, is slowly declining, possibly due to improving diagnostics of the primary underlying enteropathy.

Nevertheless, continuous itching feeling in addition to blister and lesion formation in the skin can be unbearable to live with and the quality of life may suffer. In many of the skin related-disease cases the diagnosis and especially finding the right treatment is not always straightforward. Therefore, it is important to understand the mechanisms behind the pathogenesis and symptoms of these disorders so that better prevention and early recognition could be achieved.

In this thesis, I have attempted to elaborate the pathogenic mechanisms of DH. By using immunohistochemistry methods, I examine molecular interactions between autoimmune responses and the skin to clarify their role in the pathogenesis of DH.

LITERATURE OVERVIEW

1.1 Overview to Dermatitis Herpetiformis

Dermatitis herpetiformis is considered to develop from untreated celiac disease, an autoimmune-mediated enteropathy. The incidence of CD is increasing and though the opposite is happening in DH, still around 10% of CD patients develop DH (Reunala et al. 2018, Reunala et al. 2021). Both disorders are driven by the ingestion of dietary gluten present in wheat, barley and rye. Therefore, the only treatment is a lifelong gluten-free diet (GFD). It is worth noting, that in both diseases there is an increased risk of lymphomas, lymphocyte originated malignancies and the risk is even higher when GFD treatment is neglected in the first years after diagnosis (Hervonen et al. 2005).

Symptoms of DH include blistering and rash in the skin, particularly in the elbows, knees and buttocks. Along with the symptoms, DH is diagnosed based on the detection of granular deposits of immunoglobulin A (IgA) class autoantibodies in skin biopsies taken from healthy skin, adjacent to symptomatic areas, by direct immunofluorescence staining. Previous research has shown that IgA autoantibodies bind to their autoantigen, transglutaminase 3 (TG3) enzyme and localize persistently in DH patients' skin, where they are found as granular immunocomplexes in the dermal-epidermal junction (DEJ) and/or in the papillary dermis (Sárdy et al. 2002). Thus, it is of note that in DH, TG3 localizes abnormally in the papillary dermis, while it is present predominantly in the cornified layer of the epidermis also in healthy individuals.

Interestingly, IgA has been found in the skin although a DH patient has been asymptomatic for up to 19 years with strict gluten-free diet treatment (Hietikko et al. 2018a). Reunala et al. (Reunala et al. 2015b) have proposed strong binding of TG3-IgA immunocomplexes to the extracellular matrix (ECM) in papillary dermis to explain this. Potential binding site for TG3-IgA immunocomplexes could be for example members of the collagen family, structural proteins present in the ECM of the skin. Of these collagen type IV and type VII have been connected to other skin-related diseases (Gelse et al. 2003, Sitaru et al. 2002a, Sitaru et al. 2002b). In addition, fibrinogen is worth considering as it is a structural protein also present in the papillary dermis and it has been shown to serve as an enzymatic substrate for TG3 and thus interact with TG3-IgA immunocomplexes (Taylor et al. 2015). It has been suggested that activated fibrinogen takes part in blister formation and might attract neutrophils, T cells, and macrophages to the site (Donaldson et al. 2007).

Unlike celiac disease-specific autoantibodies targeting transglutaminase 2 (TG2), TG3-autoantibodies have not been well characterized, and it is still unknown what precisely attracts the immunocomplexes to the papillary dermis or the dermal-epidermal junction and causes the following formation of blisters typical to DH.

1.1.1 Historical background

DH was first presented and named by Louis Duhring at the university of Pennsylvania in 1884 (Duhring 1983). Over 60 years later, sulphones were discovered as treatment to symptoms of DH, then called Duhring's disease (Esteves and Brandao 1952). In the early 1960s Pierard and Whimster (Pierard and Whimster 1961) reported first histological skin biopsy findings for DH, and eight years later, van der Meer (van der Meer 1969) made a breakthrough in immunological and diagnostic aspects of DH by discovering immunoglobulin A (IgA) deposits at the dermal papillae using direct immunofluorescence methods.

Furthermore, in 1960s and 1970s the link between DH and celiac disease was discovered, first by similar findings of villous atrophy in DH and CD patients and then by responses to gluten-free diet, which eased both skin symptoms and enteropathy (Marks et al. 1966, Fry et al. 1973, Reunala et al. 1977). Gluten challenge studies confirmed this later by demonstrating that relapse of skin symptoms and enteropathy reoccurred when gluten was introduced into the patients diet again (Leonard et al. 1983).

Around the same time, the genetic linkage connecting CD and DH was discovered for the first time (Katz et al. 1972). Human leukocyte antigen (HLA) class II pattern, especially HLA-DQ2 or HLADQ8 haplotypes play a part in the pathogenesis of both diseases (Balas et al. 1997).

Other significant pathogenetic discovery for CD and further to DH was finding the autoantigen for IgA autoantibodies. Dieterich et al. (Dieterich et al. 1997) found IgA deposits targeting tissue transglutaminase (TG2) in the small bowel mucosa of CD patients, whereas Sárdy et al. (Sárdy et al. 2002) discovered TG3 acting as a main target for IgA in DH.

1.1.2 Clinical presentation

Dermatitis herpetiformis is characterized by an itchy and blistering rash and symmetrically distributed lesions, especially in the elbows, buttocks, and knees and in some cases on the abdomen, shoulders, upper back, scalp, face and groin (Collin and Reunala 2003, Collin et al. 2017) (Figure 1). Because of pruritus, patients tend to scratch the lesions, which then frequently excoriate. In addition, lesions can lead to post inflammatory pigment changes (Collin et al. 2017). Furthermore, continuous itching has been reported to cause sleeping disturbances (Kulczycka-Siennicka et al. 2017).

Localized rash predominantly in extensor skin areas is the most common presentation of DH, but also other sites have been reported. Oral manifestations are extremely rare and consist of erosions, vesicles and erythematous macules on the mucosa and tongue. Yet, the direct connection between these oral manifestation and DH is still unclear and most likely they are related to DH-associated diseases. (Bolotin and Petronic-Rosic 2011) Other clinical findings include acral purpura, blood spots in the peripheral areas of the body, which are considered as a leading manifestation of DH (Bolotin and Petronic-Rosic 2011, Tu et al. 2013). Also, dental defects, including enamel pits, horizontal grooves and colour changes are linked to CD and DH (Bolotin and Petronic-Rosic 2011).

Clinical presentation of DH can be similar with other skin related diseases. Therefore, difference between diseases like linear IgA disease, bullous pemphigoid, atopic, contact or nummular dermatitis, or scabies and DH should be made in diagnosis (Bolotin and Petronic-Rosic 2011).

Usually, DH patients show similar intestinal symptoms as patients suffering from celiac disease. These symptoms are caused by similar pattern of villous atrophy in the small intestine or milder mucosal changes. (Collin et al. 2017) About three quarters of DH patients show classical gluten-sensitive villous atrophy, and the rest have more moderate changes, mostly seen as increased number of intraepithelial γδ T lymphocytes. Therefore, the most common intestinal symptoms of DH are only discomfort in gastrointestinal area and occasional digestive tract symptoms. Much rarer in DH are the severe symptoms of CD, significant weight loss and steatorrhea. (Collin and Reunala 2003)

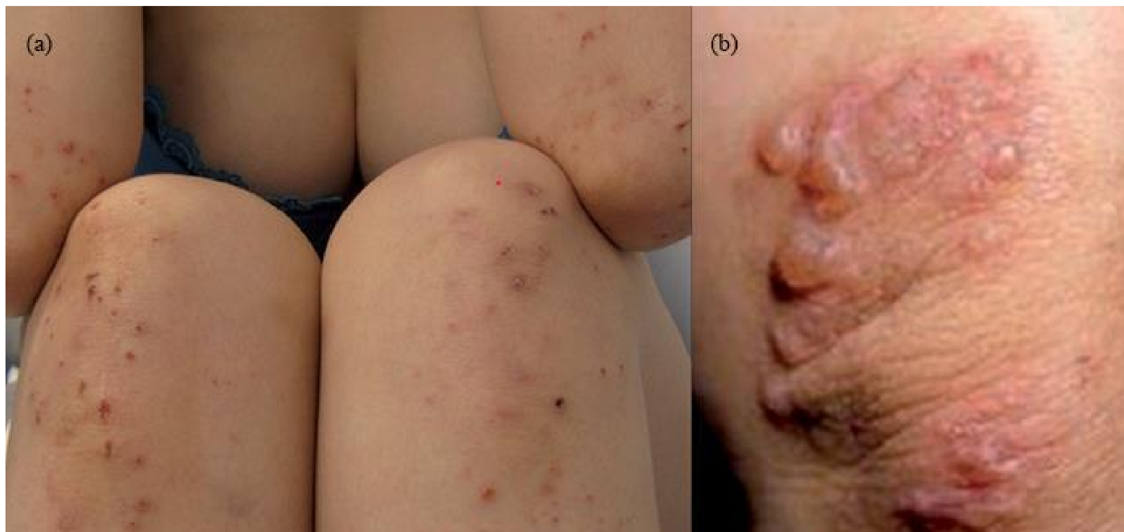

Figure 1. *Typical sites of the symptoms of DH. (a) symmetrical rash and erupted blisters on the knees and the elbows. (b) closer view of the blisters on the elbow. Figure adapted from Reunala et al. (2015b)*

1.1.3 Epidemiology

Dermatitis herpetiformis is an autoimmune disease with gradually decreasing incidence (Reunala et al. 2021). It occurs mostly in Northern Europe and in the USA (Bolotin and Petronic-Rosic 2011), while it is rare amongst African and Asian populations, mainly because of genetic and food consumption differences compared to the Western world (Zhang et al. 2012).

