TIINA RAIVIO

Whole Blood Self-Tissue Transglutaminase-based Antibody Testing in Coeliac Disease

From an in-house method to
commercial rapid test applications

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UNIVERSITY OF TAMPERE

To my family

ABSTRACT

Coeliac disease is an autoimmune disorder in which small-bowel mucosal damage and circulating coeliac autoantibodies against the autoantigen, tissue transglutaminase (tTG), arise as a result of gluten ingestion. Application of serum autoantibody tests, for example immunoglobulin (Ig) A-class endomysial (EMA) and tissue transglutaminase antibody (tTG-ab), has contributed to our knowledge that the condition presents with a variety of symptoms ranging from abdominal complaints to extraintestinal manifestations of variable degree, or the disease can even be totally silent. Additionally, the disease has proved to be nowadays common, affecting approximately 1% of the population worldwide. However, up to 90% of patients remain undiagnosed and may thus have coeliac-related symptoms unawares or be exposed to the late complications of untreated coeliac disease. The coeliac disease diagnosis is based on findings of mucosal villous atrophy with crypt hyperplasia in a small-bowel biopsy sample. Serological coeliac tests are thus needed and widely used as a first-step screening method among coeliac risk groups and subjects evincing symptoms indicative of the disease, in selecting patients to undergo invasive and diagnostic small-bowel biopsy. Nonetheless, conventional EMA and tTG-ab testing requires serum samples, and external coeliac antigen is used in test kits. Additionally, for accurate serum EMA and tTG-ab testing, expert personnel and centralized special laboratory facilities are needed, this rendering testing relatively costly, and test results are available for decision-making only after a time lag.

The aim of the present study was to evaluate a novel whole blood method utilizing the patient's own fresh tTG, self-tTG, found in erythrocytes of the sample, as the coeliac autoantigen for detecting IgA-class coeliac autoantibodies. For this purpose four whole blood self-tTG-based prototypes - a proof-of-concept in house point-of-care test (POCT), commercial enzyme-linked immunosorbent assay (ELISA) and two commercial rapid tests with minor differences, rapid test 1 and rapid test 2 - were used. The results obtained were further compared to those from traditional serum indirect immunofluorescence-based EMA and ELISA-based tTG-ab tests and to diagnostic small-intestinal biopsy, respectively.

Utilizing tTG-deficient tTG knockout mice-derived red blood cells and normal mice red blood cells together with coeliac patients' sera for point-of-care testing, the whole blood IgA self-tTG method was first shown to detect antibodies specifically targeted to tTG (**I**). Subsequently, stored samples from altogether 268 untreated and 143 treated coeliac disease patients and 186 non-coeliac disease controls were studied in the laboratory (**I-IV**). Further, fresh whole blood samples from 315 patients with coeliac disease suspicion (**I, IV**)

and from 263 known coeliac patients on a gluten-free diet were studied prospectively on site in an office setting (**I**). On stored whole blood samples tested in the laboratory the in house POCT showed 92% sensitivity and 98% specificity for untreated coeliac disease and agreed well with the traditional serum EMA (agreement 92%) and tTG-ab tests (95%) (**I, II**). In a selected patient series, where the EMA prevalence was low among untreated coeliac disease patients, the in house POCT (sensitivity 82%) worked equally to the serum EMA test (80%), whereas the serum tTG-ab test (88%) was superior (**II**). The in house POCT was applicable in the follow-up of the treatment, as positive test results weakened in 90% of coeliac patients after a one-year gluten-free diet (**II**). Importantly, the in house POCT also proved highly reliable in on site detection of IgA-class tTG-ab in the office among patients with coeliac disease suspicion and among coeliac disease patients on the diet. Additionally, the in house POCT was even able to pick out patients with selective IgA deficiency, when total serum IgA detection was used as positive test control (**I**).

The commercial whole blood IgA self-tTG ELISA showed 91% sensitivity and 98% specificity for untreated coeliac disease when stored whole blood samples were applied (**III**). The corresponding figures for rapid test 1 were 93% and 94% (**III**) and for rapid test 2 97% and 93%, respectively (**II, IV**). When retrospectively studied coeliac disease patients commenced a gluten-free diet, rapid test 2 results converted from positive to negative in 87% of patients after a one-year follow-up (**II, IV**). Moreover, rapid test 2 was negative in 97% of long-term treated coeliac disease patients (**IV**). Rapid test 2 was easy to perform in five minutes, as was rapid test 1, and had 100% intra- and interobserver agreement. In prospective IgA-class tTG-ab detection in the office rapid test 2 agreed with the serum EMA and tTG-ab tests carried out in specialized laboratory facilities in 97% of the 150 cases with coeliac disease suspicion (**IV**). Rapid test 2 was positive in 31% of 150 patients, and all rapid test-positive patients (n=44) who agreed to undergo small-bowel biopsy showed coeliac-type mucosal lesion (positive predictive value 100%).

It was shown here for the first time that the novel and simple whole blood IgA self-tTG method performs comparably to the traditional serum EMA and tTG-ab tests in coeliac disease case finding, and can also be used in coeliac disease dietary follow-up. Additionally, the principle offers a means of picking out IgA-deficient samples. Unlike the serum EMA and tTG-ab tests, self-tTG testing requires no external coeliac autoantigen, as the patient's own endogenous erythrocyte self-tTG, present in a whole blood sample, is utilized in IgA-class tTG-ab detection. The test principle can be applied as the in house POCT, the commercial ELISA or the commercial rapid test kits which give a test result with minimal workload from a fresh or stored finger prick or venous whole blood sample within five minutes. Essentially, using the rapid tests, tTG-ab can also be determined in office facilities without any additional laboratory equipment and the result can be used for immediate decision-making. It is thus established that application of whole blood IgA self-tTG testing serves as an appropriate alternative to usage of the cumbersome and time-consuming serum EMA and tTG-ab tests.

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TIIVISTELMÄ

Keliakia on autoimmuunisairaus, jossa ravinnon gluteeni aiheuttaa ohutsuolen limakalvovaurion ja autovasta-aineiden muodostumisen verenkiertoon keliakian autoantigeenia, kudostransglutaminaasia (tTG), vastaan. Seerumin autovasta-aineita, esimerkiksi immuuniglobuliini (Ig) A-luokan endomysium- (EMA) ja kudostransglutaminaasivasta-aineita (tTG-ab), mittaavien seulontatestien käytön myötä on paljastunut, että keliakiassa taudinkuva voi vaihdella eriasteisina vatsavaivoina ja suoliston ulkopuolisena oireiluna, tai potilas voi olla jopa täysin oireeton. On myös selvinnyt, että keliakia on yleinen sairaus, jota esiintyy noin prosentilla väestöstä maailmanlaajuisesti. Toisaalta jopa 90 % keliaakikoista on diagnosoimatta. Sen vuoksi potilailla voi olla tietämättään keliakiaan liittyviä oireita, ja he mahdollisesti altistuvat hoitamattoman keliakian myöhäiskomplikaatioille. Keliakian diagnoosi perustuu ohutsuolikoepalassa todettuun villusatrofiaan ja kryptahyperplasiaan. Näin ollen serologisia keliakiatestejä tarvitaan ja käytetäänkin laajasti keliakian ensimmäisen vaiheen seulonnassa keliakian riskiryhmissä ja oireisilla potilailla, minkä jälkeen potilaat voidaan tarvittaessa lähettää edelleen diagnostiseen ja invasiiviseen ohutsuolikoepalatutkimukseen. Perinteiseen EMA- ja tTG-ab-testaukseen tarvitaan kuitenkin seeruminäyte, ja testeissä käytetään ulkopuolista keliakia-antigeenia. Lisäksi testaus vaatii asiantuntijuutta ja keskitettyjä erikoislaboratorio-olosuhteita luotettavan testituloksen saamiseksi. Täten vasta-ainetestaus on suhteellisen kallista, ja testitulos on käytettävissä viiveellä hoitopäätöksen tekemisessä.

Tutkimuksen tarkoituksena oli arvioida uudenlaista menetelmää, jossa keliakia-autoantigeenina käytetään potilaan omaa kokoverinäytteen punasolujen sisäistä tuore-tTG:a, oma-tTG:a, IgA-luokan keliakia-autovasta-aineiden mittaamisessa. Tutkimuksessa käytettiin neljää oma-tTG:n käyttöön perustuvaa kokoverikeliakiavasta-ainetestiä: omatekoista vieritestiä (POCT), kaupallista ELISA-testiä sekä kahta lähes samanlaista kaupallista pikatestiä (pikatesti 1 ja pikatesti 2). Saatuja tuloksia verrattiin perinteisiin serologisiin (immunofluoresenssimenetelmään perustuvaan EMA ja ELISA-menetelmään perustuvaan tTG-ab) testituloksiin sekä diagnostiseen ohutsuolibiopsiaan.

Ensiksi tutkimuksessa todistettiin oma-tTG-metodin mittaavan spesifisesti tTG:a kohtaan syntyviä vasta-aineita käyttämällä tTG-puutteisen hiiren punasoluja ja normaalin hiiren punasoluja yhdessä keliakiapotilaiden seeruminäytteiden kanssa POCT-testauksessa (**I**). Sen jälkeen tutkittiin säilöttyjä näytteitä yhteensä 268 hoitamattomalta ja 143 hoidetulta keliaakikolta ja 186 ei-keliakiakontrollilta laboratoriossa (**I-IV**). Tuorekokoverinäytteitä tutkittiin 315 keliakiaepäilyksen alaiselta potilaalta (**IV**) ja 263 gluteenittomalla dieetillä

olleelta potilaalta prospektiivisesti paikan päällä toimisto-olosuhteissa (**I**). POCT:n sensitiivisyys hoitamattomalle taudille oli 92 % ja spesifisyys 98 %, kun säilöttyjä kokoverinäytteitä testattiin laboratoriossa. Lisäksi POCT-tulokset olivat varsin yhtäpitäviä perinteisten seerumin EMA- (konkordanssi 92 %) ja tTG-ab-testituloksien (95 %) kanssa (**I, II**). Valikoidussa potilasmateriaalissa, jossa hoitamattomien keliakiapotilaiden EMA-esiintyvyys oli matala, POCT (sensitiivisyys 82 %) toimi kuten seerumin EMA-testi (80 %), mutta seerumin tTG-ab-testi (88 %) oli näitä parempi (**II**). POCT soveltui myös hoidon seurantaan, sillä 90 %:lla keliaakikoista positiivinen testitulos heikkeni vuoden gluteenittoman dieetin jälkeen (**II**). Tärkeä havainto oli, että POCT määritti tTG-ab:t keliakiaepäilyksen alaisilta ja gluteenittomalla dieetillä olleilta potilailta erittäin luotettavasti myös toimisto-olosuhteissa paikan päällä käytettynä. Lisäksi POCT jopa tunnisti IgA-puutteiset potilaat, kun testin positiivisena kontrollina käytettiin seerumin kokonais-IgA:n mittausta (**I**).

Kaupallisen oma-tTG:n käyttöön perustuvan ELISA-testin sensitiivisyys hoitamattomalle keliakialle oli 91 % ja spesifisyys 98 % säilöttyjä kokoverinäytteitä käytettäessä. Vastaavat luvut kaupalliselle pikatesti 1:lle olivat 93 % ja 94 % (**III**) ja pikatesti 2:lle 97 % ja 93 % (**II, IV**). Retrospektiivisesti tutkituilla keliaakikoilla positiivinen pikatesti 2-tulos muuttui negatiiviseksi 87 %:lla potilaista vuoden gluteenittoman dieettihoidon jälkeen (**II, IV**). Lisäksi pikatesti 2 oli negatiivinen 97 %:lla pitkäaikaisdieettihoidolla olleista keliakiapotilaista (**IV**). Pikatesti 2 oli helppo suorittaa viidessä minuutissa, samoin kuin pikatesti 1, ja tulokset olivat sataprosenttisesti toistettavia ja yhteneviä eri tutkijoiden suorittamana. Kun 150 potilaalta, joilla epäiltiin olevan keliakia, määritettiin IgA-luokan tTG-ab pikatesti 2:lla prospektiivisesti toimisto-olosuhteissa, tulokset olivat yhteneviä erikoislaboratoriossa tehtyjen seerumin EMA- ja tTG-ab-testien kanssa 97 %:ssa tapauksista (**IV**). Pikatesti 2 oli positiivinen 31 %:lla 150 potilaasta, ja kaikilla pikatesti 2-positiivisilla potilailla (n = 44), jotka suostuivat menemään ohutsuolitutkimukseen, todettiin koepalassa keliakia (positiivinen ennustearvo 100 %).

Tämä tutkimus osoitti ensimmäisen kerran, että uudenlainen ja yksinkertainen kokoveren IgA oma-tTG-metodi paljastaa hoitamatonta keliakiaa kuten seerumin EMA ja tTG-ab testit ja että metodia voidaan myös käyttää keliakian dieetin seurannassa. Lisäksi oma-tTG menetelmä mahdollistaa IgA-puutteisten näytteiden tunnistamisen. Toisin kuin seerumin EMA- ja tTG-ab-testeissä, oma-tTG testauksessa ei tarvita ulkopuolista keliakia-autoantigeenia, sillä potilaan omaa kokoverinäytteen punasolujen sisäistä oma-tTG:a käytetään IgA-luokan tTG-ab:n määrittämisessä. Testimetodia voidaan soveltaa käyttämällä POCT:a, kaupallista ELISA-menetelmää tai kaupallisia pikatestejä. Pikatesteillä testivastaus saadaan minimaalisella työllä, joko tuoreesta tai säilötystä sormenpää- tai laskimoverinäytteestä, jopa viidessä minuutissa. Ennen kaikkea, pikatestien avulla tTG-ab voidaan määrittää toimisto-olosuhteissakin ilman lisälaboratoriovälineitä, ja testitulos on saatavilla välittömästi hoitopäätöksen tekoa varten. Näin ollen voidaan todeta, että kokoveren IgA oma-tTG testaus on varteenotettava vaihtoehto perinteisten ja vaivalloisten seerumin EMA ja tTG-ab testien käytölle.

CONTENTS

ABBREVIATIONS

AGA	gliadin antibody
APC	antigen-presenting cell
ARA	reticulin antibody
CD	coeliac disease
CrD	crypt depth
DGP-ab	deamidated gliadin peptide antibody
DH	dermatitis herpetiformis
EATL	enteropathy-associated T-cell lymphoma
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	endomysial antibody
ESPGAN	European Society for Paediatric Gastroenterology and Nutrition
GFD	gluten-free diet
gp	guinea pig
HLA	human leukocyte antigen
hr	human recombinant
HUC	human umbilical cord
IF	immunofluorescence
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
JEA	jejunal antibody
ME	monkey oesophagus
MHC	major histocompatibility complex
MICA	MHC class I chain-related gene A
nh	native human red blood cell
NHL	non-Hodgkin's lymphoma
POCT	point-of-care test
TcR	T cell receptor
Th	helper T lymphocyte
TMB	tetramethylbenzidine
tTG	tissue transglutaminase
tTG-ab	tissue transglutaminase antibody
U	unit value
VA	villous atrophy
Vh	villous height

LIST OF ORIGINAL PUBLICATIONS

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

I Korponay-Szabó IR, Raivio T, Laurila K, Opre J, Király R, Kovács JB, Kaukinen K, Fésus L and Mäki M (2005): Coeliac disease case finding and diet monitoring by point-of-care testing. Aliment Pharmacol Ther 22: 729-37.

II Raivio T, Korponay-Szabó IR, Collin P, Laurila K, Huhtala H, Kaartinen T, Partanen J, Mäki M and Kaukinen K (2007): Performance of a new rapid whole blood coeliac test in adult patients with low prevalence of endomysial antibodies. Dig Liver Dis 39: 1057-63.

III Raivio T, Korponay-Szabó IR, Paajanen T, Ashorn M, Iltanen S, Collin P, Laurila K, Nemes É, Kovács JB, Carrard G, Saramäki M, Mäki M and Kaukinen K (2008): Comparison of a novel whole blood self tissue transglutaminase-based ELISA with a whole blood rapid test and established serological assays. J Pediatr Gastroenterol Nutr. In press.

IV Raivio T, Kaukinen K, Nemes É, Laurila K, Collin P, Kovács JB, Mäki M and Korponay-Szabó IR (2006): Self transglutaminase-based rapid coeliac disease antibody detection by lateral flow method. Aliment Pharmacol Ther 24: 147-54.

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INTRODUCTION

Coeliac disease is a lifelong autoimmune disorder induced by dietary gluten, found in wheat, rye and barley, in genetically susceptible individuals. Classical coeliac disease is characterized by severe gastrointestinal complaints, malnutrition and failure to thrive (Visakorpi et al. 1970, Young and Pringle 1971, Cooke and Holmes 1984). Nowadays, however, patients suffer more often from milder or atypical, extraintestinal, symptoms, or the disease can even be completely silent (Mäki et al. 2003, Tommasini et al. 2004). Population-based screening studies have revealed that the disease affects approximately 1% of the European population and high prevalences are also reported elsewhere in the world (Fasano et al. 2003, Mäki et al. 2003, Pratesi et al. 2003, Tommasini et al. 2004, Ben Hariz et al. 2007). Due to its protean presentation, however, coeliac disease is difficult to recognize and up to 90% of patients may remain undiagnosed (Ravikumara et al. 2007). Diagnosis of coeliac disease is based on the finding of coeliac-type mucosal injury, villous atrophy with crypt hyperplasia, in a small-bowel biopsy sample (Walker-Smith et al. 1990). The diagnosis is further confirmed by clinical or histological response to treatment, a strict permanent gluten-free diet. Early recognition and dietary treatment of the disease is justified, since untreated coeliac disease may involve complications such as osteoporosis or malignancies (Kemppainen et al. 1999, Mustalahti et al. 1999, Askling et al. 2002, Green et al. 2003). Moreover, upon adoption of a diet coeliac-related symptoms are normally alleviated, and the quality of life may improve (Mustalahti et al. 2002).

In coeliac disease ingestion of gluten-containing food triggers a response to self-proteins, chiefly to tissue transglutaminase (tTG), resulting in the formation of primarily immunoglobulin (Ig) A-class circulating autoantibodies such as endomysial (EMA) and tissue transglutaminase antibodies (tTG-ab) (Dieterich et al. 1997). Detection of the autoantibodies has been recommended and widely used as a first-step non-invasive screening method in coeliac disease case finding among patients evincing symptoms indicative of the disease and in coeliac disease risk groups (Hill et al. 2005, Rostom et al. 2006). EMA is detected by an indirect immunofluorescence (IF) method using for example monkey oesophagus or human umbilical cord sections as substrate (Mäki 1995, Kolho and Savilahti 1997). Although highly specific for coeliac disease, the EMA test is, nonetheless, observer-dependent and laborious (Mäki 1995, Rostom et al. 2005). Following the recognition of tTG as the major coeliac autoantigen objective and easier enzyme-linked immunosorbent assay (ELISA)-based coeliac autoantibody tests, using guinea pig-derived tTG as antigen, were developed (Dieterich et al. 1998, Sulkanen et al. 1998b). Later, these tTG-ab tests were

proved to be even more sensitive and specific for untreated coeliac disease when human recombinant or red blood cell-derived tTG was used as antigen (Rostom et al. 2005). The aforementioned EMA and tTG-ab tests are, however, carried out by expert personnel using serum samples and are centralized in specialized laboratories. Hence testing is costly and laborious, and results are available for decision-making only after a time lag. There thus remains a need for a widely accessible, easy-to-perform and quick coeliac antibody test to reveal unrecognized coeliac disease. Importantly, a few rapid coeliac antibody tests have also been devised (Corazza et al. 1997, Baldas et al. 2000, Sorell et al. 2002, Ferre-Lopez et al. 2004, Baviera et al. 2007). These use external guinea pig-derived or recombinant tTG or wheat gliadin as antigen in antibody detection. Moreover, the tests are mainly conducted with serum samples and require a readily accessible centifuge.

The coeliac autoantigen, tTG, is a ubiquitous enzyme also present in erythrocytes of whole blood (Bergamini et al. 1999, Lorand and Graham 2003). A patient's own endogenous erythrocyte fresh tTG, self-tTG, has thus the potential to bind and thereby detect tTG-ab in the serum of a whole blood sample after it has been liberated by haemolysis (Mäki and Korponay-Szabó, Patent application PCT/FIO2/00340). The aim of this study was to evaluate this innovative means, the whole blood IgA self-tTG method utilizing a whole blood sample self-tTG as antigen, in detecting coeliac autoantibodies. First, the specificity to detect tTG-targeted antibodies was studied. Subsequently, the test principle was evaluated using a proof-of-concept test, the in house point-of-care test (POCT), and a commercial ELISA and lateral flow immunochromatographic-based rapid test application in untreated and treated coeliac disease and in non-coeliac disease controls not only in a laboratory but also prospectively on site in an office setting. Self-tTG test results obtained were further compared to those of the conventional serological tTG-ab and EMA tests and to small-bowel mucosal morphology.

REVIEW OF THE LITERATURE

1. DEFINITION AND HISTORY OF COELIAC DISEASE

Coeliac disease can be defined as a life-long autoimmune-type enteropathy caused by ingestion of gluten in genetically predisposed individuals. The disease was first described by Aretaios in the second century B.C. (Adams 1856). Thereafter, Gee (1888) characterized the condition accurately in his prestigious paper "On the Coeliac Affection". He described the disease at all ages, but especially childhood, as chronic indigestion resulting in loose stools, cachexia and distended abdomen. Sixty years later a Dutch paediatrician, Dicke (1950), noted the harmful effect of wheat, rye and barley in coeliac disease and as a result the treatment, a strict gluten-free diet, was conceived (van de Kamer et al. 1953). In the nineteen-fifties small-bowel mucosal damage indicating coeliac disease was revealed by Paulley (1954), and a peroral apparatus for obtaining an intestinal biopsy was developed to diagnose the condition correctly (Shiner 1957). It has since been realised that coeliac disease is very considerably underdiagnosed. The disorder presents with a variety of symptoms or can even be silent, and is not only an intestinal disease, one well-known extraintestinal manifestation being a gluten-triggered blistering skin disorder called dermatitis herpetiformis (DH) (van der Meer 1969, Reunala et al. 1977).

2. CLINICAL FEATURES OF COELIAC DISEASE

2.1. Classical symptoms

Classically coeliac disaese was thought to manifest mainly before two years of age after the introduction of gluten into the diet. The common symptoms were diarrhoea, steatorrhoea, failure to thrive, malnutrition and abdominal distension and pain (Visakorpi et al. 1970, Young and Pringle 1971, Cooke and Holmes 1984). Additionally, older children were described as frequently suffering from short stature, anaemia and rickets (Visakorpi et al. 1970, Young and Pringle 1971). In the late 1970s and early 1980s coeliac disease was assumed to be disappearing (Challacombe and Bayliss 1980, Stevens et al. 1987). Subsequently, however, it was shown that the number of cases was in fact

increasing, but appearing now at later ages and patients were often monosyptomatic or presented only with milder symptoms (Logan et al. 1983, Mäki et al. 1988). Nowadays it is realised that the clinical spectrum of the disease is wide. Typically it presents with only mild forms of abdominal complaints such as borborygmus, abdominal pain, diarrhoea, constipation, bloating and malaise (Bodé and Gudmand-Høyer 1996, Zipser et al. 2003). Anaemia, tiredness or weight loss are also common symptoms leading to the diagnosis (Zipser et al. 2003). Isolated malabsorbtion of iron, folic acid, calcium, vitamin D or B12 can be present, but does not necessarily lead to clinical manifestations (Kupper 2005, Tikkakoski et al. 2007). Secondary lactose intolerance often emerges as a result of decreased lactase enzyme activity in the chronically inflamed small-bowel mucosa (Bodé and Gudmand-Høyer 1988, Nieminen et al. 2001). However, clinically silent coeliac disease is also frequently detected by screening and today's coeliac patients can feel healthier than non-coeliacs or even be overweight (West et al. 2003, Dickey and Kearney 2006).

