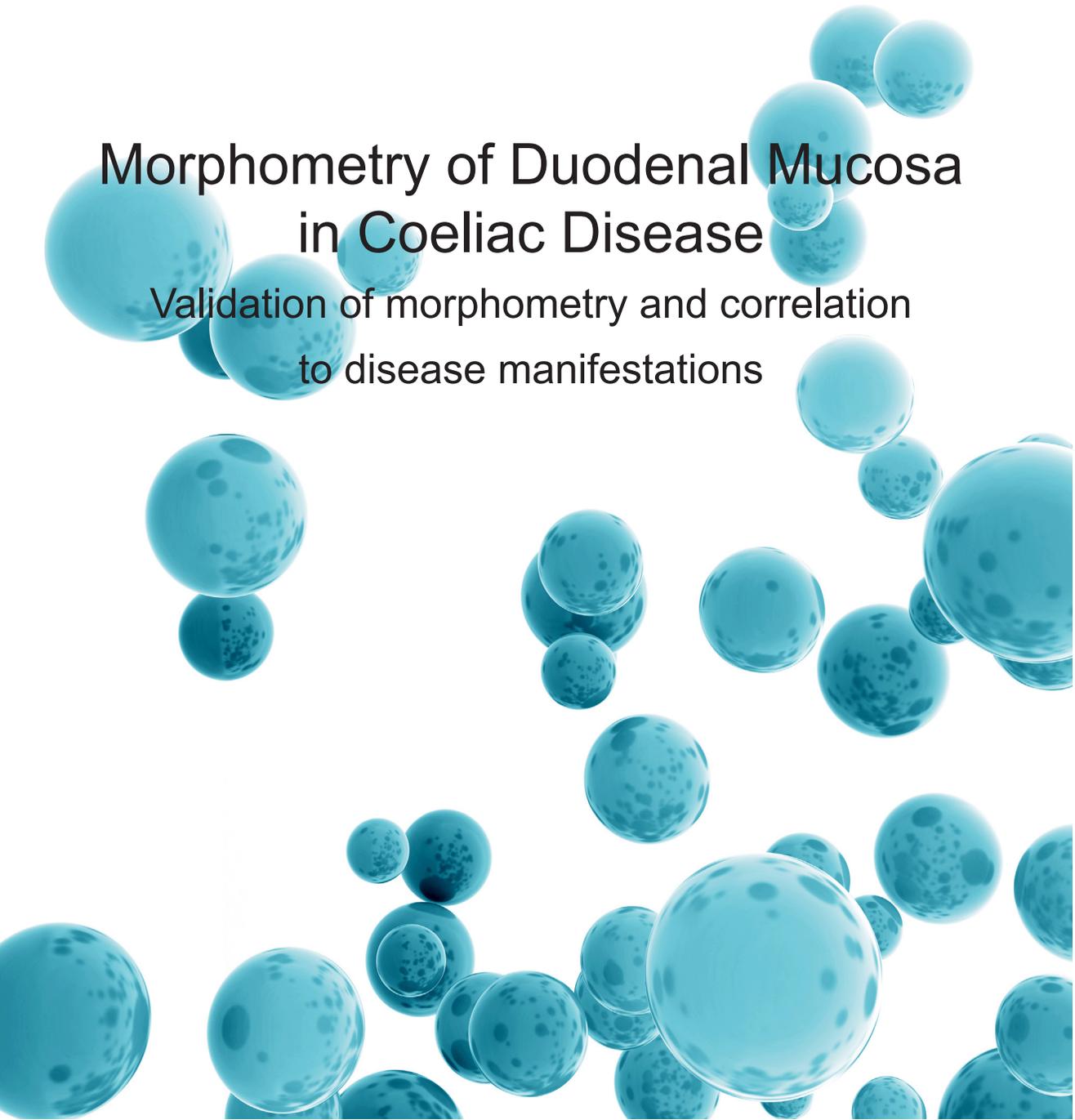


JUHA TAAVELA

Morphometry of Duodenal Mucosa in Coeliac Disease

Validation of morphometry and correlation
to disease manifestations





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ACADEMIC DISSERTATION

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the Board of the School of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 5,
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UNIVERSITY OF TAMPERE

JUHA TAAVELA

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University of Tampere, School of Medicine
Tampere Center for Child Health Research
Finland

Supervised by

Professor Markku Mäki
University of Tampere
Finland
Docent Kalle Kurppa
University of Tampere
Finland

Reviewed by

Professor Kaija-Leena Kolho
University of Helsinki
Finland
Docent Jukka Laine
University of Turku
Finland

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“There is nothing more deceptive than an obvious fact.”

-Arthur Conan Doyle

ABSTRACT

Coeliac disease is an autoimmune reaction triggered by dietary gluten in the small-bowel mucosa of genetically susceptible patients. The clinical presentation is diverse, ranging from classical gastrointestinal symptoms to extraintestinal manifestations, mild laboratory abnormalities or seemingly asymptomatic patients who are found by screening in at-risk groups. Antibody assays from serum are nowadays widely used in screening patients, but the final diagnosis requires villous atrophy and crypt hyperplasia in small-bowel biopsy. It has however recently been recommended in the new European guidelines that children with very high antibody titres could be diagnosed without biopsy. The biopsies are taken via oesophagogastroduodenoscopy from the second part of the duodenum, but according to recent guidelines also from the proximal duodenal bulb. The specimens are usually graded with a categorical variable such as the Marsh classification as this suits for quick visual estimates. However, the reliability and reproducibility of such classifications has been found to be poor in many studies and they are thus too rough and inaccurate for academic, clinical and pharmacological studies. Quantitative histological measurements such as the villous height crypt depth ratio (VH: CrD) and intraepithelial lymphocyte (IEL) density would more accurately represent the spectrum of mucosal damage as a continuum. The aim of this study was to assess the reliability and reproducibility of the reading of biopsy specimens with specific emphasis on the use of these quantitative methods in coeliac disease. Additionally, the link between the degree of mucosal damage and symptoms and signs of coeliac disease and the reliability of bulb biopsies in coeliac disease was further evaluated.

The dissertation comprises three separate studies. Study **I** investigated the reliability and reproducibility of quantitative histological measurements and possible pitfalls due to specimen sectioning in coeliac disease diagnostics. Altogether 93 samples with different grades of mucosal injury were evaluated in blinded fashion. In study **II** symptoms and signs of coeliac disease were assessed against the degree

of mucosal damage and serum antibody titres. Duodenal samples were collected from 638 consecutive oesophagogastroduodenoscopies and the results were compared to patient reported symptoms, quality of life, laboratory parameters and bone mineral density. Finally, study **III** was a prospective multicentre study assessing the reliability of duodenal bulb specimens in coeliac disease diagnostics in children. Sera and biopsy samples were collected from 22 coeliac disease patients and 22 non-coeliac disease controls.

Study **I** showed that the VH:CrD is a reliable and reproducible method in measuring small-bowel mucosal damage. A new cut-off for clinically significant change was set at 0.4, which may be used in future clinical trials. The reason for faulty diagnoses from tangential cuttings was apparent in the 3D models of mucosal damage presented. The results from study **II** showed significant correlations between VH:CrD and gastrointestinal symptoms, quality of life, laboratory parameters, mucosal inflammation and serum antibody titres. Also, serum antibody titres correlated with almost all the same parameters as the VH:CrD. In study **III**, the anatomical duodenal bulb samples were of inadequate quality and mucosal damage was also present in bulb specimens from disease control patients. Bulb mucosal transglutaminase 2 targeted intestinal IgA deposits found coeliac disease patients in all.

The results of this investigation proved that quantitative small-bowel morphometric measurements are a reliable method in evaluating mucosal injury in coeliac disease. The samples must be well oriented and of good quality to ensure reliable results. The degree of small-bowel mucosal damage correlated with most of the patient-reported symptoms and signs, thus adding informational value to the pathological diagnostics, while on the other hand the symptoms may be used as an indicator of mucosal status in large population studies. Damage in anatomical bulb specimens must be interpreted with caution and in uncertain cases analysis of IgA deposits is an effective tool in finding coeliac disease patients. More studies are needed to assess the diagnostic yield acquired with the addition of bulb biopsies.

TIIVISTELMÄ

Keliakia on autoimmuunisairaus ohutsuolen limakalvolla, jonka laukaisee ravinnon gluteeni geneettisesti alttiilla potilailla. Sairauden ilmiasu voi vaihdella klassisista suolisto-oireista suoliston ulkopuolisiin oireisiin, pelkkiin verenkuvamuutoksiin ja jopa oireettomiin potilaisiin, jotka löytyvät vain seulomalla riskiryhmiä. Taudin seulonnassa käytetään seerumin keliakiavasta-ainetutkimuksia, mutta lopulliseen diagnoosiin vaaditaan ohutsuolen koepalassa todettu limakalvovaurio. Mielenkiintoisesti uusimmissa eurooppalaisissa suosituksissa ehdotetaan, että hyvin korkean vasta-ainetason omaavilla lapsilla voitaisiin jättää koepala ottamatta keliakian diagnostiikassa. Koepalat otetaan ruuansulatuskanavan tähytyksen avulla ohutsuolen alkuosasta ja nykyään myös pohjukaissuolen alkuosasta eli heti mahaportin jälkeen. Limakalvovauriota arvioidaan yleensä käyttäen luokiteltuja muuttujia, esimerkiksi ns. Marsh-luokitusta, sen helpon ja nopean suorittamisen vuoksi. Luokiteltujen muuttujien toistettavuus on kuitenkin todettu huonoksi, ja ne ovat liian karkeita kliiniseen ja farmakologiseen tutkimuskäyttöön. Koepaloista tehdyistä leikkeistä voidaan antaa vaihtoehtoisesti jatkuvat histologiset suureet, jolloin päämuuttujana toimivat suolinukan pituuden ja suolikuopakkeiden syvyyden suhde (VH:CrD) ja toissijaisena muuttujana limakalvotulehduksen aste, jotka kuvaisivat limakalvomutosten jatkuvuutta todellisemmin. Tämän tutkimuksen tarkoituksena oli tutkia jatkuviin muuttujiin perustuvien histologisten mittareiden luotettavuutta keliakian diagnostiikassa ja tutkimuskäytössä. Lisäksi tutkittiin pohjukaissuolen alkuosan eli bulbuksen näytteiden merkitystä keliakian diagnosoinnissa sekä arvioitiin histologisen vaurion korrelaatiota keliakian kliiniseen taudinkuvaan.

Väitöskirjatyö koostui kolmesta erillisestä osatyöstä. Osatyössä **I** tutkittiin histologian luotettavuutta ja näytteiden leikkaussuunnan vaikutusta keliakian diagnostiikkaan. Kaikkiaan 93 potilaan näytteet arvioitiin sokkoutetusti käyttäen jatkuvia muuttujia. Osatyössä **II** tutkittiin oireiden vakavuuden yhteyttä histologisen

vaurion asteeseen ja seerumin vasta-ainetasoihin. Pohjukaissuolen limakalvon koepalat kerättiin 638 peräkkäisestä ruoansulatuskanavan tähystyksestä ja tuloksia verrattiin ruoansulatuskanavan oireisiin, elämänlaatuun, sairauteen liittyviin laboratorioarvoihin ja luuntiheyteen. Väitöskirjan **III** osatyössä tutkittiin prospektiivisesti pohjukaissuolen alkuosan näytteiden merkittävyyttä keliakian diagnostiikassa. Seerumi-näytteet ja koepalat kerättiin 22 keliakikolta ja 22 tautikontrolli-potilaalta.

Osatyö **I** osoitti standardoidun toimintatapamme ja VH:CrD:n luotettavaksi tavaksi arvioida limakalvovauriota. Työssä määritettiin VH:CrD:n uudeksi raja-arvoksi 0.4 merkitseville limakalvomuutoksille, mikä on erityisen tärkeä tieto tulevaisuuden kliinisille kokeille. Osatyön **II** tulokset osoittivat ohutsuolen VH:CrD:een korreloivan merkitsevästi potilaiden kokemuksiin suolisto-oireisiin ja elämänlaatuun, laboratorioarvoihin, limakalvotulehduksen voimakkuuteen sekä keliakiavasta-ainetasoihin. Myös seerumin vasta-ainetasot korreloivat merkitsevästi lähes samoihin muuttujiin kuin VH:CrD. Osatyön **III** tulokset osoittivat pohjukaissuolen alkuosan näytteiden olevan huonolaatuisia ja siellä esiintyvän limakalvovauriota monien muidenkin tautitilojen yhteydessä. Kaikilla keliakikoilla oli nähtävissä vasta-ainekertymät bulbusen koepaloissa ja toisaalta kellään kontrolli-potilaalla ei kertymiä ollut.

Väitöskirjan tulokset osoittivat VH:CrD:n ja tulehdussolujen laskennan luotettaviksi keliakian diagnostiikassa ja tutkimustyössä. Näytteiden pitää olla oikein leikatut ja hyvänlaatuiset diagnostiikan luotettavuuden varmistamiseksi. Limakalvovaurio korreloi potilaiden itsekokemuksiin oireisiin ja elämänlaatuun, mikä toisaalta lisää biopsian tietoarvoa ja toisaalta kuvastaa myös oireiden seurannan tärkeyttä. Pohjukaissuolen alkuosan limakalvovauriota pitää tulkita varoen ja epävarmoissa tapauksissa vasta-ainekertymien arviointi koepaloista vaikuttaa tehokkaalta keinolta keliakikkojen löytämiseksi. Bulbus-näytteiden tuoma diagnostinen lisäarvo perinteisiin koepaloihin verrattuna vaatii lisätutkimuksia.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals **I-III**:

I Taavela J, Koskinen O, Huhtala H, Lähdeaho ML, Popp A, Laurila K, Collin P, Kaukinen K, Kurppa K, Mäki M. Validation of morphometric analyses of small-intestinal biopsy readouts in celiac disease. *PLoS One*. 2013;8:e76163.

II Taavela J, Kurppa K, Collin P, Lähdeaho ML, Salmi T, Saavalainen P, Haimila K, Huhtala H, Laurila K, Sievänen H, Mäki M, Kaukinen K. Degree of damage to the small bowel and serum antibody titers correlate with clinical presentation of patients with celiac disease. *Clin Gastroenterol Hepatol*. 2013;11:166-71. (Reprinted with permission from Elsevier)

III Taavela J, Popp A, Korponay-Szabo IR, Ene A, Vornanen M, Saavalainen P, Lähdeaho M, Laurila K, Parvan A, Anca I, Kurppa K, Mäki M. Prospective study on usefulness of duodenal bulb biopsies in coeliac disease diagnosis in children – urging caution. *American Journal of Gastroenterology*. In Press.

ABBREVIATIONS

AGA	antigliadin antibodies
APC	antigen-presenting cell
ARA	antireticulin antibodies
BMI	body mass index
BMD	bone mineral density
CI	confidence interval
DGP	deamidated gliadin peptides
DH	dermatitis herpetiformis
EC	enterocyte
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibodies
ESPGHAN	European Society of Pediatric Gastroenterology, Hepatology and Nutrition
GSRS	Gastrointestinal Symptom Rating Scale
HLA	human leukocyte antigen
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
IFN	interferon
NSAID	non-steroidal anti-inflammatory drug
PGWB	Psychological General Well Being
RCD	refractory coeliac disease
SD	standard deviation
TCR	T- cell receptor
TG2-ab	transglutaminase 2 antibody
TNF	tumour necrosis factor
VH:CrD	villous height crypt depth ratio

INTRODUCTION

Coeliac disease is a chronic immune-mediated disorder triggered by dietary gluten in genetically predisposed individuals. The diagnosis of coeliac disease requires villous atrophy and crypt hyperplasia in the biopsy and there is usually also an increase in mucosal inflammation (Walker-Smith et al. 1990). The classical symptoms are diarrhoea and malabsorption (Visakorpi et al. 1970). A changing pattern of disease symptoms towards extraintestinal manifestations and higher age at diagnosis were observed in the 1970s and 1980s in both adults and children (Logan et al. 1983, Mäki et al. 1988). Clinical presentations with minor symptoms or those detected by screening in at-risk groups are nowadays frequent (Kaukinen et al. 2010). Interestingly, the diagnostic criteria for children defined by the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) have recently changed fundamentally and now it is an option to diagnose symptomatic children with coeliac disease if antibody titres are particularly high. However, in children with low and moderate titres biopsy and in all adult cases biopsy is still considered mandatory (Husby et al. 2012). Today, additional tests are required in cases of lesser degrees of mucosal injury (Husby et al. 2012, Ludvigsson et al. 2014). The biopsies may be classified according to categorical classifications or by direct quantitative measurements. However, as the damage develops gradually from mild inflammation to overt villous atrophy and crypt hyperplasia, categorical classification is subject to certain limitations (Kurppa et al. 2009).

The best known grouped classifications in histological assessment are those described by Marsh and modified by Oberhuber and another by Corazza and Villanacci (Marsh 1992, Oberhuber 2000, Corazza and Villanacci 2005). They are practical and quick in routine practice and have good inter-observer reliability in clear cases presenting a manifest mucosal lesion (Marsh 3c) and in healthy mucosa with high villi (Marsh 0) (Corazza and Villanacci 2005, Arguelles-Grande et al. 2012). However, allocation of samples between these two clear-cut groups has shown

marked variability between observers due to the continuous spectrum of mucosal damage in coeliac disease and also as it comprises a subjective visual estimate of a sample (Corazza et al. 2007, Mubarak et al. 2011, Arguelles-Grande et al. 2011). The quantitative measurements, VH:CrD and counting of IELs, overcome certain of these problems and are suitable for detecting small but significant changes in mucosal architecture (Kuitunen et al. 1982, Lähdeaho et al. 2012). Both of these classification systems must always be undertaken with reliable sectionings, as tangential cutting may cause both false-negative and false-positive findings and therefore cutting perpendicular to the mucosal surface has been of crucial importance from the outset (Thurlbeck et al. 1960, Risdon and Keeling 1974, Shidrawi et al. 1994).

Mucosal injury in coeliac disease develops gradually and one would logically assume the severity of clinical presentation to follow the status in the mucosa, but many studies have given inconsistent results (Dorn et al. 2010, Thomas et al. 2009, Weizman et al. 1997). However, all the studies in question were made in patients with classical gastrointestinal or severe symptoms, in tertiary centres and with the rather inaccurate categorical classifications. If histological and serological values correlated with the severity of symptoms, they could be used as an indirect sign of disease activity.

A new biopsy location, the duodenal bulb, has emerged in the most recent coeliac disease guidelines as diagnostic accuracy has increased with the inclusion of this site (Bonamico et al. 2008, Evans et al. 2011, Weir et al. 2010). However, up to 2005 guidelines did not allow the taking of biopsies from the bulb (Hill et al. 2005). The most common disturbing factors mentioned in the literature are Brunner's glands, lymphoid follicles and a high gastric acid load, which may cause lesions mimicking coeliac disease (Trier 1971, Hasan et al. 1989, Chang et al. 2005). Specific morphometrical studies are lacking in this area and there are also studies showing no increased detection rate with bulb biopsies (Ravelli et al. 2005 and 2010).

The use of specific tools in studies and routine should be well validated and investigated before use to avoid pitfalls in diagnostics. Hence, the purpose of this present study was to evaluate the use of quantitative morphometric variables, the VH:CrD and IEL density. First, the morphometrical variables were validated and compared to establish the reliability and reproducibility of the methods in use. Second, the possible link of histology and serology to symptoms was sought in order to gain possible new tools for assessing disease status. Last, bulb biopsies were compared between coeliac disease patients and disease controls to assess the reliability and specificity of this area in diagnostics. All measurements were made according to standard operating procedure, which requires strict assessment of the quality of specimens.

REVIEW OF THE LITERATURE

1. COELIAC DISEASE

Coeliac disease is a life-long intolerance of wheat, rye and barley gluten in genetically predisposed individuals. It is nowadays one of the most common food-related disorders in Western countries (Mäki et al. 2003), although it was long considered a rather rare condition which began only in childhood and could even be lethal before the discovery of the gluten-free diet. The first description of the disease was written as far back as the 1st century BC by Aretaeus of Cappadocia, and the first scientifically recognized report was that by Samuel Gee (1888). At that time coeliac disease was a disorder involving severe gastrointestinal symptoms, diarrhoea, cachexia and distended abdomen. Gee already stated that if these patients can be cured at all it must be by means of diet, though he had no conception as to what type of diet. Half a century later the trigger of the disease was found: gluten (Dicke et al. 1953). The next year the characteristic mucosal villous atrophy and crypt hyperplasia were found in the small bowel (Paulley 1954) and soon thereafter the per-oral biopsy techniques emerged (Royer et al. 1955, Shiner 1956). In 1964 it was shown that the small-bowel mucosa heals on a gluten-free diet (Collins et al. 1964), but it was not until 1969 that it became evident that the link is permanent and that a life-long diet must be followed (Sheldon 1969). The first antibodies, antigliadin antibodies (AGA), were already described in 1958 (Berger 1958), while the culmination of this development was in a way the finding of tissue transglutaminase as the coeliac disease-associated endomysial autoantigen (Dieterich et al. 1997). Interestingly, no postulations implying that autoimmune mechanisms are implicated in coeliac disease were made until the 1990s (Mäki et al. 1991a, Mäki 1994). Nowadays this is widely accepted as an unquestioned fact (Sollid 2000, Catassi and Fasano 2008a). At present it is understood that coeliac disease may present with symptoms and signs at any age and that the symptoms may range from very mild to overt disease.