In Finland, the prevalence of DH has increased from 10.4 per 100 000 to 75.3 per 100 000 between years 1980 and 2009, and about one in eight celiac patients have DH (Reunala and Lokki 1978, Salmi et al. 2011). In contrast, annual incidence of DH has decreased from 5.2 to 2.7 per 100 000 in Finland between 1980 and 2009 and from 1.8 to 0.8 per 100 000 person-years in the UK between 1990 and 2011 (Salmi et al. 2011, West et al. 2014), whereas in case of CD the incidence has increased fourfold during the follow up. (West et al. 2014, Virta et al. 2017). This could be explained by growing awareness of CD and DH, and evolved screenings of both diseases (Salmi et al. 2011).

Dermatitis herpetiformis is not an age-related disease, but it is rarely diagnosed in children. From 477 patients, only 4% where under 16 years at the age of DH diagnosis. In the 2000s, the mean age of diagnosis was 48.8, which is around 12 years more than it was 1970s. (Salmi et al. 2011) Incidence of DH was highest in the age-group 50-69 in the UK and in Finland (West et al. 2014, Salmi et al. 2011).

Population-based studies before the 21st century have shown a slight difference in the prevalence between genders, men to female ratio being between 1,5:1 and 2:1 (Bolotin and Petronic-Rosic 2011). However, in the latest studies, the results were more balanced with male to female ratio of 1.1:1 (Salmi et al. 2011) and 1:1 (West et al. 2014).

1.1.4 Diagnosis

Along with skin symptoms, DH is diagnosed by detecting granular deposits of IgA autoantibodies in the papillary dermis using direct immunofluorescence staining (DIF) (Figure 2). Skin biopsies should be taken from healthy skin, adjacent to symptomatic areas due to appearance of the IgA deposits. (Zone et al. 1996, Collin and Reunala 2003). About 5% of DIF results are false-negative, mostly because of the site of the biopsy (Zone et al. 1996). In some cases, particularly in Japan, IgA deposits have been detected in fibrillar form instead of granular (Ohata et al. 2012, Makino and Shimizu 2019).

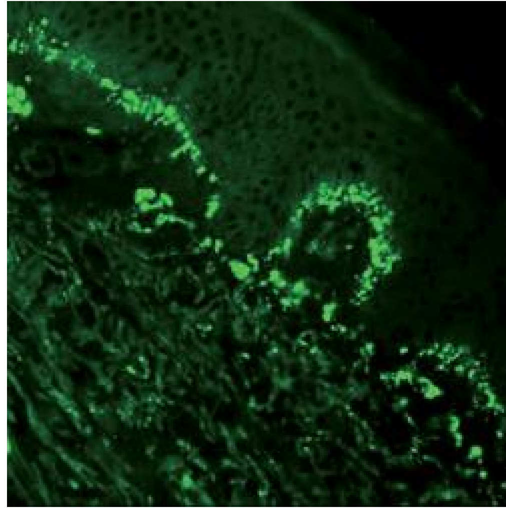

Figure 2. *Direct immunofluorescence (DIF) of DH patients skin showing granular immunoglobulin A (IgA) deposits (green) at the dermal papillae. Picture adapted from Reunala et al. (2015b)*

DIF is often enough to confirm the diagnosis of DH with the right clinical picture. Still even DIF, the golden standard of DH diagnostics, has its limitations. Studies have shown negative result for DH with DIF even though other marks of DH are clearly present (Alonso-Llamazares et al. 2007, Bresler and Granter 2015). Factors affecting DIF results can be time and site of the biopsy, which can give false-negative results (Beutner et al. 2000, Huber et al. 2013). On the other hand, IgA deposits can persist in the papillary dermis even years after gluten-free diet treatment (Leonard et al. 1983, Hietikko et al. 2018a).

In addition to DIF, histopathological methods can reveal subepithelial blisters and neutrophil infiltrate in dermal papillae (Warren and Cockerel 2002). However, even though the specificity of commonly used histological diagnostic method, hematoxylin and eosin (HE) staining has been shown to be high (0.95), the sensitivity is only 0.75. That means HE stain gives true negatives, but not always correct positive test results. Also, HE staining results can be easily mixed with other bullous dermatoses. (Bresler and Granter 2015) Advantage of HE staining is its cost-effectiveness compared to other diagnostic methods (Caproni et al. 2009, Bresler and Granter 2015).

Some problems related to the diagnosis of DH is delay (Mansikka et al. 2018, Salmi et al. 2011) and misdiagnosis to other inflammatory skin diseases like atopic dermatitis and eczema (Bonciolini et al. 2018). According to Mansikka et al. (Mansikka et al. 2018), diagnosis can be delayed up to years after first symptoms appear, although growing knowledge about DH and gluten sensitivity has cut down the average delay.

Several studies have shown IgA deposits in the papillary dermis in healthy skin of individuals diagnosed with CD patients or other skin related disease (Caproni et al. 2009, Bonciolini et al. 2018, Antiga et al. 2021), which is why novel, more sensitive diagnostic methods might be needed. In a recent clinical study (n=45), 64% of CD patients without DH had granular IgA or

IgA1 deposits in the DEJ, 93% showed positive anti-TG2 antibodies and 22% had anti-TG3 antibodies (Antiga et al. 2021).

Serological analysis is thought to be a supplemental diagnostic tool for DH since CD and DH are characterized with specific autoantibodies, especially IgA class anti-TG2 antibodies are often found in the circulation in both conditions (Dieterich et al. 1997). Anti-TG2 is easily and often tested with commercial enzyme-linked immunosorbent assay (ELISA) method. Majority of DH patients have IgA anti-TG2 autoantibodies in their serum (Dieterich et al. 1999). Other potential markers, such as IgA anti-TG3, have been suggested, but they are still missing standardized and available testing methods or lack the ability to distinct DH from other CD related skin disorders. (Antiga et al. 2019)

1.1.5 Treatment

Like for celiac disease, a life-long gluten-free diet (GFD) is the most potent treatment for dermatitis herpetiformis (Ciacci et al. 2015). In GFD, gluten containing cereals like wheat, rye and barley, as well as foods containing these are strictly avoided. With the diet, symptoms in the skin and gastrointestinal area ease relatively fast, the mucosa of the small intestine recovers in a year or so, and the TG2- and TG3 autoantibodies decrease from blood circulation along with small bowel atrophy recovery (Fry et al. 1973, Reunala et al. 1977, Reunala et al. 2015a). Also, cutaneous deposits of IgA and TG3 gradually disappear (Hietikko et al. 2018a, Mansikka et al. 2019).

Few individuals have refractory DH, as in some celiac disease cases. In CD that means that the GFD treatment does not affect the symptoms nor histology, whereas in DH the gastrointestinal symptoms may disappear, and refractory characteristics are seen as persistent rash and IgA deposits in the dermis (Hervonen et al. 2016). On the contrary, few cohort studies indicate that some patients go to remission with GFD and can introduce gluten to their diet again (Leonard et al. 1983, Garioch et al. 1994, Bardella et al. 2003). Nevertheless, because of controversial conclusions, it is highly recommended to adhere to the lifelong GFD (Mansikka et al. 2019).

Refractory and many symptomatic DH patients, whose rash and pruritus stay persistent even for several years on a strict GFD, need additional treatment (Hervonen et al. 2016). Dapsone (4,4-diaminodiphenylsulfone) eases the rash and itching. It affects to the inflammatory responses by inhibiting neutrophil and eosinophil- mediated tissue damage and inflammation (Zhu and Stiller 2001). It relieves the symptoms in a few days but does not affect the IgA deposits or enteropathy. The dose (25-100mg/day) is adjusted by the intensity of the itch and development of new lesions, and gradually decreased when skin has healed from the rash. (Zhu and Stiller 2001) The time period of the dapsone usage has a wide spectrum between patients, mostly because of the relapses from the strict GFD (Reunala et al. 2021).

Usually, dapsone is well tolerated, and with correct dosing, seldomly causes side-effects. With higher doses (over 200mg/day) the risk for methemoglobinemia, hemolysis, agranulocytosis and other rarer and untraceable side-effects rises (Zhu and Stiller, 2001). Option, without the risk of the side-effects is topical 5% dapsone gel, which has shown its potential to ease the rash (Burbidge and Haber 2016).

1.2 Pathogenesis of Dermatitis Herpetiformis

Pathogenesis of DH has similarity to the initial stage of CD. Autoimmune reaction in the small bowel mucosa activated by dietary gluten drives the production of autoantibodies against peptides of gluten and tissue transglutaminase (TG2). The specificity of antibodies to the main autoantigen of DH, epidermal transglutaminase (TG3), is thought to develop from the initial TG2 responses. T cell and B cell mediated responses result in autoantibodies circulating in the blood stream and IgA anti-TG3 antibodies aggregating with TG3 enzyme in the papillary dermis of the skin, where these TG3-IgA immunocomplexes likely mediate local immunoreactions leading to blister and lesion formation in the lamina lucida of the skin. (Antiga et al. 2019) (Figure 3)

1.2.1 Genetic and environmental factors

Both DH and CD have same strong genetic predisposition. In a Finnish family study, over 18% of the 281 DH patients had a first-degree relative affected by CD or DH, and the risk of developing either disease was significantly higher with an affected relative (Hervonen et al. 2002). In other small-scale epidemiological study from the USA, 6,5% of DH patients had a first-degree relative that had a DH diagnosis as well (Meyer and Zone 1987). Whereas in case of CD, results were similar with a larger, meta-analysis study of genetic susceptibility, in which 7,5% of CD patients had also first-degree relatives diagnosed with CD (Singh et al. 2015).