2.2. Extraintestinal manifestations and complications

It has become apparent that coeliac disease cannot be regarded as a purely gastrointestinal disease. Numerous extraintestinal manifestations of the condition have been reported. As coeliac disease is a common disorder some associations reported might be merely coincidental findings. Moreover, whether these conditions are consequences of malabsorbtion or immunological responses to gluten ingestion in coeliac disease remains obscure.

Gluten-sensitive skin disease, DH, is one classical extraintestinal presentation of coeliac disease. Although DH was already described in 1884, the association between enteropathy and DH was not detected until 1966 (Duhring 1884, Marks et al. 1966). DH is characterized by itching blisters especially on elbows, knees, buttocks and scalp (Collin and Reunala 2003). DH patients also evince coeliac-type symptoms, although these are, in general, milder in patients with DH (Collin and Reunala 2003).

Bone-related disorders such as osteomalasia, osteoporosis or osteopenia, bone pain and fractures have long been linked with coeliac disease (Cooke and Holmes 1984). Nowadays untreated coeliac disease patients are known to be frequently affected by decreased bone mineral density, irrespective of age and clinical picture (Mora et al. 1998, Kemppainen et al. 1999, Mustalahti et al. 1999). Conversely, 1-3.4% of patients with osteoporosis are affected by coeliac disease (Drummond et al. 2003, Stenson et al. 2005). Kaukinen and associates (2001) reported that bone mineral density can even be decreased without overt villous atrophy in patients with early developing coeliac disease. Due to the decrease in bone mineral density the risk of fractures increases. In untreated coeliac disease the fracture risk seems to be slightly increased (Vazquez et al. 2000, Thomason et al. 2003), and in a study by Vazquez and colleagues (2000) the increased risk was associated with poor dietary treatment and late diagnosis

of coeliac disease. The pathogenesis of disturbed bone metabolism in coeliac disease is multifactorial, but remains somewhat unclear. Decreased intake and absorption of calcium results in secondary hyperparathyroidism and, additionally, production of proinflammatory cytokines affecting bone turnover might at least partly explain bone-related manifestations in coeliac patients (Corazza et al. 2005).

Hepatic disorders may also occur concurrently with coeliac disease. In fact, in a study by Farre and colleagues (2002) hypertransaminasaemia was present in 32% of coeliac cases at diagnosis and in 4.3% cases it was the only manifestation of the disease. Vice versa, approximately 9% of patients with unexplained elevation of liver transaminases (Volta et al. 1998a) and 3.4% of patients having non-alcoholic fatty liver disease (Bardella et al. 2004) may suffer from untreated coeliac disease, or the disease can even manifest as liver failure (Kaukinen et al. 2002a).

In a study by Luostarinen and associates (1999) 10% of coeliac disease patients were detected due to neurological symptoms. Of neurological manifestations coeliac disease is most commonly associated with peripheral neuropathy (Hadjivassiliou et al. 1996), cerebral ataxia (Hadjivassiliou et al. 1996) and memory impairment (Luostarinen et al. 1999). Coeliac disease prevalence might also be increased in patients suffering from epilepsy (Luostarinen et al. 2001) and migraine (Gabrielli et al. 2003). Mental disorders such as depression are further suggested to be overrepresented among coeliac disease patients (Pynnönen et al. 2004, Ludvigsson et al. 2007).

Reproductive disorders are associated with coeliac disease and can be among the first signs of it (Collin et al. 1997). The prevalence of unrecognised coeliac disease among women with unexplained infertility is thought to be increased up to 4.1% (Collin et al. 1996b), though not always significantly (Kolho et al. 1999). Untreated coeliac disease is also considered to be a risk factor for unfavourable pregnancy outcome, for instance for recurrent spontaneous abortion (Gasbarrini et al. 2000), intrauterine growth retardation (Gasbarrini et al. 2000) and low birth weight (Ludvigsson et al. 2005), but not all studies agree (Greco et al. 2004). Undiagnosed coeliac disease might further result in later menarche, secondary amenorrhoea and earlier menopause (Smecuol et al. 1996).

Coeliac disease can be manifested in the mouth. In a study by Aine and colleagues (1990) dental enamel defects were present in 83% of coeliac disease patients. Additionally, 4.9% of patients suffering from oral mucosal disorders, particularly recurrent oral ulcerations, are reported to have coeliac disease (Jokinen et al. 1998). Occasionally untreated coeliac disease can present only with arthritis or arthralgia (Collin et al. 1992a). Also hyposplenism (Corazza et al. 1999) and exocrine pancreatic insufficiency (Carroccio et al. 1997) can occur concomitantly with coeliac disease.

The most severe complications of coeliac disease are various malignancies. Coeliac patients are known to carry an increased risk of non-Hodgkin's lymphoma (NHL), especially enteropathy-associated T-cell lymphoma (EATL), the relative risk ranging from 3.2 to 42.7 (Holmes et al. 1989, Askling et al. 2002, Green et al. 2003, Viljamaa et al. 2006). Conjunctions with other

malignancies, for instance small-bowel adenocarcinoma, oropharyngeal and oesophageal carcinomas, colorectal and primary liver cancers, have also been reported (Askling et al. 2002, Green et al. 2003). The mortality rate among coeliac disease patients has ranged from 1.3 to 2.0, when compared to that of the general population (Logan et al. 1989, Corrao et al. 2001, Viljamaa et al. 2006). The increased mortality among coeliac patients is due mainly to malignancies and is mostly related to late diagnosis, poor adherence to a gluten-free diet and severe symptoms (Logan et al. 1989, Corrao et al. 2001, Viljamaa et al. 2006). Interestingly, a recent study suggests that life expectancy in DH may even be increased (Viljamaa et al. 2006).

Coeliac disease can also be complicated as refractory sprue, in which small-intestinal mucosal damage with or without symptoms does not heal upon a strict gluten-free diet or coeliac-type mucosal lesions reappear despite good diet adherence (Biagi and Corazza 2001). Additionally, ulcerative jejunoileitis, another rather rare complication of coeliac disease, is characterized by chronic idiopathic ulcerations in the small bowel (Biagi et al. 2000). The prognosis of both refractory sprue and ulcerative jejunoileitis is rather poor, and evidence suggests that these conditions and intestinal lymphoma, EATL, constitute a neoplastic continuum (Cellier et al. 2000).

2.3. Silent coeliac disease and associated conditions

Recently conducted screening studies on coeliac disease have revealed that the condition is often clinically more or less silent (Fasano et al. 2003, Mäki et al. 2003, Tommasini et al. 2004). Asymptomatic coeliac disease is detected especially among coeliac disease at-risk groups. A wide range of associations, for instance autoimmune disorders, with increased risk of coeliac disease have been described (Table 1). Subsequently, as the high prevalence of coeliac disease has been unveiled, some of the associations detected, for example asthma, have been shown to be probably coincidental (Collin et al. 1994a). Furthermore, sometimes it is obscure whether a condition is an association or an extraintestinal manifestation of coeliac disease. Some of the associated conditions can be explained by similar genetic factors. For instance, coeliac disease is overrepresented among first-degree relatives of coeliac disease patients, the prevalence of the condition being approximately 10% in this group (Stokes et al. 1976, Mäki et al. 1991, Fasano et al. 2003). Moreover, in monozygous twins the concordance rate for coeliac disease or DH is at least 75% (Hervonen et al. 2000, Greco et al. 2002). Additionally, patients with selective IgA deficiency run an at least tenfold risk of coeliac disease (Collin et al. 1992b, Meini et al. 1996). Other patient groups where the association with coeliac disease seems to be real are presented in Table 1.

Table 1. *The prevalence of coeliac antibody positivity and frequency of biopsy-proven coeliac disease in associated disorders.*

Associated condition Reference	Country	Study material	Screening method	Antibody positivity (%)*	Frequency of biopsy-proven coeliac disease (%)
Type I diabetes mellitus					
(Mäki et al. 1984a)	Finland	215 children	ARA	4.2	2.3
(Collin et al. 1989)	Finland	195 adults	ARA	4.1	4.1
(Sigurs et al. 1993)	Sweden	436 children and adolescents	AGA, ARA	4.6	3.4
(Not et al. 2001)	Italy	491 children and adults	EMA	5.7	5.7
(Hansen et al. 2006)	Denmark	269 children	AGA, EMA, tTG-ab	12.3	12.3
(Mankaï et al. 2007)	Tunisia	205 children	EMA	8.3	5.3
Autoimmune thyroid diseases					
(Collin et al. 1994b)	Finland	83 adults	AGA, ARA, EMA	6.0	4.8
(Volta et al. 2001)	Italy	220 adults	AGA, EMA, tTG-ab	2.7	3.2
(Ch'ng et al. 2005)	UK	111 cases	AGA, tTG-ab	7.2	4.5
Primary Sjögren's syndrome					
(Iltanen et al. 1999)	Finland	34 adults	AGA, EMA	8.8	14.7
(Szodoray et al. 2004)	Hungary	111 adults	AGA, EMA, tTG-ab	5.4	4.5
Alopecia areata					
(Corazza et al. 1995)	Italy	256 children and adults	AGA, EMA	1.2	1.2
Addison's disease					
(O'Leary et al. 2002)	Ireland	44 adults	EMA, tTG-ab	4.9	12.2
(Myhre et al. 2003)	Norway	76 children and adults	AGA, EMA, tTG-ab	6.7	7.9
(Betterle et al. 2006)	Italy	109 children and adults	tTG-ab	4.6	2.8
Primary biliary cirrhosis					
(Dickey et al. 1997)	Northern Ireland	57 adults	EMA	10.5	7.0
(Volta et al. 2002)	Italy and Spain	173 adults	AGA, EMA, tTG-ab	4.0	4.0

Autoimmune hepatitis					
(Volta et al. 1998b)	Italy	181 children and adults	AGA, EMA	4.4	2.8
(Villalta et al. 2005b)	Italy	47 cases	tTG-ab	6.4	6.4
Down's syndrome					
(Bonamico et al. 2001a)	Italy	1,202 children and adults	AGA, EMA	5.4	4.6
(Carnicer et al. 2001)	Spain	284 children and adolescents	AGA, EMA	6.0	6.3
(Agardh et al. 2002)	Sweden	48 children and adolescents	AGA, EMA, tTG-ab	14.6	18.8
Turner's syndrome					
(Ivarsson et al. 1999b)	Sweden	87 children	AGA, EMA	4.6	4.6
(Bonamico et al. 2002)	Italy	389 children and adults	AGA, EMA	5.7	6.4
Juvenile chronic/idiopathic arthritis					
(Lepore et al. 1996)	Italy	119 children	EMA	3.3	2.5
(Stagi et al. 2005)	Italy	151 children	AGA, EMA, tTG-ab	ND	6.7
Autoimmune myocarditis					
(Frustaci et al. 2002)	Italy	187 adults	EMA, tTG-ab	4.4	4.4
IgA-nephropathy					
(Collin et al. 2002)	Finland	168 adults	EMA, tTG-ab	1.8	3.6

ARA=reticulin antibody, AGA=gliadin antibody, EMA=endomysial antibody, tTG-ab=tissue transglutaminase antibody

* Reported antibody positivity among patients studied is primarily of EMA positivity or, in the absence of EMA test results, of ARA or tTG-ab positivity

3. DIAGNOSIS OF COELIAC DISEASE

The first diagnostic criteria for coeliac disease in children, and later also for adult patients, were given by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) in Interlaken, Switzerland, in 1969 (Meeuwisse 1970). The criteria, often referred to as the Interlaken statement, suggested that coeliac disease was a permanent gluten-intolerance which could be diagnosed by three small-intestinal biopsies taken at different time points. Firstly, the coeliac-type mucosal lesion with subtotal villous atrophy and crypt hyperplasia in a small-intestinal biopsy while on a gluten-containing diet had to be shown. Secondly, the mucosal damage healed on a gluten-free diet, and finally the mucosa again deteriorated when gluten was reintroduced. The Interlaken criteria were reconsidered by ESPGAN in 1990 (Walker-Smith et al. 1990). In the revision the small-intestinal biopsy-based finding of typical mucosal injury with villous atrophy and crypt hyperplasia is still requisite, and also clinical remission on a strict gluten-free diet is required. However, a control biopsy to prove mucosal improvement during the diet is requisite only when clinical response is equivocal, for example, among asymptomatic patients. Moreover, a gluten challenge with biopsies is no longer considered mandatory, being advocated only if there are any doubts as to the diagnosis or if older children or teenagers intend to abandon the diet. In the absence of clinical or histological response to the diet differential diagnoses of coeliac disease have to be reconsidered. The conditions appearing with coeliac-type small-bowel mucosal lesion – for example collagenous sprue, soy or cow's milk intolerance, giardiasis, cryptosporidiosis, infectious gastroenteritis, tropical sprue, Whipple's disease, immunodeficiency syndromes, Crohn's disease, autoimmune enteropathy – are nevertheless more infrequent than coeliac disease (Freeman 2004). The presence of coeliac antibodies, gliadin antibody (AGA), reticulin antibody (ARA), EMA and later on also tTG-ab, in a patient's circulation during a gluten-containing diet and disappearance on a gluten-free diet add weight to the diagnosis. Additionally, antibody tests are considered possible guides to dietary compliance (Walker-Smith et al. 1990).

DH, a dermatological variant of coeliac disease, is diagnosed by demonstration of pathognomic granular IgA deposits in the papillary dermis in unaffected skin close to the active lesion by direct IF (van der Meer 1969, Collin and Reunala 2003). It is recommended that DH patients undergo small-intestinal biopsy before starting a gluten-free diet, as approximately 75% present with gluten-sensitive villous atrophy. Moreover, the remainder have at least small-bowel mucosal inflammation with an increased number of $\gamma\delta+$ intraepithelial lymphocytes (IELs) compatible with early developing coeliac disease (Collin and Reunala 2003). Circulating coeliac antibodies can also be detected in roughly 70-80% of DH patients (Hällström 1989, Dieterich et al. 1999).

The diagnostic criteria revised in 1990 are still applied to both children and adults (Hill et al. 2005, Rostom et al. 2006). However, a new revision has been declared as the nature of coeliac disease has gradually been clarified (Kaukinen et al. 2001). Nowadays it is understood that gluten intolerance in genetically susceptible individuals does not present solely with so-called small-bowel mucosal flat lesion with villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis. Rather, the mucosal damage develops gradually from an early lesion of lymphocyte infiltration in the epithelium and lamina propria (Marsh I) to crypt hyperplasia (Marsh II) and subsequently to severe partial, subtotal or total villous atrophy (Marsh III) and borderline cases are also to be found (Marsh 1992). In early stages of the disease, DH being an example, a possibly symptomatic patient might present with only an increased number of small-bowel mucosal IELs, γδ+ and villous tip IELs being the most coeliac-specific, albeit not pathognomic (Järvinen et al. 2004, Salmi et al. 2006a). In contrast, the full-developed flat lesion and so-called latent coeliac disease may emerge later if the patient continues on a gluten-containing diet (Järvinen et al. 2004, Salmi et al. 2006a). Moreover, highly coeliac-specific autoantibodies, for example EMA and tTG-ab, found in patient serum and also deposited in the small intestine, can predict forthcoming coeliac disease and are hence helpful in the diagnostic work-up in borderline cases (Järvinen et al. 2004, Korponay-Szabó et al. 2004, Salmi et al. 2006a). Human leukocyte antigen (HLA) typing can further be used in exclusion of coeliac disease and DH in equivocal cases, as nearly all coeliac disease patients share alleles which encode for HLA DQ2 or DQ8 proteins, whereas approximately 40% of the general population share these alleles (Sollid et al. 1989, Karell et al. 2003, Mäki et al. 2003). All in all, establishing gluten intolerance in genetically predisposed individuals is a complex procedure and cannot be based solely on small-bowel mucosal morphology. In some circumstances starting treatment of coeliac disease before the development of overt villous atrophy may be reasonable (Kaukinen et al. 2001).

4. COELIAC ANTIBODY TESTS

Mucosal surfaces are the major sites at which the body encounters foreign antigens. Logically, 80% of all immunoglobulin-producing cells in humans are located in the intestinal mucosa, producing dimers chiefly of IgA (Mäki 1995). Consequently, also the coeliac antibodies are produced in untreated disease in the mucosa (Marzari et al. 2001) and can present as mucosal extracellular deposits in the small bowel (Korponay-Szabó et al. 2004, Salmi et al. 2006a, Salmi et al. 2006b) and also in the jejunal juice (Mawhinney and Love 1975). The production of the antibodies additionally results in elevation of coeliac disease-specific, most importantly IgA-class, antibodies in the circulation and also in saliva in untreated coeliac disease. Of these, AGA and deamidated gliadin peptide antibody (DGP-ab) are targeted to dietary gliadin, whereas the autoantibodies, for instance ARA, jejunal antibody (JEA), EMA, tTG-ab and

antiactin antibody (Clemente et al. 2000), are formed against the patient's own tissue structures, endogenous antigens.

According to the diagnostic criteria for coeliac disease a small-intestinal biopsy is still requisite. However, the presence of circulating coeliac antibodies during a gluten-containing diet and their disappearance on a gluten-free diet add weight to the diagnosis (Walker-Smith et al. 1990). Indeed, application of the highly sensitive and specific non-invasive coeliac disease antibody tests has, in large part, revealed the protean picture and commonness of the disease. The non-invasive tests facilitate the diagnostic work-up of coeliac disease especially among patients evincing minimal or atypical symptoms or conditions associated with the disease. Additionally, the antibody tests are considered possible guides to dietary compliance, as discussed in a later section (paragraph 7.2.) (Walker-Smith et al. 1990).

A wide range of methods and test kits with different characteristics and accuracies are nowadays available for coeliac antibody testing. Assessment of accuracies reported may be challenging, as results might have been evaluated in coeliac disease patients and controls without small-intestinal mucosal examination. Besides, the sensitivity of the gold standard of coeliac disease diagnosis, small-bowel mucosal histology, has itself lately been questioned as to its revealing genetic gluten intolerance (Kaukinen et al. 2001), and serum coeliac-specific antibodies may in fact indicate early developing coeliac disease in patients still having intanct small-bowel mucosal morphology (Kaukinen et al. 2001, Järvinen et al. 2004, Salmi et al. 2006a). Moreover, coeliac patients might have initially been selected based on positive coeliac serology, and some tests have been evaluated in cases of more severe small-intestinal damage than others, leading probably to overestimation of sensitivity (Rostami et al. 1999, Abrams et al. 2004, Abrams et al. 2006). It has further been suggested that the accuracies of antibody tests might vary in different groups and sensitivities might drop significantly when the tests are applied in a clinical setting (Abrams et al. 2006).

Over and above these considerations, one challenge in antibody testing is seronegative coeliac disease, where coeliac antibodies cannot be detected during a gluten-containing diet. Seronegativity is estimated to affect 6-22% of coeliac patients (Dickey et al. 2000b, Collin et al. 2005). As the antibodies are usually measured in IgA class, false-negative results occur among untreated coeliac patients with selective IgA deficiency, a condition more common in coeliac patients than in the general population (Collin et al. 1992b, Cataldo et al. 1998). In this patient group coeliac antibodies should instead be determined in IgG class (Cataldo et al. 2000, Korponay-Szabó et al. 2003a). However, seronegative coeliac disease also occurs in patients having normal serum IgA level (Dickey et al. 2000b, Collin et al. 2005). Although no unambiguous evidence is on record, seronegative coeliac disease might be associated with older age and more severe clinical picture (Salmi et al. 2006b). On the other hand, the condition has also been suggested to be more prevalent among coeliac disease patients with a lesser degree of villous atrophy (Rostami et al. 1999, Abrams et al. 2004, Abrams et al. 2006).

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4.1. Gliadin and deamidated gliadin peptide antibodies

In untreated coeliac disease serum antibodies against gliadin, the ethanol-soluble coeliac disease-toxic part of wheat, increase (Ferguson and Carswell 1972). Various methods – for example serum-based IF (Stern et al. 1979), ELISA (O'Farrelly et al. 1983), diffusion-in-gel ELISA (Kilander et al. 1983) and solid radio-immunoassay (Ciclitira et al. 1983), whole blood-based strip ELISA (Not et al. 1993), stool-based ELISA (Kappler et al. 2006) and saliva-based ELISA (Rujner et al. 1996) - have been developed to detect AGA. Of these, the serum-based ELISA method is widely accepted, and it is recommended that AGA be determined in both IgA and IgG class (Troncone and Ferguson 1991). The sensitivities of IgA-class AGA have ranged widely from 31% to 100% and IgG-class AGA from 35% to 100% in untreated coeliac disease in different studies, and the specificities from 46% to 100% and from 36% to 97%, respectively (Table 2). In addition to untreated coeliac disease, serum AGA can also be detected in other disorders (O'Farelly et al. 1988) and gastrointestinal diseases (Unsworth et al. 1983) and even in healthy individuals (Uibo et al. 1993). AGA might increase with age in non-coeliac individuals (Uibo et al. 1993) and elevated serum AGA does not seem to be associated with coeliac disease-type genetics in relatives of coeliac disease patients (Mäki et al. 1991). For these reasons, application of the conventional AGA assays is not at present recommended in coeliac disease case finding (Hill et al. 2005, Rostom et al. 2006), although the AGA test appears to be in fact more accurate in detecting untreated coeliac disease among young children (Savilahti et al. 1983).

In coeliac disease certain glutamine residues of gliadin peptides are deamidated to negatively charged glutamic acid residues by the intestinal coeliac autoantigen, tTG (Dieterich et al. 1997, Molberg et al. 1998). Deamidation renders the gluten peptides more antigenic and thus suitable for binding with high affinity to HLA DQ2- and DQ8-heterodimers expressed by antigen-presenting cells (APCs), this resulting in enhanced T-cell response (Molberg et al. 1998) and presumably also deamidated gliadine peptide-specific B-cell response. Accordingly, more coeliac disease-specific circulating antibodies, DGP-ab, are produced if compared to native gliadin peptide antibodies (Aleanzi et al. 2001, Schwertz et al. 2004). Recently, a commercial ELISA for detecting IgA- and IgG-class DGP-ab has been developed (Prince 2006). The novel DPG-ab test is significantly superior compared to the conventional AGA tests, its sensitivity ranging from 84% to 98% in IgA-class and from 84% to 97% in IgG-class and specificity from 86% to 98% in IgA-class and from 86% to 100% in IgG-class (Table 2).