2. EPIDEMIOLOGY

Historically, coeliac disease was regarded as a rare disease of infancy. One of the oldest epidemiological reports from 1950 approximated the incidence of coeliac-like sprue syndrome as between 1:10000 and 1:5000 in Great Britain (Davidson and Fountain 1950). Reports since have varied depending on the diagnostic criteria and nation in question but a considerable change took place in the 1980s, when the clinical presentation was found to be milder and the age had shifted to older groups (Mäki et al. 1988). Recent screening studies with serum autoantibodies and biopsies have shown an approximate prevalence of 1% in both children (Mäki et al. 2003, Korponay-Szabo et al. 2007) and adults (West et al. 2003) in Europe and the United States. However, some regional differences are present for unexplained reasons, as in Finland and Sweden the current prevalence is estimated to be 2-3% and in Germany only 0.3% (Mustalahti et al. 2010). The prevalence seems to increase with age, as shown by the higher seropositivity in the elderly (up to 2.7%) (Vilppula et al. 2009). In a study by Lohi and colleagues (2007) it was shown that coeliac seropositivity had increased from one to two per cent in the Finnish population between 1980 and 2000, indicating that this is not the result of better diagnostics but that the prevalence is actually rising.

3. COELIAC DISEASE ANTIBODIES

3.1 Serological tests

The importance of serological tests has risen as their accuracy has improved and in recent diagnostic criteria high levels (>10 times the upper limit of normal) of immunoglobulin A anti-tissue transglutaminase (IgA) type 2 antibodies (TG2-ab) are considered adequate for a coeliac disease diagnosis even without biopsy in

symptomatic and endomysium antibody (EmA) -positive patients (Husby et al. 2012).

In contrast to the autoantibodies against self-antigens next presented, the first antibodies, the AGA, are targeted against the gliadin part in gluten. These were tested for by enzyme-linked immunosorbent assay in the early 1980s, but unfortunately they were found to be present also in other conditions such as food-allergies and post-infectious sprue (Unsworth et al. 1983, Lindberg et al. 1985). The sensitivity and specificity of the AGA was also markedly variable, ranging from about 30% to 97% (Mäki et al. 1991b, Sulkanen et al. 1998a, Kaukinen et al. 2007a).

The first autoantibodies against the connective tissue component were the R1-type antireticulin antibodies (ARA) discovered in 1971, which reacted against the reticular fibres of the endomysium. The test for them had several drawbacks, but as specificity was more than 90% in most studies it was widely used (Mäki 1995, Lock et al. 1999).

Endomysial antibodies were found in 1983 and are to this day the mainstay in the diagnosis of coeliac disease due to their excellent accuracy (Chorzelski et al. 1984). EmA is detected by immunofluorescence assay using monkey oesophagus or human umbilical cord as substrate (Ladinser et al. 1994).

The discovery of tissue transglutaminase as the autoantigen of ARA and EmA and the subsequent development of TG2-ab immunoassays changed the diagnostics of coeliac disease (Sulkanen et al. 1998b, Dieterich et al. 1998). TG2-ab is more sensitive than EmA in most studies but less specific, and low false-positive TG2-ab results may be found in inflammatory bowel disease, infections and chronic liver disease (Di Tola et al. 2002, Ferrara et al. 2010, Bizzarro et al. 2006).

The current guidelines for coeliac disease diagnostics recommend the use of EmA and/or TG2-ab in serologic screening. None of the guidelines now recommend the use of ARA or AGA tests in the screening. EmA may be used for screening but TG2-ab is usually the first-line serologic test, as EmA has limited availability, involves subjectivity and costs more. Some median sensitivities and

specificities of the most common serum antibodies are presented in Table 1. To rule out false-negative findings, the IgA-class immunoglobulins should always be tested to exclude selective IgA deficiency (Savilahti et al. 1971) and if negative, the antibodies should be measured in IgG class (Collin et al. 1992, Sulkanen et al. 1998a, Korponay-Szabo et al. 2003).

The previously mentioned AGA have been replaced by antibodies against deamidated gliadin peptides (DGP), which are much superior in accuracy (Kaukinen et al. 2007a). These antibodies have proved to be almost as good as TG2-ab in children and adults, and especially IgG-DGP is useful as it detects IgA-deficient patients (Kurppa et al. 2011, Dahle et al. 2010). Combination of DGP to EmA or TG2-ab has been shown to increase accuracy even further (Kurppa et al. 2011, Schuym and Rumessen 2013).

Table 1. Median accuracies of serum antibody tests for the diagnosis of coeliac disease in some meta-analyses and systematic reviews.

Test	Sensitivity %	Specificity, %
IgA AGA	85 ^c	90 ^c
EmA	84 ^d , 93 ^a , 95 ^c	99 ^a , 99 ^c , 100 ^d
IgA TG2-ab	93 ^b , 93 ^a , 93 ^d , 98 ^c	95 ^d , 96 ^b , 98 ^c , 98 ^a
IgG TG2-ab	63 ^d , 70 ^c	95 ^c , 99 ^d
IgA DGP	88 ^b , 88 ^c , 88 ^d	94 ^b , 95 ^c , 97 ^d
IgG DGP	80 ^c , 88 ^d	98 ^c , 99 ^d

Data adapted from Lewis and Scott (2006^a, 2010^b), Leffler and Schuppan (2010^c) and Schuym and Rumessen (2013^d).

Interesting, albeit of little clinical use, are the anti-actin antibodies and the measurement of TG2-ab from saliva and stool (Brusca 2011 et al., Husby et al. 2012). The anti-actin antibodies cannot replace the previously mentioned antibodies but have shown yielded good results in detecting severe mucosal damage (Marsh 3c) (Achour et al. 2010).

3.2 Small-bowel mucosal antibodies

The extracellular deposition of IgA in the small-bowel mucosa was first observed by Shiner and Ballard in 1972 and later shown to precisely target TG2 (Korponay-Szabo et al. 2004). These deposits have had almost perfect sensitivity for coeliac disease and also high specificity, though weak deposits are reported in treated coeliacs and in some non-coeliac control patients (Koskinen et al. 2008). They are reported in developing coeliac disease before villous atrophy (Kaukinen et al. 2005) and also in seronegative coeliac disease (Salmi et al. 2006). Use of IgA deposits requires frozen sectionings and specialized knowledge of the method and may thus be an excellent additional tool in diagnostics in cases of strong disease suspicion but negative serum antibodies and normal histology (Salmi et al. 2010).

IgA deposits are not a feature solely of the intestine, being present also in other tissues (Korponay-Szabo et al. 2004), of which the most obvious is the skin in dermatitis herpetiformis (Reunala 1978, Reunala et al. 2001). The autoantigen in dermatitis herpetiformis is transglutaminase 3 instead of TG2, suggesting that the specificity of the autoimmune response may be the reason for the heterogeneity of the extraintestinal manifestations (Sárdy et al. 2002). IgA deposits have also been accumulated in the cerebellum and brainstem vessel endothelium in patients with gluten ataxia (Hadjivassiliou et al. 2006). These findings might indicate that the autoantibodies have a role in the development of extraintestinal disease development (Korponay-Szabo et al. 2004).

3.3 Point-of-care tests

Rapid on-site testing for coeliac antibodies would reduce costs, make testing possible without a specialized laboratory and shorten diagnostic delays in primary care (Korponay-Szabo et al. 2005, Raivio et al. 2006, Popp et al. 2013). Three interesting point-of-care tests are available; one tests IgA TG2, one IgA, IgG and IgM TG2 and one tests for IgA and IgG anti-DGP. All of these are

immunochromatographic tests and all are performed in similar manner with a whole blood sample from the fingertip. The accuracy of all these tests has been very good and almost similar to those of IgA anti-TG2 and EmA in laboratories; however, the recommendation at this point is to confirm results with laboratory tests (Nemec et al. 2006, Popp et al. 2013, Mooney et al. 2015).

4. GENETICS

Certain genes play an important role in predisposition to coeliac disease as shown MacDonald and colleagues as far back as 1965. The prevalence of the disease is about 10% in first-degree relatives (Mäki et al. 1991b, Högberg et al. 2003) and in monozygotic twins the concordance rate is up to 90% (Hervonen et al. 2000). Human leukocyte antigen (HLA) genes are polymorphic genes located in a gene cluster called the major histocompatibility complex on chromosome 6p21.3. Several of these HLA genes have been found to be associated with more than 100 diseases, most of which are autoimmune disorders (Shiina et al. 2004). In coeliac disease, the association of HLA-A1 and B8 was identified in the 1970s (Stokes et al. 1972) and later an even stronger association was found for Dw3 (presently DRB1*03 or DR3), which often coexists with B8 (Keuning et al. 1976). This identification of haplotypes allowed genetic testing even before the finding of the actual genes. Subsequently, the primary associated locus was found to be the specific alleles encoding HLA DQ2 and DQ8 (Sollid et al. 1989, Sollid et al. 2000). Approximately 90% of coeliac disease patients express the HLA-DQ2 haplotype (DQA1*0501/DQB1*0201), though it is also present in about one third of the general population. Of the remaining coeliacs, 5% have the HLA-DQ8 haplotype (DQA1*0301/DQB1*0302) and almost all the remainder have at least one of the two genes encoding the DQ2 $\alpha\beta$ -heterodimer (DQB1*0201 or DQA1*0501) (Karell et al. 2003). These haplotypes are necessary to the development of coeliac disease, but not sufficient. About 57 non-HLA

variants have been identified which are also involved in the disease and these are a part of the inflammatory and immune responses (Romanos et al. 2014).

5. PATHOGENESIS

Both environmental and immunologic factors are needed to develop coeliac disease. Dietary prolamines in wheat, rye and barley are the most important environmental factors. The term gluten is a general heading for insoluble prolamine peptides, namely gliadin in wheat, hordein in barley and secalin in rye (Platt and Kasarda 1971). Gluten enhances the baking properties of dough, so that, unfortunately for coeliacs, cereals containing these have come to be favoured (Molberg et al. 2003).

Also other factors, for example common viral infections with adeno- and rotaviruses, have been suggested to increase the risk of coeliac disease through molecular mimicry (Kagnoff 1987). As coeliac disease prevalence has increased the conception known as the “hygiene hypothesis” has gained popularity as a possible cause. In this hypothesis, the improvement of hygiene in modern countries and, thus, the lack of microbial exposure increases the prevalence of autoimmune diseases, since the human immune system reacts to self-antigens in the lack of infectious agents (Lohi et al. 2007, Kondrashova et al. 2008). Breastfeeding is another factor studied in this context and has been thought to offer protection against coeliac disease development (Ivarsson et al. 2002). However, in two recent multi-centre randomized double blinded studies breastfeeding had no effect on coeliac risk (Lionetti et al. 2014, Vriezinga et al. 2014).

Gluten is rich in proline, making it resistant to luminal digestion, this in turn generating an inflammatory reaction in the small bowel. This insufficient degradation generates immunogenic and toxic gliadin peptides in the small-bowel lumen (Shan et al. 2002, Jabri 2009). The immunogenic peptides may cross the epithelial barrier due to increased epithelial permeability by paracellular or transcellular route

(Rauhavirta 2014). There are two peptides which are mainly active *in vivo*: the 33-mer (P55–87) and the 25-mer (P31–55) (Nanyakkara et al. 2013).

After entrance to the lamina propria, the 33-mer induces activation of the adaptive Th1 pro-inflammatory response (Figure 1) (Sollid 2000). TG2 is a multifunctional enzyme present in the basement membrane, and in coeliac disease it converts, by deamidation, glutamine residues to glutamic acid residues, which have higher affinity to HLA-DQ2/8 (Dieterich 1997, Molberg 1998, Henderson 2007). The gliadin peptides are processed by the antigen-presenting cells (APCs), and presented on these in the context of HLA DQ8 or DQ2 are then recognized by the gluten-specific CD4+ T cells (Molberg et al. 1998). Activated CD4+ cells produce pro-inflammatory cytokines such as IFN- γ and TNF- α through the Th1 pathways, this contributing to the development of crypt hyperplasia and villous atrophy through direct cytotoxicity and matrix metalloproteinases (Nilsen et al. 1995, Pender et al. 1997, Jabri 2009). Additionally, in the Th2 pathway the CD4+ T cells promote the activation of B-cells into antibody-producing plasma cells (Figure 1) (Sollid et al. 1997). The antibodies are also suggested to cause extraintestinal manifestations by depositing in different tissues such as the liver and brain (Korponay-Szabo et al. 2004, Hadjivassilou et al. 2006). The TG2-ab may also promote coeliac disease progression as they have been shown to inhibit the differentiation of epithelial cells (Halttunen and Maki 1999), to affect epithelial cell proliferation (Barone et al. 2007), to modulate epithelial cell permeability (Zanoni 2006 et al.) and to disturb angiogenesis (Myrsky et al. 2008).

In addition to adaptive immunity, the non-immunodependent peptide 31-43 (p31-43) induces mucosal damage via a non-T-cell-dependent pathway or so-called innate immunity (Maiuri et al. 2003). The main mediator of these effects is interleukin 15 (IL-15), which up-regulates the expression of two receptors in the IELs (CD94

and NKG2D) and in the enterocytes (ECs) their ligands (MICA and HLAE) resulting in apoptosis of these ECs (Figure 1) (Hue et al. 2004, Jabri et al. 2000).

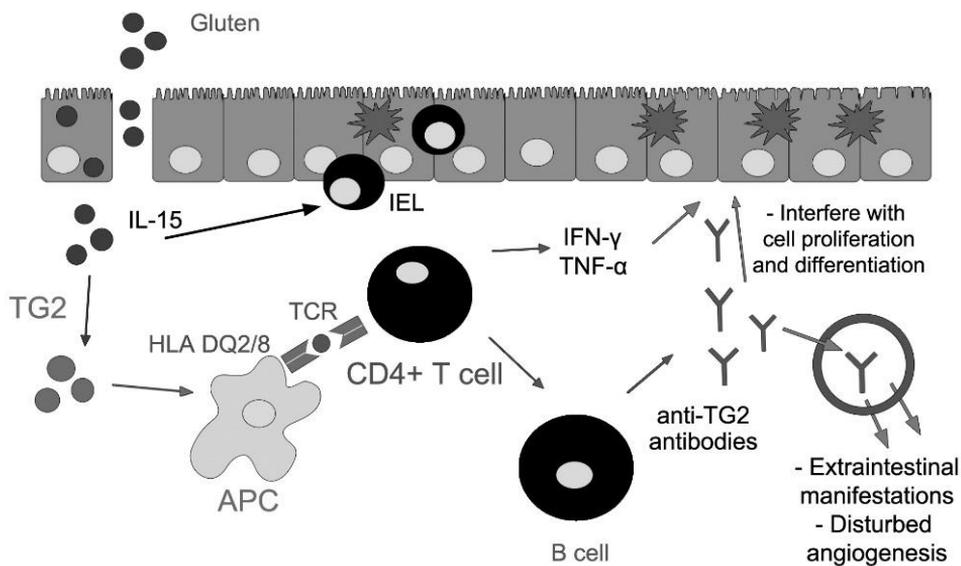


Figure 1. Pathogenic mechanisms in coeliac disease. Gluten peptides enter the lamina propria, where the adaptive and innate immunity proceed. IL-15, interleukin-15; TG2, transglutaminase 2; APC, antigen-presenting cell; HLA, human leukocyte antigen; TCR, T-cell receptor; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor α . Adapted and modified from Kurppa et al. 2014

6. CLINICAL FEATURES OF COELIAC DISEASE

6.1 Classical manifestations and changing pattern of coeliac disease

The gastrointestinal signs of diarrhoea, malnutrition, weight loss and growth retardation already described by Gee in 1888 are considered the classical signs of coeliac disease. Also, deficiencies of fat-soluble vitamins D, E, A and K, iron, folic acid, calcium, folate and B12-vitamin were reportedly common; and have as a result symptoms and signs such as anaemia, poor bone density, growth retardation and failure to thrive (Visakorpi and Mäki 1994). These classical symptoms and signs became rarer in the 1980s following the introduction of serological tests, and patients with only one symptom, extraintestinal symptoms such as arthralgia or anaemia, or even no symptoms at all, were increasingly found (Logan et al. 1983, Mäki et al. 1988). The changing pattern of coeliac disease was obvious. In addition, coeliac disease was long regarded as a children's disease, while nowadays it is known to be a disease of the whole lifespan (Mäki et al. 1988, Lohi et al. 2007, Vilppula et al. 2011).

6.2 Extra-intestinal manifestations and clinically silent coeliac disease

Several extraintestinal manifestations have been described and this has been referred to as atypical presentation; however, such findings have become so common that this term can no longer be considered valid (Collin et al. 1999, Kaukinen et al. 2010). The most common extraintestinal phenomenon is dermatitis herpetiformis (DH), which was first considered to be a disease occurring concomitantly with coeliac disease, but nowadays seen to be a specific manifestation of coeliac spectrum. The

signs are polymorphic blistering rash on elbows, knees, buttocks and scalp. The diagnosis is made from IgA deposits seen by direct immunofluorescence in the uninvolved skin next to the rash (Collin and Reunala 2003). Acquisition of a small-bowel biopsy is recommended, as villous atrophy and crypt hyperplasia in the small bowel is present in 60-80% of DH patients (Reunala et al. 1978, Karpati 2012, Salmi et al. 2014).

Neurologic manifestations are said to be present in up to 22 per cent of coeliac disease patients, and one Finnish study reported that 7% of coeliacs were found upon such findings (Luostarinen et al. 1999, Briani et al. 2008). Most common disorders are ataxia, peripheral neuropathy, encephalopathy and myopathy (Hadjivassiliou et al. 2010). Variable results have been seen with a gluten-free diet in the case of neurological manifestations, but it should be tried as a treatment in gluten ataxia, peripheral neuropathy, encephalopathy and myopathy (Kaplan et al. 1988, Hadjivassiliou et al. 2006 and 2010).

Affective disorders such as anxiety disorders and depression have been linked as symptoms of coeliac disease, as a gluten-free diet seems to alleviate them (Hallert 1982, Addolorato et al. 2001, Viljamaa et al. 2005). In a recent meta-analysis depression was found to be more prevalent in coeliacs than in healthy controls. On the other hand, the severity of anxiety was comparable to that in controls (Smith and Gerdes 2012). As to psychiatric disorders the data are scarcer but a slightly increased risk of schizophrenia and major depression has been proposed (Addolorato 2008).

Untreated coeliac patients have been shown to have reproductive and gynaecological disorders such as unexplained infertility (Collin et al. 1996), recurrent miscarriages, intrauterine growth restriction (Tersigni et al. 2014), older age at menarche, younger age at menopause and secondary amenorrhoea (Ferguson et al. 1982, Smecuol et al. 1996, Moleski et al. 2015); the risk is significantly lower with a gluten-free diet (Tersigni et al. 2014). These disorders may be without common gastrointestinal signs and in such cases serologic screening is therefore suggested (Tersigni et al. 2014). However, some studies have also shown similar rates of fertility

in coeliacs and controls and no difference in pregnancy complications (Kolho et al. 1999); similar results were recorded in a study by Tata and associates (2005) but there was a significant difference in miscarriages.

Coeliac disease is a known cause of hepatic disorders and according to a recent meta-analysis 3 to 4 per cent of cryptogenic hypertransaminasaemia cases are caused by undetected coeliac disease (Hagander et al. 1977, Sainsbury et al. 2011). In most cases the liver enzymes are within normal range but decrease with the introduction of a gluten-free diet (Korpimäki et al. 2011). However, autoimmune liver diseases and also severe liver failures requiring liver transplantations have been described (Kaukinen et al. 2002a).

An effect on bone mineral density (BMD) is a common and seemingly logical manifestation of coeliac disease. Lowered BMD is common in both children (Tau et al. 2006) and adults (Valdimarsson et al. 1994, Kempainen et al. 1999, Vilppula et al. 2011). In accord, the risk of osteoporotic fractures is increased in untreated coeliac disease patients (Vasquez et al. 2000, West et al. 2003). Specifically, in a recent meta-analysis the risk of any fracture was increased by 30% and of hip fracture by 69% (Heikkilä et al. 2015). The evident cause of osteoporosis in coeliacs would be malabsorption, which causes reduced intestinal calcium and D-vitamin absorption, this leading to secondary hyperparathyroidism (Corazza et al. 1995). However, osteopenia can also be present in coeliac disease patients with normal mucosal architecture (Mustalahti et al. 1999, Kaukinen et al. 2001, Kurppa et al. 2010a). Other possible causes of lowered BMD might be the chronic release of proinflammatory cytokines (Fornari et al. 1998) or changes in the osteoprotegerin and receptor activator of nuclear factor kappa-B ligand ratio (Fiore et al. 2006). Antibodies against osteoprotegerin have also been proposed (Riches et al. 2009). However, a more recent study by Larussa and colleagues (2012) found no evidence of these antibodies.