Especially human leukocyte antigen class II, more precisely HLA-DQ2 (DQA1*0501 and DQB1*02 alleles) and HLA-DQ8 (DQA1*03 and DQB1*0302 alleles), haplotypes are carried by basically every DH and CD patient and are clearly involved in the pathogenesis of DH and CD (Spurkland et al. 1997). Yet, it is good to take notice that around 30% of the general population have HLA-DQ2 or HLA-DQ8 haplotypes and minority of them develop DH or CD. A genetic study on monozygotic twins showed that even with identical genetic background, individuals can develop either DH or CD, or even change the phenotype. (Hervonen et al. 2000, Kurppa et al. 2008) Therefore other genetic and non-genetic factors may contribute to the pathogenesis of DH and CD, beyond HLA-DQ genes and gluten. An example of another disease susceptibility gene, linked to CD, is myosin IXB (MYO9B), which regulates the permeability of the gut barrier through the actin cytoskeleton. Yet, more genetic research is needed. (Bolotin and Petronic-Rosic 2011)

The main exogenous factor in DH and CD is dietary gluten. Gluten is a general concept for storage proteins, prolamin glycoproteins present in wheat, barley and rye. In wheat, it is present in an alcohol-insoluble form glutenin and alcohol-soluble form gliadin, in barley as hordein and in rye as secalin (Wieser 2007). Due to the high amount of glutamine- and especially proline-residue rich areas, gluten is partially resistant to degradation by gastrointestinal protease-enzymes. Remaining, non-degraded long polypeptides from gluten act as antigens in the small intestine where they cause immunological reactions leading to DH or CD. (Shan et al. 2002)

In addition to gluten, other environmental factors have been suggested to contribute to the pathogenesis of DH. Potassium iodide has been proven to trigger the clinical skin reactions, vesicular lesions and cellular infiltrates when topically or orally introduced to DH patients (Taylor and Zone 2018). Taylor and Zone (Taylor and Zone 2018) suggest on the grounds of immunohistochemistry test results that the response to potassium iodide is dependent on TG3 activity when aggregated to IgA. In addition to these, cases have been reported where development of DH has been linked to certain hormone treatments or stress and inflammation reaction chain caused by enteral surgical operations (Antiga et al. 2019).

1.2.2 Intestinal manifestations

It is believed that like in celiac disease, the origin of DH development lies in the gluten intolerance derived from the gut. Therefore, the pathogenesis somewhat overlaps with CD. The main autoantigen of CD, tissue transglutaminase (TG2), catalyses the deamidation (glutamine to glutamate) of gliadin, component of gluten into a form that is recognized by the HLA-DQ2/8-molecules on the surface of antigen presenting cells (APCs). This leads to immune responses which manifest typically as gastrointestinal symptoms, small intestine damage, inflammation, villous atrophy, and increased level of gamma/delta type intraepithelial lymphocytes. Though in DH these occur in varying degrees and are in many cases much more moderate than in CD (Marks et al. 1966, Alakoski et al. 2012, Reunala et al. 2015b). According to studies, gluten dependent TG2-IgA deposits are found in the small bowel mucosa of around 80% of DH patients (Koskinen et al. 2010, Salmi et al. 2014).

Immune responses activated by gluten drive the production of anti-endomysial IgA autoantibodies, anti-TG2, and anti-TG3 antibodies, against the main autoantigen of DH. Even though TG3 has similar conserved domains of enzymatic functions with TG2 (Sárdy et al. 2002), it has notably lower efficiency to form enzyme-peptide -complexes with gluten, and only via thioester linkages, compared to TG2 which has the ability to form iso-peptide and thioester bonds with gluten (Stamnaes et al. 2010). Even though both CD and DH patients can produce antibodies against both transglutaminases, the TG3-specific antibodies seem to bind more selectively and with higher

avidity to TG3 in DH. Additionally, studies have not been able to show cross-reactivity of antibodies between different transglutaminase isoforms. Moreover, the mechanisms for anti-TG3 IgA autoantibody production is still somewhat unclear. More studies are needed to clarify whether it happens through primary antigen presentation or by epitope spreading, where existing autoimmune response expands to target addition antigens similar to the original one. (Reunala et al. 2015, Kárpáti et al. 2018)

One *ex vivo* study with small-intestinal mucosal cultures revealed that TG3 antibody secreting plasma cells can be found in the intestine, especially in patients with active DH (Hietikko et al. 2018b). This was supported by another study, which followed the occurrence of TG3 antibody secreting plasma cells of DH and CD patients through gluten challenge. TG3 secreting plasma cells were found in the small intestine, and they increased in number with gluten intake suggesting they are gluten dependent. Also, these cells were not detected with similar degree in CD patients suggesting strong DH-specificity (Sankari et al. 2020).

1.2.3 Cutaneous manifestations

Skin has three main layers, outermost is the protective, keratinocyte containing epidermis, middle is the functional dermis and under those lies the subcutaneous fat containing hypodermis. The dermis has two layers, the reticular layer and the upper one, papillary layer stretches to epidermis with vascularized villus-like dermal-papillae. The area between epidermis and dermis is called the basement membrane or the dermal-epidermal junction (DEJ), which is further divided to dermal side lamina densa and epidermal side lamina lucida (Breitkreutz et al. 2013).

It is important to note that TG3 is expressed in the cornified envelope, the uppermost layer of epidermis in normal skin (Zone et al. 1996). The distinguishing mark of DH are the IgA deposits found specifically in the papillary dermis together with TG3 enzyme (Sárdy et al. 2002). The mechanisms of the accumulation of these TG3-IgA complexes and further blister and lesion development in DH are weakly understood.

Nevertheless, there is a clear correlation between the circulating anti-TG3 IgA antibodies and skin lesions in DH (Görög et al. 2016a) but the role of TG3-IgA deposits in the pathogenesis is still unclear, particularly since they can also be found in asymptomatic areas of skin in DH patients. The deposited and enzymatically active TG3-IgA complexes have been hypothesized to induce inflammation resulting in blister and lesion formation in multiple ways. TG3 has potentially the ability to bind and fix fibrinogen, thus promoting its proinflammatory properties (Taylor et al. 2015). The IgA deposits may also attract neutrophils expressing Fc IgA receptors on their surfaces (Smith et al. 2002). Gornowicz-Porowska et al. (Gornowicz-Porowska et al. 2017) found significant increase in cutaneous expression of Fc IgA receptor CD89 in active DH and suggested that together with Fc IgA receptor CD71 it modifies the neutrophilic immune responses in DH. Fc

IgA receptors are expressed in several cell types. CD89 is commonly expressed in neutrophils while CD71 is abundantly expressed in cells with high proliferation rate, like intestinal epithelium and basal epidermis (Gornowicz-Porowska et al. 2017).

Then again, few issues stand against these indications. For instance, TG3-IgA deposits are also found in the healthy skin of DH and CD patients (Donaldson et al. 2007, Bonciolini et al. 2018) and the deposits can persist in the skin even after years of GFD and the disappearance of active rash (Reunala et al. 2015, Hietikko et al. 2018a) For these reasons, it is possible that other stimulating factors play a part in the blistering rash. These factors could be other components of the skin driving the immunological response in addition to TG3-IgA complexes or activation by external mechanical force like pressure or stretching (Reunala et al. 2015b).

1.3 Immunopathogenic aspects of Dermatitis Herpetiformis

DH is still lacking detailed insight of the immunopathogenic cascades taking place in the small intestine and in the DEJ. In the small bowel mucosa, APCs begin the cascade by presenting gluten derived peptides and TG2 to CD4+ helper T cells, which then promote the B cell differentiation to antibody producing plasma cells (Figure 3). T helper (Th) cells can be divided to several subclasses with distinct cytokine profiles. Interestingly, celiac disease is strongly considered as Th1-mediated (Nilsen et al. 1995), while based on the finding of Th2-related cytokines, interleukin (IL)- 4 and IL5 in DH patients' skin and serum, indicate that DH might be mediated by Th2 or both Th1 and Th2 cells. In addition, in the same study the level of a typical Th1 cytokine, interferon γ (INF- γ) was weak. (Caproni et al. 1998) Yet in a later study, circulating gliadin-responsive IFN-γ expressing cells were found using the ELISpot method in both CD and DH patients (Kalliokoski et al. 2020). Also, elevated levels of proinflammatory cytokines IL-8 and TNF-α have been reported in DH patients' serum and IL-8 levels positively correlate to dietary gluten intake (Hall et al. 2006, Hall et al. 2007).

Regardless, the main autoantigen of DH is TG3 while in case of CD it is TG2, both DH and CD patients have been found to have autoantibodies targeting both autoantigens. But in DH only TG3 forms granular deposits with IgA in the papillary dermis and moreover only DH patients have IgA antibodies that bind selectively and with high affinity to TG3 (Sárdy et al. 2002, Sankari et al. 2020). The mechanism for the development of autoantibody production against TG3 in both CD and DH patients is still unknown. Yet, in the light of current knowledge, epitope spreading is one possible mechanism since genetic sequences of TG2 and TG3 are highly homologous. In addition, autoantibodies against a third transglutaminase, similar to TG2 and TG3, the neuronal transglutaminase (TG6) have been found in DH and CD patients. Thirdly, the epidemiology of DH, more specifically the low incidence of DH and low prevalence of IgA autoantibodies against TG3 in children supports this theory. (Antiga et al. 2019). Therefore, two or more subpopulations

of plasma cells, clonally related to each other could produce autoantibodies in CD and DH. Site of these IgA autoantibody-secreting plasma cells is also unclear. Few studies indicate that anti-TG3 IgA antibody -secreting plasma cells in small intestine (Hietikko et al. 2018b, Sankari et al. 2020).