Table 2. *Sensitivity and specificity of serum IgA-class and IgG-class gliadin (AGA) and deamidated gliadin peptide (DGP-ab) antibodies for untreated coeliac disease.*

Reference	Patients	Controls	IgA-class AGA		IgG-class AGA	
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(Mäki et al. 1991)	13 adults and children	109 adults and children	31	87	46	89
(McMillan et al. 1991)	28 adults	68 adults	100	100	57	87
(Ferreira et al. 1992)	21 adults	160 adults	91	85	76	88
(Lerner et al. 1994)	28 children	41 children	52	94	88	92
(Sacchetti et al. 1996)	32 children	42 children	78	98	94	79
(Bottaro et al. 1997)	50 children	25 children	92	68	100	36
(Sulkanen et al. 1998a)	92 adults	95 adults	80	86	35	97
(Sulkanen et al. 1998b)	136 children and adults	207 children and adults	85	82	69	73
(Lock et al. 1999)	27 adults	65 adults	93	95	77	91
(Tesei et al. 2003)	248 adults	176 adults	64	92	85	86
(Mankaï et al. 2005)	143 children and adults	74 children and adults	95	85	ND	ND
(Kaukinen et al. 2007a)	44 children and adults	46 adults	52	46	ND	ND
(Volta et al. in press)	128 adults	134 adults	73	79	73	60
			IgA-class DGP-ab		IgG-class DGP-ab	
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(Agardh 2007)	119 children	57 children	91	91	95	86
(Kaukinen et al. 2007a)	44 children and adults	46 adults	91	98	ND	ND
(Niveloni et al. 2007)	60 adults	81 adults	98	94	97	100
(Volta et al. in press)	128 adults	134 adults	84	86	84	100

ND=no data

4.2. Reticulin, endomysial and jejunal antibodies

In untreated coeliac disease and DH more coeliac-specific tissue antibodies are also produced. In 1971 an IF method was introduced to detect serum ARA in coeliac disease and DH patients using unfixed cryostat sections of rat kidney,

liver and stomach tissue as antigens (Seah et al. 1971a, Seah et al. 1971b). Thereafter, Rizetto and Doniah (1973) described five different IF patterns, and R_1-type reticulin antibodies were reported to be the most clearly coeliac disease- and DH-specific (Magalhaes et al. 1974, Eade et al. 1977). Unlike serum AGA, ARA-positivity seems to be associated with coeliac-type HLA (Mäki et al. 1991). When used by expert laboratory personnel, especially the IgA-class ARA test is reported to be highly sensitive and specific for untreated coeliac disease (Seah et al. 1971b, Mäki et al. 1984b, Hällström 1989). The overall sensitivity of the IgA-class ARA test is, however, reported to vary from 47% to 97% and specificity from 72% to 100% (Table 3). The IgG-class ARA test is even less sensitive, ranging from 13% to 59% for untreated coeliac disease, even if it seems to be coeliac-specific, 96-97% (Mäki et al. 1984b, Sulkanen et al. 1998a).

Sera from coeliac disease patients react not only with rodent tissues but also with primate and human tissues. Chorzelski and colleagues (1983) showed that IgA-class coeliac disease-specific antibodies, then named EMA, were directed against the 'reticulin-like' silver stain-positive fibres in connective tissue of the monkey oesophagus smooth muscle, termed the endomysium, and EMA was shown to correlate well with serum ARA (Hällström 1989). Since then the IgA-class EMA IF test using monkey oesophagus as substrate has gained wide acceptance due to its good sensivity and specificity, nearing 100% (Table 3 and Table 4). However, the EMA test has been suggested to be age-dependent and less sensitive among coeliac children under two years of age (Bürgin-Wolff et al. 1991). In 1989 Hällström (1989) reported ARA-positive serum to give a moderate to strong immunofluorescent reticular network pattern also in human jejunum, liver, lung, spleen, thymus and pancreas. Additionally, Kárpáti and associates (1986) showed that DH patients had ARA- and EMA-like IgA-class antibodies against human jejunum and named these antibodies JEA. Later the EMA test was further improved to offer a more ethical and less expensive screening tool for untreated coeliac disease, as monkey oesophagus was replaced by an easily available substrate, human umbilical cord, and the new EMA test was found to give as sensitive and specific results as the former EMA test (Table 3 and Table 4) (Ladinser et al. 1994). The serum human umbilical cord-based IgA-class EMA test has been standardized in Europe and is nowadays widely applied in detection of coeliac antibodies (Stern 2000). However, in IgG class both EMA tests have significantly lower sensitivity (McMillan et al. 1991, Sulkanen et al. 1998a), except in coeliac patients suffering from selective IgA deficiency (Cataldo et al. 2000, Korponay-Szabó et al. 2003a). Although the serum EMA IF test offers good sensitivity and a specificity of 100% for untreated coeliac disease, it can also be criticized in that the testing is expensive, subjective and labour-intensive, requiring experienced personnel to perform it (Mäki 1995).

Table 3. *Sensitivity and specificity of serum IgA-class reticulin (ARA) and endomysial (EMA) antibodies using human umbilical cord (HUC) or monkey oesophagus (ME) as substrate for untreated coeliac disease.*

Reference	Patients	Controls	ARA		EMA	
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(Mäki et al. 1984b)	29 children	245 children	97	98	ND	ND
(Hällström 1989)	50 children and adults	69 children and adults	94	100	94 (ME)	100 (ME)
(Mäki et al. 1991)	13 children and adults	109 children and adults	92	95	92 (ME)	95 (ME)
(Ferreira et al. 1992)	21 adults	160 adults	91	99	100 (ME)	99 (ME)
(Lerner et al. 1994)	28 children	41 children	65	100	97 (ME)	98 (ME)
(Volta et al. 1995)	60 adults	100 adults	ND	ND	95 (ME) 95 (HUC)	100 (ME) 100 (HUC)
(Sacchetti et al. 1996)	32 children	42 children	94	100	97 (ME) 93 (HUC)	100 (ME) 100 (HUC)
(Bottaro et al. 1997)	50 children	25 children	74	100	96 (ME) 94 (HUC)	96 (ME) 100 (HUC)
(Kolho and Savilahti 1997)	53 children	114 children	96	92	94 (ME) 94 (HUC)	100 (ME) 100 (HUC)
(Sulkanen et al. 1998a)	92 adults	95 adults	78	100	85 (HUC)	100 (HUC)

ND=no data

4.3. Tissue transglutaminase antibodies

In 1997 Dieterich and associates (1997) reported that tTG is the major autoantigen targeted by EMA in coeliac disease, as the EMA staining pattern was abolished by absorption of untreated coeliac patient serum with tTG. tTG is a ubiquitous, calcium-dependent enzyme mainly stored intracellularly in an inactive form, for instance in fibroblasts, endothelial, red blood and epithelial cells, and is released to the extracellular space especially upon mechanical and inflammatory stress (Bergamini et al. 1999, Lorand and Graham 2003, Koning et al. 2005). tTG is activated, chiefly in the extracellular space, by calcium to stabilize the matrix by catalyzing the covalent, irreversible cross-linking of proteins via creation of isopeptide bonds between a donor glutamine residue and an acceptor lysine residue. In the absence of available glutamine acceptors or at low pH, tTG has the ability to deamidate the glutamine residues of proteins, also of gluten peptides, to negatively charged glutamic acid residues. Logically, tTG is upregulated in wound healing, angiogenesis and apoptosis (Koning et al. 2005).

After recognition of the coeliac autoantigen, it was further shown that IgA-class tTG-ab could be detected in untreated coeliac patient but not in control serum samples by ELISA using guinea pig liver-derived tTG as antigen, and that the test agreed well with the serum EMA test (Dieterich et al. 1997, Dieterich et al. 1998, Sulkanen et al. 1998b). Thereafter, Korponay-Szabó and associates (2000) demonstrated that ARA, EMA, JEA and tTG-ab were virtually identical by first showing that monoclonal tTG-ab bound to the tissue sections in EMA, ARA and JEA patterns using both rodent and primate tissues as substrates. Additionally, they demonstrated that guinea pig-derived tTG added to fibronectin could replace the chemically removed endomysial target antigen, which appears to be bound to fibronectin (Korponay-Szabó et al. 2000). Secondly, by utilizing tTG-deficient tissue sections from tTG knockout mice Korponay-Szabó and group (2003b) proved that serum ARA, EMA and JEA binding patterns were exclusively tTG-dependent in coeliac disease and DH and targeted the fibronectin-bound extracellular tTG in normal human and rodent tissues.

Since the aforementioned findings numerous less expensive and simpler diagnostic coeliac disease antibody tests, based on specific antigen and thus suitable for automation, have been developed. tTG-ab has been measured most commonly by serum-based ELISA (Dieterich et al. 1997, Dieterich et al. 1998, Sulkanen et al. 1998b), but also by serum-based radioimmunoassay (Bonamico et al. 2001b), stool-based ELISA (Kappler et al. 2006) or saliva-based ELISA (Baldas et al. 2004) or radioimmunoassay (Bonamico et al. 2004). The first-generation serum IgA-class tTG-ab ELISA tests using guinea pig liver tTG as antigen turned out to have poorer sensitivity and especially specificity than the second-generation tTG-ab assays utilizing human recombinant or purified tTG as antigen (Table 4) (Martini et al. 2002, Wong et al. 2002, Rostom et al. 2005). However, a user should be aware of differences in the test accuracies of both guinea pig tTG- and human tTG-based kits. Firstly, there are discrepancies for some kits between cut-offs suggested by the manufacturers and receiver-operating characteristic-optimized cut-offs and the test performance is dependent, in addition to source of tTG, for instance on the extracting method and purity of tTG and the production and processing of recombinant tTG (Wong et al. 2002, Van Meensel et al. 2004, Reeves et al. 2006). Furthermore, false-positive tTG-ab test results may occur for instance among patients suffering from chronic liver disease (Carroccio et al. 2001, Villalta et al. 2005a), albeit that the accuracies of the tTG-ab tests in this patient group have also been shown to be kit-dependent (Villalta et al. 2005a). Nevertheless, tTG-ab agrees markedly with coeliac-type HLA genetics (Mäki et al. 2003) and overall sensitivities and specificities of the different IgA-class tTG-ab ELISA kits are good and similar to those of the serum IgA-class EMA test (Table 4). The serum IgG-class tTG-ab test offers a sensitive and specific tool in selective IgA deficiency to detect untreated coeliac disease (Cataldo et al. 2000, Korponay-Szabo et al. 2003). In patients without IgA deficiency, however, the test is unsensitive (Troncone et al. 1999, Sblattero et al. 2000, Agardh 2007).

Table 4 *Sensitivity and specificity of serum IgA-class endomysial antibodies (EMA) using human umbilical cord (HUC) or monkey oesophagus (ME) as substrate and of IgA-class tissue transglutaminase antibodies (tTG-ab) using either guinea pig (gp)-, human recombinant (hr)- or native human erythrocyte (nh)-derived tissue transglutaminase as antigen for untreated coeliac disease.*

Reference	Patients	Controls	EMA		tTG-ab	
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(Sulkanen et al. 1998b)	136 children and adults	207 children and adults	93 (HUC)	100 (HUC)	95 (gp)	94 (gp)
(Lock et al. 1999)	27 adults	65 adults	100 (ME) 96 (HUC)	100 (ME) 98 (HUC)	85 (gp)	97 (gp)
(Troncone et al. 1999)	48 children	63 children	96 (ME)	98 (ME)	92 (gp)	98 (gp)
(Sblattero et al. 2000)	65 children and adults	20 children and adults	93 (HUC)	100 (HUC)	84 (gp) 91 (hr)	100 (gp) 100 (hr)
(Dickey et al. 2001)	73 adults	58 cases	81 (ME)	97 (ME)	75 (gp)	98 (gp)
(Bürgin-Wolff et al. 2002)	208 children and adults	157 children and adults	97 (ME)	100 (ME)	96 (hr)	99 (hr)
(Carroccio et al. 2002)	24 adults	183 adults	100 (ME)	100 (ME)	100 (gp) 100 (hr)	92 (gp) 97 (hr)
(Tesei et al. 2003)	248 adults	176 adults	86 (ME)	100 (ME)	91 (hr)	95 (hr)
(Collin et al. 2005)	126 children and adults	106 children and adults	89 (HUC)	98 (HUC)	94 (hr)	99 (hr)
(Mankaï et al. 2005)	143 children and adults	74 children and adults	96 (HUC)	100 (HUC)	86 (gp)	96 (gp)
(Agardh 2007)	119 children	57 children	ND	ND	97 (nh)	96 (nh)
(Kaukinen et al. 2007a)	44 children and adults	46 adults	80 (HUC)	100 (HUC)	89 (hr)	98 (hr)
(Niveloni et al. 2007)	60 adults	81 adults	ND	ND	95 (nh)	98 (nh)
(Volta et al. in press)	128 adults	134 adults	94 (HUC)	100 (HUC)	97 (hr)	91 (hr)

ND=no data

4.4. Rapid antibody tests

Although serum tTG-ab ELISA-based testing has significantly facilitated the diagnostic work-up of coeliac disease, testing is somewhat time-consuming and has to be carried out by trained staff in centralized laboratories, which are not available globally. There has thus been a growing interest for rapid and easy-to-perform coeliac antibody tests, and different tests have been developed for tTG-ab and AGA detection (Table 5). The tests use recombinant human tTG, guinea pig-derived tTG or wheat gliadin as antigen and are mostly carried out from patients' serum samples. Rapid test results can be read visually in five minutes to one hour. Data obtained hitherto suggest that these tests are accurate in detecting untreated coeliac disease (Table 5).

Table 5. *Coeliac antibody rapid test characteristics and sensitivities and specificities of the tests for untreated coeliac disease.*

Reference	Test principle, sample needed for testing	Test antigen	Outcome of the test	Testing time	Sensitivity (%)	Specificity (%)
(Corazza et al. 1997)	Micro enzyme-linked immunosorbent assay, serum sample	Wheat gliadin	IgA- and IgG-class AGA	> one hour	91	80
(Garrote et al. 1999)	Immunochromatographic assay, serum sample	Wheat gliadin	IgA- and IgG-class AGA	< one hour	96	94
(Baldas et al. 2000)	Dot blot assay, serum or whole blood sample	hr-tTG	IgA- and IgG-class tTG-ab	20 minutes	100	98*
(Sorell et al. 2002)	Immunochromatographic assay, serum or plasma sample	gp-tTG	IgA- and IgG-class tTG-ab	< 10 minutes	100	100
(Ferre-Lopez et al. 2004, Baviera et al. 2007)	Immunochromatographic assay, serum sample	hr-tTG	IgA- and IgG-class tTG-ab	5-10 minutes	95-96	99
(Ferre-Lopez et al. 2004, Baviera et al. 2007)	Immunochromatographic assay, serum sample	hr-tTG and Wheat gliadin	IgA-class tTG-ab and AGA	5-10 minutes	93-95 / 63-88	99 / 95-96

AGA=gliadin antibody, hr-tTG= human recombinant tissue transglutaminase, tTG-ab=tissue transglutaminase, gp-tTG=guinea pig tissue transglutaminase
* Coeliac disease was not excluded by small-bowel biopsy among the non-coeliac disease controls

5. EPIDEMIOLOGY OF COELIAC DISEASE

Before the nineteen nineties coeliac disease was considered to be a relatively rare disorder with a prevalence of approximately 1:1000 and considerable regional differences (Greco et al. 1992). Thereafter, prevalence figures have significantly increased worldwide due to the application of sensitive and specific serological coeliac antibody tests suitable for screening coupled with a higher index of suspicion of the disease and availability of open access endoscopy with routine small-intestinal biopsy. For example, in 1997 the prevalence of coeliac disease in Finland was reported to be 1:370, where it was actively sought in patients having coeliac-related typical or atypical symptoms or being at-risk of having coeliac disease (Collin et al. 1997). Subsequently, Mäki and associates (2003) screened Finnish schoolchildren and revealed a coeliac disease prevalence of 1:99. A recent population-based screening study carried out in Finland further revealed that the prevalence of coeliac disease, detected solely on clinical grounds, had risen from 1:3497 in the years 1978-80 to 1:200 in the years 2000-01 among adults (Lohi et al. 2007). Moreover, the total prevalence of adult coeliac disease, including both clinically detected coeliac disease and patients found by screening had also increased from 1:92 in the years 1978-80 to 1:52 in 2000-01 (Lohi et al. 2007). Additionally, studies conducted elsewhere around the world have revealed that coeliac disease is a common disorder affecting approximately 1% of the population and is considerably undiagnosed (Table 6). Until now the highest seroprevalence of coeliac disease, 1:18, has been reported among Saharawi children in North-Africa (Catassi et al. 1999). On the other hand, the disorder remains fairly uncommon among sub-Saharan Africans and in yellow races, especially among the Japanese, who lack the coeliac-type HLA (Kagnoff 2007). Nonetheless, it has been suggested that this is partly due to underestimation of the disease in areas where no large epidemiologic studies have been carried out (Fasano and Catassi 2001). Findings would indicate that the disease is more predominant in women (Cooke and Holmes 1984, Tikkakoski et al. 2007). However, DH is diagnosed slightly more often in men and the prevalence of the disease is up to 1:1515 (Collin and Reunala 2003).

Table 6. *The prevalences of coeliac disease detected before screening and of biopsy-proven coeliac disease after screening and of coeliac antibody positivity according to recent studies.*

Author	Area	Study population	Screening method	Prevalence of coeliac disease		
				Detected before screening	All biopsy-proven	Antibody positivity*
(Johnston et al. 1997)	Ireland	1,823 adults	AGA, ARA, EMA	1:912	1:122	1:16
(Kolho et al. 1998)	Finland	1,070 adults	EMA	0	1:130	1:97
(Catassi et al. 1999)	Saharawi	989 children	EMA	ND	ND	1:18
(Csizmadia et al. 1999)	Netherlands	6,127 children	EMA	ND	1:198	1:82
(Ivarsson et al. 1999a)	Sweden	1,894 adults	AGA, EMA	1:947	1:189	1:210
(Korponay-Szabó et al. 1999)	Hungary	427 children	EMA	0	1:85	1:71
(Riestra et al. 2000)	Spain	1,170 children and adults	AGA, EMA	1:1170	1:390	1:585
(Carlsson et al. 2001)	Sweden	690 children	AGA, EMA	ND	1:53	1:86
(Gomez et al. 2001)	Argentina	2,000 adults	AGA, EMA	1:2000	1:182	1:167
(Hovell et al. 2001)	Australia	3,011 adults	EMA	1:1506	1:335	1:301
(Mäki et al. 2003)	Finland	3,654 children	tTG-ab, EMA	1:365	1:99	1:68
(Pratesi et al. 2003)	Brazil	4,405 children and adults	EMA	0	1:294	1:275
(West et al. 2003)	UK	7,550 adults	EMA, tTG-ab	1:328	ND	1:87
(Bingley et al. 2004)	UK	5,470 children	tTG-ab, EMA	1:1368	ND	1:101
(Neri et al. 2004)	USA	2,000 blood donors	tTG-ab, EMA	ND	ND	1:125
(Tommasini et al. 2004)	Italy	3,188 children	tTG-ab, EMA	1:1594	1:106	1:103
(Ress et al. 2007)	Estonia	1,160 children	tTG-ab	0	1:290	1:232
(Ben Hariz et al. 2007)	Tunisia	6,286 children	tTG-ab, EMA	1:3143	1:225	1:157

AGA=gliadin antibody, ARA=reticulin antibody, EMA=endomysial antibody, ND=no data, tTG-ab=tissue transglutaminase antibody
* Reported antibody positivity among the patients studied is primarily EMA positivity or, in the absence of EMA test results, ARA or tTG-ab positivity

6. PATHOGENESIS OF COELIAC DISEASE

Despite great advances recently in the field of coeliac disease research, much of the pathogenetic mechanism underlying the condition remains to be elucidated. It is known that coeliac disease results from the interaction between environmental, genetic and immune factors. Ingestion of gluten, found in wheat, rye and barley, is clearly a requisite factor for initiation of the disease. Other environmental elements affecting disease development have also been proposed. Duration of breast-feeding, introduction of gluten-containing foods in relation to weaning, amount of gluten given and timing of gluten introduction to an infant might contribute to the risk (Greco et al. 1988, Ivarsson et al. 2000, Peters et al. 2001). Additionally, gastrointestinal infections such as adenovirus (Kagnoff et al. 1987) and rotavirus (Stene et al. 2006) have also been proposed to trigger coeliac autoimmunity. A recent study carried out among people from Russian Karelia and Finland, who share identical geographical and climatic factors and have partly the same ancestry and are equally exposed to grain products, showed remarkable prevalence differences in coeliac disease between the two populations (Kondrashova et al. in press). Hence lower economic status and inferior hygienic environment was suggested to protect from coeliac disease in Karelia (Kondrashova et al. in press).

The importance of the genes in the development of coeliac disease has been acknowledged for decades, as MacDonald and associates (1965) already in 1965 revealed an increased coeliac disease prevalence of 11.5% among relatives of coeliac disease patients. Subsequently a high concordance rate of coeliac disease, at least 75%, in monozygotic twins has been revealed (Hervonen et al. 2000, Greco et al. 2002). The disease is strongly associated with major histocompatibility complex (MHC) genes and more precisely HLA class II DQ genes located on the short arm of chromosome six (6p21). These genes encode DQ-heterodimers of α and β chains, for instance to surfaces of APCs. Approximately 90% of coeliac disease patients carry the HLA DQ2 heterodimer encoded by the alleles DQA1*05 (α) and DQB1*02 (β) (Sollid et al. 1989, Karell et al. 2003). When these alleles are inherited in *cis*, they are located in the same chromosome (DR3 or more recently termed the DR17 haplotype) (Sollid et al. 1989). Functionally the same DQ2 heterodimer can also be formed in *trans* in alleles located in two different choromosomes in DR5 (more recently termed DR11 or DR12) and DR7 haplotypes (Sollid et al. 1989). DQ2-negative coeliac disease patients carry mostly the DQ8 heterodimer, encoded by DQA1*0301 (α) and DQB1*0302 (β) in the DR4 haplotype, or half of the DQ2 heterodimer, encoded either by DQA1*05 (α) or DQB1*02 (β) (Karell et al. 2003). In a multinational study by Karell and associates (2003) coeliac disease patients not possessing any of these alleles accounted for only 0.4% of the disease population. Thus, HLA DQ2 and DQ8 are necessary but not sufficient for the development of coeliac disease, as about 40% of healthy Caucasians carry these same heterodimers (Mäki et al. 2003). There is a gene dosage effect of the MHC genes, and those patients who are DR3 homozygous carry the highest genetic

risk of developing coeliac disease (Ploski et al. 1993, Bourgey et al. 2007). Furthermore, it was recently suggested that the clinical picture of the condition is also affected by the patient's genotype, as homozygosity for the DQB1*0201 allele is associated with more severe forms of the disease (Karinen et al. 2006). However, no correlation between the genotype and the phenotype was found in coeliac disease in an earlier study (Greco et al. 1998).

HLA DQ loci are estimated to account for some 40% of inherited coeliac disease susceptibility (Bevan et al. 1999). Thus, other genes predisposing to the disease have been actively screened by genome-wide linkage analysis, and several candidate regions have been found. For instance, gene regions 5q31-33 (Greco et al. 2001, Liu et al. 2002, Percopo et al. 2003) and 2q33 (Djilali-Saiah et al. 1998, Holopainen et al. 2004) have been shown to be associated with an increased risk of coeliac disease in some populations. One promising candidate gene, MYO9B on chromosome 19p13.1, encoding an unusual myosin molecule affecting the actin remodelling of epithelial enterocytes, was found in the Dutch population (Monsuur et al. 2005). This result, however, has not been confirmed in coeliac disease patients from other regions (Amundsen et al. 2006, Giordano et al. 2006), suggesting that MYO9B might not be a coeliac risk factor, at least not in all populations. All in all, the rather inconsistent evidence so far obtained would imply that the noted candidate susceptibily genes in coeliac disease do not play as prominent a role as the DQ loci. Besides, the role of these genes in coeliac pathogenesis remains for the most part unsolved.