In addition to extraintestinal manifestations also clinically silent coeliac disease with positive coeliac antibodies and small-bowel mucosal lesion is nowadays

a frequent phenomenon (Mäki et al. 1991b, Collin et al. 1999, Vilppula et al. 2008). Such patients are found especially in at-risk groups and in family members (Mäki 1991b). Treatment of these asymptomatic subjects with the restrictive and burdensome gluten-free diet is not as straightforward as in symptomatic patients (Hoffenberg and Liu 2011, Ukkola et al 2011). However, increasing data suggest that screen-detected patients benefit from a gluten-free diet in the same way as symptomatic patients (Kinos et al. 2012, Kurppa et al. 2014a). Also, BMD and quality of life have been shown to be enhanced with a gluten-free diet (Mustalahti et al. 1999 and 2002, Kurppa et al. 2014a). The general consensus at the moment would accept that screening in high risk groups might be appropriate, but further evidence for mass screenings of the general population is still needed (Ludvigsson et al. 2015).

6.3 Degree of mucosal injury and symptoms and signs of coeliac disease

The diverse range of symptoms in coeliac disease, from overt gastrointestinal symptoms to minor laboratory abnormalities or even no symptoms at all, is an interesting phenomenon. Although the diversity would logically be explained by the degree of mucosal damage, studies have been inconsistent in finding a correlation between clinical presentation and degree of injury (Weizman et al. 1997, Thomas et al. 2009, Dorn et al. 2010). A link between anaemia and total villous atrophy is the most evident finding in these studies, in addition to the well-evinced correlation between the degree of histological damage and the levels of antibody titres (Donaldson et al. 2007, Thomas et al. 2009, Husby et al 2012, Abu Daya et al. 2013). Indirect evidence of a correlation between clinical presentation and degree of damage is seen in a parallel alleviation of histological and clinical symptoms on a gluten-free diet (Kurppa et al. 2010b). Furthermore, psychological complications such as anxiety and depression have also been alleviated with dietary treatment (Addolorato et al. 2001, Viljamaa et al. 2005, Ludvigsson et al. 2007). In the absence

of correlation it has been proposed that severity of symptoms is related more to the length of bowel affected (Rubin and Dobbins 1965). However, a study by Murray and colleagues, using video capsule endoscopy showed no effect of the length of injured bowel on the clinical presentation (Murray et al. 2008).

6.4 Early-developing coeliac disease

The previous sections have focused on the wide spectrum of clinical manifestations in coeliac disease, but there would seem also to be a continuum in the diagnostic findings. The intestinal lesion gradually worsens with ongoing gluten consumption, but prior to the development of actual lesion is the so-called latent or early developing coeliac disease phase in which coeliac disease antibodies, mucosal inflammation or IgA-deposits are usually already seen (Mäki et al. 1990, Collin et al. 1993, Kurppa et al. 2010b). The term latent coeliac disease was first used by Ferguson and group in 1993, describing patients who had at first normal small-bowel mucosa but who later developed villous atrophy and crypt hyperplasia (Ferguson et al. 1993).

Patients with positive antibodies but normal mucosal architecture are increasingly found in serological screening, and such subjects have long been a common finding among dermatitis herpetiformis patients (Reunala 2001, Mäki et al. 2003, Kurppa et al. 2010b). The positivity of the coeliac disease antibodies may be false especially in low titres (Husby et al. 2012), but in many cases they would appear to be an early phenomenon in the coeliac disease continuum (Korponay-Szabo et al. 2004, Simell et al. 2010, Agardh et al. 2015).

7. TREATMENT OF COELIAC DISEASE

7.1 Dietary treatment

A gluten-free diet was found to be an effective treatment of coeliac disease in the early 1960s and it is still the only treatment available (Collins et al. 1964, Mäki 2014). It consists in lifelong strict avoidance of wheat, rye, barley and products containing added gluten. The use of oats (avenin) was also banned until prospective studies found no unfavourable effect on the mucosa or symptoms in adult (Janatuinen et al. 1995) or childhood celiac disease (Högberg et al. 2004, Holm et al. 2006) or dermatitis herpetiformis (Hardman et al. 1997, Reunala et al. 1998). Recent studies have nevertheless suggested possible immunologic alterations in patients consuming oats, though the role of these is unknown as long-term results seem to be even positive as a result of increased fibre intake (Tjellström et al. 2014, Sjöberg et al. 2014, Kaukinen et al. 2013). There has also been the questions of trace amounts of gluten in industrially purified wheat starch, but these are nowadays deemed safe for coeliac patients (Kaukinen et al. 1999).

While a strict diet alleviates symptoms in a few days, lowers antibody titres within weeks to months, mucosal recovery may often take longer than 12 months (Haines et al. 2008, Collin et al. 2004); studies from the United States show incomplete mucosal recovery after even 4 years on a gluten-free diet (Lee et al. 2004, Rubio-Tapia et al. 2010a). The diet is difficult to adhere to, costly and socially restrictive and compliance varies between countries, while adherence of up to 96% is achievable (Viljamaa et al. 2005, Ukkola et al. 2012). In addition to gastrointestinal symptoms the diet alleviates extraintestinal symptoms, increases bone mineral density (Valdimarsson et al. 1996), and removes the excess risk of mortality, malignancies and gynaecological disorders (West et al. 2004, Tersigni et al. 2014). These beneficial effects are also evident in patients found through screening (Vilppula et al. 2011). In many cases, the diet is not sufficient in dermatitis

herpetiformis and additive anti-inflammatory dapson medication is often used in the first years after diagnosis (Reunala 2001).

The response to a gluten-free diet needs to be monitored and serum antibodies are used to reveal dietary transgressions, although they may be absent despite persisting mucosal damage (Kaukinen et al. 2002b). Follow-up biopsies are suggested in adults to assure mucosal healing, but in children routine follow-up biopsies are no longer the practice (Collin et al. 2010, Husby et al. 2012, Rubio-Tapia et al. 2013). As dietary lapses are frequent, however, recent guidelines from the British Society of Gastroenterology do not consider follow-up biopsy mandatory if patients are asymptomatic on a gluten-free diet and evince no signs suggestive of complications (Ludvigsson et al. 2014).

7.2 Novel therapies

Gluten is nowadays added to baking products, nutritional values are good and products containing it are wide. Hence, a gluten-free diet is burdensome to maintain and cross-contamination easily occurs. The costs of the diet are also high and the dietary nutrient composition may be unhealthy (Wild et al. 2010, Singh et al. 2011). Such are the main reasons for poor adherence, which is most evident in adolescent and screen-detected patients (Kurppa et al. 2012). Hence, coeliac disease patients would welcome drugs to ease the burden of the diet, especially those frequently visiting restaurants, dissatisfied with the costs and low quality of life (Tennyson et al. 2013).

As coeliac disease constitutes a new field in drug development there are no agreed endpoints for studies. Targets need to be validated and both objective and symptom-based measures must be taken into account (Gottlieb et al. 2015). Biopsy is an important objective endpoint in the disease itself, being the basis of the diagnosis, and the healing of the mucosa upon a gluten-free diet is essential for the well-being of the patient (Mäki 2014). A continuous mode of measurement such as

VH:CrD would be useful, as it can detect small but significant changes in the small-intestinal mucosa (Lähdeaho et al. 2011). Another endpoint advocated by the United States Food and Drug Administration is the use patient-reported outcomes which reflect the direct manifestations of the disease and are therefore important (FDA 2006). Whatever endpoints are used, they must be tailored to the drug in question and the phase of the drug trial, and must also be acceptable to regulatory bodies (Gottlieb et al. 2015).

7.2.1 Drug studies

Interestingly, as early as 1964 an enzyme in crude papain was found to detoxify gluten (Messer et al. 1964). At the moment, several new drugs and devices have entered phase 1 and 2 stages in clinical trials and many are also in the discovery phase. The therapies now developed affect different parts of the pathogenetic cascade, from the ingestion of gluten to the biological functions of the mucosa. All are planned to be used as therapy adjunctive to the gluten-free diet, except for a vaccine in development which is intended to restore oral tolerance to gluten and replace the gluten-free diet (Mäki 2014). Among the most promising candidates are the glutenases, which degrade gluten into non-toxic degradation products in the bowel in a similar fashion to lactase enzymes in lactose intolerance (Khosla and Sollid 2011, Kurppa et al. 2014b). Numerous other candidates are also under development to affect the different stages of the pathogenetic cascade in coeliac disease (Molberg et al. 1998, Kurppa et al. 2014, Mäki 2014).

7.3 Refractory coeliac disease

Refractory coeliac disease (RCD) is a possible diagnosis in a clinical presentation of continuing symptoms, signs of malabsorption and persistent villous atrophy despite a strict gluten-free diet of more than a year's duration and where other possible

aetiologies of villous atrophy have been excluded (Rubio-Tapia and Murray 2010). There are type I and type II RCD, of which type II has significantly more severe presentation and poorer prognosis. The poorer prospects here are predominantly due to nutritional complications and a high risk of enteropathy-associated T-cell lymphoma (Malamut et al. 2009). RCD I is usually treated with topical steroids and budesonide, but also more potent treatments may be needed. In comparison, treatment of RCD II requires cytotoxic chemotherapeutic agents (Kelly et al. 2015). One candidate novel treatment, anti-IL 15, has been suggested as a possibility in the future (Abadie and Jabri 2014).

8. DIAGNOSTIC CRITERIA

The terminology in the field of coeliac disease was long diverse and inexact by reason of multiple synonyms and general headings in use (Rubin and Dobbins 1965). Definitions and guidelines were eventually collectively agreed upon at the Interlaken meeting by ESPGHAN in a round table discussion in 1969 (Meeuwisse 1970). The diagnosis of coeliac disease was restricted to patients who evince a permanent intolerance to gluten. The old diagnostic requirements were the finding of subtotal villous atrophy in a small-bowel mucosa biopsy while on a gluten-containing diet, improvement of mucosal structure on a gluten-free diet and then again destruction of it upon gluten challenge. These criteria were revised in 1990 by Walker-Smith and associates and clinical recovery in symptomatic patients with at least partial villous atrophy was concluded to be sufficient for diagnosis. In asymptomatic patients histological recovery was still needed, with gluten challenge in uncertain cases. Antibodies were regarded as supportive evidence. The recent guidelines of ESPGHAN (Husby et al. 2013) now give an option in a subgroup of children to diagnose coeliac disease without biopsy if immunoglobulin A TG2-ab titres are high (>10 times the upper limit of normal) and in further testing the patient has positive

endomysial antibodies and the relevant HLA. However, by far the majority of diagnoses are still made with biopsy, as this possibility to make a nonbiopsy diagnosis applies only to a subgroup of children in Europe. In other parts of the world, and in adults who form the majority of patients, biopsy is still considered the cornerstone of coeliac diagnostics (Ludvigsson et al. 2014, Rubio-Tapia et al. 2012).

9. MORPHOMETRIC MEASUREMENTS OF THE SMALL-BOWEL MUCOSA

9.1 History

The first reports of villous clubbing in small-bowel mucosa came at the beginning of the 20th century from autopsy reports in patients with tropical or non-tropical (=coeliac disease) sprue (Beneke 1910, Manson-Bahr 1924). Later, in 1954, the finding of morphologically differing villi were proven accurate by Paulley by biopsies obtained via laparotomy samples. Specific examination and multiple specimens became possible only when methods for peroral biopsy had been developed (Shiner 1956, Crosby et al. 1957). In addition to histologic examination, such evidence was also found under the dissection microscope (Holmes et al. 1961). Using this approach normal finger-like villi can be seen but also normal variants like leaf-shaped broad villi (Figure 2). In a histological survey under the microscope these two variants do not differ and both represent healthy mucosa (Holmes et al. 1961). In a case of flat mucosa the specimen seems featureless or has a mosaic pattern in which deep grooves separate flat mosaic tiles. These findings follow the grading set by Doniach and Shiner in 1957, as moderate findings of convolutions or ridges correspond to partial villous atrophy and flat mucosa to subtotal villous atrophy (Figure 2).

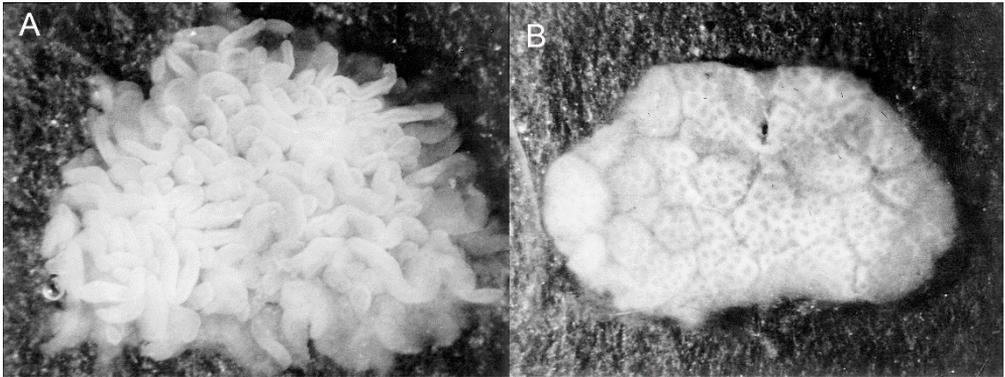


Figure 2. Dissection microscope photographs of normal mucosa with mostly leaf-shaped but also a few finger-like villi (A) and flat mucosa with mosaic pattern and crypt orifices clearly visible in a coeliac disease patient (B). Originals taken by Markku Mäki.

9.2 Site of biopsy

Biopsies were first taken with suction capsules such as the Watson or Crosby-Kuegler from the distal duodenum or proximal jejunum immediately adjacent to the ligament of Treitz. In the procedure no anaesthesia was required for children. The patient swallowed the capsule and it was ensured by X-ray that the capsule was correctly positioned. The biopsy was then aspirated with a syringe to the capsule. The most dreaded consequences were massive haemorrhage and perforation of the intestinal wall. However, the number of perforations dropped markedly as the procedure became familiar (Greene et al. 1974).

Biopsies began to be undertaken with endoscopic forceps in the 1980s, mainly to avoid radiologic screening, which was troublesome especially in young children and pregnant women (Scott and Jenkins 1981, Mee et al. 1985). Also, with endoscopy the mucosa of the oesophagus, stomach, pylorus and duodenum can be evaluated. These forceps biopsies were smaller and orientation could not be ensured as well as with capsule biopsies (Figure 2). However, if at least 4 samples were taken this was considered adequate in light of other benefits (Mee et al. 1985). Nowadays the acquisition of at least 4 duodenal biopsies is still found useful, as the diagnostic

yield increases significantly and sensitivity reaches even 100% (Lebwohl et al. 2011, Ludvigsson 2014). The endoscopist can see markers of coeliac disease through fiberoptics such as scalloping or reduction of duodenal folds, though this is not sensitive or specific enough to dispense with biopsy (Dickey and Hughes 2001).

9.3 Histological classifications of mucosal sections

As already said above, the first classification of mucosal sections was set by Doniach and Shiner in 1957. The same grouped classification of normal, partial villous and subtotal villous atrophy was subsequently acknowledged by ESPGHAN to be the standard (Meeuwisse 1970). In the 1990s a new grouped classification emerged as Marsh presented three phases in the development of coeliac disease. Type III, which represents diagnostic lesion, was later modified by Oberhuber by dividing it into three subgroups depending on the degree of villous atrophy (Table 2). In all of these type III classes there are hyperplastic crypts and an infiltration of IELs (Marsh 1992, Oberhuber et al. 1999).

Table 2. Categories used in Marsh-Oberhuber classification to describe numerically the degree of small-bowel mucosal damage in coeliac disease.

	0	1	2	3a	3b	3c
		<u>Normal villi</u>		<u>Villous shortening^a</u>		
<i>Architecture</i>	normal	normal	crypt hyperplasia	mild	moderate	atrophy
<i>IEL density</i>	normal	increased	increased	increased	increased	increased

^a with crypt hyperplasia
IEL, intraepithelial lymphocyte.

In addition to the above categorical classifications, Shiner and Doniach in 1960 gave precise values for villous height and crypt depth (Shiner and Doniach 1960, Doniach and Shiner 1960). This was further developed by Kuitunen and

associates in 1966 and 1982, and the ratio of villous heights and crypt depths was found more specific for coeliac disease as it also evaluates crypt depth (Figure 3).

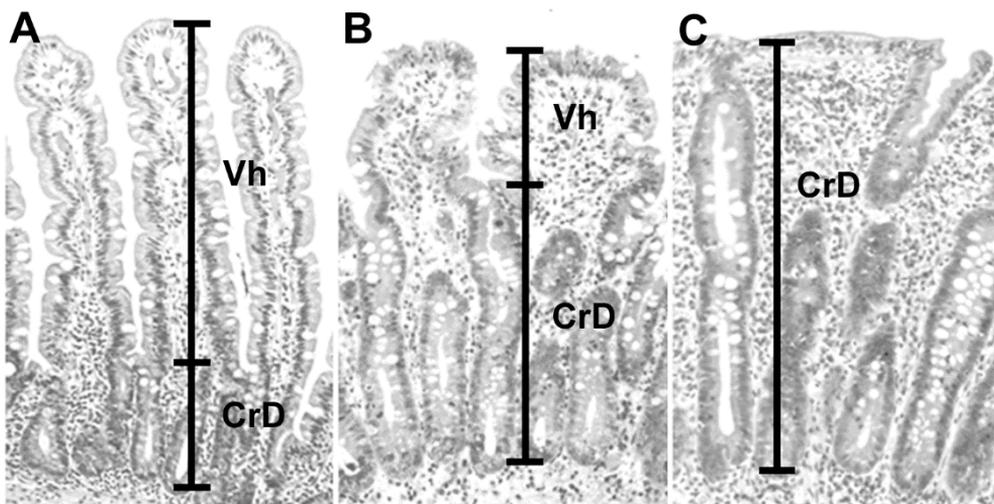


Figure 3. Measurement of villous height crypt depth ratio in different degrees of damage. VH, villous height; CrD, crypt depth. Images by Juha Taavela.

This ratio has been in use in the Tampere coeliac disease research group since 1990 and the first publications came in 1993 (Holm 1993). The normal ratio has been a matter of some controversy and different values have been suggested, mostly arbitrarily. VH:CrD above 3.0 has been considered normal in many articles, while some denote even values of 1.0 as normal (Chang et al. 2005, Villanacci and Corazza 2005, Wahab et al. 2002). The 1982 study by Kuitunen and colleagues found a median of 3.3 in controls and 2.3 in treated coeliacs and a study by a group under Hayat (2002) found a median value of 1.8 in controls. This laboratory has regarded a ratio above 2.0 as a cut-off for normal values (Kuitunen et al. 1982, Kaukinen et al. 2007b). This cut-off has proved accurate and in-line when comparing with mucosal inflammatory markers, serology and mucosal IgA deposits (Koskinen et al. 2010). It also differs from capsule biopsies as these have significantly higher VH:CrD ratios (Holm et al. 2006). The specific measurement of villous height and crypt depth

and their ratio is of superior accuracy, as in categorical classifications significant changes may remain hidden among one group. It also gives the VH:CrD and IEL separately, whereas these are grouped in categorical classifications (Figure 4). However, as these continuous variables require measurement they are somewhat more burdensome than the categorical classifications, in which evaluation is done upon personal estimate under the microscope.

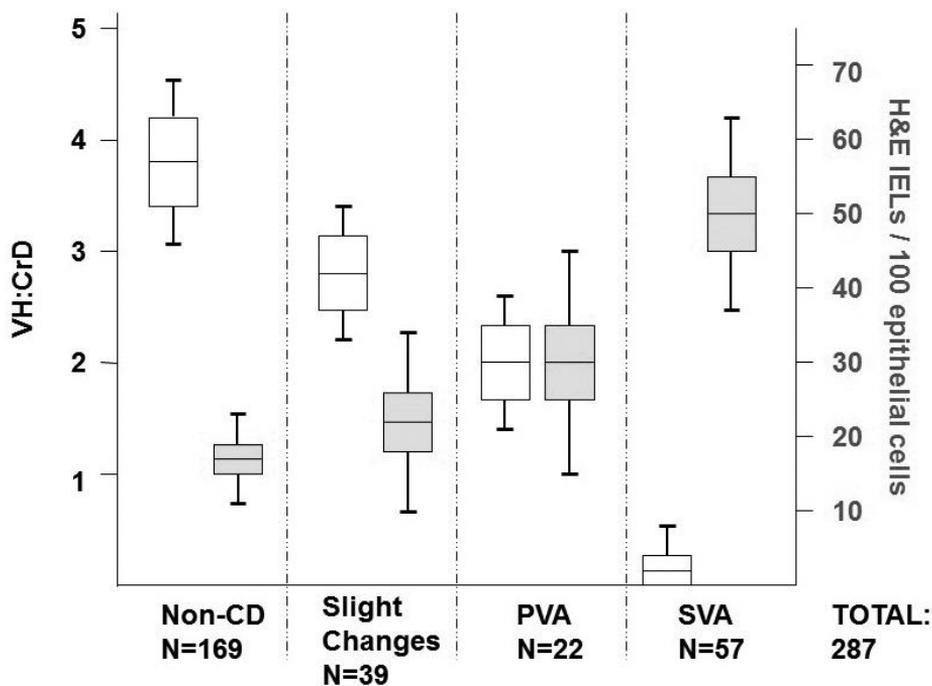


Figure 4. Comparison of continuous villous height crypt depth ratio (VH:CrD) and categorical classification. The VH:CrD is shown as white boxes and the intraepithelial lymphocyte (IEL) density as grey boxes with 95% confidence intervals. As one may observe, the categories overlap significantly in both VH:CrD and mucosal inflammation. In addition, the range in VH:CrD and IEL density is marked in each class. The specimens were randomly selected jejunal biopsies from a local repository which were stored between 1976 and 1986. Adapted from Hartikainen 1994. Non-CD, non-coeliac control patient; PVA, partial villous

atrophy; SVA, subtotal villous atrophy, H&E, haematoxylin eosin-stained paraffin-embedded specimens.