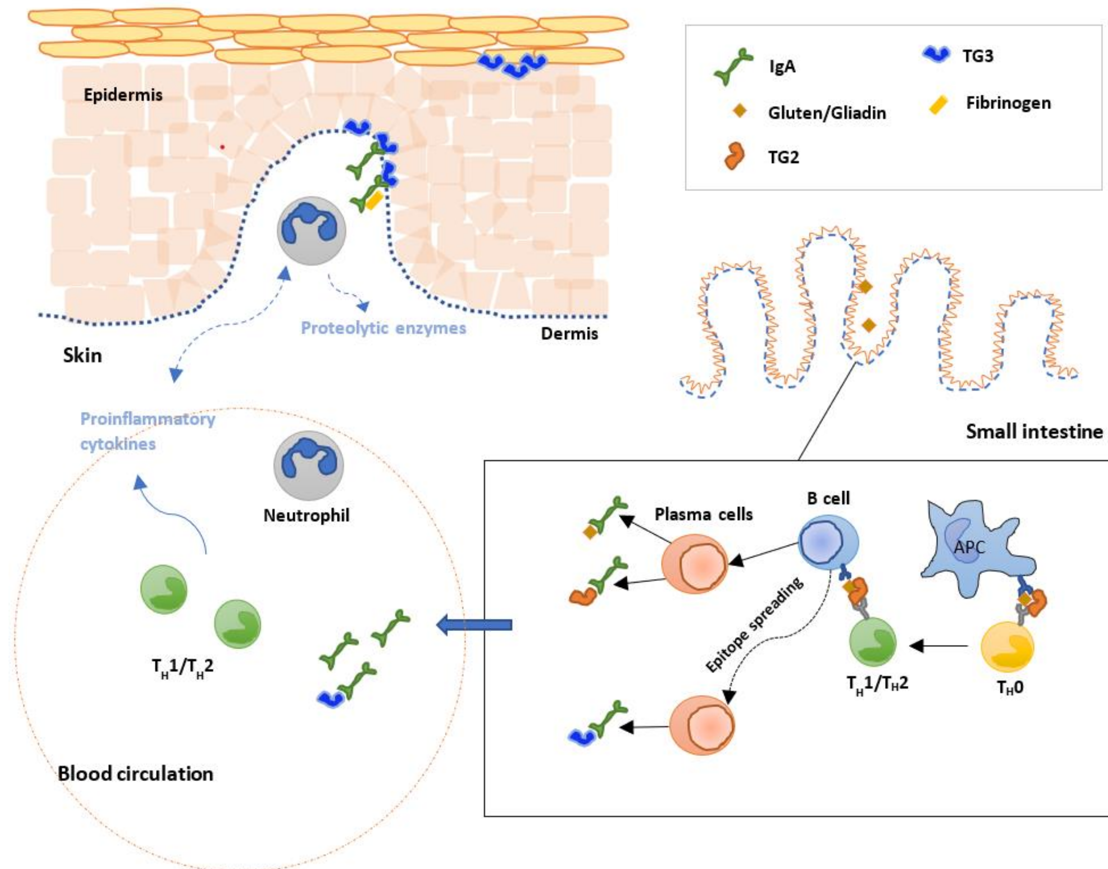

Figure 3. *Potential pathogenic mechanisms of dermatitis herpetiformis. In the small intestine, gluten and its derivate gliadin peptides induce autoimmune reaction and production of autoantibodies mediated by T helper cells, subtypes 1 or 2 (T_H1/T_H2). Main autoantibodies in DH, anti-epidermal transglutaminase (TG3) IgA antibodies could emerge from B cells initially producing autoantibodies against tissue transglutaminase (TG2) through epitope spreading. In the skin IgA-TG3 immunocomplexes accumulate in the dermal papillae, which could result from either circulating immunocomplexes binding to the site or TG3 diffuses from upper layers of epidermis where it is normally present. In the papillary dermis, immunocomplexes also cross-link with fibrinogen and attract neutrophil infiltration. Complex interplay between cytokines produced by T helper cells and possibly neutrophils, neutrophil derived proteolytic enzymes targeting structural proteins in the basement membrane, lead to cleavage of basement membrane connecting dermis and epidermis and further to blister formation. APC: antigen presenting cell. Figure drawn based on original sources Antiga et al. (2019) and Kemppainen et al. (2021).*

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1.3.1 IgA and TG3 immunocomplexes

In normal skin, epidermal transglutaminase (TG3) is highly expressed in the epidermal cornified layer, where its function is to crosslink proteins and stabilize the cornified envelope. However, in DH it is also present in the dermal papillae with IgA and especially in the perilesional areas of the skin (Sárdy et al. 2002, Zone et al. 1996).

IgA deposits in papillary dermis are the hallmark of DH. Studies have characterized the IgA found in the skin as dimeric (Unsworth et al. 1982) and mainly as the IgA1 subclass, which is interesting because both IgA subclasses, IgA1 and IgA2, are found in the blood circulation of the skin (Preisz et al. 2005). It is still to be solved whether IgA antibodies form typical aggregates with TG3 before entering to the papillary dermis or colocalize only on site of DEJ.

There are two theories explaining the occurrence of TG3-IgA complexes in papillary dermis. One is the shedding of TG3 from the epidermis, the endogenous site of expression of TG3 in the skin, to papillary dermis caused by trauma or damage. To investigate this theory, study was made with human skin grafted mice, in which passive transfer of goat anti-TG3 or DH patients' sera with IgA anti-TG3 and granular deposits occurred (Zone et al. 2011). Other theory suggests that IgA-TG3 complexes circulate in the bloodstream and deposit into dermal papillae and cutaneous vessels by binding to structural proteins (Görög et al. 2016a, Preisz et al. 2005). Taylor et al. (Taylor et al. 2015) already demonstrated by DIF that TG3-IgA complexes bind to fibrinogen, one potential substrate for TG3 binding in papillary dermis.

1.3.2 TG3 substrates

Fibrinogen has a vital function in its active form fibrin as a clotting factor and therefore it normally circulates in the blood stream. In addition to blood clot formation, fibrinogen has inflammatory capabilities to activate immune responses, including T cells, macrophages, and neutrophils (Reunala et al. 2015b). In DH studies, fibrinogen, fibrin and fibronectin, other important factors in wound healing and cell adhesion have been recognized close to IgA in dermal papillae. Furthermore, TG3 enzyme has been shown to keep its activity in complex with IgA and fibrinogen. (Taylor et al. 2015) Fibrinolysis is a process where formed fibrin clots are broken down. More recently, Görög et al. (Görög et al. 2016b) studied the differences in fibrinolytic potential between untreated DH patients, dapsone treated DH patients, and healthy controls, and observed weaker plasma fibrinolysis in untreated DH patients than treated patients or control subjects in addition to differentiated fibrin structure.

Another potential group of TG3 binding partners are collagens. Older investigations of blister formation in DH and other blistering diseases have reported destruction of collagen proteins, especially collagen types IV, VII and XVII leading to separation of epidermis and dermis (Airola

et al. 1995, Sitaru et al. 2002a, Nishie 2020). Collagens are most common proteins and important structural components of extracellular matrix. For example, collagen VII, has important role in keeping the integrity of skin and dermal-epidermal adhesion with anchoring fibrils. Collagens, especially collagen IV, release physiologically and pathologically active components called matricryptins during proteolytic degradation. (Gelse et al. 2003, Ricard-Blum 2011) There is not much evidence of the relevance of collagens in pathogenesis of DH, but few indications give reason to investigate those connections more.

In immunomapping study of one DH patient's lesions demonstrated collagen IV and collagen VII staying in the dermal side of the blister, under IgA (Suzuki et al. 2014) Russo et al. (Russo et al. 2018) showed indications of collagen VII taking part of the blister formation. Also, two other skin diseases; epidermolysis bullosa acquisita (EBA) and bullous pemphigoid are characterized by autoantibodies targeting collagen VII and collagen IV, respectively, and these antibodies potentially take part in dermal-epidermal separation in these disorders (Sitaru et al. 2002a, Sitaru et al. 2002b). The role of collagen XVII in the development of blistering diseases have been studied to some extent, and its role in bullous pemphigoid has been suggested (Nishie 2020). Moreover, Raghunath et al. (Raghunath et al.1996) have demonstrated cross-linking between tissue transglutaminase and collagen VII.

1.3.3 Neutrophilic infiltrate

Neutrophils, the most common granulocytes, and their infiltrate have been recognized to accumulate to the papillary dermis and to have a key role in the inflammatory reactions associated with DH (Caproni et al. 2009). Characterization studies of circulating neutrophils in active DH patients versus inactive DH or healthy controls revealed increased binding ability to IgA antibodies via Fc IgA receptors, increased CD11b expression and decreased L-selectin expression. CD11b and L-selectin are thought to be necessary for the activation of neutrophils and suggests neutrophil priming, enhancement of responsiveness to an activating stimulus later in the inflamed site, in this case skin, occurring in the intestine. (Smith et al. 2002) To support this, another study demonstrated elevated expression of dermal endothelial E-selectin, which mediates the migration of neutrophils to the skin (Hall et al. 2006). Chemokine IL-8 upregulates the expression of CD11b and L-selectin in neutrophils and might be one proinflammatory factor in DH originating from the small bowel mucosa (Hall et al. 2007).

Already in early studies, matrix metalloproteinases (MMPs) collagenase and stromelysin-1 were found in the same site as neutrophil accumulations in the lesions of DH patients' basal keratinocytes of the skin. Proteases are capable to degrading structural proteins, collagenase fibrillar type collagens and stromelysin-1 basement membrane components, including collagen IV and laminin. Neutrophils could take part in the production of these proteases either directly with neutrophil

cytokines or through urokinase-type plasminogen activator -plasmin system, which activates collagenases. (Airola et al. 1995). In addition, Granzyme B (GzmB), another protease enzyme capable of cleaving basement membrane proteins has been found to accumulate in the papillary dermis with neutrophils in DH. GzmB was linked to the degradation of structural proteins collagen VII, collagen XVII and α6/β4 integrin and further to epidermal separation and blister formation (Russo et al. 2018).

Despite the amount and long timeline of studies trying to solve intriguing questions about the pathogenesis of the skin in DH, they have failed to give unambiguous answers. Reasons seems to be the complexity in the immunological responses and versatile nature of DH. Therefore, more elaborative studies are needed for better understanding of the pathogenetic mechanisms in DH.

OBJECTIVES

This study has two aims:

I. First to verify if TG3 and IgA binds to fibrinogen, collagen IV and/or collagen VII in the papillary dermis in DH patients.

II. Second, to investigate the potential role of neutrophils in the dermal-epidermal separation mechanism that leads to blister formation in DH.