In coeliac disease the gluten-derived glutamine- and proline-rich peptides, which are resistant to degradation by gastrointestinal proteases (Shan et al. 2002), elicit both adaptive and innate mucosal immune responses (Figure 1). It remains obscure whether these peptides use paracellular or transepithelial passages in crossing the epithelial barrier, or whether for example they pass through the mucosa to the subepithelial region with the help of dendritic cells (Rescigno et al. 2001). Fasano and colleagues (2000) suggested that upregulation of zonulin, which contributes to tight junction regulation, might in coeliac disease lead to enhanced permeability of tight junctions between enterocytes, thus opening a paracellular route for incompletely digested gluten peptides to enter the lamina propria. It has also been speculated whether transient intestinal infections could lead to increased intestinal permeability allowing antigens such as intact gliadin molecules to enter the mucosal immune system of the lamina propria. After entering the lamina propria especially the coeliac disease-toxic peptide 31-43 (p31-43) of gliadin has been shown to stimulate factors of innate immunity (Maiuri et al. 2003). Cytokine interleukin (IL) 15 expression is thereby rapidly increased in cells of the lamina propria and in the epithelium (Maiuri et al. 2003, Hüe et al. 2004). IL-15, in turn, activates and expands IELs (Mention et al. 2003) and, additionally, induces the expression of the cell-surface antigen, MICA, induced by stress on enterocytes (Hüe et al. 2004, Meresse et al. 2004) and the MICA receptors, NKG2D, on $\alpha\beta+$ and $\gamma\delta+$ IELs (Roberts et al. 2001, Mention et al. 2003). Ultimately, the activated IELs are thought to become cytotoxic, killing the enterocytes expressing MICA by apoptosis and hence causing intestinal mucosal damage (Hüe et al. 2004).

The coeliac-immunogenic fragments of gluten, particularly the digestion-resistant 33-mer peptide fragment (p56-89), in turn activate the adaptive immune response in genetically susceptible individuals (Shan et al. 2002) (Figure 1). After entry from the lumen to the lamina propria, the glutamine residues of the gluten peptides are deamidated by the intestinal tTG to negatively charged glutamic acid residues (Molberg et al. 1998). The deamidation is essential in rendering the peptides more antigenic and suitable for binding with high affinity to HLA DQ2- and DQ8-heterodimers expressed by APCs such as dendritic cells. Subsequently, gluten-reactive CD4+ helper T lymphocytes (Th) recognise and are activated by the gluten peptides bound to HLA DQ2 and DQ8 (Molberg et al. 1998). As a result, Th1 cell proliferation and production of proinflammatory cytokines such as tumour necrosis factor α and, most importantly, interferon γ are induced (Nilsen et al. 1995). This further activates fibroblasts and inflammatory cells to produce for example matrix metalloproteinases, which are at least partly responsible for mucosal matrix degradation and thus the coeliac disease-type small-intestinal lesion (Pender et al. 1997, Daum et al. 1999). Th2 cell activation, in turn, leads to the activation and proliferation of B cells, which produce antibodies against gluten and tTG. However, the mechanism of tTG-ab formation has not been fully explained. It has been proposed that tTG-ab production is driven by the gluten-specific T cell response (Sollid et al. 1997). According to this hypothesis tTG cross-links itself to gluten peptides. Subsequently, the gluten peptide-tTG-complexes are taken up by tTG-specific B cells and after intracellular degradation of the complexes the B cells present the gluten peptides on surface HLA DQ2 and DQ8 molecules to gluten-specific T cells, this leading to T cell activation. The activated T cells, in turn, help the tTG-specific B cells in activating to produce tTG-ab. If gluten is withdrawn from the diet in coeliac disease no gluten peptide-tTG complexes are formed and hence no gluten-specific Th cell activation is continued, resulting in cessation of tTG-ab formation by the unactivated B cells. According to this model the T cell immune response to gliadin would also elicit antibody production against other gliadin peptide-cross-linked proteins (Sollid et al. 1997). It is also of note that the role of the antibodies in the disease pathogenesis remains somewhat equivocal. It has been suggested that IgA-class tTG-ab inhibits the epithelial cell differentiation by interference with tTG-mediated activation of transforming growth factor β (Halttunen and Mäki 1999). Furthermore, tTG-ab may have an important role in epithelial cell proliferation (Barone et al. 2007). For these reasons tTG-ab could play a part in generating and maintaining the coeliac-type small-bowel mucosal lesion, villous atrophy and crypt hyperplasia.
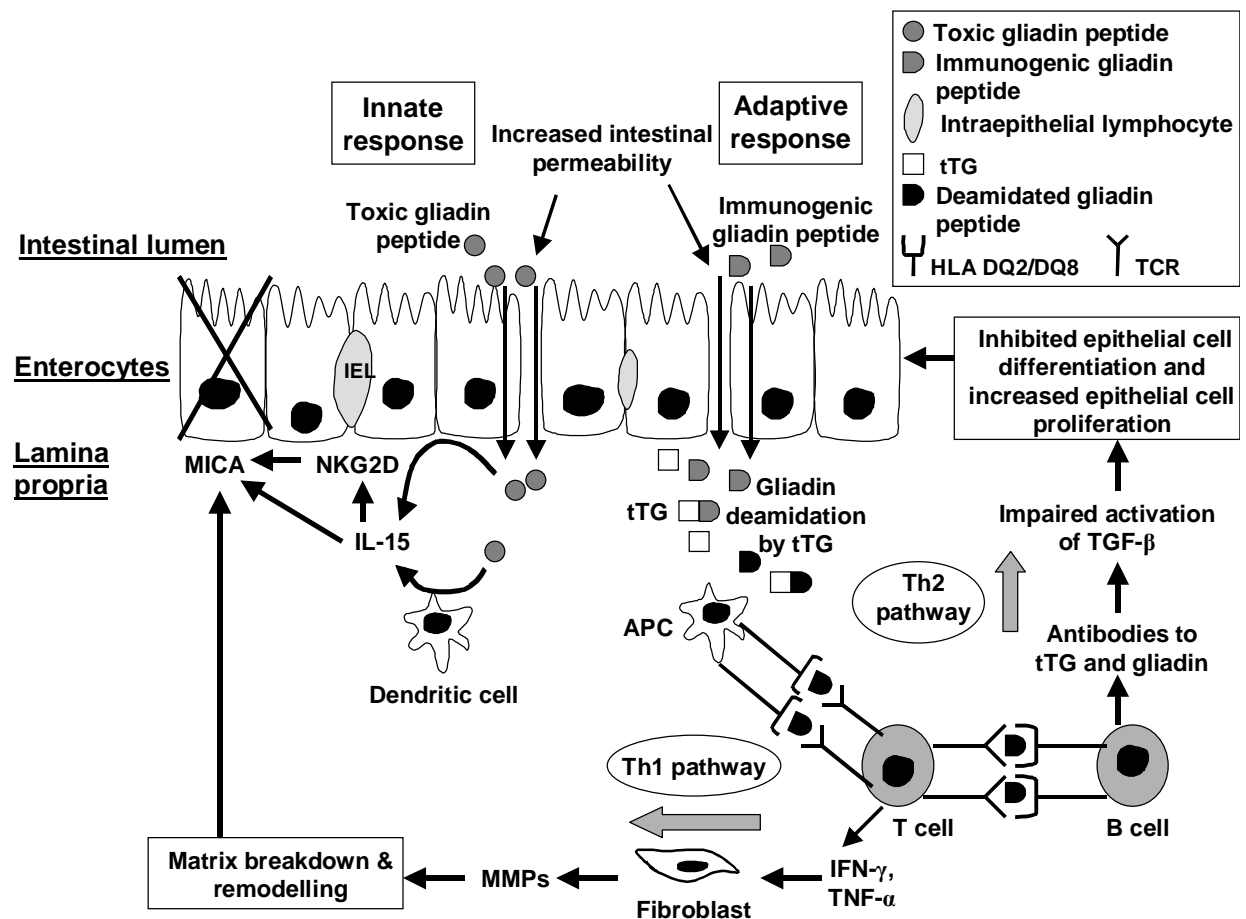
Figure 1. *Summary of underlying mechanism in coeliac disease pathogenesis leading to small-intestinal mucosal damage. IL-15=interleukin 15, tTG=tissue transglutaminase, APC=antigen-presenting cell, HLA=human leukocyte antigen, TCR=T cell receptor, INF-γ =interferon γ, TNF-α=tumour necrosis factor α, Th=helper T lymphocyte, MMP=matrix metalloproteinase, TGF-β=transforming growth factor β.*

7. TREATMENT OF COELIAC DISEASE

To this day coeliac disease is treated with strict permanent adherence to a gluten-free diet, where wheat, rye and barley should be avoided. The toxic fractions of these cereals in coeliac disease are alcohol-soluble proline- and glutamine-rich prolamins named gliadin in wheat, secalin in rye and hordein in barley (Kagnoff 2007). Traditionally oats have also been excluded from the diet. However, oats do not belong to the same tribe, the triticeae, and hence the prolamins of oats (avenin) differ from those of wheat, rye and barley (Kagnoff 2007). In 1995 Janatuinen and associates (1995) showed that adult coeliac disease patients can tolerate oats. Later several studies further proved that oats are safe for both

adults (Janatuinen et al. 2002) and children (Högberg et al. 2004) suffering from coeliac disease and also for DH patients (Reunala et al. 1998). However, some sporadic coeliac disease cases sustaining a histological relapse after consuming oats have been reported (Lundin et al. 2003).

Controversy remains as to whether a gluten-free diet should be naturally gluten-free or whether industrially purified wheat starch-based gluten-free products containing trace amounts of gluten could also be consumed in coeliac disease. In several studies the wheat starch-based diet has not proved harmful for coeliac disease patients (Kaukinen et al. 1999a, Peräaho et al. 2003, Collin et al. 2004). Moreover, trace amounts of gluten can be measured, not only in wheat starch-based gluten-free products, but also in naturally gluten-free products in consequence of contamination (Collin et al. 2004). Further, occasional dietary transgressions seem to cause more small-intestinal mucosal damage than trace amounts of gluten (Kaukinen et al. 1999a).

DH is also treated with a strict gluten-free diet, and DH patients might even be more sensitive to ingested gluten than coeliac disease patients. Both the small-intestinal damage and the blisters in DH improve on a diet. However, the cutaneous disease in DH responds slowly to dietary treatment, taking some months or even years, and additional treatment with an anti-inflammatory drug, dapsone, is often needed. (Collin and Reunala 2003)

Some coeliac disease patients fail to improve in symptoms and small-intestinal mucosal damage despite a strict gluten-free diet. If differential diagnoses and inadequate dietary treatment of coeliac disease have been ruled out, the patient may suffer from refractory sprue, ulcerative jejunoileitis or small-intestinal lymphoma. No conclusive evidence is available, but patients suffering from refractory sprue might benefit from immunosuppressive treatment. (Biagi and Corazza 2001)

It should be kept in mind that coeliac disease patients may evince nutritional deficiencies such as folate and B-vitamin deficiencies, despite adhering long-term to a strict gluten-free diet (Hallert et al. 2002). In these circumstances additional vitamin or mineral supplementation is obviously indicated.

7.1. Benefits of dietary treatment

In most coeliac disease patients a strict gluten-free diet normally results in alleviation of symptoms within two weeks (Pink and Creamer 1967). Small-bowel mucosal damage recovers more slowly and might take over a year despite dietary treatment (Grefte et al. 1988, Kaukinen et al. 1999a, Wahab et al. 2002). Early adherence to a diet also reduces the risk of malignant complications such as NHL and gastrointestinal cancers, and the overall risk of malignant diseases in coeliac disease is nowadays close to that in the general population (Holmes et al. 1989, Collin et al. 1996a, Askling et al. 2002, West et al. 2004, Viljamaa et al. 2006). However, it has not been proved that patients with untreated silent or early developing coeliac disease carry an increased risk of a malignant condition.

In contrast, several studies have shown that impaired bone mineral density found in untreated coeliac disease frequently normalizes on a gluten-free diet, also in the clinically silent form (Mora et al. 1998, Mustalahti et al. 1999). The risk of fractures might also be lower in coeliac disease patients diagnosed early and effectively treated than in those with late diagnosis or poor dietary adherence (Vazquez et al. 2000). Liver manifestations such as elevated liver transaminases or even liver failure can in some cases be reversed on a strict diet (Volta et al. 1998a, Farre et al. 2002, Kaukinen et al. 2002a, Bardella et al. 2004). In studies which have revealed associations or extraintestinal manifestation of coeliac disease, other conditions such as arthritis (Lepore et al. 1996), migraine (Gabrielli et al. 2003), arrythmia (Frustaci et al. 2002) and alopecia areata (Barbato et al. 1998) have also in some cases resolved after dietary treatment. Additionally, a gluten-free diet may have a positive effect in cases of delayed menarche, secondary amenorrhoea, early menopause (Smecuol et al. 1996), infertility (Collin et al. 1996b) and unfavourable foetal outcome (Ludvigsson et al. 2005) among women suffering from coeliac disease. Controversy prevails as to whether gluten exposure time and diagnostic delay contribute to other later associated autoimmune diseases (Ventura et al. 1999, Viljamaa et al. 2005b). No conclusive evidence is on record as to whether metabolic control of type one diabetes mellitus improves when a gluten-free diet is commenced in coeliac disease (Kaukinen et al. 1999b, Amin et al. 2002). Futhermore, it is a matter of some debate whether dietary treatment leads to resolution of neurological manifestations of coeliac disease (Bushara 2005). In contrast, quality of life seems to increase among coeliac disease patients when they exclude dietary gluten, and this might also be the case in silent coeliac disease detected by screening (Mustalahti et al. 2002, Johnston et al. 2004).

7.2. Follow-up and dietary compliance in coeliac disease

After prescription of a gluten-free diet to a coeliac disease patient, a regular, long-term follow-up should also be commenced in order to confirm the diagnosis and clinical and histological response to treatment, and to ensure good dietary compliance (Bardella et al 1994). Nutritional deficiencies should be detected and treated. Untreated coeliac disease patients are also recommended to be screened for osteoporosis (National Institutes of Health Consensus Development Conference Statement on Celiac Disease 2005). Dietary compliancy is promoted by information on the gluten-free diet provided by a dietician and on the disease and its complications (Ljungman and Myrdal 1993). Local coeliac disease societies are further valuable sources of information and thus promote dietary adherence among their members (Green et al. 2001). There is no unambiguous evidence on the most effective method to follow up coeliac disease patients, and no standardized guidelines exist for dietary monitoring. Follow-up small-intestinal biopsy is recommended, though not nowadays necessary for the diagnosis, especially in the case of adult coeliac patients after commencing

dietary treatment (Walker-Smith et al. 1990, Rostom et al. 2006, Kaukinen et al. 2007b). Coeliac patients respond to the diet clinically and histologically with great heterogeneity. Wahab and associates (2002) reported that histologic remission, referring to Marsh 0, I or II, was seen in 65% of patients after two years of dietary treatment and in 90% in long-term follow-up, whereas among children full recovery was seen in 95% within two years of treatment and in all child patients on the long-term. The decline of coeliac disease-specific serum antibodies, EMA, tTG-ab and now also DPG-ab, during a diet is further an indication of dietary adherence and antibody testing is therefore recommended in the dietary monitoring of coeliac disease (Hill et al. 2005, Rostom et al. 2006). However, antibody tests might not reveal slight dietary transgressions (Troncone et al. 1995). Secondly, antibody test results may seroconvert to negative despite ongoing small-intestinal mucosal damage (Kaukinen et al. 2002b). The sensitivities of the tTG-ab, EMA or DGP-ab tests have varied from 29% to 100% in detecting dietary transgressions and from 16% to 91% in revealing small-intestinal villous atrophy in coeliac disease patients on a gluten-free diet, as shown in Tables 7 and 8 (Troncone et al. 1995, Dickey et al. 2000a, Fabiani et al. 2000). On the other hand, antibody tests are of great value in revealing major dietary transgressions (Troncone et al. 1995). A positive test result can be considered an indication of both poor dietary compliance and unsatisfactory mucosal response in coeliac disease, and hence the tests may be helpful in the timing of small-intestinal biopsy (Troncone et al. 1995, Dickey et al. 2000a, Kaukinen et al. 2002b). All in all, use of the coeliac antibody tests in the dietary monitoring of coeliac disease is reasonable, albeit of limited value (Hill et al. 2005, Rostom et al. 2006).

Dietary compliancy varies a great deal in coeliac disease around the world, as shown in Table 8. Studies report that 17-88% of coeliac disaese patients adhere strictly to the diet, while 0-30% do not follow the diet at all (Ljungman and Myrdal 1993, Greco et al. 1997, Kaukinen et al. 1999a, Ciacci et al. 2002, Högberg et al. 2003, Viljamaa et al. 2005a). Female gender especially among adolescents (Ljungman and Myrdal 1993, Greco et al. 1997), education (Ciacci et al. 2002) and diagnosis during early childhood (Högberg et al. 2003) may influence dietary compliancy positively. Some studies suggest that compliancy is poor among screen-detected, asymptomatic coeliac disease patients (Fabiani et al. 2000). However, a recent report from Finland revealed that dietary compliancy may also be excellent among screen-detected coeliac disease patients, as 96% of the patients studied did not consume gluten more than four times per month (Viljamaa et al. 2005a). In contrast, the corresponding figure for symptom-detected coeliac patients was 93% (Viljamaa et al. 2005a).

Table 7. *Serum endomysial (EMA), tissue transglutaminase (tTG-ab) or deamidated gliadin peptide antibody (DGP-ab) positivity in proportion to small-bowel mucosal morphology among coeliac disease patients on a gluten-free diet (GFD).*

Reference	Duration of GFD	Patients	Patients evincing VA* n (%)	Coeliac antibody positivity n (%)	
				In patients with VA*	In patients with normal histology
(Troncone et al. 1995)	≥ 10 years	23 adolescents	11 (48)	10 (91) (EMA)	1 (8) (EMA)
(Sategna-Guidetti et al. 1996)	ND	47 adults	38 (81)	9 (24) (EMA)	0 (0) (EMA)
(Dickey et al. 2000a)	One year	53 cases	32 (60)	5 (16) (EMA)	0 (0)
(Kaukinen et al. 2002b)	Median one year	87 adults	27 (31)	11 (41) (tTG-ab) 7 (26) (EMA)	7 (12) (tTG-ab) 4 (7) EMA
(Volta et al. in press)	One year	53 adults	15 (28)	10 (67) (DGP-ab)	8 (21) (DGP-ab)

* VA=small-bowel mucosal villous atrophy indicating Marsh IIIa-c type lesion
ND=no data

Table 8. *Serum endomysial (EMA), tissue transglutaminase (tTG-ab) or deamidated gliadin peptide antibody (DGP-ab) positivity in proportion to dietary adherence among coeliac disease patients on a gluten-free diet (GFD).*

Reference	Duration of GFD	Patients	Patients having dietary lapses n (%)	Coeliac antibody positivity n (%)	
				In patients having dietary lapses	In patients with a strict GFD
(Troncone et al. 1995)	≥ 10 years	23 adolescents	19 (83)	11 (58) (EMA)	0 (0) (EMA)
(Dickey et al. 2000a)	One year	53 cases	4 (8)	4 (100) (EMA)	1 (2) (EMA)
(Fabiani et al. 2000)	Five years	44 adolescents	24 (55)	7 (29) (EMA)	0 (0) (EMA)
(Fabiani and Catassi 2001)	≥ one year	149 children and adults	25 (17)	14 (56) (EMA) 10 (40) (tTG-ab)	1 (1) (EMA) 21 (17) tTG-ab
(Kaukinen et al. 2002b)	Median one year	87 adults	11 (13)	5 (45) (EMA) 5 (45) (tTG-ab)	6 (8) (EMA) 13 (17) (tTG-ab)
(Vahedi et al. 2003)	≥ one year	95 adults	55 (58)	34 (62) (EMA)	1 (3) (EMA)
(Viljamaa et al. 2005a)	5-21 years	97 adults	19 (20)	7 (37) (EMA and tTG-ab)	1 (1) (EMA and tTG-ab)
(Volta et al. in press)	One year	53 adults	14 (26)	10 (71) (DGP-ab)	8 (21) (DGP-ab)

7.3. New aspects of treatment

Therapies alternative to a gluten-free diet in coeliac disease are constantly being explored. One future approach might be degradation and thus detoxication of T cell stimulatory peptides in cereal products, in which intestinal proteases fail to digest by using for example combination enzyme supplement therapy of glutamine-specific endoprotease coupled with bacterial prolyl endopeptidase (Shan et al. 2002, Gass et al. 2007). This enzyme therapy might raise the safe threshold of gluten intake in coeliac disease. However, human experiments are still lacking. Other targets of therapy, to name a few, might be generation of detoxified cereals by selective breeding or genetic modification (Vader et al. 2003, Molberg et al. 2005), inhibition of presentation of the gluten peptides to T cells by blocking the binding sites of HLA DQ2 or DQ8 molecules (Xia et al. 2007), inhibition of intestinal tTG responsible for deamidating gluten peptides (Choi et al. 2005), generation of immunotolerance to gluten by gluten challenge implementented with for example antibodies to CD3 inducing T cell silencing (Chatenoud 2003) and inhibition of the zonulin effect on intestinal permeability (Paterson et al. 2007). Additionally, use of cytokine therapy such as IL-10 (Salvati et al. 2005) or of a blocking antibody to IL-15 (Villadsen et al. 2003) is also a possible future means to avoid the need for a gluten-free diet. However, more evidence is needed to ensure the safety and efficiency of the alternative therapies in the treatment of coeliac disease.

THE PRESENT STUDY

1. PURPOSE

The aim of the present study was to evaluate a novel method utilizing whole blood sample endogenous erythrocyte self-tTG as antigen in detecting IgA-class coeliac autoantibodies, and to compare this method to the conventional serological, tTG-ab and EMA, tests and to diagnostic small-intestinal biopsy.

The specific objectives were:

1. To assess a proof-of-concept test, the whole blood IgA self-tTG in house POCT, utilizing Nunc-Immunosticks
   a. by first proving the specificity of the new method to detect autoantibodies against tTG using tTG knockout mice-derived erythrocytes and coeliac disease patients' sera (**I**)
   b. in untreated and treated coeliac disease patients and non-coeliac disease controls in a laboratory setting (**I, II**)
   c. in rapid case finding and monitoring of dietary treatment of coeliac disease on site in an office setting (**I**)

2. To study the whole blood IgA self-tTG method in detecting untreated coeliac disease in the laboratory also using ELISA, suitable for large-scale screening for the disease (**III**)

3. To evaluate whole blood IgA self-tTG commercial rapid test applications using the lateral flow immunochromatographic strip system
   a. in untreated and treated coeliac disease patients and non-coeliac disease controls in a laboratory setting (**II-IV**)
   b. in rapid case finding of coeliac disease on site in an office setting (**IV**)

2. PATIENTS

The subjects investigated in studies **I-IV** are presented in Table 9. They were examined at the Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Hungary (**I, III, IV**), the Department of Paediatrics, University of Debrecen, Hungary (**I, IV**), the Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Finland (**I, II, IV**), the Department of Paediatrics in Tampere University Hospital, Finland (**III**) and in Hatanpää Health Centre, Finland (**I, II**) between the years 1998-2005.

2.1. Untreated and treated coeliac disease patients in laboratory testing (I-IV)

Altogether 268 untreated coeliac disease patients studied retrospectively were included in studies **I-IV**. All of these patients fulfilled the revised ESPGAN diagnostic criteria for coeliac disease (Walker-Smith et al. 1990). The whole blood IgA self-tTG in house POCT was evaluated in 150 of these patients (**I, II**), the whole blood IgA self-tTG ELISA in 150 and the two different whole blood IgA self-tTG rapid tests, rapid test 1 and rapid test 2, in 150 (**III**) and in 121 (**II, IV**), respectively.