9.4 Intraepithelial lymphocytes

The first criterion for normal cut-off in IELs was 40/100 ECs. However, this estimate was based on studies made in the 1970s, when capsule biopsies were used (Ferguson et al. 1971, Mavromichalis et al. 1976). More recent studies by oesophagogastroduodenoscopy suggest a value of 25 IELs/100 ECs as cut-off for abnormality (Hayat et al. 2002, Walker et al. 2010). Even as low as 20 IELs/100 ECs thresholds for haematoxylin-eosin-stained specimens have been proposed (Veress et al. 2004). In normal mucosa the amount of IELs decreases from the base to the tip of the villi and a change in this pattern in the other direction is suggestive of gluten-sensitive enteropathy (Goldstein 2004, Järvinen et al. 2004). The sensitivity and specificity of measurement of the villous tip IELs were 0.84 and 0.95, which are very good values and comparable to those obtained from $\gamma\delta$ receptor-positive T cells (Mäki et al. 1991c, Iltanen et al. 1999, Järvinen et al. 2003).

The oldest mode of staining used for the measurement of IELs is that with haematoxylin-eosin stained paraffin embedded specimens. However, values obtained are rather unreliable and better methods are available. The most widely used and more reliable and reproducible approach is the immunohistochemical CD3-staining from paraffin-embedded specimens (Veress et al. 2004, Mino et al. 2003). CD3 staining from frozen biopsies is also used as it is a by-product of $\gamma\delta$ -cell staining. A rise in $\gamma\delta$ -cells in the mucosa is not specific for coeliac disease but increases its likelihood (Mäki et al. 1991c, Holm et al. 1992, Järvinen et al. 2003). The measurement of frozen embedded CD3 and $\gamma\delta$ -staining are reliable and have both shown a sensitivity of 93% and specificities of 73% and 88%, respectively, for coeliac disease in our laboratory (Järvinen et al. 2003). The use of immunohistochemistry is

suggested in borderline cases and in academic and pharmacological studies (Järvinen et al. 2003, Collin et al. 2005).

9.5 Reliability and misinterpretation of biopsy cuttings

The correct interpretation of histologic specimens has been of crucial importance from the beginning of peroral biopsies (Rubin et al. 1960a and b). However, some investigators have shown wide intraobserver and especially interobserver variation in classifications of biopsy sections (Corazza et al. 2007, Mubarak et al. 2011, Arguelles-Grande 2011, Picarelli et al. 2014). The coeliac disease lesion may be patchy and hence at least four biopsies are advocated (Lebwohl et al. 2011).

9.5.1 Plane of cutting

Although the importance of correct orientation of the specimen has long been recognised (Rubin et al. 1959), the matter is ignored or not taken into account in routine practice (Collin et al. 2005, Ravelli et al. 2012). The sectionings should be perpendicular to the mucosal surface and cuttings even slightly askew may cause false diagnoses (Brandborg 1958 et al., Rubin et al. 1959). Incorrect biopsy orientation results in cross-sectioning of the crypts and thus loss of evidence of crypt hyperplasia, and is one of the main reasons for missing a coeliac disease diagnosis (Risdon and Keeling 1960, Thurlbeck et al. 1960, Shidrawi 1994, Rostom et al. 2006, Arguelles-Grande et al. 2011). As far back as 1964 a specimen which had broad stunted villi histologically exhibited in the dissecting microscope no villi at all and only a convoluted appearance of the mucosa, and blunted villi were falsely suggested (McCarthy et al. 1964). The proportion of unreadable samples may be about 10% (Collin et al. 2005), but figures as high as 70% have also been presented (Gonzalez et al. 2009). In tangential cuttings, recuttings must be requested and no reading is permissible in these sections.

9.5.2 Brunner glands and bulb biopsies

Whereas capsule biopsies were taken after the ligament of Treitz, nowadays endoscopy biopsies are taken with forceps from the second or last part of the duodenum, while a new trend has emerged which suggests that the gluten sensitive mucosal villous atrophy and crypt hyperplasia is first seen in the anatomical duodenal bulb (Bonamico et al 2004). Interestingly, when publications state that a biopsy is obtained from bulb, it may have been taken more distally and regrouped by the pathologist as a bulb specimen on the basis of Brunner's glands. Hence, the entity "anatomical duodenal bulb" biopsy is defined here as seen by the endoscopist and the "functional duodenal bulb" is then defined by the pathologist because of presence of Brunner's glands. The bulb biopsy was avoided in the guidelines until 2005 (Hill et al. 2005) and even in 2011 the Italian pathologist consensus report strongly discourages the use of bulb biopsy as a potential source of error (Hill et al. 2005, Villanacci et al. 2011). The new guidelines starting from 2012 suggest the acquisition of a bulb biopsy (Husby et al. 2012, Rubio-Tapia et al. 2013, Bai et al. 2013, Ludvigsson et al. 2014). However, these guidelines have not taken account of the special features of bulb samples which differentiate these from duodenum samples. Avoidance was prompted by the abundant presence of Brunner's glands and lymphoid follicles in the duodenal bulb, the possible detrimental effects of nearby stomach acid and susceptibility to mucosal damage caused by gastrointestinal infections, all of which could cause blunted, shortened or widened villi in the duodenal bulb, mimicking coeliac disease enteropathy while the distal duodenum remained normal (Rubin 1960a et al., Jeffers et al. 1993, Oberhuber et al. 1997, Villanacci et al. 2011). False-positive cases have been reported in bulb specimens which were at first deemed to be coeliac disease, while subsequently the cause was found to be *H. pylori* and immunodeficiency syndrome (Gonzalez et al. 2010). Biopsies from the bulb have also been deemed more fragile and orientation is more difficult (Gonzalez et al. 2010, Evans et al. 2011). Current data from bulb studies show that 0-10% of coeliac disease patients have Marsh III lesions only in the bulb,

as presented in Table 3 (Bonamico et al. 2004, Mangiavillano et al. 2009, Evans et al. 2011). There is evidence to indicate that there could be proximal-distal severity, as gluten reaches the bulb first, causing the worst damage there (Vogelsang et al. 2001, Ravelli et al. 2010, Evans et al. 2011). On the other hand, some studies report no increased detection rate with the acquisition of bulb biopsies (Ravelli et al. 2005, Ravelli et al. 2010). Some have also found that the bulb is always involved in coeliac disease (Nenna et al. 2011). Bulb biopsy is now suggested in many major guidelines, but prospective studies are few.

Table 3. Studies investigating the diagnostic yield of bulb biopsies.

	Coeliac disease patients	Lesion only in bulb
Vogelsang et al. 2001	21	2 (10 %)
Bonamico et al. 2004	95	4 (4 %)
Ravelli et al. 2005	110	0 (0 %)
Bonamico et al. 2008	665	16 (3 %)
Hopper et al. 2008	56	1 (2 %)
Gonzales et al. 2009	40	5 (13 %)
Rashid and MacDonald 2009	35	4 (11 %)
Ravelli et al. 2010	686	0 (0 %)
Mangiavillano et al. 2010	47	5 (11 %)
Weir et al. 2010	101	12 (10 %)
Evans et al. 2011	126	9 (11 %)
Levinson-Castiel et al. 2011	87	6 (7 %)
Nenna et al. 2011	43	1 (2 %)
Tanpowpong et al. 2012	103	6 (6 %)
Kurien et al. 2012	28	5 (18 %)
Sharma et al. 2013	101	8 (8 %)
Valitutti et al. 2014	41	0 (0 %)
Caruso et al. 2014	25	0 (0 %)

9.6 Other causes of lymphocytic duodenosis and villous atrophy

Lymphocytic duodenosis or, in other words, an increase in intraepithelial lymphocyte density, is a common finding and present in up to 3.8% of the population negative for coeliac serology (Walker et al. 2010). It has an association with many infections, for example *H. pylori* and giardiasis, diseases of immunity such as the common variable immune deficiency, autoimmune and chronic inflammatory disorders, together with drugs and neoplasia; see Table 4 (Hammer and Greenson 2013). This explains why although intraepithelial lymphocytosis is an early marker of coeliac disease, its specificity remains limited (Chang et al. 2005).

Table 4. Cause of lymphocytic duodenosis in architecturally normal mucosal biopsies in some large studies.

Disease association	Mahadeva et al. 2002 (%) n=14	Kakar et al. 2003 (%) n=43	Hammer et al. 2010 (%) n=100	Aziz et al. 2010 (%) n=100	Shmidt et al. 2014 (%) n=322 ^a
Coeliac disease	21	9	18	21	15
<i>H. pylori</i>	0	0	6	14	3
Bacterial overgrowth	0	4	3	2	15
Gastrointestinal infection ^b	0	0	0	5	0
Drugs (NSAID, ASA)	0	14	8	13	13
IBD	0	12	8	2	9
Autoimmune	0	14	6	4	0
Unexplained	21	-	26	34	29
IBS	14	23	20	18	0
Microscopic colitis	0	7	0	2	2
Other ^d	43	14	4	0	2

a, the last year, 2010, is presented only because study had 11 years in total and each is presented separately.

b, giardia, threadworm, campylobacter.

c, systemic sclerosis, hypothyroidism, hyperthyroidism, rheumatoid arthritis, primary biliary cirrhosis, psoriasis.

d, chronic liver disease, chronic pancreatitis, anaemia.

NSAID, nonsteroidal anti-inflammatory drug; ASA, acetylsalicylic acid; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.

In addition to the unspecific nature of intraepithelial lymphocytosis, villous atrophy may be the result of many other diseases or conditions even though coeliac disease is the most common cause in the developed countries (Freeman 2004). Table 5 gathers some conditions referred to in publications as underlying non-coeliac enteropathy causing possible villous atrophy.

Table 5. Diseases and conditions other than coeliac disease which according to the literature may cause villous atrophy.

Immune disorders	Common variable immunodeficiency syndrome, hypogammaglobulinaemia
Autoimmune diseases	Autoimmune enteropathy, IBD
Infection	<i>H. pylori</i> , giardiasis, cryptosporidiosis, AIDS, SIBO, tropical sprue, viral gastroenteritis
Nutrient deficiency	Malnutrition, B12-vitamin, folic acid, zinc
Other	Peptic duodenitis, collagenous sprue, eosinophilic gastroenteritis, olmesartan, cow's milk enteropathy
Neoplasia	Enteropathy-type intestinal T cell lymphoma

IBD, inflammatory bowel disease; AIDS, acquired immune deficiency syndrome; SIBO, small-intestinal bacterial overgrowth.

Data from Freeman 2004, Serra and Jani 2006 and Pallav et al. 2011.

The epidemiology of causative agents in non-coeliac villous atrophy remains obscure, but the most common causes seem to be autoimmune enteropathy, inflammatory bowel disease, *H. pylori* infections or peptic duodenitis (Ludvigsson et al. 2009, Pallav et al. 2014). Autoimmune enteropathy has a histology similar as to that of coeliac disease, but is unresponsive to a gluten-free diet; it involves anti-EC antibodies, persistent diarrhoea and a response to immune-suppressive treatment (Serra and Jani 2006). Tropical sprue is an intestinal malabsorption syndrome of unknown cause which affects residents or travellers in tropical countries. It usually starts with acute diarrhoea and patients may develop malabsorption of nutrients. It is regarded as an infectious process since there is usually a rapid response to

antibiotics (Baker et al. 1986, Greenson 2015). Collagenous sprue is another entity which has even been proposed to be a variant of coeliac disease (Jain et al. 2014). It presents as villous atrophy, but the biopsy also contains subepithelial collagen deposits and there is usually a slight response to a gluten-free diet; immunosuppressive drugs are nevertheless needed in most cases (Rubio-Tapia et al. 2010b). Small-intestinal bacterial overgrowth (SIBO) is defined as a condition with increased numbers or change in type of bacteria (Bures et al. 2010). Histologically, more villous blunting was observed by Lappinga and associates (2010) in SIBO patients than in controls. More than half, however, had normal mucosa in the study. Antibiotics are the drugs of choice (Greenson 2015).

Gastrointestinal infections may also induce coeliac-like changes in the duodenal mucosa. *H. pylori* is a common cause of duodenal inflammation and it may give rise to changes in the anatomical duodenal bulb (Voutilainen et al. 2003, Gonzalez et al. 2010). Giardiasis may even cause villous atrophy, although this is uncommon and usually mild (Oberhuber et al. 1997). The rare Whipple's disease caused by *Tropheryma whipplei* may also have in the histological picture variable villous atrophy in addition to the pathognomic periodic acid Schiff-positive macrophages in the lamina propria (Ratnaike 2000). Many opportunist infections may likewise underlie villous blunting in human immunodeficiency virus-positive patients (Serra and Jani 2006).

THE PRESENT STUDY

1. AIMS

As the coeliac disease diagnosis is still based upon evaluation of histologic specimens it is of prime importance to acquire reliable and reproducible results from high-quality sectionings. The aim of this study was to explore the VH:CrD and its reliability, correlation to clinical parameters and use in different biopsy sites. The benefits of this inquiry may be seen in academic and pharmacologic studies but also in routine practice.

The specific aims were to:

1. Assess the use of VH:CrD and density of IELs and their reliability and reproducibility in coeliac disease patients. **(I-III)**
2. Detect and confirm potential pitfalls in histological evaluation. **(I, III)**
3. Establish whether there is a correlation between the degree of mucosal injury and coeliac disease antibodies and clinical parameters. **(II)**
4. Evaluate the reliability of bulb biopsies in coeliac disease diagnostics in children. **(III)**

2. PATIENTS AND SAMPLES

2.1 Samples in study I

The samples in question were obtained from a prospectively collected database maintained by the coeliac study group. Altogether 93 small-intestinal mucosal specimens from 72 coeliac disease patients were selected to represent various stages of mucosal injury ranging from normal control to total villous atrophy and crypt hyperplasia (Table 5); non-readable samples were also included (n=12). The samples were read by two observers to acquire interobserver variation in the parameters. One observer read the samples twice to obtain intraobserver variation with a period of 6 months between measurements. All slides were read in blinded and randomized fashion.

2.2 Patients in study II

The participants here comprised 445 adult coeliac disease patients on whom 638 consecutive upper gastrointestinal endoscopies had been performed (Table 6). Newly diagnosed patients and patients on a gluten-free diet for at least one year were included; some patients had undergone an initial diagnostic endoscopy and later a second, which is the reason for the higher number of endoscopies than patients. All underwent clinical and serological evaluations and endoscopy in the Department of Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital. Patients with inadequate histologic samples, immunosuppressive medication or refractory coeliac disease were excluded. The baseline characteristics of the participants in study **II** are shown in Table 6. The majority of the small-bowel mucosal biopsy specimens were from coeliac patients with gastrointestinal symptoms, but up to 44% originated from subjects diagnosed by screening in at-risk groups or on the basis of extraintestinal manifestations of coeliac disease.

2.3 Patients in study III

Forty-four children were recruited prospectively from the Paediatrics Departments in Tampere University Hospital, Tampere, the Institute of Mother and Child Care, Bucharest, and the University of Medicine, Cluj-Napoca. Clinical and demographic data are presented in Table 6 and original article **III**. The patients were suspected of coeliac disease due to suggestive symptoms or signs or as being involved in screening for the disease in at-risk groups. Upon consent, biopsies for morphometry and evaluation of IgA deposits were collected from the anatomical bulb in addition to the samples taken from the distal duodenum for clinical diagnosis. All duodenal bulb specimens were centralized in Tampere for biopsy handling and reading was done according to the standard operating procedure, see original article **I**. Morphometric measurements were made in blinded fashion parallel to histopathology assessment of the distal duodenal samples. The coeliac disease diagnoses were based on serum autoantibody positivity together with the demonstration of distal duodenal mucosal villous atrophy with crypt hyperplasia as evaluated by experienced local pathologists. Cases not fulfilling the diagnostic criteria for coeliac disease were grouped as non-coeliac disease controls.

Table 6. Demographic and clinical data on patients in studies **I-III**.

	<u>Study I</u>		<u>Study II</u>	<u>Study III</u>	
	Specimens from	Specimens from	Endoscopies in	CD patients	Non-CD control
	CD patients	control patients	CD patients		patients
	n=76 ^a	n=17	n=638 ^b	n=22	n=22
Age, median (range), years	54 (15-81)	50 (21-73)	52 (16-81)	5 (3-18)	5 (2-15)
Female, n (%)	37 (67)	10 (59)	(68)	16 (73) ^c	11 (50) ^c
Positive family history, %	-	-	56	50 ^b	5
Main reason for investigations, %					
Gastrointestinal symptoms	-	-	56	64	95
Extraintestinal symptoms	-	-	14	23	5
Screen-detected	-	-	30	14 ^d	0
Newly diagnosed patients, %	21	0	35	100	0
Patients on a gluten-free diet, %	58	0	65	0	0
Gluten challenge, %	21	0	0	0	0

^a Specimens were from a total of 56 separate patients.

^b Endoscopy was performed in a total of 445 separate patients.

^c p=0.122

^d two asymptomatic patients were screened for coeliac disease due to previous type 1 diabetes and as carrying family risk. CD, coeliac disease.

3. METHODS

3.1 Biopsies

All small-bowel mucosal biopsy specimens were taken during upper gastrointestinal endoscopy using forceps. Specimens were taken from the distal duodenum (**I-III**) but also from the anatomical bulb (**III**). Cutting and embedding of biopsy blocks was done at the Tampere Centre for Child Health Research, University of Tampere (**I-III**), but for diagnostic purposes separate additional distal duodenal biopsy blocks were handled on-site (**III**). The mucosal morphometrical variables pertinent to morphology and inflammation were assessed (**I-III**), as well as TG2-specific IgA deposits (**III**). For these purposes, both standard paraffin and also frozen embedded biopsies were acquired (**I-III**). All evaluations were made blindly without prior knowledge of the patients' medical history, laboratory values or dietary status (**I-III**).

3.1.1 Morphometrical and categorical evaluation (I-III)

For morphological analyses duodenal mucosal specimens were paraffin-embedded and standard 5- μm -thick sections cut and stained with haematoxylin-eosin. From well-oriented specimens villous heights (μm) and crypt depths (μm) were measured and VH:CrD calculated from at least three separate villus-crypt units, as described by Kuitunen and colleagues in 1982. A ratio of 2.0 or more was considered normal (Kuitunen et al. 1982, Kaukinen et al. 2007b). Categorical evaluation (**II**) was made according to Marsh–Oberhuber classification, see Table 2.

To highlight the importance of correct sectioning, a case from routine clinics with subsequent recutting and the effect on morphological biopsy readouts is presented. Additionally, a formalin-fixed and paraffin-embedded biopsy specimen was cut twice so that the plane of sectioning was both perpendicular and tangential

to the luminal surface. These biopsy specimens were HE-stained and grouped in Marsh-Oberhuber classes (0, 1, 2, 3a, 3b, and 3c) by five independent pathologists. The pathologists were blinded to each other's statements and to the fact that they were dealing with the same biopsy block.

In addition, a 3D computer model (Autodesk Inventor 2008, Autodesk, San Rafael, CA) was created to evaluate the effect of different planes of sectioning on the morphology of the mucosa. Three computerized blocks were made, each representing the small-intestinal mucosa in a different stage of morphological injury. Sections of these blocks were cut both perpendicular and tangential to the luminal surface.

3.1.2 Biopsy readout standard operating procedure (I-III)

According to the findings of Kuitunen and colleagues the Tampere Coeliac Disease Study Group has formed the currently used standard operating procedure (SOP) for histological evaluations. The SOP involves teaching technicians to obtain correctly oriented cuttings of biopsy specimens for histological evaluation. A vital step in the procedure is that an accredited evaluator, besides producing acceptable interobserver and intraobserver morphometric results, be able to identify cases with an inadequate specimen and/or poor biopsy orientation, where measurements of villus-crypt units are not viable. It is essential that only biopsies in which the plane of sectioning is perpendicular to the luminal surface be considered, as judged by the fact that the crypts of Lieberkühn are cut longitudinally and not in cross-section (Rubin 1960a and b, Thurlbeck 1960, Risdon and Keeling, 1974). In a case of poor orientation resulting in tangential cuttings the evaluator asks for recuttings until reliable morphological readouts can be obtained.