MATERIALS AND METHODS

1.4 Immunohistochemistry of patient skin cryosections

Study included a total of 8 DH patient skin biopsies taken from perilesional skin. Samples were obtained by a collaboration with clinicians T. Salmi, T. Reunala and K. Hervonen. Frozen biopsy samples from following patients were used: two gluten challenge patients (post-challenge samples), two DH patients on a gluten-free-diet – treated but refractory to treatment and thus showing still symptoms, two untreated DH patients and two gluten-free-diet treated patients who were in remission. (Table 1) Gluten challenge patients were in remission with DH and after gluten challenged symptoms appeared again (Mansikka et al. 2019). Presence of TG3 and IgA immunocomplexes were determined in previous studies.

Prior to staining, cryosections were defrost and air-dried for 45min at room temperature and washed 2 x 3mins in phosphate-buffered saline (PBS). All used antibodies were diluted to 1% bovine serum albumin (BSA)-PBS if not noted otherwise (Table 1). Sections were stained with Fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal TG3 antibody (1:100) (A030, Zedira, Darmstadt, Germany) or with Rabbit anti-Collagen IV alpha 1 (1:500) (NB120-6586SS, Novus biologicals) overnight at 4 °C as primary antibody, followed by Alexa Fluor 555-conjugated donkey anti-rabbit antibody (1:500) (A32794, Thermo Fisher Scientific) 1h in room temperature as secondary antibody. In case of fibrinogen staining, FITC-conjugated rabbit polyclonal TG3 antibody (1:100) (A030, Zedira, Darmstadt, Germany) were diluted to 1% BSA PBS-Tween 20 (0.05%).

For double staining's, sections were further incubated with tetramethylrhodamine (TRITC)-conjugated goat anti-human IgA (1:50) (A18786, Life Technologies, USA) 1 h in room temperature, Mouse monoclonal Anti-Collagen Type VII (1:500) (SAB4200686-25UL, Sigma-Aldrich), Sheep polyclonal Anti-fibrinogen (1:500) (ab118533, Abcam) in 1% BSA PBS-Tween 20 (0.05%) or with FITC-conjugated rabbit polyclonal TG3 antibody (1:100) (A030, Zedira, Darmstadt, Germany) overnight at 4 °C as second primary. Respectively Alexa Fluor 568-conjugated donkey anti-mouse (A10037, Thermo Fisher Scientific) (1:500) or Alexa Fluor 555-conjugated donkey anti-sheep (1:500) (A21436, Thermo Fisher Scientific) in 1% BSA PBS-Tween 20 (0.05%) were used for 1 h in room temperature as secondary antibodies. Sections were washed with PBS between antibodies. Finally, sections were mounted under coverslips using coating agent containing 4′,6-diamidino-2-phenylindole (DAPI) for staining nuclei.

Stainings were analysed at 20x and 40x magnification with immunofluorescence microscope (Olympus BX60F5, Olympus Optical CO. LTD, Japan) and further processed with Fiji Image J and Photoshop Adobe Creative Cloud.

Table 1. *Primary and secondary antibodies used in immunofluorescence stainings.*

Primary Antibody	**Manufacturer**	**Dilution**	**Secondary Antibody**	**Manufacturer**	**Dilution**
Rabbit polyclonal FITC-labelled anti-human TG3	(A030, Zedira)	1:100 in 1% BSA-PBS			
Goat TRITC-conjugated anti-human IgA	(A18786, Life technologies)	1:50 in 1% BSA-PBS			
Rabbit anti-Collagen IV alpha 1	(NB120-6586SS, Novus biologicals)	1:500 in 1% BSA-PBS	Alexa Fluor 555-conjugated donkey anti-rabbit	(A32794, Thermo Fisher Scientific)	1:500 in 1% BSA-PBS
Mouse monoclonal Anti-Collagen Type VII	(SAB4200686-25UL, Sigma-Aldrich)	1:500 in 1% BSA-PBS	Alexa Fluor 568-conjugated donkey anti-mouse	(A10037, Thermo Fisher Scientific)	1:500 in 1% BSA-PBS
Sheep polyclonal Anti-fibrinogen	(ab 118533, Abcam)	1:500 in 1% BSA-PBS-Tween 20 (0.05%)	Alexa Fluor 555-conjugated donkey anti-sheep	(A21436, Thermo Fisher Scientific)	1:500 in 1% BSA-PBS-Tween 20 (0.05%)

1.5 Fibrinogen protein western blot and DH serum treatment

Fibrinogen from human plasma (F3879, Sigma-Aldrich) was diluted to 0,2 μg/μl in PBS and further mixed with loading buffer, 2x sodium dodecyl sulphate (SDS)-buffer (2% SDS, 40% glycerol, 10% 2-mercaptoethanol, 62,5mM Tris-HCl; pH 6,8 and 0,01% Bromophenol Blue) or native sample buffer (40% glycerol, 62,5mM Tris- hydrochloride (HCl); pH 6,8 and 0,01% Bromophenol Blue) for denatured or native protein electrophoresis, respectively. SDS-fibrinogen was denatured in heat block in 90 ºC for 5min before running on gel. For denatured fibrinogen, SDS-running buffer (25mM Tris, 192mM glycine and 0,1% SDS) was used and for native fibrinogen, Tris-buffer without SDS (25mM Tris and 192mM glycine).

Two amounts: 0,5μg and 1μg of SDS-denatured and native fibrinogen proteins were loaded into two Mini-Protean TGX Stain-Free Precast Gel (456-8126, Bio-Rad Laboratories Inc, USA). Denatured protein was run with Prestained PageRuler Plus ladder (#26619, Thermo Scientific) and native protein with same Prestained PageRuler Plus ladder (#26619, Thermo Scientific) as well as with Unstained protein ladder PageRuler Broad Range (#26630, Thermo Scientific). Gels were run separately using a Mini-Protean Tetra Vertical Electrophoresis Cell (Bio-Rad, USA).

Gels were scanned with ChemiDoc XRS+ System (Bio-Rad, USA) and Image Lab 6.0.1 for 2,5 mins before blotting. After they were blotted to Midi Format 0.2 µm PVDF Trans-Blot Turbo (#1704157, Bio-Rad) membrane using Trans-Blot Turbo transfer system (Bio-Rad). Manufacturers recommended transfer protocol of Mixed MW was used (7 min, 1.3 A, up to 25 V).

Prior to blocking, ladders were marked with WesternSure pen (Li-Cor). Denatured protein was blocked with 1% tris buffered saline (TBS)-BSA and native protein with Odyssey blocking buffer (927-50100, Li-Cor, USA) for 1h at room temperature. Membranes were incubated with DH patient serum previously tested and confirmed to contain a high level of TG3-antibodies (patient serum 1:50 in 1% BSA- tris buffered saline Tween 20 (TBST)) overnight on a shaker at +4°C.

For antibody processing, membranes were washed 3x5min with TBST. Then incubated with primary antibody, rabbit anti-human IgA (A0262, DAKO) 1:1000 in 1% BSA-TBST for 2 h on a shaker at room temperature and washed with TBST 3x5min. Secondary antibody, goat anti-rabbit IRDYE 680RD (Li-Cor, USA) 1:5000 in TBST was added and incubated for 1,5h on a shaker at room temperature. Between and after antibodies, membranes were washed with TBST for 3x5min, rinsed and left in TBS. Results were imaged with Li-Cor Odyssey CLx imager with both channels, 700nm and 800nm using pre-setting's for protein gel.

1.6 Dermal-epidermal separation with granulocytes

First whole blood samples from healthy donors were treated with standard Ficoll-Paque method for isolation of peripheral blood mononuclear cells (PBMCs) from Miltenyi Biotec Inc. Layer of granulocytes were extracted from centrifuged Ficoll reagent-tube by pipetting and resuspended with 5x volume of balanced salt solution and centrifuged 400 x g for 15 min. After this red blood cell (RBC) lysis was performed and centrifuged again 400 x g for 15min at room temperature. Red blood cells were removed with the supernatant and remaining granulocytes were resuspended to balanced salt solution. After third centrifuge of 400 x g for 10 min at room temperature, rest of the supernatant was removed, and granulocyte cell pellet was resuspended in RPMI with 1% human serum.

DH patient's biopsy sections, from untreated patient and GFD-treated but refractory patient, were treated with granulocyte (containing approximately $1x10^6$ cells) solution in at 37°C for 2 h. Finally, sections were stained with DAPI and observed with fluorescence microscope Olympus BX60F5 (Olympus Optical CO. LTD, Japan). Alternatively, skin sections were incubated with DH patient serum (diluted 1:1 with PBS) at 37°C for 1 h and subsequently washed 3x5 min with PBS, prior to granulocyte treatment.

RESULTS

Focus on this study was to identify if TG3 binds to structural proteins, collagen IV or collagen VII and/or to fibrinogen in the papillary dermis of DH patient's skin. To assess that, frozen DH patient sections (n=8) were stained with immunofluorescence antibodies to visualize possible colocalization between TG3 and IgA, collagen type IV, collagen type VII and fibrinogen. Summary of the results is presented in Table 2. In immunofluorescence staining's six out of eight patients showed DH typical TG3 deposits in papillary dermis. Those two patients not having visible TG3-deposits, both were gluten-free diet treated, one noted as refractory, having still skin symptoms and other normal without any symptoms. In addition, in two samples, one untreated patient and one GFD-treated normal, the deposits were not as clear or TG3 stain was sparser in the dermis. In addition, in all eight samples anti-TG3 showed normal cornified layer of TG3 in epidermis. Also, in all samples where TG3 was clear, also IgA was shown colocalized with TG3 (Figure 4).