Further, whole blood and serum samples obtained from 143 coeliac disease patients during a gluten-free diet were evaluated in the laboratory retrospectively (**II, IV**). Altogether 48 of the patients were studied both before and after one-year dietary treatment with the self-tTG in house POCT (**II**). The strictness of the diet was evaluated by inquiry and a four-day record of food intake. Likewise, self-tTG rapid testing was carried out among 15 coeliac patients both before and after one-year dietary treatment and among 91 long-term treated (median of strict gluten-free diet 9 years, range 1-24 years) coeliac patients (**II, IV**).

Whole blood anticoagulated with ethylene diamine tetra-acetic acid (EDTA) or sodium sitrate and serum samples were obtained from all the aforementioned patients and the non-coeliac disease controls at the time of biopsy and stored at -20°C until tested for tTG-ab and EMA.

2.2. Non-coeliac disease control patients in laboratory testing (I-IV)

Altogether 186 non-coeliac disease controls were included in studies **I-IV**. Of these, 67 suffered from dyspepsia (**I-IV**), 30 from inflammatory bowel disease (**I, III, IV**), 18 from gastroesophageal reflux disease (**I, III, IV**), 15 from congenital sucrase-isomaltase deficiency (**I, III, IV**), eight from nutritive allergy (**I**) and seven from intestinal polyposis (**I, III, IV**). Altogether 41 of the controls had other miscellaneous reasons for coeliac disease antibody testing (**I, III, IV**). All non-coeliac disease controls evinced normal villous morphology.

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2.3. Prospectively enrolled patients (**I, IV**)

Whole blood IgA self-tTG-based testing was also carried out on fresh whole blood samples prospectively on site in an office setting in altogether 315 new patients consuming gluten-containing food. Subsequently, samples for serological coeliac antibody testing were also obtained from these patients.

Of the aforementioned patients 165 were tested with the whole blood IgA self-tTG in house POCT (**I**). The group included 46 patients with gastrointestinal symptoms and a high suspicion of enteral disorder such as coeliac disease, 84 subjects being at risk of coeliac disease, for example patients with various autoimmune diseases, diabetes mellitus, eating disorders or patients who were first-degree relatives of known coeliac patients, and 35 adults with dyspepsia and with a low suspicion of coeliac disease. Patients with clinical suspicion of upper gastrointestinal disease underwent endoscopy and small-intestinal biopsy.

Further, the whole blood IgA self-tTG rapid test 2 was evaluated in 150 of the 315 prospectively enrolled subjects in a tertiary gastroenterology centre (**IV**). Of these subjects 78 had symptoms suggestive of coeliac disease and 72 were first-degree family members of coeliac patients. Subjects yielding a positive coeliac disease antibody test result were invited to ungergo endoscopy with small-intestinal biopsy.

Moreover, 263 consecutive patients with previously diagnosed biopsy-proven coeliac disease and with known serum total IgA levels were tested prospectively in the office with the whole blood IgA self-tTG in house POCT and samples for serum-based testing were also obtained. The patients had been on a gluten-free diet for two months to 21.4 years (median 3.9 years). The strictness of the diet was assessed prospectively at the time of the interview by structured questionnaire, discussion with the patient, clinical findings and history (**I**). Intensified dietary instructions were given, also by a dietician, to all in house POCT-positive treated patients. Altogether 16 of these patients underwent a repeat test in the office after 3-6 months from the dietary intervention. Correspondingly, 14 serum EMA-positive coeliac subjects who received the information on their positive antibody test results and the instructions to improve the diet by mail and did not participate in the on site in house point-of care testing, were used as controls for the intervention (**I**).

Table 9. *Demographic data of patients assessed in studies **I-IV**.*

Setting	Study **I**		Study **II**		Study **III**		Study **IV**	
Subjects	n (female)	Median age, range (years)	n (female)	Median age, range (years)	n (female)	Median age, range (years)	n (female)	Median age, range (years)
<u>Laboratory testing</u>								
Untreated coeliac disease	99 (67)	10 (1.4-59)	51 (38)	48 (24-69)	150 (96)†	9 (1.4-40)	121 (85)¶	12 (1.6-68)
Treated coeliac disease	-	-	48 (35)*	46 (28-70)	-	-	106 (68)§	56 (9-82)
Non-coeliac disease controls	65 (29)	15 (3.3-67)	36 (23)	56 (23-73)	107 (41)‡	12 (1-55)	107 (46)**	15 (0.9-72)
<u>On site testing prospectively</u>								
Patients with coeliac disease suspicion	165 (85)	13 (1.2-72)	-	-	-	-	150 (86)	9 (0.9-72)
Coeliac disease patients on GFD	263 (163)	13 (2.8-76)	-	-	-	-	-	-

* The same coeliac patients were evaluated before and after one-year dietary treatment
† Fifty-two of the patients were also included in study **I**
‡ Thirty-two of the patients were also included in study **I**
§ Fifteen coeliac disease patients were evaluated before and after one-year dietary treatment
¶ Altogether 101 of the patients had also participated in studies **I**, **II** and/or **III**
** Altogether 97 of the patients had also participated in studies **I**, **II** and/or **III**

3. METHODS

3.1. Whole blood IgA self-tTG antibody tests (*I-IV*)

All whole blood IgA self-tTG tests used in the present study were based on the same innovation of using the patient's own endogenous tTG, self-tTG, found in the erythrocytes of a whole blood sample in IgA-class coeliac autoantibody detection (Mäki and Korponay-Szabó, Patent application PCT/FIO2/00340). In whole blood self-tTG testing self-tTG is first liberated from the erythrocytes by haemolysing the sample. Thereafter, if tTG-ab is present in the serum of the sample it complexes with the liberated autoantigen, self-tTG. tTG-tTG-ab-complexes are then captured from the haemolysed sample by tTG-binding proteins to a solid surface. The IgA-class tTG-ab of the complexes is further detected by labelled antihuman IgA in the testing (Figure 1 in original publication **II**). The whole blood IgA self-tTG tests were performed on thawed or fresh venous whole blood samples anticoagulated with EDTA or sodium citrate or fresh fingertip capillary whole blood samples. All tests were carried out blindly without prior knowledge of serological coeliac antibody test results. The different whole blood IgA self-tTG antibody tests used in autoantibody evaluation are presented in Table 10.

Table 10. *Different IgA-class self-tissue transglutaminase (self-tTG)-based methods used in the present study to measure coeliac antibodies in whole blood samples.*

Abbreviations, used in studies	Commercial name and producer	Test principle	Testing time	Cut-off level
Whole blood IgA self-tTG in house POCT, **I, II**	-	Nunc-Immunostick (Nunc A/S, Roskilde, Denmark) *	35 minutes	Visible colour formation
Whole blood IgA self-tTG ELISA, **III**	Celiac IgA EIA, catalog number 6300100, Ani Labsystems Ltd. Oy, Vantaa, Finland	ELISA	> 1 hour	5.0 U/ml
Whole blood IgA self-tTG rapid test 1, **III**	Biocard™ Celiac Test, catalog number 3-027-000, Ani Biotech, Vantaa, Finland	Immunochromatographic assay †	5-10 minutes	Visible colour formation
Whole blood IgA self-tTG rapid test 2, **II, IV**	Biocard™ Celiac Test, catalog number 3-028-000, Ani Biotech, Vantaa, Finland	Immunochromatographic assay †	5-10 minutes	Visible colour formation

POCT=point-of-care test, ELISA=enzyme-linked immunosorbent assay, U/ml=units per millilitre
* See Figure 2
† See Figure 3

3.1.1. Self-tTG in house point-of-care testing (I, II)

The proof-of-concept test, whole blood IgA self-tTG in house POCT, for the aforementioned test principle was first assessed in the laboratory (**I, II**) and subsequently in a real point-of-care fashion in an office setting using fresh whole blood samples (**I**). The test was developed into the Nunc-Immunostick pad (Nunc A/S, Roskilde, Denmark) (Figure 1 in original publication **I**) and the test result was available within 35 minutes. First, two quadrants of the pad were precoated with gelatin (0.05% in 0.3 M bicarbonate buffer, pH 9.6) able to bind fibronectin and tTG-tTG-ab complexes from the haemolysed whole blood sample. Furthermore, we sought to investigate whether whole blood IgA self-tTG testing was also suitable for detecting the total IgA level of a patient. Hence in study **I** one quadrant of the pad was additionally coated with antibodies against human IgA (Boehringer, Mannheim, Germany) diluted 1:4000 in 0.3 M bicarbonate buffer (pH 9.6) to react with normal plasma IgA as a positive control. In study **I** one uncoated quadrant and in study **II** two uncoated quadrants served as negative controls. In the testing, a drop of whole blood (25 µl) was delivered into distilled water in order to haemolyse the sample and shortly thereafter buffer was added (hypotonic saline with 0.05M Tris, 0.01M EDTA and 0.1% Tween 20). Next, the precoated pad was inserted into the haemolysed sample dilution, where it was incubated for 15 minutes. After washing in tap water the pad was immersed for another 15 minutes in peroxidase-conjugated antihuman IgA solution (Dako, Glostrup, Denmark) diluted 1:2000 in 0.05M Tris (pH 7.4), washed again and inserted into a gel-containing colorigenic substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich, Steinheim, Germany) with Sephadex powder (Amersham Pharmacia Biotech, Uppsala, Sweden). After five minutes' incubation in the dark the test result was read visually. The results were considered negative (0) if blue colour developed in only one quadrant (the IgA-sensitive part) of the pad in study **I** or if the pad remained completely blank in study **II**. In a positive test result blue was seen in either altogether three quadrants (**I**) or two quadrants (**II**) of the pad and the positive test results were further semi-quantified by inspection as strong (deep blue, ++) or weak positive (faint blue, +) in study **II**. If no colour developed in study **I** the result was interpreted to be invalid for tTG-ab evaluation as the sample was IgA-deficient. The coating of the Nunc-Immunostick and reading of the in house POCT result when the positive control was used is shown in Figure 1 in original publication **I**. Further, comparison between the traditional serum-based EMA test principle and whole blood IgA self-tTG in house POCT and interpretation of in house POCT results when the positive control was not used is shown here in Figure 2. The substrate for whole blood self-tTG in house point-of-care testing was found to be stable for up to one month at +4°C, whereas the conjugate was made up freshly each day.

Interobserver variation in whole blood IgA in house point-of-care testing was assessed blindly with 30 randomly selected whole blood samples from the patient cohorts assessed in study **I** in the laboratory. Additionally, intraobserver variation was studied blindly with 20 whole blood samples at five different time

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points by altogether three observers. In addition, whole blood sample conservation evaluation was carried out with eight whole blood samples drawn from untreated coeliac disease patients. The samples had been stored after collection at –20°C and –80°C for 24-26 months without previous thawing. Conservation of precoated Nunc-Immunosticks was investigated with sticks which had been stored for nine months at +4°C (**I**).
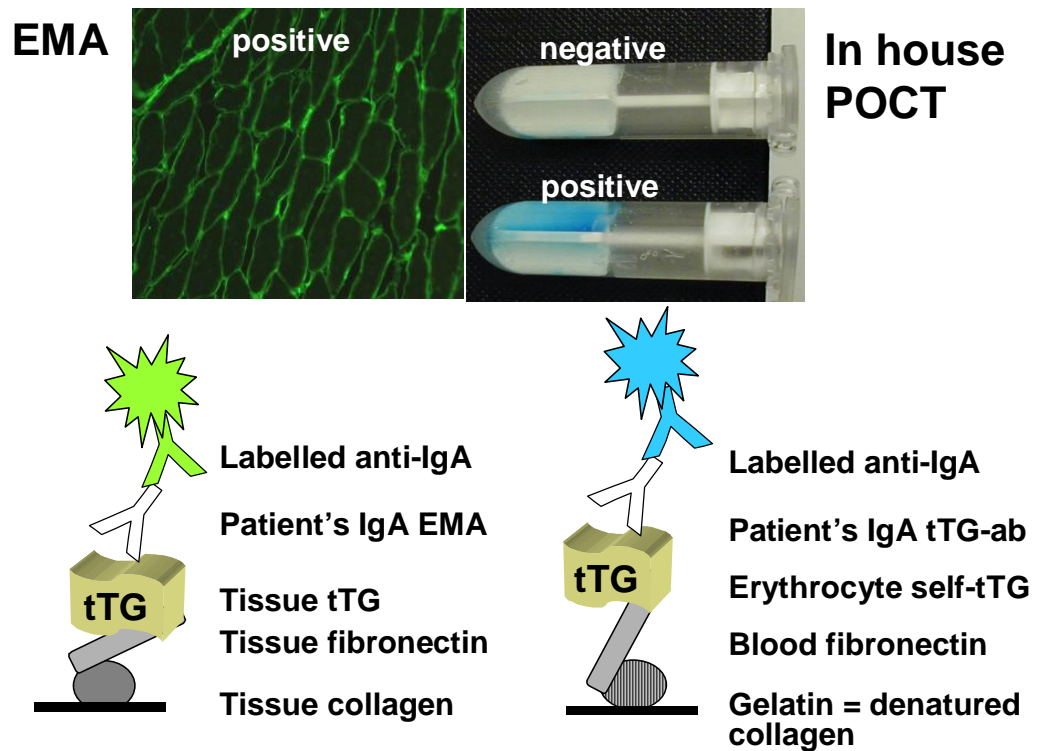
Figure 2. *Comparison of the conventional serum-based endomysial antibody (EMA) test principle and the novel whole blood-based IgA self-tissue transglutaminase (tTG) in house point-of-care test (POCT) in IgA-class coeliac autoantibody detection and interpretation of in house POCT results. In the EMA test tissue sections (containing collagen, fibronectin and tTG) such as human umbilical cord (Sulkanen et al. 1998a, Korponay-Szabó et al. 2003b) are used in coeliac antibody detection, whereas in the in house POCT fibronectin and erythrocyte self-tTG are captured from a whole blood sample by gelatin (denatured collagen). In both tests tTG-specific IgA is visualized in colour reaction by labelled anti-human IgA.*

3.1.2. Investigation of target specificity of the whole blood IgA self-tTG method to tTG (*I*)

Ten EMA- and tTG-ab-positive serum samples obtained from coeliac disease patients were used in investigating whether the colour reaction in positive POCT was due to binding of specific antibodies to tTG. The sera were tested with the in house POCT after being mixed 1:1 with washed red blood cells from normal or from tTG-deficient tTG knockout mice (De Laurenzi and Melino 2001) or they were applied without being mixed with any red blood cells. Thereafter, the testing was performed in the manner described in the previous section.

3.1.3. Commercial self-tTG ELISA testing (*III*)

IgA self-tTG-based antibody detection was also carried out blindly in the laboratory with the commercial whole blood-based ELISA method (Celiac IgA EIA, catalog number 6300100, Ani Labsystems Ltd. Oy, Vantaa, Finland, abbreviated here as IgA self-tTG ELISA) (*III*). In the IgA self-tTG ELISA, IgA-class tTG-ab, if present, is detected from a whole blood sample by binding the patient's self-tTG immunocomplexed by its autoantibodies to a specific antigen attached to the polystyrene surface of a 96-well Microstip®. IgA-self-tTG-complexes are further detected as a colour reaction using solutions containing anti-human IgA and chromogen.

Self-tTG ELISA testing was carried out according to the manufacturer's instructions. Briefly, whole blood samples mixed with a sample diluent (phosphate buffered saline with additives) (dilution 1:51) and a sample diluent and control samples (negative control, calibrator and high positive control also available in the test kit) mixed with distilled water were pipetted into the wells in the coated test plate in duplicate and the plate was incubated at room temperature for 15 minutes. After washing with washing solution (concentrated citrate buffered saline with additives), the plate was incubated with conjugate solution (horseradish peroxidase-conjugated sheep anti-human IgA) for 30 min. Next, colourless enzyme substrate (H_2O_2) containing the chromogen (TMB, a non-mutagenic chromogen for horseradish peroxidase) was added after washing, and the plate subsequently incubated 30 minutes in the dark; finally the colour formation reaction was stopped by adding stopping solution (0.45 M H_2SO_4). The colour intensity was measured at 450 nm and the result for each sample calculated according to the equation U/ml = CF x (A_{SAMPLE}-A_{BLANK}) / (A_{CAL}-A_{BLANK}), where CF refers to the calibration factor value specified in the Calibration Sheet provided with the kit, A_{SAMPLE} to the mean absorbance of the sample, A_{BLANK} to that of the reagent blank and A_{CAL} to that of the calibrator, respectively. A value $\geq$ 5.0 U/ml was considered positive according to the manufacturer's suggestion.

3.1.4. Commercial self-tTG rapid testing (II-IV)

Two different prototypes of commercial whole blood IgA self-tTG rapid tests, rapid test 1 (Biocard™ Celiac Test, catalog number 3-027-000, Ani Biotech, Vantaa, Finland; **III**) and 2 (Biocard™ Celiac Test, catalog number 3-028-000, Ani Biotech, Vantaa, Finland; **II, IV**), were applied according to the manufacturer's instructions. First, the rapid test evaluation was carried out blindly in the laboratory from thawed venous whole blood samples. Subsequently, the testing method was assessed on site in office conditions using fresh capillary or venous whole blood samples (**IV**).

In whole blood rapid testing IgA-class tTG-ab was detected by a lateral flow immunochromatographic casette using the whole blood sample's self-tTG as antigen and colloidal gold-labelled mouse antibodies to human IgA as the signal generator. The filter containing the signal generator was bound to the filter tip of a tube containing the haemolysing buffer (rapid test 1, **III**) or to the test strip (rapid test 2; **II, IV**) (Figure 3). First, 10μl of venous or capillary whole blood samples was inserted into the tube with haemolysing buffer in order to liberate self-tTG from the red blood cells. Subsequently, three drops of the sample dilution were added to the application field on the test casette (Figure 1 in original publication **IV**) and the sample migrated by capillary diffusion in the strip. If IgA-class tTG-ab was present in the serum of the sample, tTG-ab-self-tTG-immunocomplexes, further bound to labelled antihuman IgA located in the filter tip or test cassette, were formed. These complexes attached to a tTG-capturing protein test line linked to the nitrocellulose test membrane, and formed a visible red test line (Figure 1 in original publication **IV**). Additionally, a control system was integrated into the strip to ensure that the sample dilution and reagents had passed into the test area. The control line was formed if the colloidal gold-labelled mouse antibodies to human IgA not bound with tTG-ab IgA had migrated through the strip and reacted with anti-mouse IgG antibodies bound to the control line (Figure 1 in original publication **IV**). The test result was read after five minutes, not later than ten minutes. The result was interpreted as positive if both the test and the control lines were seen, and negative if only the control line was formed (Figure 1 in original publication **IV**). The result was invalid if the control line was missing.

Interobserver variation in whole blood IgA self-tTG rapid test 2 was evaluated with 20 randomly selected stored whole blood samples from the study cohort (**IV**) between two investigators in blinded fashion. Additionally, intraobserver variation was assessed blindly with the corresponding samples at different time points.
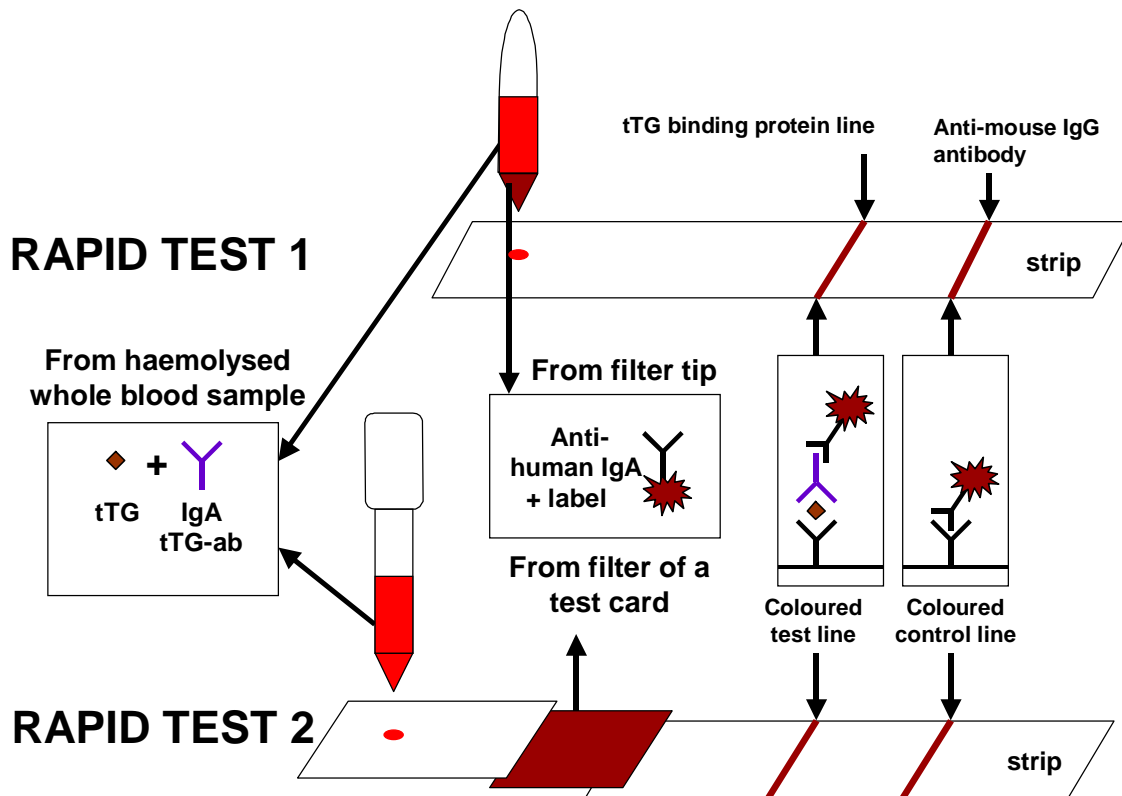
Figure 3. *Layout of the whole blood IgA self-tissue transglutaminase (tTG) rapid tests and comparison of rapid tests 1 and 2 applied in studies **II** (rapid test 2), **III** (rapid test 1) and **IV** (rapid test 2). In both tests a capillary or venous whole blood sample is used to detect IgA-class tissue transglutaminase-antibodies (tTG-ab). The sample's erythrocyte self-tTG, liberated by mixing haemolysing solution with it, serves as coeliac autoantigen. In contrast, the filter containing labelled antihuman IgA is located in the filter tip of the tube containing the haemolysing solution in rapid test 1, whereas in test 2 the filter is located on the test strip.*

3.2. Conventional serological coeliac antibody tests (*I-IV*)

tTG-ab and EMA were determined from serum samples from all subjects assessed in studies **I-IV**. The different serum-based assays used in autoantibody evaluation are presented in Table 11. All serum-based tests detected the autoantibodies in IgA class, except for the commercial EMA test, which detected both IgA- and IgG-class EMA. The commercial tTG-ab and EMA tests were applied according to manufacturers' instructions. Additionally, the in house EMA test using either monkey oesophagus or human umbilical cord as substrate

was conducted as previously described (Korponay-Szabó et al. 1997, Sulkanen et al. 1998a).