3.1.3 Intraepithelial lymphocytes (I-III)

IELs were counted in the same haematoxylin-eosin-stained specimens as those in which VH:CrD measurements were made (**I**, **II**), but also from CD3⁺ stainings in the same biopsy blocks (**I**). Another set of biopsies from the same patients were embedded in optimal cutting temperature compound, snap-frozen and stored at -70°C until used for IEL density countings or for IgA-deposit stainings (**I-III**). The CD3⁺ paraffin-embedded specimens were stained with monoclonal CD3-specific antibody (CD3, Ab-2; Thermo Scientific, Waltham, MA). IEL densities were counted under light microscopy both in haematoxylin-eosin stained sections (Kuitunen et al. 1982) and in sections where T lymphocytes were stained with monoclonal CD3-specific antibodies (Patey-Mariaud et al. 2000). At least 300 epithelial cells were counted in a continuous length of the surface epithelium, and results were expressed as number of IELs per 100 epithelial cells. Only lymphocytes on the specific plane of cutting were counted, and not those showing only unspecific stain (Figure 5).

Frozen 5- μ m-thick sections were processed and CD3⁺ IELs stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA), $\gamma\delta^+$ IELs with TCR γ antibody (Endogen). The IELs were then counted with a 100 \times flat-field light-microscope objective throughout the surface epithelium; at least 30 fields measuring 1.6 mm in epithelial length were counted and IEL density expressed as cells/mm of epithelium (Holm et al. 1992, 1993, 1994, Iltanen et al. 1999). CD3⁺ IEL densities over 37 cells/mm of epithelium and densities for $\gamma\delta^+$ IELs over 4.3 cells/mm of epithelium were considered abnormal (Järvinen et al. 2003).

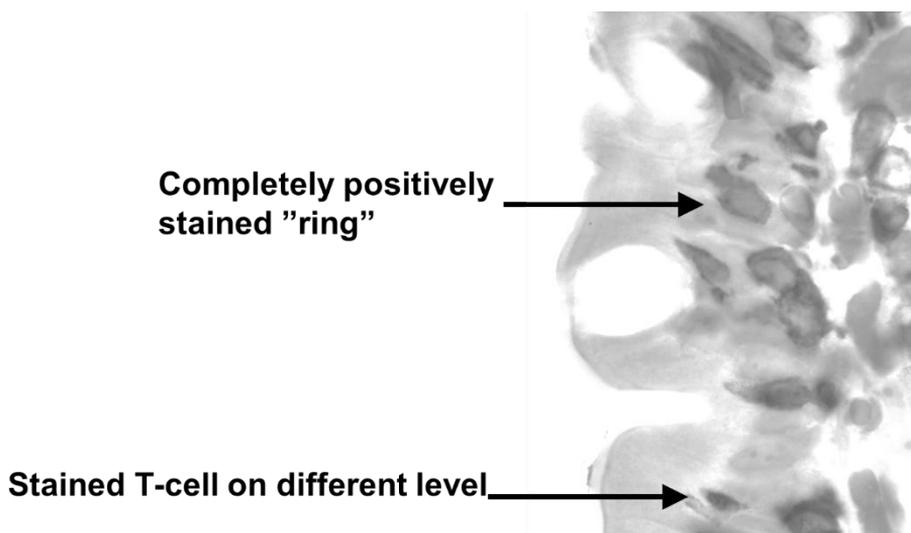


Figure 5. Counting of intraepithelial lymphocytes (IELs) in the epithelium. Only IELs with visible nucleus are counted and not those stains with no nucleus present which are on a different level of cutting.

3.1.4 TG2-specific IgA deposits (III)

IgA deposits targeting mucosal extracellular TG2 were analysed from unfixed frozen sections using double staining with fluorescein isothiocyanate-labelled rabbit antibody against human IgA (Dako, Glostrup, Denmark) and monoclonal mouse antibody against TG2 (CUB7402; NeoMarkers, Fremont, CA), followed by rhodamine-conjugated anti-mouse IgG antibodies (Dako). The coeliac disease-specific TG2-targeted subepithelial IgA deposits were graded according to their intensity along the basement membrane in the villus-crypt area as follows: negative, weak (+), moderate (++) and strong positive (+++) (Korponay-Szabo et al. 2004, Koskinen et al. 2010). All evaluations were carried out blinded without knowledge of patients' disease history or laboratory findings.

3.2 Serology (II-III)

Serum IgA-class endomysial antibody (EmA) titres were determined by an indirect immunofluorescence.in-house method using human umbilical cord as substrate (Ladinser et al. 1994, Sulkanen et al. 1998b). A serum dilution of 1:≥5 was considered positive and reciprocal titres were used: 5, 50, 100, 200, 500, 1000, 2000, 4000 (**III**). In addition, serum TG2-ab titres were measured by a commercial enzyme-linked immunosorbent assay (ELISA) (Celikey, Phadia, GmbH, Freiburg, Germany) using human recombinant TG2 as antigen. A cut-off level of ≥5 units/ml was considered positive according to the manufacturer's recommendations (**II-III**).

3.3 Laboratory parameters (II)

Laboratory measurements were made using the standard laboratory methods of Tampere University Hospital. The following laboratory values were measured: blood haemoglobin (reference value for men, 13.4–16.7 g/dL; reference value for women, 11.5–15.5 g/dL), serum iron (reference value, 50–190 µg/dL), erythrocyte folic acid (reference value, 200–700 nmol/L), and serum vitamin B12 (reference value, 150–740 pmol/L).

3.4 Genetic markers (II-III)

Genotyping of the alleles encoding the αβ DQ2 and DQ8 heterodimer molecules was performed using either the DELFIA Coeliac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) according to the manufacturer's instructions. In part of the samples (**II**) typing was based on HLA tagging of single-nucleotide peptides genotyped according to Koskinen and associates (2009).

3.5 Clinical symptoms and quality of life (II)

Self-perceived gastrointestinal symptoms and health-related quality of life were assessed by means of structured and validated questionnaires widely applied in the context of coeliac disease (Lohiniemi et al. 1998, Hallert et al. 1998, Mustalahti et al. 2002, Ukkola et al. 2011). The Gastrointestinal Symptoms Rating Scale (GSRS) questionnaire comprises 15 separate items, a total score and 5 subdimensional scores measuring abdominal pain, gastro-oesophageal reflux, indigestion, diarrhoea and constipation. The average values are calculated in each subdimension and the total score is the average of all items. The scoring is based on a 7-grade Likert scale in which higher scores indicate more severe gastrointestinal symptoms (Svedlund et al. 1988).

The Psychological General Well-Being (PGWB) questionnaire was used to assess health-related quality of life (Dupuy, 1984). It is validated and widely used in coeliac studies (Mustalahti et al 2002, Kurppa et al. 2010a). The questionnaire comprises 22 separate items and 6 different subdimensions: anxiety, depression, well-being, self-control, general health and vitality. For each dimension, the score is given by the sum of the relevant items. Similarly, the global score is calculated from the sum of the 6 dimension scores. Scoring is based on a 6-grade Likert scale, with higher scores indicating better psychological well-being.

3.6 Bone assessment and body mass index (II)

Bone density was measured in the lumbar spine and left femoral neck by dual-energy X-ray absorptiometry according to our standard procedures (Sievänen et al. 1996). BMD values were expressed as standard deviation scores. The scores compare individual BMD values either with those of sex-matched healthy young adults (T-score), or with that of the age- and sex-matched population (Z-score). Body mass index (BMI) was calculated as weight per square of height (kg/m^2).

3.7 Statistical analyses (I-III)

Quantitative data were expressed as number of subjects (n) and/or percentages, means or medians and ranges or 95% confidence intervals (**I-III**). Intraobserver and interobserver variations were analysed by the Bland-Altman method, linear regression analyses and intraclass correlation coefficients (ICC) (Bland and Altman 1986, Bartko 1966) (**I**). In the Bland-Altman method, the differences between two quantitative measurements are plotted against the averages of the two measurements, and results are reported as the mean difference between the two measurements and limits of agreement, which are defined as the mean difference plus and minus twice the standard deviation of the differences. Twice the standard deviation was used as margin of error and a change exceeding this would thus be considered clinically significant. In the Bland-Altman plot, the x axis shows the mean of the results of the two measurements and the y axis the absolute difference between the two measurements. When there is an increase in the variation of differences as the magnitude of the measurements increases, differences on the y axis are recommended to be expressed as percentages of the values on the axis (i.e. proportionally to the magnitude of the measurements) (Pollock et al. 1992). The mean difference should be approximately 0 and the 95% confidence intervals on both sides of 0 so that no systematic error has affected the measurements. Inter-method agreement was assessed with ICC. ICC values were interpreted as follows: >0.75 was excellent, 0.40–0.75 fair to good and <0.40 poor (Fleiss 1986). When appropriate, the Pearson or Spearman correlation coefficients were applied to assess correlations between different variables (**II**). A correlation coefficient value below 0.35 is considered poor, between 0.35 and 0.5 fair, between 0.5 and 0.8 moderately strong and over 0.8 very strong (Chan 2003). A P value less than .05 was considered significant in all studies (**I-III**). Two-tailed Student's t-test was used to compare differences between groups and receiver operating curves (ROC) to assess sensitivity and specificity of various cut-off points for coeliac disease (**III**).

3.8 Ethical considerations

The study design and recruitment of patients were approved by the Ethics Committees of the Pirkanmaa Hospital Region, Finland (**I-III**), the Universities of Medicine and Pharmacy “Carol Davila”, Bucharest, and “Iuliu Hatieganu”, Cluj-Napoca, and by the Institute for Mother and Child Care “Alfred Rusescu”, Romania, Bucharest (**III**). All patients and/or their parents gave written informed consent.

4. RESULTS

4.1 Reliability and reproducibility of morphometric measurements (I)

Of the 93 specimens considered 12 were correctly deemed unacceptable for accurate morphometric measurements by two independent observers. The spectrum of severity of mucosal injury is presented in Table 7.

The error ranges are obtained by doubling the standard deviations (Table 8). The intraclass correlations showed excellent agreement in VH:CrD measurements, paraffin-embedded and frozen-embedded CD3⁺ stainings; in haematoxylin-eosin staining the correlation was also excellent but clearly lower. Further, IEL densities from different fixations and stainings were mutually convertible and the following equations can be obtained from the linear regression analyses: IEL densities of CD3⁺ stained T-cells in paraffin-embedded specimens (CD3Paraf) can be converted to the conventional IEL densities in HE-stained paraffin specimens (HEParaf) by the equation $HEParaf = 0.619 * CD3Paraf + 8.061$, CD3⁺ frozen specimens (CD3Fro) to HE paraffin IEL density by the equation $HEParaf = 0.442 * CD3Fro + 8.843$ and IEL densities from paraffin CD3⁺ to frozen CD3⁺ staining by the equation $CD3Fro = 1.466 * CD3Paraf + 10.416$.

Table 7. Means and ranges of readable distal duodenum specimens in morphological measurements, intraepithelial lymphocyte (IEL) densities in CD3+ staining in paraffin and frozen and haematoxylin-eosin (HE) stained specimens in coeliac disease patients.

	Mean	Range
<i>Morphology, n=81</i>		
VH, μm	322	1-595
CrD, μm	232	152-535
VH:CrD	1.39	0.01-3.23
<i>IELs</i>		
Paraffin CD3+ IELs/ 100 ECs, n=88	38	9-103
Frozen CD3+ IELs/ mm, n=86	68	12-190
HE IELs/ 100 ECs, n=88	32	8-82

VH, villous height; CrD, crypt depth; VH:CrD, villous height crypt depth ratio; ECs, enterocytes.

Table 8. Reliability and reproducibility of morphometric measurements in study I.

	Two times standard deviation	ICC
<i>VH:CrD</i>		
Intraobserver, n=81	0.318	0.983
Interobserver, n=81	0.454	0.978
<i>IELs, Intraobserver</i>		
Paraffin CD3+IELs/100 ECs, n=88	34.2 %	0.961
Frozen CD3+ IELs/ 100 ECs, n=86	33.0 %	0.956
HE IELs /100 ECs, n=88	53.2 %	0.854

VH:CrD, villous height crypt depth ratio; IELs, intraepithelial lymphocytes; HE, haematoxylin-eosin; ECs, enterocytes.

4.2 Plane of specimen cutting (I)

We picked out from routine clinics an example illustrating the importance of correct biopsy orientation: a pathologist interpreted a specimen as having normal mucosa on the basis of villous structures seen in the sample (Figure 2 in study I). However, due to a high clinical suspicion of disease, biopsy re-evaluation was requested and recuttings were undertaken in these specimens tangentially cut as judged by the cross-sections of the crypts. In the reoriented and recut specimens, coeliac disease was apparent with obvious crypt hyperplasia and villous atrophy (Table 9). To study further the effect of specimen handling, a selected block from another patient was evaluated by five independent pathologists (Table 9), with same results. In contrast, no marked differences can be seen in the IEL densities in tangential and perpendicular cuttings (Figure 3 in original publication I).

A computerized 3D-model was created to illustrate the effect of various cuttings, i.e. perpendicular and tangential, in the evaluation of small-intestinal mucosal biopsy specimens (Figure 5). The hallmark of tangential cuttings is circular cross-sectionings of the mucosal crypts. Poor biopsy orientation resulted in incorrect interpretation and incorrect VH:CrD and incorrect Marsh class (Table 9).

Table 9. Pathologists' reports on two blocks cut tangentially and perpendicular to the mucosal surface to demonstrate the effect of cutting direction (oriented vertical).

	Tangential cutting of specimen	Oriented cutting of specimen
Biopsy block I		
<i>Pathologist</i>	Marsh 0	Marsh 3c
Biopsy block II		
<i>Pathologist I</i>	Marsh 0-1	Marsh 3b
<i>Pathologist II</i>	Marsh 0-1	Marsh 3b
<i>Pathologist III</i>	Marsh 0-1	Marsh 3b
<i>Pathologist IV</i>	Marsh 1	Marsh 3c
<i>Pathologist V</i>	Marsh 1	Marsh 3c

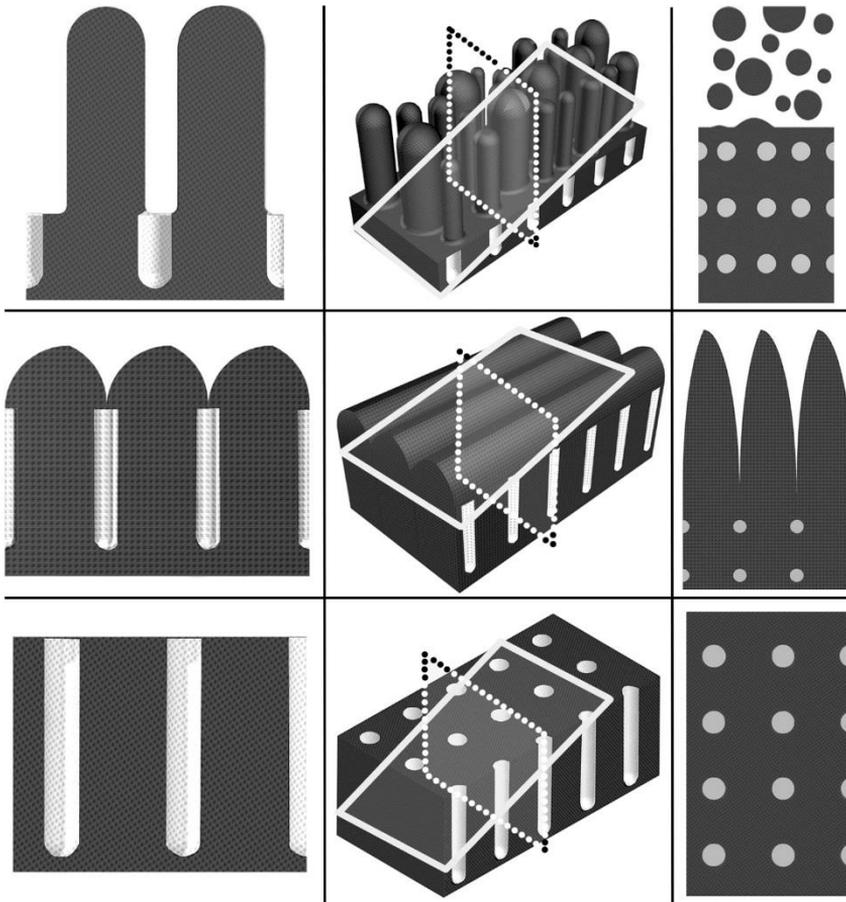


Figure 6. The three biopsy blocks are in the middle column, in which the dashed and solid lines represent planes of sectioning. Sectionings cut perpendicular to the luminal surface are in the left column and tangentially cut sectionings in the right. For example, the block in the middle row shows merged and convoluted low villous ridges, which in perpendicular cutting results in subtotal villous atrophy with deep crypts (left column middle) but in tangential cutting tall villi with only cross-sections of crypts (right column middle). Adapted from Figure 4 in original publication I.

4.3 Correlation between small-bowel mucosal damage, serological titres and symptoms (II)

The correlation coefficients and p-values for each gastrointestinal subdimension score and total score, psychological scores, laboratory values and BMD are presented in Table 10 and precise values in Tables 2 and 3 in original publication **II**. Interestingly, there was a significant p-value, though a low correlation coefficient, between VH:CrD and gastrointestinal symptoms total score in patients on a gluten-free diet ($P = .003$; correlation coefficient, 0.141). The overall correlation coefficient between VH:CrD and Marsh classification was 0.829. Marsh classification correlated significantly with almost the same parameters as VH:CrD, but not with any of the PGWB scores except self-control, (supplementary Tables 2 and 3 in original publication **II**). All patients in question had DQ2 and/or DQ8 alleles.

The mean density of mucosal CD3 + IEL was 72 cells/mm (range, 24–145 cells/mm) in untreated coeliac disease patients and 47 cells/mm (range, 1–151 cells/mm) in subjects on dietary treatment. The IEL density correlated significantly only with serum TG2-ab and erythrocyte folic acid values ($P < .001$ and $P = .008$, respectively).

At the time of the study, serum TG2-ab values were positive in 57% of the participants. The mean antibody value was 41.8 U/L (range, 0–101 U/L) in untreated coeliac patients and 2.4 U/L (0–101 U/L) in subjects on a gluten-free diet. There was a significant correlation between the measured TG2-ab values and all the gastrointestinal symptom rating scale scores except constipation. In addition, antibody values correlated significantly with the laboratory values, BMI and age of the patients, but not with BMD or any of the PGWB scores except self-control (Table 3 in original publication **II**).

Table 10. Correlation of small-bowel mucosal damage to symptoms or signs of coeliac disease in study **II**.

VH:CrD vs. $p < 0.001$	VH:CrD vs. $p < 0.05$	Not significant
GSRS total	PGWB total	PGWB subscore
GSRS subscores	PGWB subscores	<i>vitality</i>
<i>Diarrhoea</i>	<i>Anxiety</i>	GSRS subscore
<i>Indigestion</i>	<i>Depression</i>	<i>Constipation</i>
<i>Pain</i>	<i>Well-being</i>	BMI
Serum TG2-ab	<i>Self-control</i>	BMD scores
CD3+ IELs	<i>Gen.health</i>	<i>Z-score</i>
Haemoglobin	GSRS subscore	<i>T-score</i>
Serum iron	<i>Reflux</i>	Age
Erythrocyte folic acid		
B12-vitamin		

VH:CrD, villous height crypt depth ratio; GSRS, Gastrointestinal Symptom Rating Scale questionnaire; PGWB, Psychological General Well-Being questionnaire; IEL, intraepithelial lymphocyte; BMI, body mass index; BMD, bone mineral density; Z-score, patient's BMD compared to the mean for the patient's age, sex and ethnicity; T-score, patient's BMD compared to the mean for a healthy 30-year-old adult of the same sex and ethnicity of the patient.

4.4 Morphometric evaluation of duodenal bulb biopsies (III)

Twenty-two consecutive serum TG2-ab and/or EmA-positive biopsy-proven coeliac disease patients and an equal number of autoantibody-negative non-coeliac disease controls were prospectively recruited (Table 11). The quality of haematoxylin-eosin-stained anatomical bulb forceps biopsy specimens was unsatisfactory for accurate morphometric measurements even after reorientations and one or more recuttings in 20 out of 44 (45%) patients (Tables 3 and 4 in original publication **III**). There was no difference in this respect between study centres, as seven out of 17 (41%) bulb samples from Finland and 13 out of 27 (48%) from Romania were unreadable morphometrically. The bulb specimens were often small,

superficial or fragmented, revealing no crypts cut longitudinally, and thus the morphology and precise VH:CrD could not be determined. Brunner's glands were present in 23 out of 24 readable specimens upon microscopy and in 16 of these the glands crossed the muscle layer, infiltrating the mucosa (Figures 1 A, B and Figure 2B in original publication **III**).

All patients with coeliac disease and morphometrically measurable bulb specimens had villous atrophy and crypt hyperplasia in the anatomical duodenal bulb (Table 3 in original publication **III**); the mean values and 95% CIs are presented in Table 11. As expected, the bulb mucosal linings were in better condition in non-coeliac disease controls, but in 10 out of the 13 (77%) with readable samples the bulb VH:CrD was below 2.0, revealing marked crypt hyperplasia (Table 4 in original publication **III**). The ROC analysis of VH:CrD, in which the bulb cut-off of 2.0 used for coeliac-type injury gave a sensitivity of 100% but a specificity of only 23 %. Further, a VH:CrD of 1.0 gave corresponding values of 100% and 69%. The sensitivity was 100% up to a VH:CrD of 0.86, but there was still injury in controls, specificity thus remaining at only 77%. The coeliac-type crypt hyperplastic lesions of the *H. pylori* and *Giardia lamblia* are illustrated in Figure 2 in original publication **III**. The IEL density in haematoxylin-eosin stained specimens was increased in 17 out of 19 celiacs (89%) and 2 out of 19 controls (10%) (Tables 3 and 4 in original publication **III**). The final diagnoses of non-coeliac disease control patients are presented in Table 4 in original publication **III**.