Table 2. *Summary of results for TG3 and its colocalization with collagen type IV, collagen type VII and fibrinogen detected by immunohistochemistry.*

Patient type (n=8)	TG3 deposits	IgA	collagen IV	collagen VII	fibrinogen
gluten challenged	++	++*	++	++	++*
gluten challenged	++	++*	++	++	++*
GFD- refractory	++	++*	++	++	++*
GFD- refractory	-	-	++	+	+
GFD- normal	+	+*	++	++	++*
GFD- normal	-	+	+	++	+
Untreated DH	+	+*	++	++	++*
Untreated DH	-	+	++	++	++

*Abbreviations: TG3: epidermal transglutaminase, DH: Dermatitis Herpetiformis, GFD: gluten-free diet. ++: clearly present, +: faintly present, -: not visible. *: colocalization*

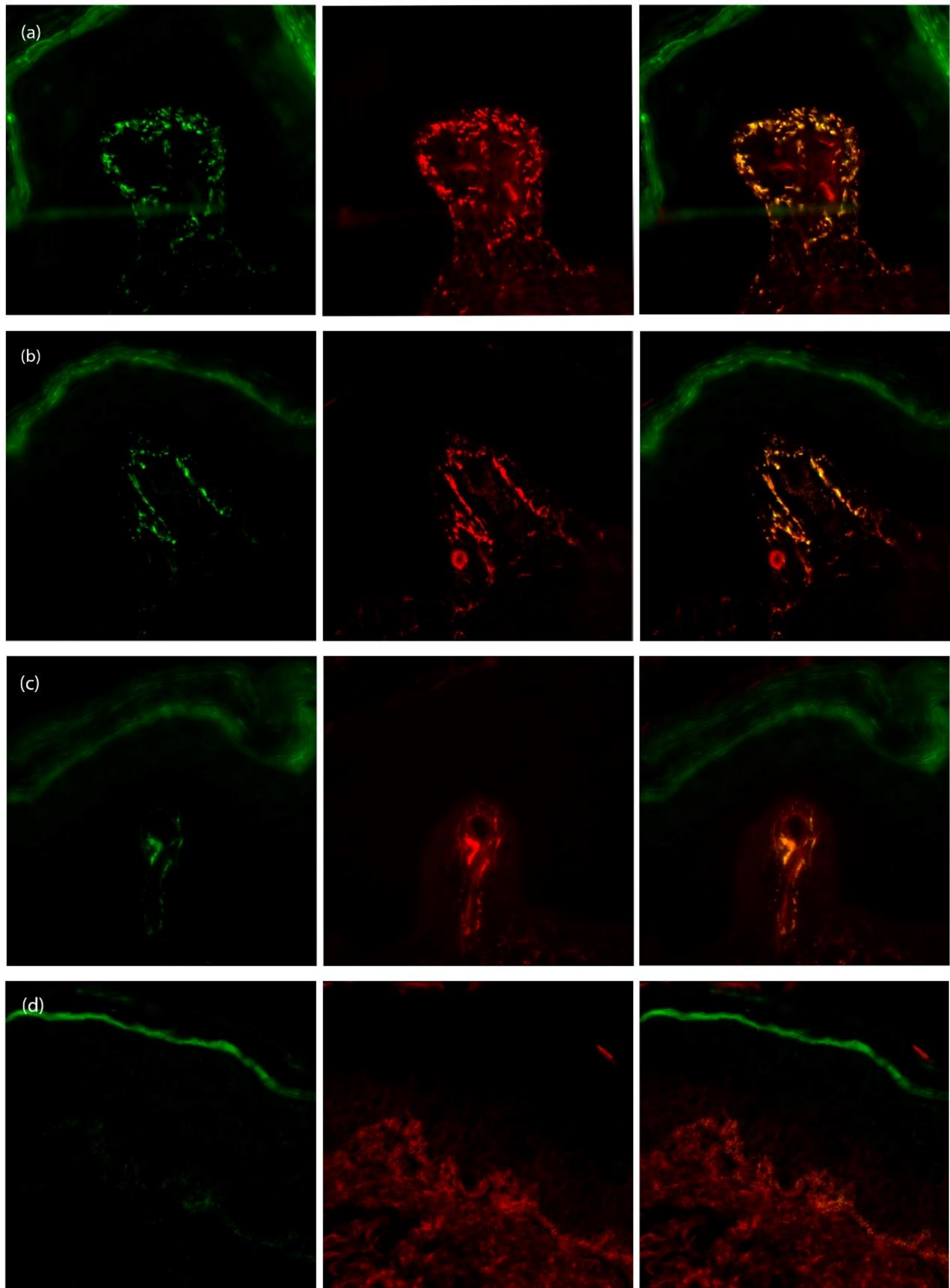

Figure 4. *Immunofluorescence staining for TG3 (green) and IgA (red) in skin sections with DH. Colocalization shown as yellow. Representative pictures of sections from (a) GFD-treated refractory DH patient. (b) gluten challenged DH patient and (c) GFD-treated patient on remission (d) untreated DH patient. Pictures taken at x40 magnification. Abbreviations: TG3: epidermal transglutaminase, IgA: immunoglobulin A, DH: Dermatitis herpetiformis, GFD: gluten-free diet.*

Both collagen antibodies showed high specificity and were clearly seen in every skin sample. Collagen type IV staining was more diffuse and spread out in the deeper levels of dermis yet saturated in the DEJ. There was no detectable colocalization between TG3 and collagen IV (Figure 5). Collagen type VII on the other hand was visible as one clear, thin layer in DEJ on top of the TG3 deposits meaning no colocalization between TG3 and collagen VII was seen either (Figure 6).

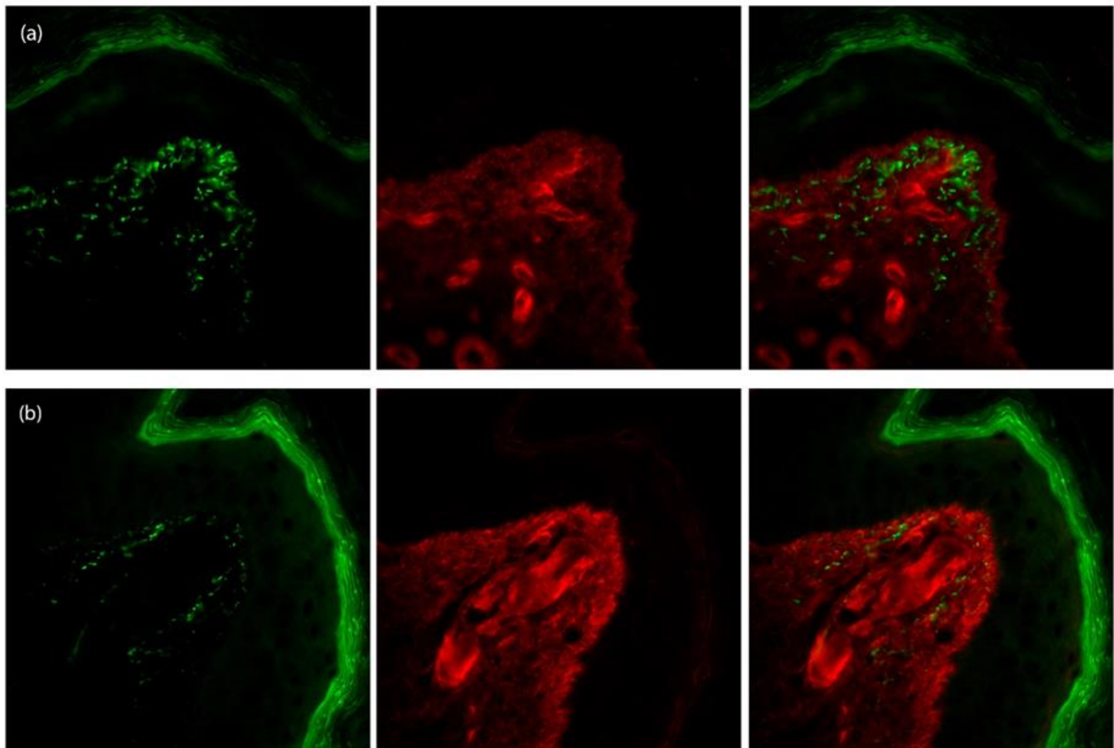

Figure 5. *Immunofluorescence staining for TG3 (green) and collagen IV (red) in skin section with DH. Representative pictures of sections from (a) GFD-treated refractory DH patient and (b) gluten challenged DH patient. Pictures taken at x40 magnification Abbreviations: TG3: epidermal transglutaminase, IgA: immunoglobulin A, DH: Dermatitis Herpetiformis, GFD: gluten-free diet.*