Table 11. *Methods used in the study to measure coeliac antibodies in serum samples.*

Abbreviations, used in studies	Commercial name and producer and/or reference	Antigen	Test principle	Cut-off level
hr-tTG ELISA, **I-IV**	Celikey™, Phadia GmbH, Freiburg, Germany	Human recombinant tTG	ELISA	5.0 U/ml
nh-tTG ELISA, **III**	QUANTA Lite™ h-tTG IgA, INOVA Diagnostics, San Diego, USA	Native human red blood cell-derived tTG	ELISA	20 U
in-house EMA, **I, IV**	(Korponay-Szabó et al. 1997)	Monkey oesophagus	indirect IF	1:2.5
in-house EMA, **I-IV**	S-EMAbA, Service Laboratory, Coeliac Disease Study Group, University of Tampere, Finland (Sulkanen et al. 1998a)	Human umbilical cord	indirect IF	1:5
commercial EMA, **III**	The ImmuGlo™ Anti-Endomysial Antibody Test System, IMMCO Diagnostics, Buffalo, USA	Primate smooth-muscle tissue	indirect IF	1:2.5

tTG=tissue transglutaminase, ELISA=enzyme-linked immunosorbent assay, U/ml=units per millilitre, U=a unit value, EMA=endomysial antibody; IF=immunofluorescence

3.3. Small-bowel mucosal biopsy (**I-IV**)

Small-bowel biopsies were taken from the distal part of the duodenum or jejunum upon upper gastrointestinal endoscopy or by Watson capsule. The specimens were processed and stained with haematoxylin-eosin and studied under light microscopy. Subsequently, morphometrical analysis, villous height (Vh) and crypt depth (CrD) determination, was made from the sections and the Vh/CrD-ratio counted (Kuitunen et al. 1982). A ratio <2 was considered compatible with coeliac disease. Finally, the coeliac disease diagnosis was based on the finding of severe partial, subtotal or total villous atrophy with crypt hyperplasia in the small bowel specimen and on clinical and/or histological response to a gluten-free diet (Walker-Smith et al. 1990, Marsh 1992). In non-coeliac disease controls the Vh/CrD-ratio was ≥2. Additionally, in three control patients in study **IV** small-bowel biopsies were also frozen and stained for γδ+ TcR-bearing IELs and the cell densities, expressed as cells per millimetre of epithelium, were calculated as previously described (Järvinen et al. 2004, Salmi et al. 2006a). The reference values were set at 4.3 cells/mm for γδ+ IELs

(Järvinen et al. 2004, Salmi et al. 2006a). Further, tTG-targeted extracellular IgA deposits were evaluated in these patients from the frozen sections in the manner described elsewhere (Korponay-Szabó et al. 2004). In coeliac disease subepithelial IgA deposits are co-localized with the extracellular tTG along the surface and the crypt basement membrane and around mucosal vessels, whereas normally IgA is only detected within plasma and epithelial cells (Korponay-Szabó et al. 2004, Salmi et al. 2006a).

3.4. HLA-typing (II, IV)

HLA DQ alleles encoding HLA DQ2 and DQ8 were analysed in all coeliac disease patients and non-coeliac controls enrolled in study II and in three non-coeliac disease controls evaluated in study IV at the Department of Tissue Typing, Finnish Red Cross Blood Service, Helsinki, Finland, using the Dynal SSP low-resolution DQ typing kit (Dynal AS, Oslo, Norway).

3.5. Statistical analysis (I-IV)

The sensitivities, specificities, negative and positive predictive values and efficiencies of the coeliac antibody tests were calculated as described in study IV. Additionally, in study I the Wilcoxon signed rank test was used in comparing serum tTG-ab levels in the hr-tTG ELISA before and after dietary intervention. A probability of <0.05 was considered significant.

3.6. Ethical considerations (I-IV)

The study protocol was approved by the Ethical Committees of Tampere University Hospital, Tampere, Finland (I-IV) and Heim Pál Children's Hospital, Budapest, Hungary (I, III, IV). Informed consent was obtained from all participants.

4. RESULTS

4.1. Whole blood IgA self-tTG in house point-of-care testing (*I, II*)

4.1.1. In house point-of-care testing in a laboratory setting (*I, II*)

The whole blood IgA self-tTG in house POCT had 92% sensitivity and 98% specificity for untreated coeliac disease when testing was carried out in the laboratory on 251 stored whole blood samples (Table 12). The corresponding figures for the serological IgA-class in house EMA were 93% (139/150) and 100% (101/101) and for hr-tTG ELISA 95% (143/150) and 100% (101/101) among the same patients (**I, II**). All patients were IgA-competent. In a selected series where EMA prevalence was low among the 51 untreated coeliac patients, the in house POCT was as sensitive (82%) as the in house EMA (80%), whereas the hr-tTG ELISA was superior to both (sensitivity 88%) (**II**). In this material one (2%) of the untreated coeliac patients did not show positivity in any of the three tests, while one (2%) was detected only by the in house POCT. All coeliac disease patients had an HLA type consistent with coeliac disease (**II**).

The in house POCT proved to be gluten-dependent, as positive test results seroconverted to negative or the test reaction at least weakened in 90% of the originally POCT-positive coeliac patients studied before and after one year's gluten-free dietary treatment (Figure 3 in original publication **II**) (**II**). The in house POCT was negative in altogether 85% of 48 treated coeliac patients (Table 12), the in house EMA in 88% and the hr-tTG ELISA in 85% (**II**). Four treated coeliac disease patients admitted dietary lapses, two of them (50%) showing positivity in the in house POCT and in the serological in house EMA and hr-tTG ELISA. Altogether 22 coeliac disease patients still evinced small-bowel mucosal villous atrophy despite the dietary treatment. In house POCT was positive in 32% of them, in house EMA in 23% and hr-tTG ELISA in 27% (**II**). None of the treated coeliac patients having normal small-bowel mucosal morphology showed positivity in the in house POCT, whereas one patient was positive in the serological in house EMA and hr-tTG ELISA (**II**).

The relation between IgA-class in house POCT and in house EMA in the patient series where EMA prevalence was low among untreated coeliac patients and in which stored samples were applied, is shown here in Figure 4 (**II**) and between in house POCT and hr-tTG ELISA in Figure 2 in original publication **II**.

Table 12. *Frequency of test positivity in untreated and treated coeliac disease (CD) and in non-CD controls studied in the laboratory in stored samples and sensitivities, specificities, positive (PPV) and negative predictive values (NPV) and efficiencies (ET) of the whole blood IgA self-tissue transglutaminase (tTG) in house point-of-care test (POCT), enzyme-linked immunosorbent assay (ELISA) and rapid test 1 and 2 and of the serum-based IgA in house endomysial antibody (EMA) and IgA/IgG commercial EMA test and IgA tTG antibody ELISA using human recombinant (hr)- or native human red blood cell (nh)-derived tTG as antigen in untreated biopsy-proven CD and biopsied non-CD controls.*

	Laboratory testing							
	Whole blood IgA self-tTG testing				Serum testing			
	in house POCT **(I, II)**	ELISA **(III)**	rapid test 1 **(III)**	rapid test 2 **(II, IV)**	in house EMA **(I-IV)**	commercial EMA **(III)**	hr-tTG ELISA **(I-IV)**	nh-tTG ELISA **(III)**
Untreated CD, positive test results, n	138/150	126*/139	129*/139	117/121	245/257	138/139	248/257	138*/139
Non-CD controls, positive test results, n	2/101	2/103	6/103	7/107	0/182	0/103	1/182	7/103
Sensitivity (%)	92	91	93	97	95	99	96	99
Specificity (%)	98	98	94	93	100	100	99	93
PPV (%)	99	98	96	94	100	100	100	95
NPV (%)	89	89	91	96	94	99	95	99
ET (%)	94	94	93	95	97	100	98	97
Treated CD, positive test results, n	7†/48	-	-	5/106	9†/139	-	8†/139	-

* One untreated CD patient yielding a negative test result was IgA-deficient
† Two patients admitted dietary lapses
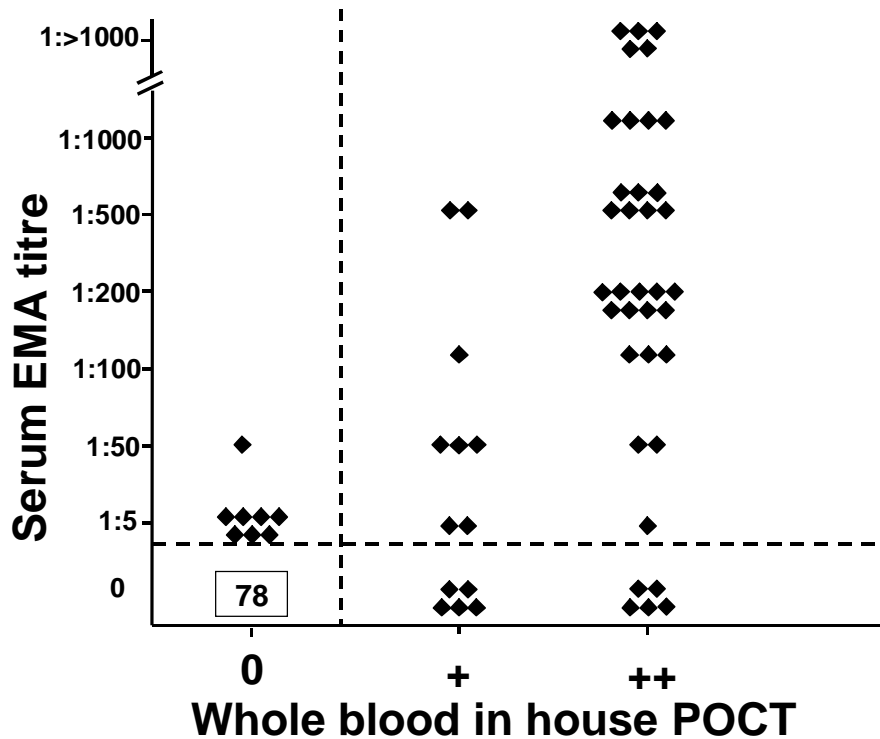
Figure 4. *Relation between the whole blood IgA self-tissue transglutaminase in house point-of-care test (POCT) and the serum IgA-class endomysial antibody (EMA) test results in altogether 135 untreated and treated coeliac disease and in non-coeliac disease controls (II). The cut-off titre for EMA positivity (1:5) is shown by a horizontal dotted line and for the in house POCT positivity by a vertical line.*

Interobserver agreement on the whole blood IgA self-tTG POCT between different evaluators was 97% when testing was carried out in the laboratory. Furthermore, POCT results were reproducible in 94% of testings, when seven positive, three weakly positive and ten negative samples were tested five times by three observers. All eight coeliac whole blood samples stored after collection frozen without thawing for up to 36 months gave a positive POCT result upon thawing. The precoated Nunc-immunosticks were functional even after nine months of storage at +4°C (**I**).

All ten IgA-class EMA- and tTG-ab-positive serum samples tested negative if the in house POCT was carried out with the sera without red blood cells. Further, POCT results were negative if testing was carried out with sera together with tTG-deficient red blood cell lysate derived from tTG-knockout mice. In contrast, positive in house POCT results were obtained when the sera were tested with normal mouse red blood cells. These findings would suggest that whole blood IgA self-tTG testing recognized specifically tTG and no other blood components or potential antibodies to them contributed to the colour reaction.

*4.1.2. Prospective in house point-of care testing in an office setting (**I**)*

Altogether 39 out of 165 (24%) new patients under suspicion of having coeliac disease were whole blood IgA self-tTG in house POCT-positive when the in house POCT was carried out prospectively on site (Table 13). Correspondingly, serum IgA-class in house EMA was positive in 37 out of 165 (22%) and serum IgA-class hr-tTG ELISA in 37 out of 147 (25%) patients in whom serological testing was also carried out. Of the 165 patients studied on site, 120 were sent for small-intestinal biopsy on clinical grounds. In the biopsied patients the in house POCT had a sensitivity of 97%, a specificity of 98%, a positive predictive value of 95% and a negative predictive value of 99% for untreated coeliac disease (Table 13). The corresponding figures for the serological IgA-class in house EMA were 97%, 100%, 100% and 99% and hr-tTG ELISA 97%, 100%, 100% and 99%, respectively (Table 13). The in house POCT correctly revealed IgA deficiency in one prospectively enrolled patient (IgA control line negative). This patient was also negative in the serological IgA-class in house EMA and hr-tTG ELISA tests and was later shown to be suffering from coeliac disease.

When 263 known coeliac disease patients on a gluten-free diet were studied on site prospectively, the in house POCT again identified all nine IgA-deficient samples by absence of the IgA-positive control line. The in house POCT was positive in 52 of the 254 (20%) IgA-competent patients, the serum in house EMA in 50 (20%) and hr-tTG ELISA in 47 (19%) (Table 4 in original publication **I**). Dietary transgressions were admitted in 17 cases and the in house POCT was positive in 15 of them (88%), the in house EMA in 16 (94%) and the hr-tTG ELISA in 16 (94%). In contrast, among those on a strict gluten-free diet for more than six months the in house POCT was positive in only 11 (6%) of 186 coeliac disease patients, the in house EMA in 9 (5%) and the hr-tTG ELISA in 10 (5%).

The aforementioned treated coeliac disease patients yielding a positive in house POCT result received intensified dietary instructions on site and 16 of them were re-evaluated 3-6 months after the initial testing. In house POCT and in house EMA test results seroconverted to negative in 12 (75%) of these patients. Likewise, the antibody levels in the serum hr-tTG ELISA decreased significantly (p<0.001) (Figure 2a in original publication **I**). In contrast, the control group of 14 serum in house EMA-positive treated coeliac disease patients who did not participate in in house point-of care testing received the information of their positive EMA results and the instructions to improve the diet by mail. Seroconversion to negative in the in house EMA was seen in only four of them (29%) when re-assessed after 3-6 months. Moreover, no significant reduction (p=0.57) in serum antibody levels in the hr-tTG ELISA was shown among these patients (Figure 2b in original publication **I**).

Table 13. *Frequency of test positivity in the whole blood IgA self-tissue transglutaminase (tTG) in house point-of-care test (POCT) and rapid test 2 and in the serum-based IgA in house endomysial antibody (EMA) test and tTG antibody enzyme-linked immunosorbent assay (ELISA) using human recombinant (hr) tTG as antigen among patients with coeliac disease (CD) suspicion studied prospectively on site.*

	On site testing * Whole blood IgA self-tTG testing		Serum testing	
	in house POCT (**I**)	rapid test 2 (**IV**)	in house EMA (**I, IV**)	hr-tTG ELISA (**I, IV**)
Untreated biopsy-proven CD, positive test results, n (%)	37/38† (97)	44/47 (94)	83/85† (98)	83/85† (98)
Non-CD controls, positive test results, n (%)	2/127‡ (2)	3/103§ (3)	0/230¶ (0)	0/212¶ (0)
Treated CD positive test results, n (%)	52/254 (20)	-	50/254 (20)	47/254 (19)

* Whole blood-based IgA self-tTG in house POCT and rapid test 2 results were available immediately on site in the office
† One untreated CD patient yielding a negative test result had selective IgA deficiency
‡ Eighty-two of the patients were biopsied and evinced normal small-bowel mucosal morphology
§ Two of the rapid test 2-negative patients were biopsied and evinced normal small-bowel mucosal morphology
¶ Eighty-four of the patients were biopsied and evinced normal small-bowel mucosal morphology

4.2. Commercial whole blood IgA self-tTG ELISA testing in a laboratory setting (*III*)

The whole blood IgA self-tTG ELISA results obtained from 242 intact stored whole blood samples are shown in Table 12. Whole blood samples from 11 untreated coeliac disease patients and from four non-coeliac controls were found to be clotted or dried after thawing, and these cases were excluded from further evaluations. The whole blood self-tTG ELISA had a sensitivity of 91% and a specificity of 98% in the laboratory, whereas the corresponding figures for the serological in house EMA were 99% and 100%, commercial EMA 99% and 100%, hr-tTG-ELISA 99% and 99% and nh-tTG ELISA 99% and 93% (Table 12). A comparison of coeliac autoantibody test results between the different tests among altogether 242 untreated coeliac disease and non-coeliac disease controls is shown in Figure 1 in original publication **III**.

4.3. Commercial whole blood IgA self-tTG rapid testing (*II-IV*)

4.3.1. Rapid testing in a laboratory setting (*II-IV*)

The commercial whole blood IgA self-tTG rapid test 1 had 93% sensitivity and 94% specificity for untreated coeliac disease when testing was carried out in the laboratory from intact 242 stored whole blood samples (Table 12) (**III**). The corresponding figures for the serological EMA and tTG-ab tests were similar or higher (Table 12). A comparison of coeliac autoantibody test results between the different tests among altogether 242 untreated coeliac disease and non-coeliac disease controls is shown in Figure 1 in original publication **III**. Moreover, sensitivity for the other prototype of the commercial whole blood self-tTG rapid tests, rapid test 2, was 97% and specificity 93% on 228 stored whole blood samples, while the serological in house EMA had 97% sensitivity and 100% specificity in this study group and hr-tTG ELISA 99% and 99%, respectively (**II, IV**).

The whole blood IgA self-tTG rapid test 2 was also assessed in treated coeliac disease in a laboratory setting (Table 12) (**IV**). Firstly, 15 of the aforementioned coeliac patients were re-evaluated after one year on a gluten-free diet (**II, IV**). In 13 (87%) the rapid test results converted from positive to negative in the follow-up, the serum in house EMA in 15 (100%) and hr-tTG ELISA in 15 (100%) (Figure 2 in original publication **IV**). When 91 long-term treated coeliac patients were studied, rapid test 2 was negative in 88 of them (97%), the in house EMA in 88 (97%) and the hr-tTG ELISA in 90 (99%) (**IV**). Only three of the long-term treated coeliac disease patients evinced small-bowel mucosal villous atrophy with crypt hyperplasia. Rapid test 2 was positive in two (67%) of them, as was the serum in house EMA, whereas the serum hr-tTG ELISA was positive in only one (33%) of them.

Both inter- and intraobserver agreement for whole blood IgA self-tTG rapid testing was shown to be 100% when testing was carried out in the laboratory with 20 randomly selected whole blood samples (**IV**).

4.3.2. Prospective rapid testing in an office setting (*IV*)

When 150 coeliac suspects were tested on site in the office, the whole blood IgA self-tTG rapid test 2 was positive in 47 (31%) of them, including 36 symptomatic patients and 11 first-degree family members, and the serological in house EMA and hr-tTG ELISA in 46 (31%) (Table 13). In relation to the serological in house EMA and hr-tTG ELISA, the rapid test 2 had a sensitivity of 96% and a specificity of 97%. Forty-four of the rapid test 2-positive patients agreed to undergo small-intestinal biopsy and all evinced mucosal atrophy with crypt hyperplasia consistent with coeliac disease (positive predictive value 100%). Additionally, three of the 103 (3%) rapid test 2-negative patients also underwent intestinal biopsy, having yielded a positive test result in in house

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EMA (n=1) or hr-tTG ELISA (n=1) or both (n=1). All of these patients were found to have coeliac-type small-bowel mucosal villous atrophy with crypt hyperplasia. A further two rapid test 2- and serum EMA and tTG-ab test-negative patients were biopsied and evinced normal small-bowel mucosal morphology.

4.4. Coeliac antibody test agreement (*I-IV*)

Agreement between the whole blood IgA self-tTG tests and the serological EMA and tTG-ab tests is shown in Table 14. Agreement between the different self-tTG tests applied in studies **I-IV** was also calculated, as some of the retrospectively enrolled patients in studies **I-IV** were the same (Table 15). In treated coeliac disease, agreement between the in house POCT and the serum in house EMA was 90% and between the in house POCT and the serum hr-tTG ELISA 92% when tested in the laboratory and 94% and 92% when testing was carried out on site (**I**). Furthermore, whole blood rapid test 2 agreed in 96% of cases with the serum in house EMA and hr-tTG ELISA when treated coeliac disease patients were tested in the laboratory (**IV**).

Table 14. *Agreement between the whole blood-based IgA self-tissue transglutaminase (tTG)-based tests - in house point-of-care test (POCT), enzyme-linked immunosorbent assay (ELISA) and rapid test 1 and 2 - and the serum-based tests - IgA in house endomysial antibody (EMA) and IgA/IgG commercial EMA test and IgA tTG antibody ELISA using human recombinant (hr)- or native human red blood cell (nh)-derived tTG as antigen - in laboratory-tested untreated coeliac diesase (CD) patients and non-CD controls and among patients under suspicion of CD studied prospectively on site.*

Setting	Serum testing			
Whole blood IgA self-tTG testing method	in house EMA agreement n (%)	commercial EMA agreement n (%)	hr-tTG ELISA agreement n (%)	nh-tTG ELISA agreement n (%)
Laboratory testing				
In house POCT	232/251 (92)	76/76 (100)	238/251 (95)	75/76 (99)
Self-tTG ELISA	227/242 (94)	226/242 (93)	228/242 (94)	221/242 (91)
Rapid test 1	226/242 (93)	226/242 (93)	225/242 (93)	220/242 (91)
Rapid test 2	215/228 (94)	128/130 (98)	216/228 (95)	123/130 (95)
On site testing				
In house POCT*	163/165 (99)	-	145/147 (99)	-
Rapid test 2*	145/150 (97)	-	145/150 (97)	-

* Whole blood-based IgA self-tTG in house POCT and rapid test 2 results were available immediately on site in the office

Table 15. *Agreement between the different whole blood IgA self-tissue transglutaminase (tTG) tests, in house point-of-care test (POCT), enzyme-linked immunosorbent assay (ELISA) and rapid test 1 and 2, in untreated coeliac disease (CD) and non-CD controls tested in the laboratory.*

	Agreement n (%)
In house POCT and whole blood self-tTG ELISA	70/76 (92)
In house POCT and rapid test 1	72/76 (95)
In house POCT and rapid test 2	106/114 (93)
Whole blood self-tTG ELISA and rapid test 1	223/242 (92)
Whole blood self-tTG ELISA and rapid test 2	124/130 (95)
Rapid test 1 and rapid test 2	122/130 (94)

5. DISCUSSION

5.1. Coeliac disease case finding with the whole blood IgA self-tTG-based and the conventional serum-based coeliac antibody tests

Coeliac disease is a common though considerably undiagnosed (Table 6) autoimmune disorder triggered by dietary gluten in genetically susceptible individuals. The disease presents with diverse symptoms and manifestations of variable degree and, when active, coeliac autoantibodies against the autoantigen, tTG, are found in the circulation (Dieterich et al. 1997, Mäki et al. 2003, Tommasini et al. 2004). The coeliac disease diagnosis itself is based on the finding of mucosal villous atrophy with crypt hyperplasia in a small-bowel biopsy sample (Walker-Smith et al. 1990). As the condition is difficult to recognize solely on clinical grounds, serological coeliac autoantibody testing by for example the accurate EMA and tTG-ab tests (Table 4) is recommended and widely used in coeliac disease case finding and referral for invasive diagnostic small-bowel biopsy (Hill et al. 2005, Rostom et al. 2005, Rostom et al. 2006). These conventional coeliac autoantibody tests, however, are performed in centralized laboratories by expert personnel from sera, and the test results are available for decision-making only after a time lag. For these reasons there has been call for a more feasible and readily available coeliac screening method, and hence a few simpler coeliac antibody rapid tests have been developed (Table 5). Nonetheless, these tests likewise require external coeliac autoantigen or wheat gliadin in the testing, and are mainly conducted using serum samples.

The coeliac autoantigen, tTG, is primarily an intracellular enzyme present in for example erythrocytes of whole blood (Bergamini et al. 1999, Lorand and Graham 2003). In the present study it was shown for the first time that coeliac autoantibodies, tTG-ab, can also be detected accurately by a novel and simple whole blood IgA self-tTG method, using a whole blood sample's endogenous erythrocyte tTG, self-tTG, as antigen. The method proved suitable for rapid on site coeliac antibody testing, utilizing either the Nunc-Immunostick or the lateral flow immunochromatographic strip system, as well as for quantitative antibody detection in a laboratory setting using the traditional ELISA format.