Table 11. Mean villous heights (VHs), crypt depths (CrDs), villous height crypt depth ratios (VH:CrDs), the mean CD3+ and $\gamma\delta$ + intraepithelial lymphocytes (IELs) in 22 coeliac disease patients and 22 non-coeliac disease control patients in Study **III**.

	Coeliac disease group	Non-coeliac disease control group
	Mean (95% CI)	Mean (95% CI)
VH, μm	80 (22 to 138)	325 (56 to 394)
CrD, μm	428 (361 to 495)	259 (218 to 299)
VH:CrD	0.2 (0.04 to 0.3)	1.3 (1.0 to 1.8)
HE IELs /100 ECs	46 (37 to 58)	16 (12 to 19)
CD3+ IELs /mm	74 (61 to 86)	23 (15 to 31)
$\gamma\delta$ + IELs /mm	26.5 (20.4 to 32.7)	3.6 (2.4 to 4.8)

$p < 0.001$ between groups in all measurements.
 CI, confidence intervals; EC, epithelial cell.

Frozen bulb samples were available from 20 out of 22 coeliac disease patients and from 17 out of 22 non-coeliac disease controls. Increased density of CD3+ IELs (>37 cells/mm) was present in 16 out of 19 (84%) coeliac patients and 6 out of 17 (35%) controls (Tables 3 and 4 in original publication **III**). The three newly diagnosed coeliac disease patients evincing a CD3+ density less than 37 cells/mm all showed increased densities of $\gamma\delta$ + IELs and positivity for IgA deposits in the bulb (Table 3 in original publication **III**) and their VH:CrDs were 0.28, 0.05, the last sample being unreadable. All coeliac disease patients showed an increased density of $\gamma\delta$ + IELs (range 8.4 - 47.3) as against only 6 out of 17 (35%) of the non-coeliac controls (range 0.7 – 8.5). All coeliac disease patients with available frozen samples had moderate to strong IgA deposits targeting TG2 in the bulb samples (Table 3 in original publication **III**). In contrast, despite findings of morphological coeliac-type injury, none of the non-coeliac disease controls had IgA deposits in their bulb tissue specimens (Table 4 in original publication **III**).

In 39 (89%) children a whole blood sample was available and all coeliac disease patients tested carried either the coeliac disease-associated HLA DQ2 or

DQ8 molecules, or both (Table 1 in original publication **III**). Interestingly, 14 out of 17 non-coeliac disease control patients also had alleles for HLA DQ2 or DQ8.

5. DISCUSSION

5.1 Biopsy and readouts of specimens

Small-bowel biopsy has been the cornerstone of coeliac disease diagnostics since the late 1950s, when the peroral biopsy technique revolutionized the clinical diagnostics of idiopathic steatorrhea (Shiner 1956). Nowadays, antibody tests are used for screening both symptomatic and asymptomatic patients and the disease is verified with a biopsy. However, the need for biopsy has been questioned especially in children with high antibody titres (Husby et al. 2012). Hence, the recent ESPGHAN guidelines state that high antibody titres are under certain conditions sufficient for diagnosis in children, for whom anaesthesia is a considerable burden (Husby et al. 2012). However, in children outside Europe and in all adults the oesophagogastroduodenoscopy and subsequent biopsy specimens still constitutes the diagnostic method (Ludvigsson et al. 2014, Rubio-Tapia et al. 2013). Biopsy is and will continue to be important especially in patients with low or mediocre antibody titres as the specificity of serological tests decreases at lower values. In addition, some adult patients may be antibody-negative and in such cases biopsy is the only means of detecting the disease (Salmi et al. 2006). Even mucosal biopsies may be normal architecturally in these seronegative cases, but the antibodies may sequester into the mucosa and be seen by IgA-deposit stainings from frozen biopsy specimens (Korponay-Szabo et al. 2004, Koskinen et al. 2008). Healing of the small-bowel mucosa on a gluten-free diet is essential to avert malnutrition and remove the excess risk of malignancies and increased mortality (Rea et al. 1996, West et al. 2004). There are several methods to assess adherence to the diet, for example visits to a doctor or dietician, serology and biopsy, while novel biological surrogates are also

being developed (Rubio-Tapia et al. 2013, Morón et al. 2013). The clearest biomarkers for coeliac disease at the moment are the antibodies, which are gluten-dependent and normalize with a strict gluten-free diet. However, serology is not sensitive enough to detect lesser degrees of mucosal damage or occasional slips in the diet, and some patients may thus evince manifest mucosal villous atrophy with crypt hyperplasia and have normal serum antibodies (Nachman et al. 2011, Kaukinen et al. 2002b). This being the case, the most reliable way to diagnose and the most accurate means of assessing disease activity is still the acquisition and study of biopsy specimens (Tursi et al. 2003, Rubio-Tapia et al. 2013).

Specimens taken for coeliac disease diagnostics are studied by pathologists who usually classify the findings according to a categorical classification system, and therefore practically make the decision of coeliac disease or not (Marsh 1992, Oberhuber 1999). The first categorical classification was that suggested by ESPGHAN, employing the following terms: normal, partial villous atrophy, subtotal villous atrophy which equaled the so-called flat lesion (Meeuwisse 1970). At that time, most coeliacs were symptomatic with predominantly flat lesions in biopsy specimens (Visakorpi 1970). As milder forms such as dermatitis herpetiformis were observed, a wider spectrum of small-bowel mucosal coeliac disease manifestations became evident (Reunala 1978, Mäki et al. 1988). Hence, in 1992 Marsh proposed a classification that better took into account the range of mucosal changes. The Marsh classification includes a lesion with lymphocytic infiltrate in the epithelium (Marsh 1), and a crypt hyperplastic lesion (Marsh 2) which precedes villous atrophy (Marsh 3) (Marsh 1992). This classification was further developed by Oberhuber (1999) with a more descriptive array dividing the destructive lesion of Marsh 3 into three subcategories 3a, b and c (Table 2). However, the most straightforward approach would be a continuous variable involving no need to classify specimens into strict categories. Such methods were already described in the 1960s as measurements of villi height, width and crypt depth (Kuitunen 1966) and later more thoroughly when the VH:CrD was posited by Kuitunen and associates in an article in 1982.

The findings in the present dissertation study confirmed for the first time the previous clinical assumption that continuous morphometric measurements are more reliable than categorical classifications. Quantitative variables measure separately gluten-induced morphological (VH:CrD) and inflammatory (IEL density) changes, which is an important feature as patients may have totally flat mucosa and IELs within normal limit, and also vice versa, marked lymphocytosis without architectural changes (Lähdeaho et al. 2011). In categorical classifications these two parameters are grouped (Figure 4), which overlooks changes in IEL densities because classification is predominantly made according to the more specific architectural changes (Arguelles-Grande et al.2011, Lähdeaho et al. 2013). In addition, in the categorical classification allocation to specific groups may be difficult and has shown high interobserver variation (Corazza and Villanacci 2005, Mubarak et al. 2011, Corazza et al. 2007). Unfortunately, this variation is specifically seen in borderline findings, which are crucial in diagnostics. In VH:CrD measurements, allocation is not a problem and the clinician attains a more precise understanding of the specimen even if the ratio is near borderline. In this study, the correlation between VH:CrD and Marsh classification was good, while the latter correlated less well with the clinical parameters. Also, an arbitrary change of 0.5 in VH:CrD was formerly regarded as a clinically relevant change in small-bowel mucosa (Kaukinen et al. 2005). This study for the first time showed this to be fairly accurate, but we found that the value could even be reduced and a new cut-off value of 0.4 could be assigned. Interestingly, as Marsh 3 lesions must by definition be under VH:CrD 2.0 (Kuitunen et al. 1982, Kaukinen et al. 2007b), and especially if a value of 3.0 is used (Villanacci and Corazza 2005), one can calculate that each of the Marsh subcategories 3a, b and c has a very broad VH:CrD range from about 0.6 even up to 1.0, and there are thus significant mucosal changes within these subcategories. These observations, together with the quantitative nature of the criteria and the reproducibility of the findings, support the use of VH:CrD as a more accurate marker of small-bowel mucosal damage.

IEL density is an unspecific marker of coeliac disease and may arise as a consequence of other diseases, infections or drug use (Walker et al. 2010, Hammer and Greenson 2013). However, it may also be the first sign of coeliac disease, as seen also here in the bulb specimens (Kaukinen et al. 1998, Collin et al. 2005). However, the correlation between IELs and clinical symptoms, serological titres and mucosal architecture was poor among coeliac disease patients, reinforcing the conception that inflammation in a mucosa with normal architecture is a poor predictor of coeliac disease (Kakar et al. 2003, Shmidt et al. 2014). Although IEL density is an unspecific marker of coeliac disease it is unaffected by the orientation of the specimen and hence is almost always measurable. The density of IELs presents a decrescendo pattern in the villi (Dickson et al. 2006, Goldstein 2004) and to enhance the diagnostic value of mucosal inflammation, IELs can be also counted from the villous tip, this having even attained a diagnostic value similar to the counting of $\gamma\delta^+$ T cells in finding coeliac disease patients (Järvinen et al. 2004). While the counting of IELs in haematoxylin-eosin-stained paraffin-embedded specimens is the basic method, the immunohistochemical CD3-staining from paraffin- or frozen-embedded specimens in this study showed better reliability and reproducibility and may even detect additional coeliacs (Mubarak et al. 2015). Further, the density of $\gamma\delta^+$ T cells was elevated in all bulb specimens from coeliac disease patients and is of use in unclear cases, as the presence of these T cells increases the likelihood of coeliac disease (Savilahti et al. 1990).

In addition to immunohistochemistry, one intriguing approach in the diagnostics is the staining of immunoglobulin A targeting transglutaminase 2 in the small-bowel mucosa (Korponay-Szabo et al. 2004). Deposits were present in all bulb specimens from coeliac disease patients and in none from controls. Previous studies have shown 100% sensitivity for overt coeliac disease and also 68-100% of potential coeliac patients evince these deposits before the development of coeliac disease, albeit much weaker in potential coeliacs than in those with coeliac disease already established (Koskinen et al. 2010, Kurppa et al. 2010b). The $\alpha\beta^+$ and $\gamma\delta^+$ T cell

densities in addition to the IgA-deposits require frozen sectionings and are therefore available only in specialized laboratories (Holm et al. 1994, Kaukinen et al. 1998, Iltanen et al. 1999).

5.1.1 Pitfalls in mucosal injury assessment

It has been known from the introduction of oesophagogastroduodenoscopy that the proper orientation of biopsy sectionings is crucial for diagnostics (Rubin 1960a and b, Shiner 1964). Orientation was easy in the era of capsule biopsies, as the specimens were large and additionally analysed under dissection microscope (Freeman 2008). When forceps biopsies became the standard the specimens obtained became much smaller and therefore difficult to orientate correctly (Mee et al. 1985, Achkar et al. 1986). Mistaken positive interpretations and misdiagnoses are burdensome and costly for the patient (Biagi et al. 2009). On the other hand, underdiagnosed coeliac disease results in persisting symptoms, an increased risk of complications and unnecessary clinic visits (Catassi and Fasano 2008b, Mattila et al. 2013).

The hallmark of incorrect biopsy cutting is seen in the cross-sectionings of crypts, which indicate that the villi have also been cut tangentially, and in such case the biopsy is not amenable to reading (Thurlbeck 1960, Villanacci 2011). Good orientation of the biopsy should not be assumed from the finding of tall villus structures but from the finding of straight crypts. Also, in tangential cuttings one may miss the first sign of coeliac disease, crypt hyperplasia (Figure 6, Arguelles-Grande et al. 2012). When cut at correct angle, the crypts of Lieberkühn are cut longitudinally and VH:CrD measurements or categorical classifications are permissible. A benefit in VH:CrD measurements is that one is forced to measure the crypt depths and hence always also to evaluate the orientation. Further, when blunted villi merge and convolute to villous ridges and are cut tangentially, the outcome is falsely normal-looking villi (Figure 6). It is also possible to misclassify normal mucosa as evincing coeliac disease when the cutting is almost fully tangential

and the villi are sliced off (Figure 6). Good-quality specimens can be obtained by orientating the biopsy on an acetate cellulose filter before cutting, but this takes additional time and requires expertise (Ravelli and Villanacci 2012). If completely tangential cuttings are seen, they must be subjected to recutting at a different angle so that the crypt depths may be evaluated and the villi represent their true height.

Other reasons for misinterpretation also exist. The samples may evince patchiness, there may be Brunner or lymphoid artefacts, in addition to which, bulb biopsies may involve their own difficulties, as discussed in the next section (Chang et al. 2005). Patchiness means that in only a few spots in the duodenum the coeliac disease lesion is evident while elsewhere the architecture is still preserved (Hopper et al. 2008, Ravelli et al. 2010); patchiness may be present even within a single biopsy specimen (Weir et al. 2010). The condition is held to be a phenomenon of developing coeliac disease which if left untreated will eventually involve the whole duodenum (Vogelsang 2001, Ravelli 2005). In such cases it is recommended to take multiple biopsies from the duodenum (Husby et al. 2012).

There is the possibility of early developing coeliac disease having no signs at all of mucosal injury or only slightly raised IELs as marks of inflammation (Mäki et al. 1990, Mäki et al. 1991b, Kakar et al. 2003, Kurppa et al. 2010b). Here the previously mentioned $\gamma\delta$ T-cells and IgA-deposits have been shown to detect coeliac disease patients in up to 96% of cases (Mäki et al. 1991c, Holm et al. 1992, Korponay-Szabo et al. 2004, Kaukinen 2005, Koskinen et al. 2008). However, high titres of TG2-ab and especially EmA are very sensitive and specific for coeliac disease and, hence, rigorous follow-up must be ensured in cases of undiagnostic serological titres and biopsy (Husby et al. 2012).

5.2 Role of anatomical duodenal bulb biopsy in coeliac disease diagnosis

Bulb biopsies were since the introduction of capsule biopsies avoided due to the unspecific flattening of villi usually found in them (Rubin 1960b). The North American Society for Pediatric Gastroenterology, Hepatology and Nutrition still stated in their guidelines in 2005 that especially the Brunner glands found in the bulb can adversely affect the interpretation of mucosal architecture, and recommended acquisition of biopsies of more distal origin (Hill et al. 2005).

Studies began to emerge at the beginning of this century on the significant diagnostic gain attained by acquisition of bulb biopsies and the approach was therefore quickly introduced in diagnostic procedure (Vogelsang et al. 2001, Bonamico et al. 2004 and 2008, Levinson-Castiel et al. 2011, Evans et al. 2012, Nenna et al. 2012). In this present study, coeliac-type mucosal lesions were present in all untreated coeliac disease patients but, even if milder, also in control patients excluded for coeliac disease. Also, specificity was only 23% with a VH:CrD of 2.0, this evidencing the unreliability of bulb specimens. Interestingly, a low VH:CrD has been suggested to constitute a possible normal variation of villous blunting within the duodenal bulb (Weir et al. 2010). As noted in earlier sections, the biopsy may be normal in developing coeliac disease (Mäki et al. 1990, Kurppa et al. 2010b) and also a tangential plane of sectioning can lead to false normal findings. This raises the question whether all mucosal damage is taken to indicate coeliac disease in cases of raised antibodies, which would thus involve overdiagnosis (Hassall, 2010). This could be especially true in the case of bulb biopsies, which have previously been avoided in view of other causes of villous flattening (Trier 1971, Hasan et al. 1989, Chang et al. 2005). Another disturbing finding in this study is the poor quality of bulb biopsies, as a substantially high proportion (45%) of specimens proved inadequate for accurate morphometric measurements. This is in accord with a previous study by Gonzalez and associates who found a proportion as high as 70% of specimens to be inadequate for morphologic assessments (Gonzalez et al. 2010).

The non-coeliac-related mucosal injury seen in the duodenal bulb can be caused by many distinct factors which were brought out by this study and hence call in question the current care guidelines (Rubin 1960b, Murray 1999, Chang et al. 2005). Most important cause is the “Brunner artefact”, i.e. stumped villi adjacent to Brunner’s glands, which are abundant in the duodenal bulb (Whitehead 1973, Kreuning et al. 1978). In the present study, 96% of anatomical bulb specimens had Brunner’s glands and in 67% the glands infiltrated the lamina propria, making morphometric analysis difficult (Figure 1 in Study **III**). The same phenomenon of stump villi has been recognized above lymphoid follicles (Chang et al. 2005). The proximity of the stomach may cause problems as the anatomical bulb faces the highest gastric acid load, and ulcer-associated and non-specific duodenitis may reduce villous height and increase crypt depth (Jeffers et al. 1993, Hasan et al. 1989). Gastrointestinal infections causing duodenitis and mucosal injury, for example *H. pylori* and giardiasis, are common pathogens in the gut in many parts of the world and must be taken into account when evaluating specimens (Ashorn et al. 1995, Oberhuber et al. 1997, Feng and Xiao 2011). Interestingly, there were 7 patients in this study with gastrointestinal infection and of these, 4/5 readable samples evinced mucosal injury up to a flat lesion (**III**). Clear crypt hyperplasia was seen in a patient with *H. pylori* infection and *Giardia lamblia* infestation (Figure 2 in study **III**). One could hypothesize that these lesions in these non-coeliac control patients were caused by the natural reaction of the small-bowel mucosa to remove the injuring agent similarly to the reaction seen in patients with infectious diarrhoea (DuPont and Hornick 1973).

One important and possibly confounding aspect in previous coeliac disease studies is the actual definition of the duodenal bulb. In many earlier studies all duodenal samples having Brunner’s glands might have been redefined as bulb specimens on a histological basis, representing “functional” bulb tissue. Subsequently, with a coeliac disease lesion in a biopsy taken from the distal duodenum at endoscopy and the presence of Brunner’s glands indicating the

specimen to be bulb tissue, it may occur that only the “functional bulb” is considered to be diseased. As far back as 1944 Landboe-Christensen in his doctoral work demonstrated that Brunner’s glands are not a histologic feature exclusive to the duodenal bulb, they are often seen extended throughout the length of the duodenum up to the jejunum, especially in children.

5.3 Degree of small-bowel mucosal damage and symptoms and signs of coeliac disease

Follow-up is required in coeliac disease to ensure proper healing of the mucosa in order to avoid complications such as intestinal lymphoma (West et al. 2004). In the follow-up multiple surrogate markers are used to assess the dietary intake of gluten: symptoms, normalization of coeliac disease serum antibody titres and structured interviews by a dietician (Ludvigsson et al. 2014). However, a biopsy is considered irreplaceable in adults, as the above markers have not been shown to reflect the healing of the mucosa well enough (Ilus et al. 2012, Rubio-Tapia et al. 2013). It has been shown that the symptoms correlated to mucosal damage in patients on a gluten-free diet, which would imply that in symptomatic patients the mucosal structure remains incompletely restored despite dietary treatment. Hence, in order to facilitate the follow-up procedure, accurate and reliable markers of mucosal status are needed but are not yet in routine use (Morón et al. 2013).

Interestingly we found here significant correlations between the degree of small-bowel mucosal morphologic damage and most of the clinical outcomes measured in coeliac disease, indicating that these could be used as reflectors of mucosal status. Of particular interest was the correlation observed between morphologic injury and patient-reported gastrointestinal symptoms and health-related quality of life. Earlier studies have shown only parallel alleviation of such symptoms as depression and anxiety (Viljamaa et al. 2005, Ludvigsson et al. 2007, Addolorato et al. 2001). As mentioned in the previous section, non-invasive markers

of mucosal health are needed and these validated questionnaires thus comprise a convenient secondary marker for assessing the status of the disease. This is in agreement with United States Food and Drug Administration recommendations to ascertain the subjective perception alongside objective clinical markers (U.S. Department of Health and Human Services FDA 2006). In addition, the correlation seen in this study between degree of mucosal damage and gastrointestinal and psychological symptoms adds value to the biopsy, as mucosal well-being reflects clinical improvement. At the same time, however, it must be acknowledged that most of the correlation coefficients measured were rather small. Thus in clinical practice patients may evince intense symptoms with only minor mucosal lesion or patients may even be asymptomatic and have “flat lesion”. Hence it is not surprising that previous studies involving patients with gastrointestinal symptoms and using the categorical Marsh classification have not been able to find significant correlations (Brar et al. 2007, Thomas et al. 2009, Dorn et al. 2010).