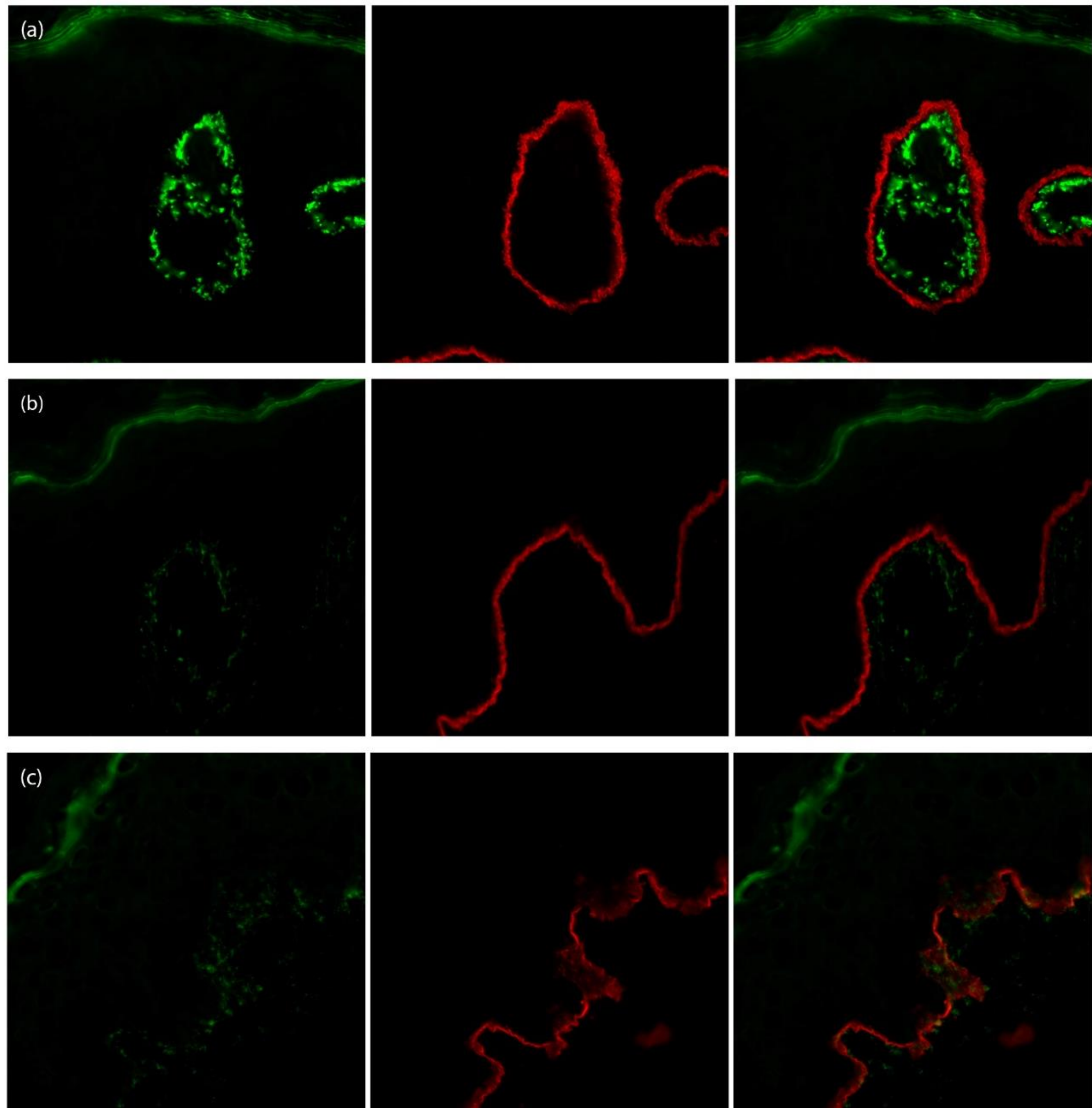

Figure 6. *Immunofluorescence staining for TG3 (green) and collagen VII (red) in skin section with DH. Representative pictures of sections from (a) GFD- treated refractory DH patient, (b) gluten challenged DH patient and untreated DH patient. Pictures taken at x40 magnification. Abbreviations: TG3: epidermal transglutaminase, IgA: immunoglobulin A, DH: Dermatitis Herpetiformis, GFD: gluten-free diet.*

Finally, fibrinogen was stained together with TG3. It is worth noting that anti-fibrinogen antibody also binds to fibrin, active form of fibrinogen. Without blocking agents, anti-fibrinogen staining was quite non-specific, but in the final staining's we managed to decrease unspecific background fluorescence sufficiently. Staining pattern was similar with collagen IV, spread out in the dermis and most brightly coloured in the DEJ. It was evident that fibrinogen is also present in the dermal papillae. Contrary to collagen colocalization was seen. Red colour for anti-fibrinogen mixed with green anti-TG3 antibody creating yellow hue can be seen in Figure 7.

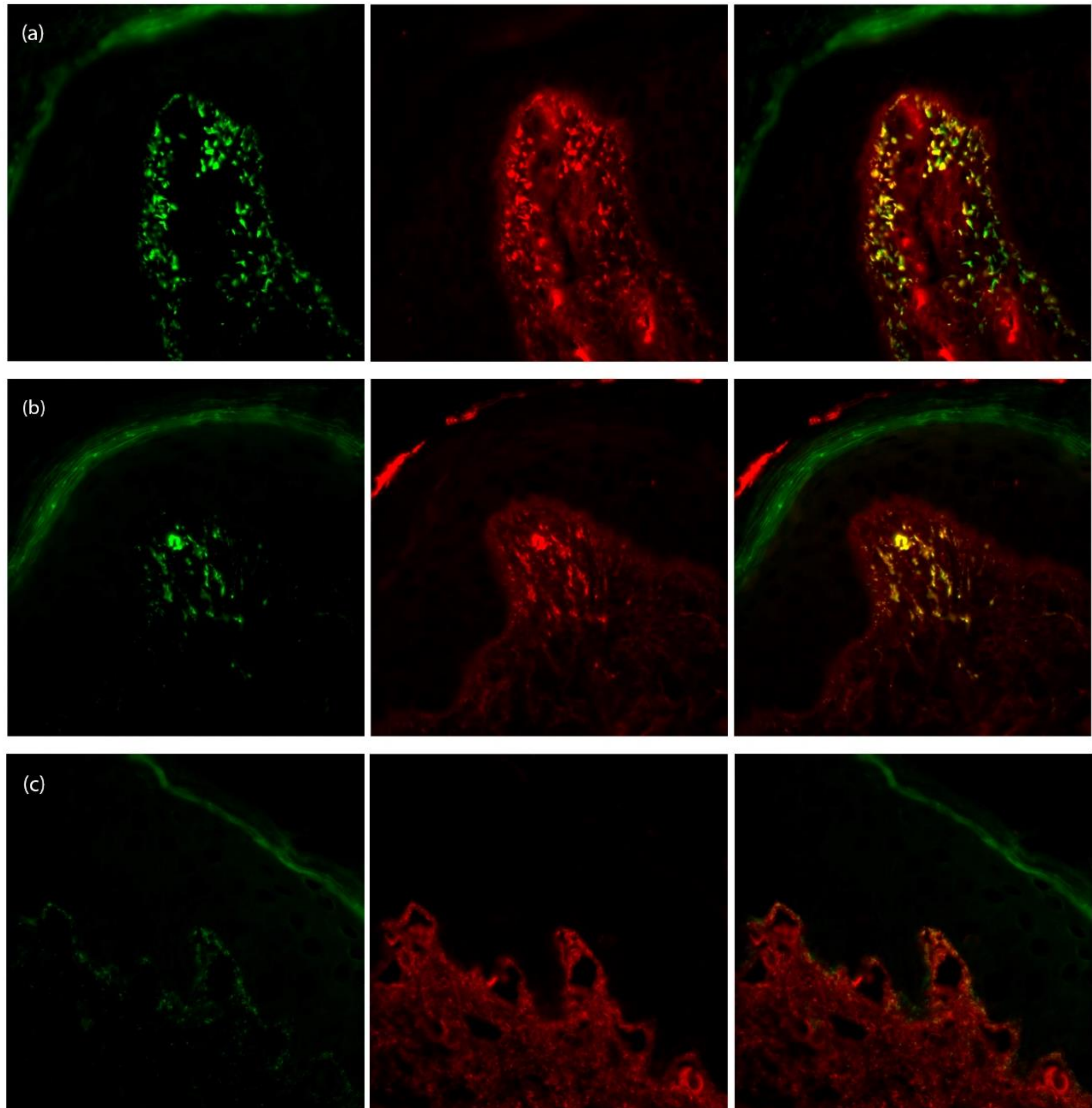

Figure 7. *Immunofluorescence staining for TG3 (green) and fibrinogen and fibrin protein (red) in skin DH. Possible colocalization shown as yellow. Representative pictures of sections from (a) GFD-treated refractory DH patient (b) gluten challenged DH patient and (c) untreated DH patient. Pictures taken at x40 magnification. Abbreviations: TG3: epidermal transglutaminase, IgA: immunoglobulin A, DH: Dermatitis Herpetiformis, GFD: gluten-free diet.*

Serum derived IgA did not bind to Fibrinogen in Western blot

Further, because of colocalized TG3 and fibrinogen, I wanted to see if serum IgA from DH patient binds to extracted fibrinogen protein in order to confirm the interaction by using another method.

Fibrinogen was separately handled in native and denatured form. After electrophoresis three specific bands were detected in gels for heat and SDS-denatured fibrinogen between 55 and 95 kilodaltons (kDa) (Figure 8b and Figure 8d) and for native fibrinogen at higher than 250kDa (Figure 9b and Figure 9d).

Membranes were incubated with DH patient serum to observe if circulating IgA binds to fibrinogen and no signs of IgA binding to denatured fibrinogen was observed (Figure 8a and Figure 8c). In gel, fibrinogen bands were shown as expected. Its molecular mass is approximately 340kDa (https://www.sigmaaldrich.com/catalog/product/sigma/f3879:18.3.2021) which is consistent with the native protein results. In denatured protein electrophoresis fibrinogens three non-identical chains were seen with molecular masses between 72kDa and 45kDa.

Then again faint stains were seen up in the lanes where native fibrinogen protein bands were detected (Figure 9a). However, it is necessary to note the background staining in membranes. Especially membrane blocked with LiCor blocking agent and incubated with gluten challenged patient's serum had fair amount of nonspecific staining (Figure 9c) Therefore with these results we cannot exclude the option of circulating IgA binding to native fibrinogen, nor it is not binding.