In whole blood IgA self-tTG testing self-tTG was first liberated from the erythrocytes of the whole blood sample by haemolysis. If IgA-class tTG-ab was present in the sample, it then immunocomplexed with the liberated self-tTG, and the complexes were captured from the sample by tTG-binding proteins onto a solid surface and detected with labelled anti-human IgA (Figure 1 in original publication **II** and Figure 2 here). This innovative self-tTG method was first proved to be tTG-specific; when tTG-deficient red blood cell lysate derived from tTG knockout mice together with coeliac autoantibody-positive sera was used for whole blood self-tTG testing, test-positivity was not seen. In contrast, when normal mouse red blood cells, containing tTG, were applied with the same sera, test results were positive (**I**). The method was, subsequently, evaluated as a means of detecting untreated coeliac disease, first with the visual proof-of-

concept test, Nunc-Immunostick-based rapid in house POCT (**I, II**). The in house POCT proved reliable in disclosing coeliac disease among both children and adults, having a sensitivity of 92% and a specificity of 98% when stored whole blood samples were employed for testing retrospectively (Table 12) (**I, II**). The test was further evaluated in finding new coeliac disease cases at point-of-care, in other words at doctor's consultation, using fresh whole blood samples from 165 patients. In this prospective assessment the in house POCT again proved accurate, having a sensitivity of 97% and a specificity of 98% for biopsy-proven coeliac disease (Table 13) (**I**). Moreover, the test was easy to carry out on site and results were available rapidly. As the self-tTG method itself proved to be sensitive and specific for untreated coeliac disease, the test was further developed by the industry to ELISA format, which is suitable for for example mass-screening purposes, and it was shown here also to be applicable for coeliac disease case finding, with a sensitivity of 91% and a specificity of 98%. Correspondingly, even more rapid and user-friendly commercial test kits, the rapid test 1 and the rapid test 2, using the lateral flow immunochromatographic strip system, were generated. In the present study the rapid tests were shown to be suitable for IgA-class tTG-ab detection first in the laboratory with a sensitivity of 93% and 97% and a specificity of 94% and 93%. Further, rapid testing turned out to be eminently repeatable and reproducible (inter- and intraobserver agreement 100%). Rapid test 2 was further performed prospectively at the point-of-care among patients with coeliac disease suspicion using fresh fingertip blood. In this setting a positive test result was shown to be highly predictive of biopsy-proven coeliac disease (positive predictive value 100%) (Table 13). Additionally, the rapid testing could be carried out quickly in office facilities, in five to ten minutes, and the test result was easy to interpret visually.

5.2. *The whole blood IgA self-tTG and the conventional serum coeliac antibody tests in dietary follow-up of coeliac disease*

Currently, coeliac disease can be treated solely with a gluten-free diet. Strict dieting is essential in order to avoid late complications of untreated coeliac disease such as osteoporosis or malignancies (Kemppainen et al. 1999, Mustalahti et al. 1999, Askling et al. 2002, Green et al. 2003). Upon initiation of a strict diet coeliac autoantibody levels have been shown to decrease in the circulation parallel with the recovery of small-bowel mucosal damage (Sategna-Guidetti et al. 1996). Therefore, autoantibody tests have been recommended and widely used in the dietary follow-up of coeliac disease (Hill et al. 2005, Rostom et al. 2006). In the current study whole blood IgA self-tTG testing was also shown to be gluten-dependent, as positive test results weakened in 90% of one-year treated coeliac patients when the in house POCT was used (Table 12 here and Figure 3 in original publication **II),** and seroconverted to negative in 87% of

cases when rapid test 2 was used (Figure 2 in original publication **IV**). Moreover, when the in house POCT was applied prospectively in an assessment of gluten-free dietary treatment of known coeliac patients, the test results were negative in 20% of IgA-competent patients (Table 13) (**I**). Additionally, rapid test 2 showed positivity in only 3% of the long-term treated coeliac patients (**IV**).

It should be noted, however, that the value of the coeliac antibody tests so far developed in the dietary follow-up of coeliac disease is limited: test results may remain negative despite occasional dietary transgressions and a test result can seroconvert from positive to negative despite ongoing small-bowel mucosal damage (Table 7 and Table 8) (Troncone et al. 1995, Dickey et al. 2000a, Kaukinen et al. 2002b). On the other hand, the antibody tests help in revealing major dietary transgressions (Troncone et al. 1995), as a positive test result implies in addition to poor dietary compliance also unsatisfactory mucosal response in coeliac disease (Troncone et al. 1995, Dickey et al. 2000a, Kaukinen et al. 2002b). Furthermore, after adoption of the diet a decrease in antibody levels or seroconvertion of a test result from positive to negative is an indication of a favourable response to the diet. Thereafter, an increase in antibody level or reappearance of coeliac antibody positivity in the tests is consistent with ongoing gluten consumption and/or small-bowel mucosal damage, a subject which should be discussed carefully with the patient.

The results of the present study show that the self-tTG tests functioned acceptably and in the same manner as the serum-based coeliac autoantibody tests in revealing dietary non-adherence or ongoing villous damage in coeliac patients on a gluten-free diet. The in house POCT was positive in two of the four treated patients who admitted dietary lapses and in one third of those who still evinced small-bowel mucosal villous atrophy despite the treatment, whereas no test positivity was registered among patients having normal small-bowel mucosal morphology (**II**). When used prospectively on site in an office, the in house POCT was positive in only 6% of prospectively studied coeliac patients who had been on a strict gluten-free diet for over half a year (Table 4 in original publication **I**). Rapid test 2, in turn, was positive in two of the three long-term treated coeliac disease patients still showing mucosal villous atrophy with crypt hyperplasia in small-bowel biopsy samples.

In the present study the whole blood IgA self-tTG ELISA was not evaluated in treated coeliac disease. However, the test can be expected to work similarly to the other self-tTG tests in dietary follow-up. The limitation of the self-tTG in house POCT and the rapid tests, unlike the self-tTG ELISA, is that they give an IgA-class tTG-ab result only as positive or negative. Therefore, seroconvertion can be detected with these tests, but not an initial decrease or increase in autoantibodies when the test result is positive.

It has already been shown that regular follow-up and guidance is important in promoting coeliac patients' dietary adherence (Ljungman and Myrdal 1993, Bardella et al. 1994). As self-tTG rapid testing can be done on site in a doctor's office for coeliac disease patients at their check-up visit, test results are available for immediate evaluation of the dietary treatment. It was shown in this series that feedback and intensified dietary advice given face to face to long-term treated

coeliac patients after yielding a positive self-tTG test result in the office encouraged them to adhere to the diet more strictly. In the follow-up antibody test results converted to negative in 75% of the patients studied with the self-tTG in house POCT. In contrast, when solely the serological tests were applied in the dietary follow-up of the long-term treated patients and information on positive test results and instructions to improve the diet were sent by mail, only 29% of the patients reverted to negative in the in house EMA.

5.3. Challenges in whole blood IgA self-tTG testing

In this study series some test positivity was detected with the whole blood IgA self-tTG tests among the non-coeliac disease control patients (Table 12), but this has often been the case when other serum-based coeliac autoantibody tests have been applied (Tables 2-5). Moreover, a false-positive test result should not be considered a major problem as, based on the current diagnostic criteria for coeliac disease, untreated coeliac disease should in any case be excluded or verified by the investigation of small-bowel mucosal morphology (Walker-Smith et al. 1990). It has been proposed that coeliac antibodies may increase in the circulation only transiently, while the small-intestinal mucosa remains intact (Simell et al. 2005, Johnston et al. 1998). However, it has also been shown that patients with positive coeliac serology but normal small-intestinal mucosal morphology may suffer from early developing coeliac disease, in which overt mucosal villous atrophy will indeed develop if gluten consumption is continued (Kaukinen et al. 2001, Järvinen et al. 2004, Salmi et al. 2006a). In the present study three rapid test 2-positive non-coeliac disease controls were investigated more carefully beyond this study and all showed signs of early developing coeliac disease: all had coeliac-type HLA and an increased density of $\gamma\delta+$ IELs or tTG-specific IgA deposits in their small-bowel mucosa (data not shown) (Korponay-Szabó et al. 2004, Salmi et al. 2006a) (**IV**). Hence it can be hypothesized that at least some individuals regarded as non-coeliac controls in the study and tested "falsely" positive in the self-tTG tests in fact suffered from genetic gluten intolerance.

As shown here (Table 12) (**II**) and also in previous studies (Dickey et al. 2000b, Collin et al. 2005), a subgroup of untreated IgA-competent coeliac patients do not shown positivity in any coeliac antibody tests. This seronegativity might be associated with a lesser degree of villous atrophy (Rostami et al. 1999, Abrams et al. 2004, Abrams et al. 2006). On the other hand, it has been suggested to affect coeliac patients of older age and with a more severe clinical picture (Salmi et al. 2006b). For these reasons a subject should be sent directly to diagnostic endoscopy despite negative coeliac serology in cases where the clinician has a strong suspicion of coeliac disease. The results of the present study show, however, that whole blood IgA self-tTG testing is as valuable as the traditional serological tests in detecting untreated coeliac disease patients with a low prevalence of coeliac autoantibodies (**II**).

A further limitation of all IgA-class coeliac antibody tests commonly used is that they miss untreated coeliac disease patients suffering from selective IgA deficiency, a condition which is more prevalent among coeliac disease patients than in the general population (Collin et al. 1992b, Cataldo et al. 1998). Information on the IgA status of a patient tested for IgA-class coeliac antibodies, or determination of the antibodies in IgG-class (Cataldo et al. 2000, Korponay-Szabó et al. 2003a), is thus crucial for valid decision-making. In study **I** total plasma IgA detection was chosen as the positive test control in the in house self-tTG POCT. This indeed helped us to pick out correctly all IgA-deficient patients; one among the prospectively studied individuals with coeliac disease suspicion who was later shown to be suffering from coeliac disease, and nine among prospectively studied known coeliac disease patients on a gluten-free diet. It is thus safe to claim that total IgA detection can be combined with the self-tTG testing and, in fact, a commercial total IgA-detecting whole blood IgA self-tTG rapid test is now available. However, this test was developed only recently and was thus not evaluated in the present study.

Notwithstanding the fact that the accuracy of the gold standard of coeliac disease diagnosis, small-bowel mucosal morphology, has itself lately been questioned (Kaukinen et al. 2001), it should be remembered that for the time being small-bowel biopsy remains requisite for the diagnosis (Walker-Smith et al. 1990). Consequently, all coeliac antibody tests serve as first step in algorithms for diagnosing coeliac disease, and the testing is effective only if action is taken on the test results. After yielding a positive coeliac antibody test result a patient should be sent for diagnostic small-intestinal biopsy and, if coeliac disease is discovered, a strict gluten-free diet should be prescribed.

In the current study altogether 15 stored and subsequently thawed whole blood samples were discarded from further evaluation as they were clearly seen to be damaged after storage due to clotting or drying (**III**). When the whole blood self-tTG tests are applied, the sample tested should be of good quality with a view to exploitation of red blood cell self-tTG. tTG is sensitive to heat, storage and oxidation, which may alter its antigenic properties, and might if damaged prove incompetent to form immunocomplexes with its serum autoantibodies (Dieterich et al. 1997, Bergamini et al. 1999, Seissler et al. 2001). In order to obtain reliable whole blood self-tTG test results multiple freezing, thawing and prolonged storage of samples should be avoided. Furthermore, clotted samples should not be used and fresh whole blood samples should in fact probably be preferred. When the self-tTG ELISA was carried out in further investigations with damaged and discarded samples, results were often negative. On the other hand, when the counterpart serum samples were mixed with fresh coeliac antibody-negative whole blood containing undamaged red blood cells, all false-negative test results, except one, were again positive (data not shown). It can thus be speculated that the accuracies of the whole blood IgA self-tTG ELISA, and rapid test 1 studied with the same material as the self-tTG ELISA might improve if whole blood samples with functional tTG able to form tTG-ab-tTG-immunocomplexes were used, similarly to POCT (**I**) and rapid test 2 testing (**IV**).

5.4. Comparison of the coeliac antibody tests

All previous coeliac autoantibody tests have used purified or human recombinant coeliac antigen in antibody detection. Additionally, serum samples are required for the testing, except in one tTG-ab dot blot assay (Baldas et al. 2000). In the traditional tTG-ab ELISA tests the plate is coated with tTG autoantigen, which may expose the protein in distorted or non-physiological ways. In contrast, in whole blood self-tTG testing the endomysial-type tissue structure to detect coeliac-specific antibodies is formed simply by a, for example, gelatin-coated test surface in which a whole blood sample's fibronectin and fresh red blood cell self-tTG are further bound (Figure 2 in the thesis and Figure 1 in original publication **II**) (Korponay-Szabó et al. 2003b). Serum separation and external coeliac antigen is thus not needed in the testing. These advantages ensure a long shelf life for the whole blood IgA self-tTG test kits and suggest that the tests using fresh tTG as antigen may overcome kit storage problems in demanding conditions such as exceptional storage temperatures and humidity.

When compared to the serum indirect IF EMA test, the whole blood self-tTG ELISA has the advantage of being objective in interpretation, and all self-tTG tests are easier and faster to carry out than the EMA test. The self-tTG in house POCT and rapid tests are observer-dependent, but nevertheless, unlike EMA testing, where test results are greatly influenced by expert reading, in house POCT and rapid test result interpretation is obviously easier. In the current series the in house POCT yielded high agreement between untrained and trained personnel and rapid test 2 showed 100% inter- and intraobserver agreement.

It was shown here that the somewhat cumbersome and complex serological EMA and tTG-ab tests, which should be done in a controlled laboratory environment by trained staff in order to obtain reliable test results, were slightly more accurate than whole blood IgA self-tTG tests in detecting untreated coeliac disease (Table 12). It is noteworthy, however, that despite the availability of today's numerous accurate coeliac antibody screening tools (Rostom et al. 2005), as many as 90% of untreated coeliac disease patients, among whom are also symptomatic patients, may still remain completely unrecognised (Korponay-Szabó et al. 2007, Ravikumara et al. 2007) or the condition is diagnosed only after a long delay (Zipser et al. 2003). One may thus speculate that a low-threshold, readily available, rapid and economical coeliac antibody test is needed to uncover these undetected coeliac disease patients. To date, a few rapid tests which can be carried out without a need for specialized personnel or laboratories have been developed (Table 5). Although more feasible than the serum indirect IF-based EMA and the ELISA-based tTG-ab, these rapid tests require mainly serum samples. This in turn involves venous puncture and a need for a centrifuge, which does not belong to the basic armoury of, for example, a family doctor's office. To the author's knowledge, only one whole blood-based rapid test, a dot blot assay using human recombinant tTG as antigen, has hitherto been developed (Baldas et al. 2000), and so far only limited data are available on the test and its value for instance in clinical practice is not known.

The whole blood IgA self-tTG method seems to be suitable for a wide range of conditions. For example, it can be used in coeliac antibody mass screening in a laboratory setting, when the ELISA format is applied, or when the in house POCT or the commercial rapid test applications are used, IgA-class tTG-ab can be determined even in remote or impoverished areas lacking centralized laboratories, sample storing possibilities or equipment for serum separation. In the present series the in house POCT and rapid test 2 were already shown to perform comparably to the well-established serum-based tests also prospectively when testing was carried out in an office setting with fresh whole blood samples and the test results were read immediately on site by health care personnel (Table 13). Conversely, samples for the serological in house EMA and hr-tTG ELISA tests had to be sent to the laboratory and the test results were available only after a time lag. As the self-tTG rapid tests have been accepted for home testing and are CE-marked, and all equipment needed for the testing is provided with the test kit, even patients themselves can use the tests at home in coeliac disease case finding or coeliac disease diet monitoring. However, to date the value of the rapid tests in detecting coeliac antibodies in a home setting has not been evaluated.

In addition to good concordance with small-bowel mucosal findings of coeliac disease (Table 12 and 13), the whole blood IgA self-tTG test results were in marked agreement with those of the established serological EMA and tTG-ab tests, especially when fresh whole blood samples were used for self-tTG testing (Table 14). Some discordance was however detected between the self-tTG tests and the serum-based EMA and tTG tests (Table 14), but also between the different serum-based coeliac autoantibody tests (Figure 1 in original publication **III**), although they are presumed to measure the same autoantibodies (Korponay-Szabó et al. 2000, Korponay-Szabó et al. 2003b). This has also been the case in previous studies, where coeliac autoantibody test results have been discrepant in 1-30% of untreated coeliac disease and control cases (Troncone et al. 1999, Dickey et al. 2001, Bürgin-Wolff et al. 2002, Tesei et al. 2003). Explanations for this have been speculated; some additional antigens other than tTG may trigger EMA formation (Dickey et al. 2001) or there may be interspecies differences in the antigens and substrates used in the testing (Troncone et al. 1999, Dickey et al. 2001). Furthermore, in different methods, for example the in EMA tests using indirect IF and the tTG-ab tests using ELISA, antigenic tTG epitopes might be exposed in different ways. On the other hand, discrepant test results are also obtained with test kits involving similar substrates or testing methods (Martini et al. 2002, Wong et al. 2002, Van Meensel et al. 2004). This was also shown here when the results of different whole blood IgA self-tTG tests were compared with each other (Table 15). The discrepancy between the self-tTG tests might partly be explained by different reagents and test formats applied. Additionally, sample quality might have influenced the result, as agreement figures shown in Table 15 and also partly in Table 14 were calculated using retrospectively studied stored whole blood sample material. All in all, however, it is obvious that no definitive coeliac antibody test exists.

5.5. Whole blood IgA self-tTG testing and future prospects

In the present series novel whole blood IgA self-tTG-based testing not requiring serum separation or external coeliac antigen was evaluated for the first time in case finding and dietary treatment of coeliac disease. The assessment was therefore first carried out in a selected high-risk population in which the prevalence of coeliac disease was markedly greater than in the general population (Table 6), and comparison was made to the traditional serum-based coeliac antibody tests and small-bowel mucosal morphology. As the self-tTG testing was here proven reliable in coeliac antibody detection in both laboratory and office setting, further studies are now needed to ascertain its accuracy in varying patient groups and situations such as a home setting. Moreover, to the author's knowledge no studies have been conducted with the total IgA-detecting self-tTG rapid test. It is further desirable that whole blood self-tTG-based tests detecting tTG-ab also in IgG-class be developed in the future.

Some additional studies with the self-tTG rapid test applications, however, have already been conducted: the method also proved accurate for Nemec and associates (2006) in prospective case finding of coeliac disease as well as in coeliac disease screening in Brazil (Crovella et al. 2007). The latter study showed, however, that the rapid test might yield false positivity among subjects suffering from parasitic infections, an issue which should be assessed more fully in the future. The method was recently also evaluated on population level among Hungarian children having their preschool physical examination, by performing the self-tTG rapid test from finger-prick blood (Korponay-Szabó et al. 2007). According to the study in question the prevalence of coeliac disease is already high (1.4%) at six years of age. Moreover, the rapid test was valuable in screening as it helped primary care nurses to detect approximately 80% of the untreated coeliac disease patients who were not picked out in clinical care. The test enabled quick tTG-ab test result evaluation and, thus, the time to the diagnostic biopsy was significantly shorter after an on site positive rapid test result than after a positive result in the serum in house EMA or hr-tTG ELISA done in the laboratory. On the other hand, the study revealed that training for self-tTG rapid testing is needed so that faint positive lines in the test window are also detected, and thus sensitivity is improved even in unexperienced hands, naturally also in home use.

In the light of the study by Korponay-Szabó and associates (2007), it may again be debated whether coeliac disease screening in the general population is justified. It has long been acknowledged that coeliac disease mostly meets the WHO (WHO Public Health Papers 1968) recommendations for disease mass screening. First of all, the disease is a common disorder (Table 6) which is often hard to recognize due to its protean clinical picture (Hill et al. 2005, Rostom et al. 2006). Besides, several accurate coeliac antibody tests suitable for early detection of the disease and selection of patients to undergo diagnostic small-intestinal biopsy are nowadays available (Rostom et al. 2005). Effective treatment, a gluten-free diet, is also available for the condition, and complications of untreated coeliac disease can thereby be avoided (Hill et al.

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2005, Rostom et al. 2006). Nevertheless, adherence of screen-detected patients to the diet can be questioned (Fabiani et al. 2000), although it was recently shown that the compliancy among these patients can also be good (Viljamaa et al. 2005a). Moreover, we still do not have adequate evidence of benefits of coeliac disease diagnosis and treatment for patients suffering from screen-detected and apparently asymptomatic coeliac disease and for the community (Mearin et al. 2005). Nonetheless, quality of life (Mustalahti et al. 2002) and bone mineral density (Mustalahti et al. 1999) may improve along with the diet also among screen-detected coeliac disease patients. It is of note, however, that in the Hungarian screening study most of the screen-detected coeliac children indeed proved symptomatic and health problems were alleviated after a half-year gluten-free diet (Korponay-Szabó et al. 2007). It can thus be suggested that a portion of unrecognized coeliac patients in fact have symptoms, which are unveiled after the disease is found, and that their health status can be improved with the diet.

In any case, more evaluations of cost-effectiveness should be made and a suitable algorithm and age for coeliac disease screening should be established. It can be foreseen that the whole blood self-tTG tests, and especially the user-friendly commercial rapid tests, could constitute better cost-benefit tools for coeliac disease screening than the conventional serum-based tests; for example the rapid test can be performed with a minimal workload from a finger-prick whole blood sample without specialized personnel or laboratory. Additionally, the testing is easy to arrange and test results are available in the screening session, enabling immediate decision-making (Korponay-Szabó et al. 2007). Moreover, as calculated by Crovella and associates (2007), savings were obtained when the self-tTG rapid test was applied instead of the serum-based tTG-ab ELISA for coeliac disease screening in Brazil.

All in all, it must still be conceded that proof of coeliac disease screening benefit is still scanty and inconsistent. Further studies are needed before screening of the general population can be undertaken.

This study series has shown that whole blood-based IgA self-tTG testing is a suitable and accurate method in detecting coeliac autoantibodies. It would appear from the knowledge obtained here of whole blood self-tTG-based testing, and previously of coeliac antibody testing, that the self-tTG tests can and should be applied for coeliac disease case finding to uncover yet unrecognized coeliac disease patients who may have coeliac-related health problems unawares. It is noteworthy that when the in house POCT or even the more convenient commercial rapid tests are applied at point-of-care, test-positive patients can be sent directly for diagnostic small-intestinal biopsy without delay. After a biopsy result indicating coeliac disease, a gluten-free diet should be prescribed, and thereafter regular, long-term monitoring should be commenced in order to confirm the diagnosis and the response to treatment, but also to obtain good dietary compliance. Subsequently, the tests can be applied in dietary assessment of patients, knowing that they, as well as the other coeliac antibody tests, may not reveal slight dietary transgression with unhealed small-bowel mucosa. On the other hand, the tests have value in that a test result is positive despite a relatively

long follow-up, indicating poor dietary adherence and/or ongoing small-bowel mucosal damage.

In whole blood IgA self-tTG test-positive subjects who do not evince small-bowel mucosal villous atrophy while consuming a gluten-containing diet, a follow-up is recommended, since overt coeliac disease can develop later in life (Kaukinen et al. 2001, Järvinen et al. 2004, Salmi et al. 2006a). Moreover, in this situation other coeliac antibody tests, for example serum EMA and tTG-ab, and additional markers of early developing coeliac disease, small-intestinal γδ+ IELs and tTG-specific IgA-deposits (Järvinen et al. 2004, Korponay-Szabó et al. 2004, Salmi et al. 2006a) can, if available, be used in the evaluation. HLA-typing can also be helpful in excluding coeliac disease, as individuals not carrying HLA DQ2 and/or DQ8 are unlikely to be affected by the condition (Karell et al. 2003). In a whole blood self-tTG test-negative case, coeliac disease can be considered unlikely unless the patient suffers from selective IgA deficiency. In such cases, IgG-class coeliac antibodies should be determined (Cataldo et al. 2000, Korponay-Szabó et al. 2003a) or the disease should be excluded by small-bowel mucosal investigation. In cases with high suspicion of coeliac disease, however, the subject should be sent for small-intestinal biopsy despite a negative result in one or other of the coeliac antibody tests.