In this present series we observed no correlation between VH:CrD and BMD in the adult population studied. Previous studies have shown that osteoporosis and osteopenia are well-recognized complications of coeliac disease (Valdimarsson et al. 1994, Kempainen et al. 1999, Vilppula et al. 2004, Tau et al. 2006). This has been linked to intestinal malabsorption causing mineral metabolism alterations and metabolic osteopathy, but other mechanisms such as chronic inflammation and serum interleukins or serum antibodies may also have a role (Corazza et al. 1995, Fornari et al. 1998, Larussa et al. 2012). Decreased BMD increases to normal levels in a few years in paediatric coeliac disease after the introduction of a gluten-free diet (Mora et al. 1998). However, in the adult population the data are controversial and although a gluten-free diet is used, the diet rarely normalizes BMD even in the long run, which is consistent with these findings (Mustalahti et al. 2002, Capriles et al. 2009, Larussa et al. 2012). One explanation could be that the gluten-free diet may not improve BMD after the crucial two first decades of life in bone mineralization, but an alternative explanation could be the increase in coeliac disease screening,

which has led to the earlier detection of coeliac disease in Finland over the past few decades. With improved diagnostic efficiency there is an earlier diagnosis and therefore a shorter duration of severe intestinal malabsorption wherein the bone has no time to weaken. In line with this, in this study even untreated patients showed only a slightly decreased age-adjusted BMD and one cannot expect significant changes on these near-normal values.

The recent ESPGHAN guidelines are the first to allow a coeliac disease diagnosis based solely on serology provided the titres are high (Husby et al. 2012). Hence the positive predictive value of TG2-ab has received attention. We found a significant correlation between serology titres and degree of mucosal damage and accordingly agree to these guidelines. Antibodies 10 times over the normal limit have been shown to predict coeliac disease almost perfectly, while low antibody titres have on the other hand been shown to be imprecise (Webb et al. 2015). Interestingly there was an inverse correlation between current age and TG2-ab titres, as also found by Vivas and colleagues (2008). It could be that the antibodies sequester in the small-bowel lamina propria in the elderly, manifesting clinically as a higher rate of seronegativity in these subjects (Salmi et al. 2006). In the future, the possibility to diagnose adults also without biopsy seems to be reasonable at least in symptomatic patients with high TG2-ab and EmA titres (Hill and Holmes 2008, Wakim-Fleming 2013).

5.4 Strengths and limitations of the study

The major strengths in this study were the use of validated and reliable outcomes. The small-bowel mucosal morphometric measurements were validated and used, the clinical markers studied are routinely utilized in clinics and the questionnaires widely applied.

Validation of morphometric measurements was done from samples chosen from a repository at the Tampere Centre for Child Health Research, so that the

specimens represented the whole spectrum of mucosal injury (VH:CrD range 0.1-3.2). The observers were blinded to the selection of samples. These were analysed again after 6 months and one possible limitation could be that the observer might remember the samples. However, the samples were blinded before the second reading and interobserver assessment was only slightly superior to the intraobserver analysis, indicating that there was no significant bias in the intraobserver readings.

The present study covered different degrees of small-bowel mucosal damage and symptoms reflecting the current situation in coeliac disease. Many previous studies have lacked patients evincing only mild mucosal changes (Marsh I and II) and the symptoms have comprised mainly classical gastrointestinal presentation. The use of well-validated and widely used questionnaires made the results reliable and reproducible. The GSRS and PGWB questionnaires are generic and thus comparable to other disease contexts. On the other hand, more coeliac disease-specific questionnaires might have detected smaller differences. As discussed above, the length of the mucosal damage could also have a role in the severity of the symptoms, but this was not analysed in this study as it would have required additional methods such as wireless capsule endoscopy or video-endoscopy which are not used in routine in coeliac disease.

Study **III** had the advantage of being a prospective multicentre study. As a limitation, the number of patients was rather low for any conclusions as to the diagnostic yield acquired with the addition of bulb biopsy. However, this was not the purpose of this study and a later large cohort will address this subject. Additionally, one might consider the high proportion of specimens dismissed from morphometric analysis to be a limitation. This was however one of the most important results, showing that bulb biopsies lack quality for precise morphometric measurements, at least in children in whom forceps samples may be quite small and superficial.

A further strength in this study was that we defined the duodenal bulb specimens on anatomical basis according to visual evaluation by the endoscopist. Previous studies have not specified whether bulb biopsies are from the anatomical

bulb or defined by the pathologist according to the presence of Brunner's glands seen in the specimen. This causes confusion in studies of the changes in bulb specimens, as it has not been routine to take samples from the bulb. This calls in question the reliability of these samples; were they acquired from the bulb or are they merely biopsies from the second or third part of the duodenum which happen to contain Brunner's glands.

6. SUMMARY AND FUTURE ASPECTS

The results of the present study show for the first time the reliability of morphometric measurements, the correlation between the degree of mucosal injury and clinical presentation and problematic aspects of bulb specimens.

The validation study proved VH:CrD to be a reliable and reproducible criterion quantitative measurement of small-bowel mucosal damage. The cut-off for significant change was set at 0.4, which can be regarded as highly precise when compared to the change required in moving from one Marsh class to another. This study also pinpointed the results of reading tangential cuttings with the computerized 3D model, where the cross-sections of crypts are the hallmarks of improper cutting direction.

The results of study **II** showed that there is a link between the degree of mucosal damage and symptoms and signs of coeliac disease. Interestingly, there was also a correlation in the gluten-free diet group possibly indicating that the gluten-free diet may not be adequate for some patients; however, the compliance of patients may be questioned. The finding of these links indicates that although direct measurement of the mucosa via biopsy remains the main method, antibody titres, laboratory parameters and patient-reported outcomes are valid secondary surrogate markers of mucosal status. Patient-reported outcomes are essential, as symptoms are a direct manifestation of the disease and any clearly perceptible effect of treatment for the patient. However, one must remember that symptoms are highly unspecific.

Additionally, in accord with many studies it was more precisely shown with VH:CrD that the degree of mucosal injury correlates to the serum antibody titres. This is adjunct to the ESPGHAN guidelines, which now approve the use of high antibody titres as diagnostic for coeliac disease in children, since mucosal lesion is practically always present in these patients.

The results of study **III** involved previous knowledge and problems in evaluating bulb biopsies. There was a clear mucosal lesion in the mucosa of the bulb in all coeliac disease patients, but interestingly 77% of non-coeliac disease control patients also had VH:CrD below 2.0, even up to a “flat lesion”. Further, even after repeated recuttings to achieve optimal orientation of the sample, 45 % of the anatomical duodenal bulb specimens were unsatisfactory for precise morphometric measurements. IgA deposits were present in bulb specimens from all coeliac disease patients and in none of the controls. This finding could be used to confirm coeliac disease in exclusively bulb injuries. Therefore, the anatomical duodenal bulb may be an untrustworthy biopsy site for coeliac disease diagnostics and special care should be taken in making decisions solely on the histology of bulb specimens. Premature conclusions might have been drawn in current care guidelines as to coeliac disease diagnoses based solely on anatomical bulb specimens. We urge caution in the assessment of bulb biopsies especially in low TG2-ab titre patients with injury solely in the duodenal bulb. In these instances, IgA deposits seem to find coeliac disease patients well. The additional benefit of duodenal bulb biopsies to the sensitivity of biopsies was not within the scope of this study and must later be investigated in large prospective studies.

These results are applicable in clinical practice and academic and pharmacological drug trials. Validated measurements with error ranges are required in gluten challenges or drug trials to assess any changes in mucosal architecture reliably. Drugs developed need to alleviate symptoms but most of all remove the excess risk of complications caused by an unhealed duodenal mucosa. New tools for monitoring mucosal status in coeliac disease are the symptoms questionnaires.

However, the reliability of these in a clinical setting is poor, as the correlation coefficients here were low, but for research purposes they are clearly of additional value.

In the future, standard operating procedures should be applied in biopsy handling and reading in routine to enhance the reliability of histological analyses. The diagnostic value of isolated lesions in anatomical duodenal bulb specimens might warrant more criticism and caution in the next coeliac disease guidelines. Mucosal IgA targeting TG2 is a powerful tool in finding coeliac disease patients in unclear cases or poor quality specimens. As the unreliability of histology has gained worldwide attention, the possibility to diagnose coeliac disease without biopsy could be more widely accepted especially in patients with high titres of coeliac disease antibodies.

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ORIGINAL PUBLICATIONS

Validation of Morphometric Analyses of Small-Intestinal Biopsy Readouts in Celiac Disease

Juha Taavela¹, Outi Koskinen¹, Heini Huhtala², Marja-Leena Lähdeaho¹, Alina Popp^{1,3}, Kaija Laurila¹, Pekka Collin⁴, Katri Kaukinen^{4,5,6}, Kalle Kurppa¹, Markku Mäki^{1*}

1 Tampere Center for Child Health Research, University of Tampere and Tampere University Hospital, Tampere, Finland, **2** Tampere School of Health Sciences, University of Tampere, Tampere, Finland, **3** Institute for Mother and Child Care "Alfred Rusescu" and University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania, **4** Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, Finland, **5** Department of Medicine, Seinäjoki Central Hospital, Seinäjoki, **6** School of Medicine, University of Tampere, Tampere, Finland

Abstract

Background: Assessment of the gluten-induced small-intestinal mucosal injury remains the cornerstone of celiac disease diagnosis. Usually the injury is evaluated using grouped classifications (e.g. Marsh groups), but this is often too imprecise and ignores minor but significant changes in the mucosa. Consequently, there is a need for validated continuous variables in everyday practice and in academic and pharmacological research.

Methods: We studied the performance of our standard operating procedure (SOP) on 93 selected biopsy specimens from adult celiac disease patients and non-celiac disease controls. The specimens, which comprised different grades of gluten-induced mucosal injury, were evaluated by morphometric measurements. Specimens with tangential cutting resulting from poorly oriented biopsies were included. Two accredited evaluators performed the measurements in blinded fashion. The intraobserver and interobserver variations for villus height and crypt depth ratio (VH:CrD) and densities of intraepithelial lymphocytes (IELs) were analyzed by the Bland-Altman method and intraclass correlation.

Results: Unevaluable biopsies according to our SOP were correctly identified. The intraobserver analysis of VH:CrD showed a mean difference of 0.087 with limits of agreement from -0.398 to 0.224 ; the standard deviation (SD) was 0.159. The mean difference in interobserver analysis was 0.070, limits of agreement -0.516 to 0.375 , and SD 0.227. The intraclass correlation coefficient in intraobserver variation was 0.983 and that in interobserver variation 0.978. CD3⁺ IEL density countings in the paraffin-embedded and frozen biopsies showed SDs of 17.1% and 16.5%; the intraclass correlation coefficients were 0.961 and 0.956, respectively.

Conclusions: Using our SOP, quantitative, reliable and reproducible morphometric results can be obtained on duodenal biopsy specimens with different grades of gluten-induced injury. Clinically significant changes were defined according to the error margins (2SD) of the analyses in VH:CrD as 0.4 and in CD3⁺-stained IELs as 30%.

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* E-mail: markku.maki@uta.fi

Introduction

In celiac disease the characteristic gluten-induced small-intestinal mucosal injury develops gradually [1,2]. The spectrum of mucosal changes contains two separate measurable parameters, inflammation reflected by intraepithelial lymphocytic infiltration and morphological damage which includes villous atrophy and crypt hyperplasia. The mucosal lesion is the gold standard for diagnosing celiac disease and is present in patients both with and without clinical symptoms or signs of the disease. Well-known grouped classifications in histological assessment are one described by Marsh and modified by Oberhuber and another produced by Corazza and Villanacci [1,3,4]. These classifications are practical

in clinical work, but allocation to specific groups may be challenging and minor histologic changes are easily missed [4]. Such small but significant changes can be caused even by relatively small amounts of gluten and are particularly important when the clinical effectiveness of pharmacological therapies is evaluated.

Also noteworthy is that only a morphologically healed mucosa, and not the disappearance of symptoms, is a prerequisite for the long-term well-being of a patient [5]. The key issue is thus whether a potential drug or vaccine is able to prevent or attenuate the gluten-induced mucosal damage [6,7]. Optimally, histological evaluation should be made with validated and reliable readout tools [4]. Unfortunately, recent studies have shown poor reproducibility when using the results of grouped classification as

the primary outcome. Moreover, evaluators have given discrepant results in borderline findings, which are diagnostically the most critical part of the deterioration [8–10]. An important reason for these diagnostic problems might be incorrect orientation of biopsy specimens, leading to tangential cuttings and faulty interpretations [11–14]. Hence, validated standard operating procedures (SOPs) are needed to ensure reliable and reproducible results.

We here evaluated the quantitative morphological (villus height-crypt depth ratio, VH:CrD) and inflammatory (density of intraepithelial lymphocytes, IEL) variables used in the assessment of different degrees of damage in small-intestinal mucosal biopsies. The aim was to provide a standardized methodology and cut-off values for significant gluten-induced changes in the small-intestinal mucosa to be employed in routine clinical practice, and in academic and pharmacological studies in celiac disease.

Materials and Methods

Study design and small-bowel mucosal specimens

The study was conducted in the University of Tampere and Tampere University Hospital. The material comprised altogether 93 small-intestinal mucosal specimens from 72 patients, which were obtained from the prospectively collected database and biobank maintained by our study group. Altogether 16 specimens were obtained from newly diagnosed untreated celiac disease patients, 44 from patients on a gluten-free diet, 16 from patients who underwent gluten challenge and 17 specimens from non-celiac disease controls. The mean age of the celiac patients was 57 years (range 15–81) and 67% were women. The mean age of the non-celiac controls was 50 years (range 21–73 and 59% of them were women. The small-bowel biopsies were selected (see later) to represent variable stages of mucosal injury ranging from completely normal histology to overt mucosal atrophy and crypt hyperplasia. Further, specimens with good ($n=81$) or poor orientation ($n=12$) were included.

The forceps biopsy specimens were formalin-fixed and embedded in paraffin and another set of biopsies from the same patient were embedded in optimal cutting temperature compound, snap-frozen and stored at -70°C until used. For morphometric analyses, paraffin-embedded standard 2- μm -thick sections were processed and stained with hematoxylin-eosin (HE) and additional sections from the same biopsy block were stained with monoclonal CD3-specific antibody (CD3, Ab-2; Thermo Scientific, Waltham, MA). The small-intestinal mucosal VH:CrD was evaluated from at least three separate VH:CrD units by measuring villi lengths (μm) and crypt depths (μm), and the result was given as the average of the ratios. IEL densities were counted under light microscopy both in HE-stained sections [15] and in sections where T lymphocytes were stained with monoclonal CD3-specific antibodies [16]. At least 300 epithelial cells were counted in a continuous length of the epithelium, and results were expressed as number of IELs per 100 epithelial cells.

Frozen 5- μm -thick sections were processed and CD3⁺ IELs stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA). The IELs were then counted with a 100 \times flat-field light-microscope objective throughout the surface epithelium; at least 30 fields measuring 1.6 mm in epithelial length were counted and the IEL density was expressed as cells/mm of epithelium [17].

The selected paraffin-embedded biopsy blocks comprised a wide spectrum of different degrees of mucosal injury and VH:CrD and density of CD3⁺ IELs were evaluated in parallel by two readers (JT,OK). Also, one evaluator had prior to the present evaluation studied and given results on all of these specimens (JT). The specimens were chosen by an independent selector (MM) and all

measurements were made by the evaluators in blinded fashion without knowing the results obtained by the other evaluator or the clinical data of the patients. One evaluator (JT) studied and reported results on the VH:CrD measurements and all IEL countings, while the other (OK) evaluated only the VH:CrD and IELs from CD3⁺-stained paraffin specimens.

Biopsy readout standard operating procedure

Our study group has adopted the morphometric small-intestinal mucosal biopsy measurements, as already described by Kuitunen and co-workers in 1982 [13], and subsequently formed the currently used SOP for histological readouts. SOP includes teaching technicians to obtain correctly oriented cuttings of biopsy specimens for morphometric evaluation. A crucial step in the procedure is that an accredited evaluator, besides producing acceptable interobserver and intraobserver morphometric results, be able to identify cases with an inadequate specimen and/or poor biopsy orientation, where measurements of villus-crypt units are not viable. It is essential that only biopsies in which the plane of sectioning is perpendicular to the luminal surface be considered, as judged by the fact that the crypts of Lieberkuhn are cut longitudinally and not in cross sections [11]. In a case of poor orientation resulting in tangential cuttings the evaluator asks for recuttings until reliable morphological readouts can be obtained. In IEL measurements the results are independent of biopsy orientation and recutting of specimens is rarely needed.

Effect of tangentially cut sections on the evaluation of villus height-crypt depth ratio

A case example from routine clinics illustrating the effect of tangential sectioning and subsequent recutting on morphological biopsy readouts is presented. To highlight the importance of correct sectioning, another formalin-fixed and paraffin-embedded biopsy specimen was cut twice so that the plane of sectioning was both perpendicular and tangential to the luminal surface. These latter biopsy slices were HE-stained and grouped in Marsh-Oberhuber classes (0, 1, 2, 3a, 3b, and 3c) by five independent pathologists who were blinded to each other's results and to the double cutting of the same biopsy block.

Further, a computerized 3D model was created to evaluate the effect of different planes of sectioning on the morphological readouts of the mucosa. First, computerized blocks were made representing small-intestinal mucosa in three different stages of morphological injury and sections of these blocks then cut in such a way that the plane of sectioning was both perpendicular and tangential to the luminal surface. The computer modelling program used was Autodesk Inventor 2008 (Autodesk, San Rafael, CA).

Statistics

Intraobserver and interobserver variations were analysed by the Bland-Altman method, linear regression analyses and intraclass correlation coefficients (ICC) [18,19]. In the Bland-Altman method, the differences between two quantitative measurements are plotted against the averages of the two measurements, and the results reported as the mean difference between the two measurements and limits of agreement, which are defined as the mean difference plus and minus twice the standard deviation of the differences. Twice the standard deviation was used as margin of error and a change exceeding this would thus be considered clinically significant. In the Bland-Altman plot, the x axis shows the mean of the results of the two measurements and the y axis represents the absolute difference between the two measurements.

When there is an increase in the variability of the differences as the magnitude of the measurements increases, differences on the y axis are recommended to be expressed as percentages of the values on the axis (i.e. proportionally to the magnitude of the measurements) [20]. Inter-method agreement was assessed with ICC. Quantitative data were expressed as number of subjects (n), mean and ranges.

Ethics

The patient recruitment and sample collection were approved by the Ethics Committee of Tampere University Hospital and all patients gave written informed consent.

Results

All 12 preselected biopsy specimens with tangential cuttings rendering them unacceptable for VH:CrD analyses were correctly identified by both study evaluators. The villus heights in the remaining 81 specimens with proper orientation ranged from 1 μm to 595 μm and the crypt depths from 152 μm to 535 μm . The subsequent mean value in VH:CrD was 1.39 (range 0.01–3.23). In IEL countings, five HE-stained and five CD3⁺ paraffin specimens and seven frozen specimens were excluded for technical reasons resulting from insufficient amounts of epithelial cells. The mean density of IELs in HE-stained specimens was 32 (range 8–82) per 100 epithelial cells, in paraffin-embedded CD3⁺-stained specimens 38 (range 9–103) per 100 epithelial cells and in CD3⁺-stained frozen specimens 68 (range 12–190) cells/mm.

The Bland-Altman plots illustrating the agreement in intraobserver and interobserver analyses in VH:CrD are shown in Figures 1A and 1B and the corresponding numerical values in Bland-Altman analyses are given in Table 1. In both intraobserver and interobserver VH:CrD analyses, the mean differences in the two measurement series compared were below 0.1, ensuring that there was no marked systematic error in the study. Twice the standard deviations, which represent the error range of the measurements, were 0.318 in intraobserver and 0.454 in interobserver analyses. The intraobserver and interobserver linear regressions are shown in Figures 1C and 1D and the ICCs in Table 1.

Bland-Altman statistics for the IEL countings are shown in Table 2. In the intraobserver analyses of IEL countings, the twice standard deviations representing the error ranges were 34.2% in CD3⁺ paraffin, 33.0% in CD3⁺ frozen and 53.2% in HE stained specimens.

The ICCs presented showed excellent agreement in the CD3⁺ stainings and the agreement was good in the HE countings (Table 2). Additionally, the inter-method comparison between frozen and paraffin-embedded CD3⁺ stainings showed an ICC of 0.679 ($p < 0.001$). These IEL densities from different fixations and stainings are mutually convertible and the following equations can be obtained from the linear regression analyses: IEL densities of paraffin-embedded CD3⁺ stainings (CD3Paraf) can be converted to the conventional IEL densities in HE-stained paraffin specimens (HEParaf) by the equation $\text{HEParaf} = 0.619 * \text{CD3Paraf} + 8.061$, CD3⁺ frozen specimens (CD3Fro) to HE paraffin IEL density by the equation $\text{HEParaf} = 0.442 * \text{CD3Fro} + 8.843$ and IEL densities from paraffin CD3⁺ to frozen CD3⁺ staining by the equation $\text{CD3Fro} = 1.466 * \text{CD3Paraf} + 10.416$.