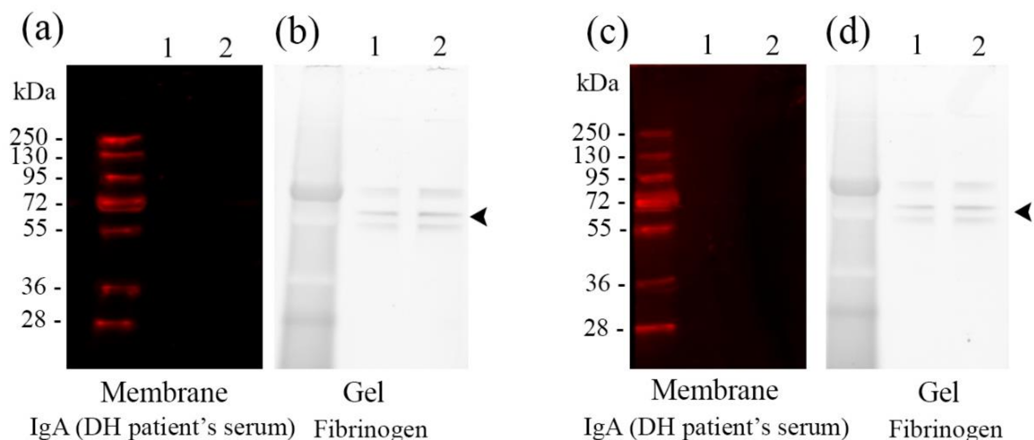

Figure 8. *Western blot to denatured fibrinogen. (a) and (c) : Membranes treated with DH patient's serum and immunoglobulin A (IgA) detected. (b) and (d): Fibrinogen protein (arrow heads) imaged in the gel after electrophoresis. Lane 1: 0.5 µg fibrinogen and Lane 2 : 1.0 µg fibrinogen*

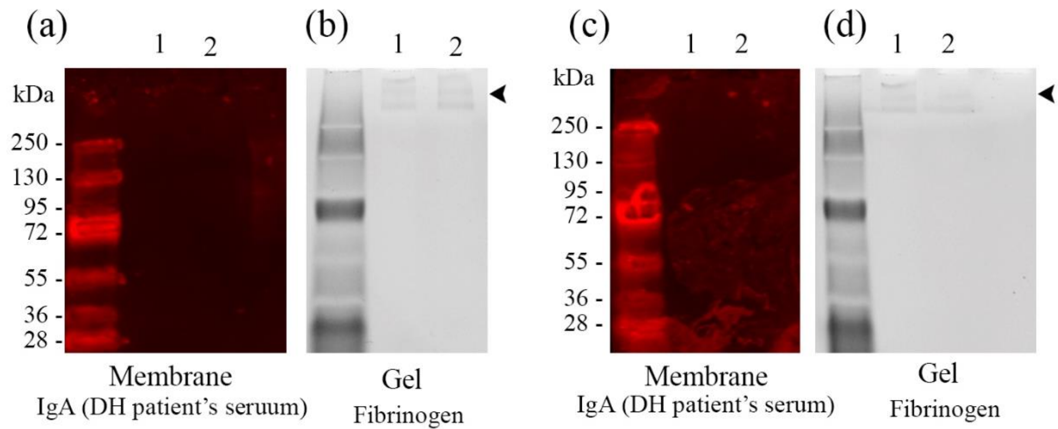

Figure 9. *Western blot to native fibrinogen. (a) and (c) : Membranes treated with DH patient's serum and immunoglobulin A (IgA) detected. (b) and (d): Fibrinogen protein (arrow heads) imaged in the gel after electrophoresis. Lane 1: 0.5 µg fibrinogen and Lane 2 : 1.0 µg fibrinogen*

Granulocyte treatment of frozen skin sections

Frozen DH patient sections were incubated with granulocyte solution. After incubation, DAPI stained sections showed no difference between the control and granulocyte treated sections in dermal-epidermal junction (Figure 10).

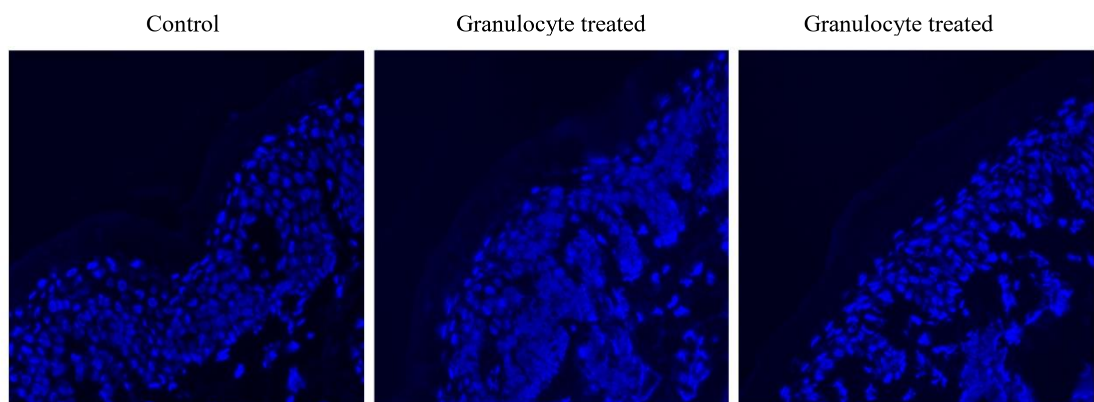

Figure 10. *Dermal-epidermal junction of DH patient section samples treated with granulocytes from healthy donor stained with DAPI (blue)*

DISCUSSION

It has been suggested that IgA-TG3 immunocomplexes are tightly bound to the ECM of the papillary dermis, potentially interacting with at least fibrinogen (Taylor et al. 2015). Yet, there are very few studies trying to identify the details of the hypothesized TG3-substrate binding in DH. For this study, a few potential TG3 substrates, namely collagen IV and collagen VII, in the ECM of the papillary dermis were selected and investigated.

The present study included verifying that the TG3 enzyme colocalizing to papillary dermis with IgA. The presence of dermal TG3-IgA immunocomplexes was basis for further investigations. Mainly, IgA stainings were coherent as seven out of eight patient cryosections showed IgA aggregates in the DEJ. However, the stain intensity varied, which could result from the age of the cryosections. There is no evidence on how stable the immunocomplexes are in the cryosections meaning there is a possibility that they disintegrate with time. Also, some sections were cut from almost finished frozen sample blocks and the DEJ area had already been used up, partly folded or torn. In addition, one GFD-normal sample, in which TG3 was not present, IgA was shown spread out in the dermis, non-specific background might be the case.

This study proved that two structural proteins can be excluded as potential TG3 substrates in the skin papillae in DH. TG3 and collagen protein family members, type IV or type VII clearly did not colocalize in immunofluorescence stainings which strongly indicates that TG3-IgA immunocomplexes do not bind directly to these. Still, with these results the possibility of these two collagen proteins being part of the separations through immune cascades cannot be ruled out. Collagens could be involved in DEJ separation via ECM destruction by matrix metalloproteinases (MPPs) and, whose expression levels have been noted higher in DH patients compared to controls (Airola et al. 1995, Zebrowska et al. 2005). In addition, expression levels of molecules controlling these degradation proteins, tissue inhibitors of matrix proteinases (TIMPs) were observed to be lower (Zebrowska et al. 2005). Increased expression of matrix metalloproteinases could be a result of neutrophil extracted cytokines. Besides in other blistering disease, epidermolysis bullosa acquisita, autoantibodies have been shown to cause neutrophil mediated inflammation and DEJ separation by binding to collagen VII protein (Sitaru et al. 2002a) and Cardoso et al. (Cardoso et al. 2015) demonstrated that tissue transglutaminase can bind to fibronectin and collagen type VI in ECM. In order to test a larger panel of possible TG3 substrates, a microplate protein-binding assay method could also be used in the future studies.

It is not a novel finding that TG3-IgA aggregates bind to fibrinogen in dermal papillae in DH. Results of this study showing colocalization between TG3 and fibrinogen support the hypothesis

of fibrinogen taking part of pathogenesis of DH. On the contrary, neither IgA nor TG3-IgA immunocomplexes in DH patient serum bound to fibrinogen in this study. This could indicate that the binding site for the fibrinogen is in TG3 or the used serum concentrations did not contain enough TG3-IgA immunocomplexes. To confirm this, the level of circulating IgA in the serum should be measured and higher concentrations should be tested. It is thus clear that optimization and repetition of fibrinogen western blot and serum handling using alternative experimental parameters are needed.

For investigation of neutrophil involvement in blister formation in DH, granulocytes were isolated from whole blood samples. The method used was not optimized for granulocyte or neutrophil extraction and therefore with commercial granulocyte isolation kit or using other separation media like dextran, better granulocyte isolation could be achieved. Further, only neutrophils could be isolated since they are considered as main granulocyte affecting in DH (Airola et al. 1995, Smith et al. 2002). Because neutrophils are easily activated during handling and they become apoptotic cells relatively fast, there is a chance that granulocyte solution did not contain any active neutrophils. Fluorescence-activated cell sorting (FACS) could be used to analyze the presence of neutrophils in the solution. In this case blood samples were from healthy donors and not from the DH patients themselves. That in mind, results could be in line with previous studies suggesting neutrophil priming before their accumulation in the DEJ (Hall et al. 2007). It should also be noted that in the case of DH, there are no reports of dermal-epidermal separation being successfully reproduced *in vitro*.

Limitation in this study was the small number of patients and size of patient groups as well as limited available resources for immunohistochemistry and neutrophil isolation. Yet, it would be interesting to solve whether TG3-IgA immunocomplexes persist in the papillary dermis due to strong binding to ECM structures in dermal papillae or just because of prolonged production and withdrawal of passive immunocomplexes. Those are left for the future studies. Better understanding to the complex immune cascades and molecular mechanisms of pathology in DH would enable new treatment and prevention strategies.

CONCLUSION

To conclude, this thesis verified the clinical characteristics of DH patients' skin and investigated hypothetical insights of the cutaneous pathology in DH. Firstly, TG3 and IgA deposits in DH patients' skin colocalize with fibrinogen, while both collagen proteins, type IV and type VII collagens were identified clearly separate from TG3-IgA immunocomplexes and these can be excluded as potential interaction sites for TG3 in the ECM of the skin. Interactions between TG3-IgA immunocomplexes and fibrinogen are more likely due to TG3 enzyme than IgA antibodies, although direct interaction with TG3-IgA immunocomplexes could not be reproduced *in vitro* using western blotting. Secondly, used methods in this study were not able to demonstrate dermal-epidermal separation mechanism led by neutrophil infiltration but the experiments do not out rule that neutrophilic activity may participate in the disease pathogenesis.

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