The effect of incorrect biopsy orientation on histological evaluation is shown in Figure 2. In routine clinics, the pathologist interpreted the specimen in Figure 2A as normal mucosa, the villous structures seen thus excluding celiac disease. However, due to a high clinical suspicion of disease, biopsy re-evaluation was

asked for and recuttings performed by reason of original tangential cutting as judged by biopsy cross-sectioning of the crypts. In these reoriented specimens (Figure 2B), crypt hyperplasia with villous atrophy compatible with celiac disease was obvious. To study further this source of diagnostic error, our selected block from another patient was evaluated by five independent pathologists. All graded the tangential sectioning showing only cross-sections of crypts but tall villi as representing Marsh 0–1 (Figure 2C). When the same block had been cut perpendicular to the luminal surface, the crypts were correspondingly cut longitudinally and the same five pathologists graded the specimen as Marsh 3a–3b (Figure 2D). In contrast, no marked differences can be seen in the IEL densities in tangential and perpendicular cuttings (Figure 3).

In Figure 4, the computerized 3D-model demonstrates the effect of differing cuttings, i.e. perpendicular and tangential, in the evaluation of small-intestinal mucosal biopsy specimens. The hallmark of tangential cuttings is circular cross-sectioning of the mucosal crypts. Poor biopsy orientation results in incorrect interpretation of VH:CrD and/or incorrect Marsh class.

Discussion

The present validation study demonstrated that quantitative variables which measure separately gluten-induced morphological (VH:CrD) and inflammatory (IEL density) changes are reliable and reproducible. Morphometric variables can be utilized for outcome measurements in both academic studies and forthcoming drug trials in celiac disease. By following our SOP, we were able to ensure strict limits of agreement in the Bland-Altman method and excellent intraobserver and interobserver ICCs in VH:CrD in biopsies with different grades of small-intestinal mucosal injury. It is obvious that the forceps biopsy specimens obtained by endoscopy are small and difficult to orientate correctly. The cornerstone of our SOP has thus been to teach the accredited evaluator when morphological variables are not amenable to measurement. In all biopsy samples the sectioning of the crypts should be inspected as a part of routine in order to avoid the reading of tangential cuttings, wherein circular cross-sections of crypts can be used as hallmark. In the present study, both accredited evaluators were able to correctly identify the specimens not suitable for subsequent morphological analyses. We believe that our methodology for biopsy orientation and measurement of morphology can be easily applied to any standard laboratory settings.

VH:CrD measurement is a sensitive tool in detecting minor changes in gluten consumption [8,21]. We have previously used a change of 0.5 in VH:CrD as clinically relevant in assessing the effect of a gluten-free diet in celiac disease patients and also during gluten challenges [22]. The present findings would argue that this value could be reduced, since the mean difference was below 0.1 and twice the standard deviation was only 0.318 in the intraobserver Bland-Altman analysis. A cautious new cut-off value of 0.4 could thus be assigned to represent a clinically relevant difference between measurements. In the interobserver analysis again, the limit needed to obtain 95% of subjects was 0.454, indicating a slight observer-dependency in VH:CrD measurements. We infer that small but significant gluten-induced mucosal architectural changes can be reliably measured by morphometry.

The IEL densities in immunohistochemical CD3 stainings here showed better limits of agreement in the intraobserver analyses than the corresponding measurements in HE-stained specimens. In contrast, the ICC between IEL measurements of the paraffin and frozen specimens was good, and either one can be used for reliable results. It should also be noted that the frozen sections are

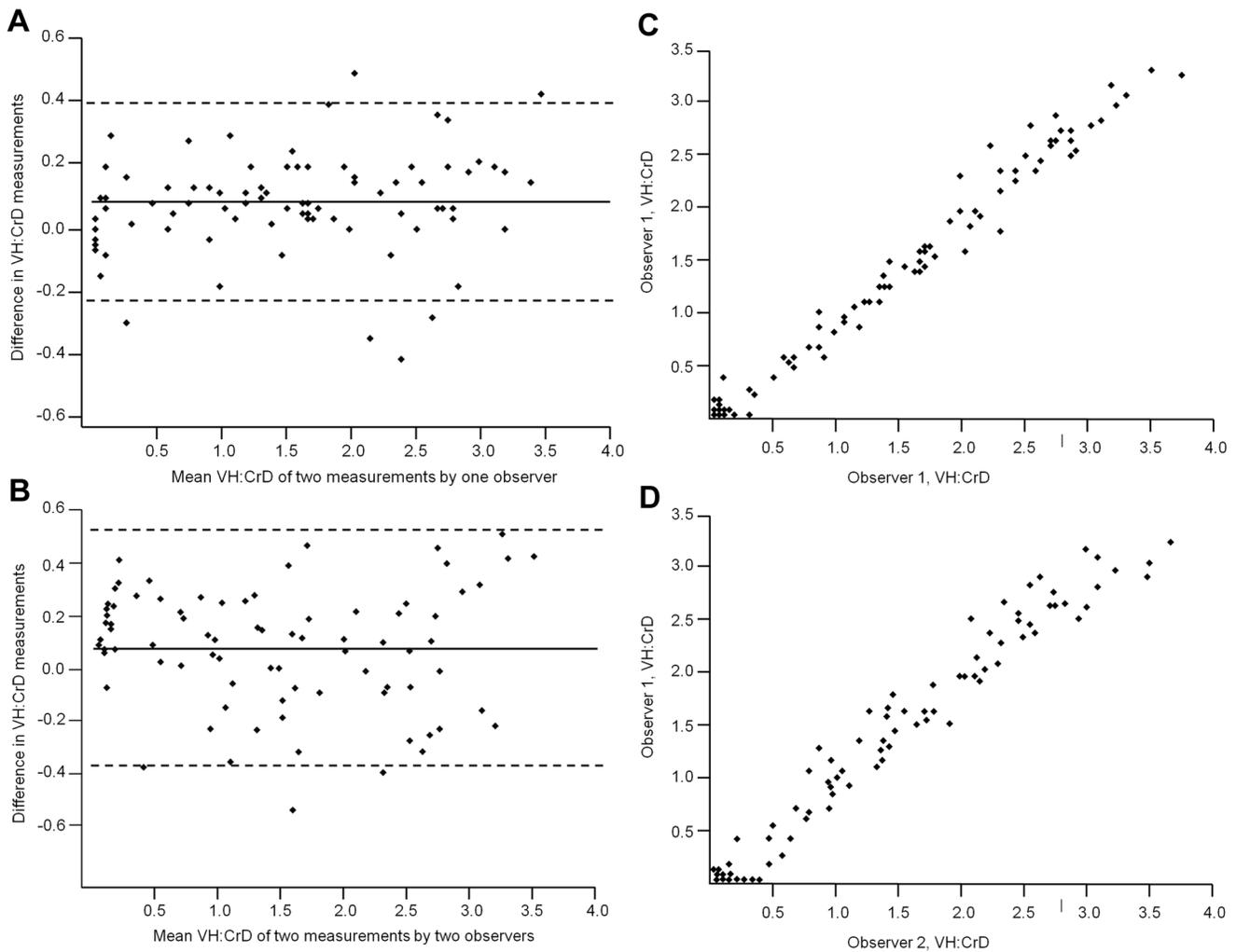


Figure 1. Graphical illustrations of the reliability and reproducibility of villus height crypt depth ratio. Panels A and B show the Bland-Altman plots of small-intestinal mucosal villus height crypt depth ratio and Panels C and D present the regression analyses for intraobserver and interobserver analyses, respectively. The solid lines in panels A and B indicate the mean difference between the measurements and the dashed lines correspond to the 95% limits of agreement.

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associated with better tissue preservation than the paraffin associated specimens. However, frozen samples are more burdensome due to specific technical requirements. We have used frozen samples in academic research in order to measure, in addition to CD3⁺ cells, the $\alpha\beta^+$ and $\gamma\delta^+$ T cell densities [16]. This also gives an opportunity for internal staining control, as the density of all T cells (CD3⁺) should be within the magnitude of the sum of $\alpha\beta^+$ and $\gamma\delta^+$ T cell densities. In the present study, twice the standard

deviation was in the intraobserver analysis in frozen specimens 33.0% and in paraffin specimens 34.2%. These findings agree well with previous studies in which a change of more than 30% in lymphocyte count has been considered clinically relevant [20]. The limits of agreement in the absolute IEL density values were wider in our study than those previously published, as we had substantially higher mean lymphocyte densities overall [23]. We thus provided values in percentages, as these are advocated when

Table 1. Bland-Altman statistics with absolute values and intraclass correlation coefficients (ICC) for analysing agreement and repeatability in small-bowel mucosal villus height crypt depth ratio (VH:CrD).

	Mean difference (95% CI)	Standard deviation	Limits of agreement	ICC
VH:CrD				
<i>Intraobserver</i>	0.087 (0.051 to 0.121)	0.159	−0.224 to 0.398	0.983
<i>Interobserver</i>	0.070 (0.020 to 0.121)	0.227	−0.375 to 0.516	0.978

CI, confidence interval.

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Table 2. Bland-Altman statistics with absolute and percentage values and intraclass correlation coefficients (ICC) for analysing agreement and repeatability in the density of intraepithelial lymphocytes (IELs) of paraffin CD3⁺, frozen CD3⁺ and hematoxylin-eosin (HE) stained small-bowel mucosal biopsy specimens.

	Mean difference (95% CI)	Standard deviation	Limits of agreement	ICC
Paraffin CD3+ IELs/100 enterocytes				
<i>Intraobserver</i>				
absolute values	1.9 (0.7 to 3.2)	5.9	-13.5 to 9.6	0.961
percentage values	5.1 (1.4 to 8.7)	17.1	-38.6 to 28.4	
<i>Interobserver</i>				
absolute values	6.9 (4.4 to 9.4)	11.6	-15.9 to 29.6	0.842
percentage values	24.1 (18.8 to 29.4)	24.7	-24.3 to 72.5	
Frozen CD3+ IELs/mm				
<i>Intraobserver</i>				
absolute values	2.0 (-0.3 to 4.3)	10.9	-19.4 to 23.4	0.956
percentage values	1.0 (-2.5 to 4.5)	16.5	-31.3 to 33.3	
HE IELs/100 enterocytes				
<i>Intraobserver</i>				
absolute values	0.7 (-2.6 to 1.3)	9.2	-17.4 to 18.7	0.854
percentage values	8.1 (-2.5 to 13.7)	26.6	-44.0 to 60.2	

CI, confidence interval.
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the variability in the measurements increases with higher IEL densities [19]. These percentage values would thus be more appropriate when assessing the effect of a gluten-free diet or novel drugs in celiac disease.

Soon after the discovery of the gluten-induced detrimental effect on the mucosal architecture in celiac disease it became clear that great care should be taken in orienting specimens in order to ensure representative vertical sections [11,24]. One of the main reasons for missing a celiac disease diagnosis is incorrect biopsy orientation, resulting in cross-sectioning of the crypts and thus loss of evidence of crypt hyperplasia [10,25]. In contrast, the IEL densities are mainly unaffected by the biopsy orientation as shown

here in Figure 3. The importance of having complete villus-crypt units and longitudinally cut crypts is highlighted by the exemplified biopsy cuttings seen in Figures 2 and 4. Further, when what are in reality low merged and convoluted villous ridges are cut tangentially the outcome is falsely normal-looking villi, as also seen in our computerized model (Figure 4). It is also possible that a normal mucosal architecture is falsely classified as celiac disease when the cutting is almost fully tangential giving the biopsy histological appearance of a “flat” lesion. Morphologically the appearance would be similar to a tangentially cut true “flat” lesion (Figure 4). False interpretation could occur especially if no cross-sections of villi are present. These examples further demonstrate

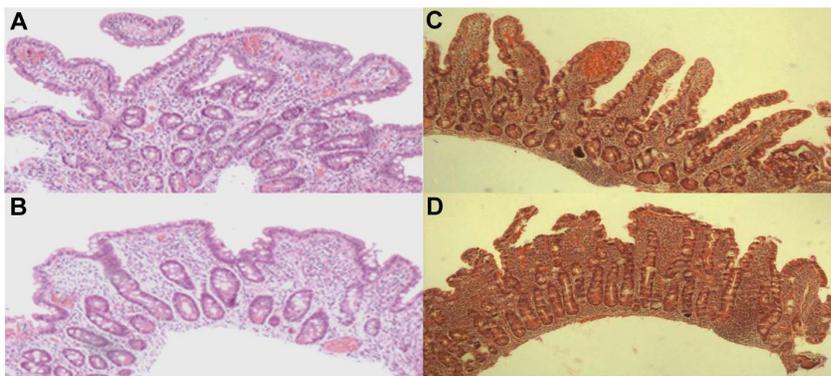


Figure 2. Two small-intestinal biopsy samples from routine clinics that show the importance of biopsy orientation in the interpretation of specimens. Sectionings A and C are cut tangential and B and D perpendicular to the luminal surface showing the effect of different orientation to the same small-intestinal mucosal biopsy block. The hallmark of tangential cutting is the cross-sectioning of the crypts while in correct vertical cutting the crypts are cut longitudinally. In routine clinics, the tangentially cut sectioning A was interpreted as normal, and on re-evaluation upon high clinical suspicion of celiac disease, the biopsy block was tilted and recut. The recut biopsy sample (B) reveals crypt hyperplasia and villous atrophy compatible with celiac disease. To further highlight this potential source of diagnostic error, five independent pathologists were asked to interpret another biopsy block with slices cut in different planes. All graded the tangential specimen C to be morphologically normal (Marsh 0–1) and the properly oriented specimen D to have villus atrophy and crypt hyperplasia (Marsh 3b or 3c).
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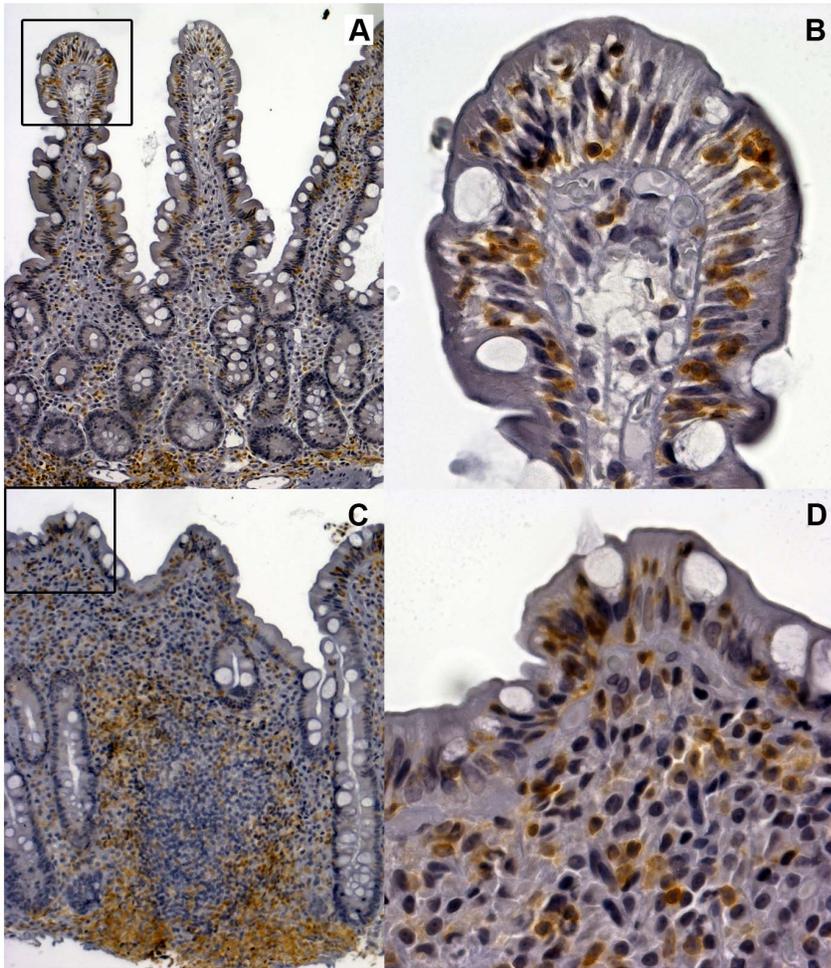


Figure 3. The intraepithelial lymphocyte densities are unaffected by the orientation of biopsy blocks. Panels A and C show tangential (A) and perpendicular (C) cuttings of paraffin embedded blocks stained for CD3⁺ T cells, i.e. the same biopsy block as in Figures 2C and 2D. The panels B and D present 40× magnified pictures of the mucosa from the places presented by the black rectangles in panels A and C. In these magnifications, the intraepithelial lymphocyte densities are 50 per 100 epithelial cells and 54 per 100 epithelial cells, respectively.
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that training of evaluators to correctly assess duodenal biopsy cuttings is essential in the diagnosis of celiac disease. One possibility to obtain good quality specimens is to orientate the biopsy on acetate cellulose filter before cutting [26].

There has recently been debate as to what should be the primary outcome when evaluating the effects of new treatments in celiac disease [6,27]. Although there is already an established mode of treatment, the gluten-free diet, there is an unmet need for novel pharmacological alternatives. Up to 60% of celiac disease patients may experience symptoms even on a strict gluten-free diet [28]. Also, mucosal healing may be incomplete with injury persisting in up to 60–80% of long-term treated patients in some series [29,30]. Such lingering mucosal damage predisposes to celiac disease-associated complications even if symptoms disappear on a gluten-free diet [5,31]. As a result, biopsy readouts should be favored in phase 2 clinical trials instead of self-perceived clinical outcomes. It is evident that if a drug is able to inhibit the gluten-induced mucosal injury similarly to a gluten-free diet, clinical symptoms, signs of malabsorption and serum celiac disease-specific antibodies will also disappear. In later phase 3 trials, well-validated patient-related and non-invasive biomarker outcomes can be

favored as endpoints and biopsies are needed only in subgroups of patients.

Conclusions

By following our SOP, excellent intraobserver and interobserver agreement in detecting small but significant changes in the small-intestinal mucosa can be achieved. The cut-off for clinically significant morphological duodenal mucosal healing or gluten-induced mucosal deterioration values as measured morphometrically (VH:CrD) was set as 0.4. A 30% or higher change in T-cell IEL densities, the marker of mucosal inflammation, can be regarded as clinically significant. The present results confirmed that proper orientation of biopsy specimens and recognition of incorrect tangential cuttings is crucial in ensuring reliable and reproducible histological results in celiac disease.

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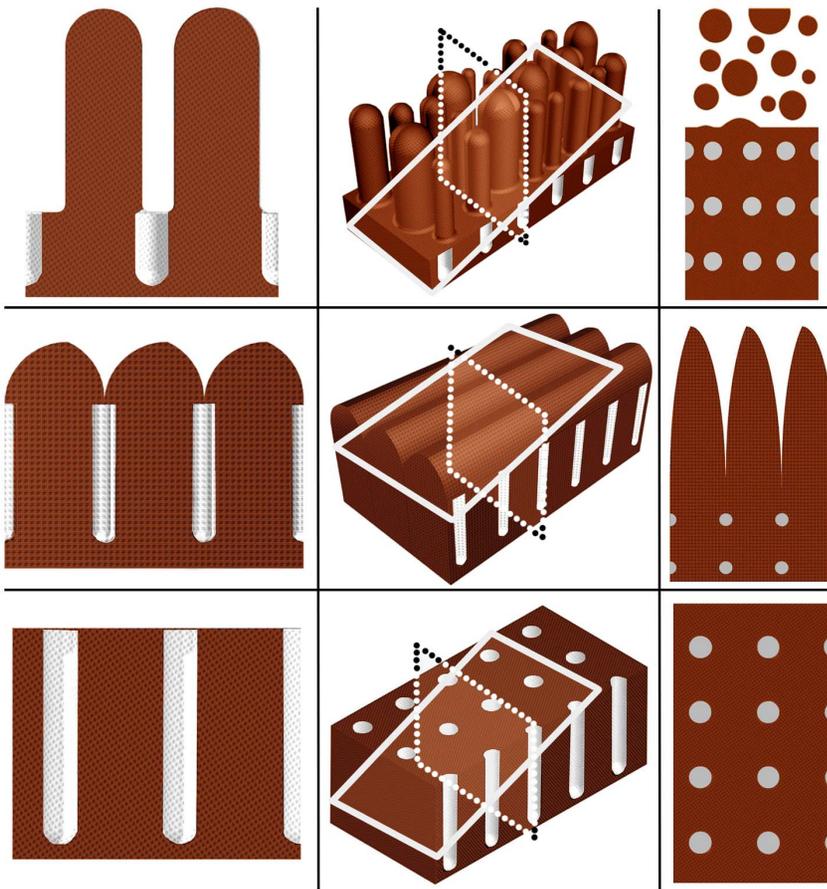


Figure 4. Computerized 3D-model demonstrates the effects of correct and incorrect planes of cutting on readout results. In the middle column are the biopsy blocks, in which the dashed and solid lines represent planes of sectioning. In the left column are sectionings cut perpendicular to the luminal surface and in the right tangentially cut sectionings. For example, in the middle row the computerized block shows merged and convoluted low villous ridges which in perpendicular cutting results in subtotal villous atrophy with deep crypts (left) but in tangential cutting in tall villi with only cross-sections of crypts (right).
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Author Contributions

Performed the experiments: JT OK. Analyzed the data: JT HH. Contributed reagents/materials/analysis tools: JT KL. Wrote the paper: JT. Conception and design of the work: JT OK HH ML AP KL PC KK

KK MM. Drafted the manuscript, revised it critically for important intellectual content and gave final approval of the version to be published: JT OK HH ML AP KL PC KK MM.

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