Lastly, it should be underlined that whole blood IgA self-tTG testing, like other coeliac antibody testing, is a first step in the coeliac diagnostic work-up and small-bowel biopsy remains requisite (Walker-Smith et al. 1990). This should be made clear also to those who use the self-tTG rapid test applications at home in coeliac disease case finding.

6. SUMMARY AND CONCLUSIONS

This study showed for the first time that coeliac autoantibodies can also be detected with a novel and extremely simple IgA self-tTG method directly from a whole blood sample. Contrary to the conventional serum EMA and tTG-ab tests, self-tTG testing required no external purified or recombinant coeliac antigen or serum separation in IgA-class tTG-ab detection. Instead, in self-tTG testing a whole blood sample's endogenous erythrocyte fresh self-tTG was utilized as coeliac antigen. The test principle could be applied as an ELISA method, suitable for mass screening purposes in the laboratory. Importantly, the method also offered a means for rapid and easy-to-perform tTG-ab testing not only in a laboratory but also in an office setting from a fresh venous or finger-prick whole blood sample with the proof-of-concept test, Nunc-Immunostick-based in house POCT, and with commercial lateral flow immunochromatographic rapid test applications. With the rapid test, tTG-ab test results were ready for decision-making within five minutes on site without need for laboratory equipment or expert reading.

The whole blood IgA self-tTG method was shown here to detect antibodies specifically targeted against tTG. Investigating untreated coeliac disease patients and non-coeliac disease controls, the whole blood self-tTG-based in house POCT, ELISA and rapid tests proved to be accurate in coeliac disease case finding, as the tests agreed well with small-bowel mucosal findings and with the conventional widely employed serum-based EMA and tTG-ab tests. Similarly to the serological coeliac antibody tests, the self-tTG tests were also gluten-dependent and were suitable for monitoring gluten-free dietary treatment in coeliac disease. The tests performed reliably in the laboratory when patients were studied retrospectively from stored whole blood samples, but also in on site testing in the office. Some stored whole blood samples were however discarded as being clearly damaged. When total IgA was used as the positive control in self-tTG testing, IgA-deficient samples were picked out correctly, and the samples were regarded as invalid for IgA-class tTG-ab determination.

To conclude, this innovative self-tTG testing provides, when samples of good quality are used, an accurate alternative to the conventional time-consuming and cumbersome serum-based EMA and tTG-ab tests to detect coeliac autoantibodies directly from whole blood. The self-tTG testing can be carried out with the self-tTG ELISA in a laboratory and, additionally, rapidly and simply by the in house POCT or the commercial rapid test applications on site in an office without need for sample transport, expert personnel or laboratory facilities. Thus self-tTG testing might be highly valuable in case finding and dietary monitoring of coeliac disease in the future. More studies, however, are needed to ascertain the accuracies of the whole blood IgA self-tTG tests obtained here, when applied for instance in a low coeliac disease prevalence series, in different populations and in a home setting.

ACKNOWLEDGEMENTS

My appreciation and admiration for the fundamental work of Emeritus Professor Jarmo Visakorpi, M.D. in the field of coeliac disease research is here acknowledged.

I also wish to thank Robert MacGilleon, M.A., for enlightening revision of the language of the original communications and this thesis.

Warmest thanks go to all members of the Coeliac Disease Study Group. It has really been a privilege to be a part of this great team! Especially, I am grateful to Teea Salmi, M.D., and Mervi Viljamaa, M.D., for the help they have offered me with this work. I appreciate our "on and off the field" talks with Kati Juuti-Uusitalo, Phil.Lic., and Kalle Kurppa, M.D. I enjoyed the company of my "roommates" in the lab. I am also thankful for friendly help given by Kaija Kaskela, the secretary of the group, and by always cheerful Anne Heimonen, Mervi Himmanka and Soili Peltomäki.

I wish to extend special thanks to all my friends outside the scientific world for supporting me with this project and in all other things in life! Especially, I want to thank Liisa Kleemola, M. Sc., for peer support, talks, laughs and tears shared and the help she has offered me during this work. I am also grateful to my dear friend Sofia Slotte, M.D., for dragging me out of the research world every now and then.

I do not have words beautiful enough to thank my dear parents and friends Raili and Antti Raivio. Without their everlasting love and support through good and bad times this thesis would never have come into existence. I also owe warm gratitude to my brother Panu Raivio and his lovely family for just being there.

Heartfelt thanks go to my dear companion Tuomo Thesleff, M.D., for supporting and encouraging me with this work and sharing medical and most importantly non-medical life with me.

Tampere, April 2008

Tiina Raivio

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ORIGINAL PUBLICATIONS

Coeliac disease case finding and diet monitoring by point-of-care testing

I. R. KORPONAY-SZABÓ*·†, T. RAIVIO*, K. LAURILA*, J. OPRE†, R. KIRÁLY‡, J. B. KOVÁCS§, K. KAUKINEN*·¶, L. FÉSÜS‡ & M. MÄKI*

*Paediatric Research Centre, Medical School, University of Tampere and Tampere University Hospital, Tampere, Finland; †Department of Paediatrics, Medical and Health Science Centre, University of Debrecen, Debrecen, Hungary; ‡Department of Biochemistry and Molecular Biology, Medical and Health Science Centre, University of Debrecen, Debrecen, Hungary; §Department of Gastroenterology and Nephrology, Heim Pál Children's Hospital, Budapest, Hungary; ¶Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, Finland

SUMMARY

Background: Immunoglobulin A class transglutaminase autoantibodies are highly predictive markers of active coeliac disease, a disorder difficult to recognize solely on clinical grounds.

Aims: To develop and evaluate a simple rapid test for point-of-care detection of coeliac autoantibodies.

Methods: The novel whole blood test utilizes the patient's endogenous transglutaminase in red blood cells for detection of transglutaminase-specific immunoglobulin A antibodies present in the blood sample, with normal plasma immunoglobulin A detection as positive test control. We evaluated 284 patients under suspicion of coeliac disease and undergoing jejunal biopsy, and 263 coeliac patients on a gluten-free diet, 383 being tested prospectively in a point-of-care setting. Results were compared with histology, conventional serum autoantibody results and dietary adherence.

Results: The rapid test showed 97% sensitivity and 97% specificity for untreated coeliac disease, and identified all immunoglobulin A-deficient samples. Point-of-care testing found new coeliac cases as efficiently as antibody tests in laboratory. Coeliac autoantibodies were detected onsite in 21% of treated patients, while endomysial and transglutaminase antibodies were positive in 20% and 19%, respectively. The positivity rate correlated with dietary lapses and decreased on intensified dietary advice given upon positive point-of-care test results.

Conclusions: Point-of-care testing was accurate in finding new coeliac cases and helped to identify and decrease dietary non-compliance.

INTRODUCTION

Coeliac disease is an autoimmune gastrointestinal disorder induced by ingestion of gluten found in wheat, rye and barley.[1, 2] The active disease is characterized by gluten-dependent autoantibodies against endomysium (EMA), a complex connective tissue structure surrounding smooth muscle cells, and more precisely, against the protein type 2 ('tissue') transglutaminase (TG2), the coeliac autoantigen anchored to endomysial collagen by fibronectin.[3, 4] Detection of these autoantibodies in the serum is a useful means of identifying new coeliac patients presenting with only mild gastrointestinal symptoms, non-specific general complaints or extraintestinal manifestations, or in populations in general.[1, 5–8] A further important application of serological tests is the regular monitoring of dietary adherence in treated patients, as the autoantibodies

Correspondence to: Dr M. Mäki, Coeliac Disease Study Group, Paediatric Research Centre, Building FM3, FIN-33014 University of Tampere, Tampere, Finland.
E-mail: markku.maki@uta.fi

disappear from the serum on a strict gluten-free diet.[9] There is thus call for quick, easy-to-perform, economical and widely accessible coeliac antibody tests which can be carried out at the first care-taking level locally.

Currently, coeliac-specific serum antibody tests are centralized in specialized laboratories to ensure appropriate sensitivity and specificity.[9] Testing is costly and the turnaround time of results may be relatively long.

Natural human TG2 protein is also found within the red blood cells,[10] and thus in any diagnostic blood specimens comprising whole blood. This easily available endogenous TG2 antigen has, after liberation by haemolysis, the potential to bind to and thereby detect coeliac autoantibodies present in the same sample without need for purified, external TG2 antigen,[11] serum separation, and possibly even without a laboratory reader. This innovative means of detection thus offers an opportunity for point-of-care testing (POCT), defined as performing a diagnostic procedure in a variety of environments outside the central laboratory.[12]

In the present study, we showed a simple self-TG2-based rapid whole blood test to be accurate in detecting untreated coeliac disease. The performance of the test was further evaluated in point-of-care (POC) settings in finding new cases and monitoring treatment.

METHODS

Patients

The patients included in the present study were investigated at the Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest and Department of Paediatrics, University of Debrecen, Debrecen, Hungary and at Tampere University Hospital, Tampere, Finland in 2002–2004.

To assess (a) whether the self-TG2-based rapid whole blood test detects antibodies to TG2, 164 stored samples from patients undergoing small intestinal biopsy because of gastrointestinal symptoms were evaluated in blinded fashion in the laboratory. Results were compared with small bowel histology as the gold standard. Coeliac disease was diagnosed in 99 patients (median age 10.2 years, range: 1.4–59) according to European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria and based on the presence of Marsh type III histological lesions.[13, 14] The 65 patients without villous atrophy (median age

14.7 years, range: 3.3–67) were diagnosed with conditions shown in Table 1. The blood samples had been collected with ethylene diamineteraacetic acid (EDTA) or sodium citrate and stored frozen as whole blood at −20 °C until use. Serum immunoglobulin (Ig) A EMA, TG2 antibodies and total serum IgA were determined independently. Patients with total serum IgA < 0.05 g/L were considered IgA-deficient.

To evaluate (b) whether POCT can be used for finding new coeliac cases, 165 new patients (median age 13 years, range: 1.2–72) were prospectively enrolled. This cohort comprised (i) 46 patients with gastrointestinal symptoms admitted to the secondary level referral centre with a high suspicion of coeliac or other enteral disease, (ii) 84 subjects at risk for coeliac disease (patients with various autoimmune diseases, diabetes mellitus, eating disorders, first-degree relatives of known

Table 1. Clinical diagnoses in control patients with normal jejunal histology results

Diagnosis	Number of patients
Gastro-oesophageal reflux disease	19 (6)
IBD*	16 (6)
Nutritive allergy	8 (8)
Lactase deficiency	5 (1)
Postinfectious disaccharidase deficiency	5 (3)
Congenital sucrase–isomaltase deficiency	12 (10)
Familiar adenomatosus polyposis	5 (2)
Helicobacter pylori infection/duodenal ulcer disease	5 (2)
Recurrent abdominal pain	4 (2)
Irritable bowel syndrome	2
Cystic fibrosis	2 (2)
Shwachman-Diamond syndrome	1 (1)
Intestinal lymphangiectasia	1 (1)
Helminthiasis	1
Duodenum stenosis	1
Bacterial overgrowth syndrome	1
Meckel diverticulum bleeding	1 (1)
Myopathy	1 (1)
Autoimmune disease†	4 (4)
Non-specific diarrhoea/dyspepsia	35 (6)
Non-specific rash	3
First-degree relatives of known coeliac patients	6 (4)
No gastrointestinal disease	9 (5)
Total	147 (65)

Values in parentheses indicate patients studied on stored blood samples.
* Crohn's disease: 15, ulcerative colitis: 1.
† Type 1 diabetes mellitus: 3, autoimmune thyreoiditis: 1.
IBD, inflammatory bowel disease.

coeliac patients) and (iii) 35 consecutive adult primary care patients coming to open-access endoscopy who had low suspicion of coeliac disease. All consumed normal, gluten-containing food. Patients with previously known EMA or TG2 antibody results were excluded. Serum antibody measurements were carried out as in the previous group. Patients with clinical suspicion of upper gastrointestinal disease underwent endoscopy and small intestinal biopsy irrespective of the antibody results.

(c) In the prospective evaluation of POCT to monitor dietary compliance, 263 consecutive patients (median age 13 years, range: 2.8–76) with previously diagnosed biopsy-proven coeliac disease and known serum total IgA levels took part. They had followed a gluten-free diet for 2 months to 21.4 years (median: 3.9 years).

Point-of-care testing

Out-patient or ward staff performed the rapid whole blood test on drawn EDTA blood after receiving the patients' consent. The test result was read on site and was always available before that of the serum antibody tests.

Point-of-care testing was similarly performed on the 263 treated coeliac patients at their scheduled check-up visit. Dietary compliance was estimated prospectively at the time of the interview on the basis of a structured questionnaire, discussion with the patient, clinical findings and history as follows: (i) strict adherence to the diet for <6 months, (ii) strict diet over 6 months, (iii) suspected but not admitted lapses and (iv) admitted dietary lapses. Diet failure was suspected clinically in patients with persistent iron deficiency, gastrointestinal complaints, retarded growth or known psychosocial problems or who previously had positive antibody results despite a diet followed for over 1 year.

Dietary intervention

If POCT gave positive results in treated patients, means of improving the diet were immediately discussed with the patient, this also involving a dietician. POCT was repeated after 3–6 months following the dietary intervention. As controls for the intervention, we used serum antibody-positive coeliac subjects who had had their check-up visit in the same year before the POCT study began and who received their results and the instructions to improve the diet by mail. The control coeliac patients were also offered a consultation with a dietician when the positive antibody results became available.

Ethical permission for the study was obtained from the Ethical Committee of the Heim Pál Children's Hospital, Budapest and of Tampere University Hospital, Tampere.

Self-TG2-based coeliac antibody testing from whole blood

In previous laboratory studies, we found that in whole blood samples anticoagulated with EDTA or sodium citrate, antibodies against TG2 form complexes with self-TG2 liberated from red blood cells upon haemolysis. These complexes can be detected by binding TG2 to a solid surface using capture proteins such as fibronectin or gelatine (denatured collagen) which binds fibronectin.[11] Based on this principle, a rapid coeliac antibody test was developed into a Nunc-Immunostick (Nunc A/S, Roskilde, Denmark) format (Figure 1), and gives results in approximately 30 min. The test requires only minimal handling and no laboratory expertise in its execution, as all reagents can be prepared in advance. Two wings of the stick were precoated with gelatine (0.05% in 0.3 M bicarbonate buffer, pH 9.6) to capture self-TG2/anti-TG2 antibody complexes from the haemolyzed patient blood sample,[11] and one wing is coated with antibodies against human IgA (Boehringer, Mannheim, Germany) diluted 1:4000 in 0.3 M bicarbonate buffer (pH 9.6) to react with normal plasma IgA as a positive control. The fourth, uncoated wing serves as negative control. For testing, one drop of whole blood (approximately 25 μL) was delivered into the haemolyzing solution (hypotonic saline with 0.05 M Tris, 0.01 M EDTA and 0.1% Tween 20) and incubated with the stick for 15 min. The stick was then washed under tap water, immersed for another 15 min in peroxidase-labelled antihuman IgA solution (Dako, Glostrup, Denmark), diluted in 1:2000 of 0.05 M Tris (pH 7.4), washed again, inserted into a gel-containing colorigenic substrate, 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, Steinheim, Germany) and stirred with 0.12 g/mL of Sephadex 100 (Pharmacia, Uppsala, Sweden), and evaluated on site by inspection. The test was read as negative, if only one quadrant (the IgA-sensitive part) developed a blue colour, and positive if also both gelatine-coated (altogether three) quadrants became blue within 5 min. If no colour developed, the sample was labelled IgA-deficient and the test invalid (Figure 1). The substrate was stable for up to 1 month at +4 °C, the conjugate was made up freshly each morning.

To investigate whether the colour developing was only due to binding of specific antibodies to TG2, 10 EMA-
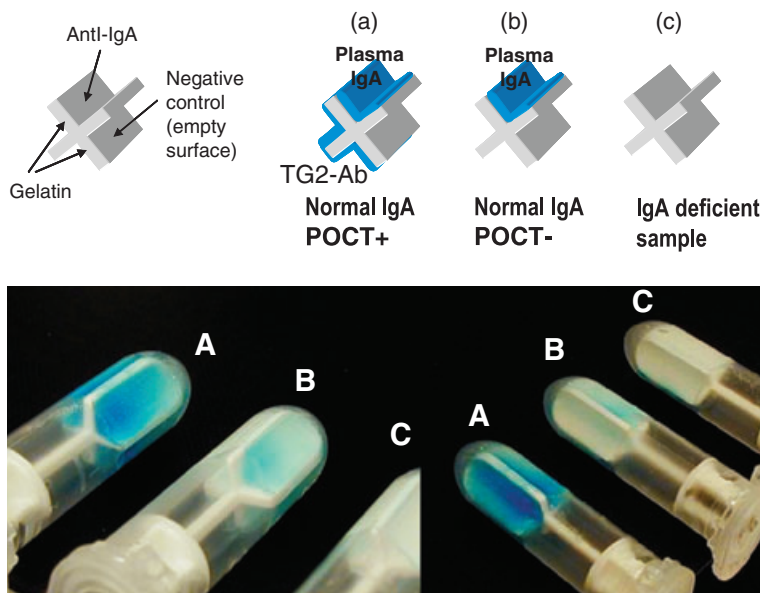
Figure 1. Layout of the self-transglutaminase (TG2)-based rapid whole blood test stick and interpretation of the point-of-care testing (POCT) result. Two quadrants of the stick are coated with gelatine to capture TG2–coeliac antibody (Ab) complexes from haemolyzed patient blood, one quadrant is coated with anti-immnoglobulin A to detect normal plasma IgA. The fourth quadrant is uncoated and serves as negative control. (a) Positive POCT result in an IgA-competent patient with blue colour reaction for TG2-Ab complexes and plasma IgA. (b) Negative POCT result in a subject with normal plasma IgA. (c) Invalid POCT result (IgA-deficient sample).

positive coeliac serum samples were mixed 1:1 with washed red blood cells from normal or TG2-null mice[15] and then tested in the same manner as the patient whole blood samples.

For the assessment of interobserver variation, 30 randomly selected EDTA blood samples from the patient cohorts evaluated at the POC were tested again in the laboratory in a blinded fashion. Further, quality control evaluations were conducted with whole blood samples thawed after various lengths (1–36 months) of storage at −20 or −80 °C and with Nunc-Immunosticks stored at +4 °C for up to 9 months after coating.

Serum antibody measurements

The IgA class serum antibodies against TG2 were measured with human recombinant TG2 using the Celikey (Pharmacia Diagnostics, Freiburg, Germany) enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions. Cut-off for positivity was 5 U/mL. EMA was determined on monkey oesophagus sections by indirect immunofluorescence as described elsewhere.[16] Samples reactive at a serum dilution of 1:≥2.5 were considered positive.

Statistical analysis

The McNemar test was used to determine that differences observed between assays were not due to chance. A probability of <0.05 was considered significant. The degree of agreement between any two tests or between

rapid test results by two different observers was calculated with fourfold contingency tables using κ-statistics. A κ-value of >0.75 indicates excellent, 0.4–0.75 good and <0.4 poor agreement. Serum TG2 antibody levels before and after dietary intervention were compared by the Wilcoxon signed rank test.

RESULTS

Use of the POCT kit to detect antibodies to TG2

The endogenous TG2-based whole blood rapid test showed 97.0% sensitivity and 96.9% specificity for untreated coeliac disease when applied to the stored 164 samples (Table 2), and performed comparably with serum EMA and TG2 antibody measurements. The results were reproducible in 94% of testings when seven positive, three weakly positive and 10 negative samples were investigated five times by altogether three observers. All eight coeliac blood samples stored after collection frozen without thawing for 24–36 months gave positive results upon thawing, and there was no interference with haemolysis if repeated freezing and thawing was avoided. Immunosticks coated with gelatine were working even after 9 months of storage at +4 °C.

All the 10 serum samples from coeliac patients containing EMA and TG2 autoantibodies tested negative if applied without red blood cells (thus without TG2 antigen) or together with TG2-deficient red blood cell lysate derived from TG2 knockout mice. Nonetheless, the test was positive if normal mouse erythrocytes were

Table 2. Positivity of the whole blood rapid test, serum IgA endomysial antibody (EMA) and serum IgA transglutaminase antibody (TG2-Ab) test in untreated coeliac disease patients and controls

	Rapid test+	Rapid test−	EMA+*	EMA−	TG2-Ab+†	TG-Ab−
Stored samples tested at the laboratory						
Untreated coeliac disease ($n = 99$)	96	3	98	1	98	1
Controls ($n = 65$)	2	63	0	65	0	65
Total ($n = 164$)	98	66	98	66	98	66
Sensitivity	97.0% (93.6–100)		99.0% (97.0–100)		99.0% (97.0–100)	
Specificity	96.9% (92.7–100)		100%		100%	
Positive predictive value	98.0%		100%		100%	
Negative predictive value	95.5%		98.5%		98.5%	
Prospectively tested patients (point-of-care case finding)						
Untreated coeliac disease ($n = 38$)	37	1‡	37	1‡	37	1‡
Controls ($n = 82$)	2	80	0	82	0	82
Total ($n = 120$)	39	81	37	83	37	83
Sensitivity	97.4% (94.3–100)		97.4% (94.3–100)		97.4% (94.3–100)	
Specificity	97.6% (94.5–100)		100%		100%	
Positive predictive value	94.9%		100%		100%	
Negative predictive value	98.8%		98.8%		98.8%	

95% confidence intervals are shown in parentheses.
* Positive if binding is seen at a serum dilution of 1:2.5 or more.
† Cut-off for positivity: 5 U/mL.
‡ Patient with selective immunoglobulin A deficiency.
There were no significant differences by the McNemar test for the rapid test results between point-of-care and laboratory testing and vs. EMA or TG2-Ab.

used. This shows that the antigen specifically recognized in the rapid test was TG2, and other blood components or potential antibodies to them did not contribute to the colour reaction even in coeliac subjects.

Use of POCT for finding new coeliac cases

As laboratory evaluation showed the rapid whole blood test to recognize patients with coeliac disease with high accuracy, we sought to establish whether the test also identifies coeliac cases when applied at the point of care, i.e. at doctor's consultation. Rate of POCT positivity was 58.7% (27 of 46) in the high clinical suspicion group, 13.1% (11 of 84) among at-risk subjects and one patient (2.8%) tested positive from the 35 primary care patients. Altogether 120 patients (all 39 with positive and 81 of the patients with negative POCT results) underwent small intestinal biopsy on clinical grounds and only these were used for the calculation of sensitivity and specificity (Table 1). Antibody-negative low risk people who did not have gastrointestinal symptoms were not eligible to biopsy.

Thirty-seven of the 39 patients with positive POCT results had small intestinal villous atrophy confirming coeliac disease (Table 1). One coeliac patient was negative in POCT for both the test and the IgA control line and was later shown to have IgA deficiency. Thus, POCT found 97.4% of coeliac patients with 97.6% specificity. However, if the IgA-deficient patient correctly picked out by the IgA control line and not having IgA autoantibodies is excluded from the calculations, the sensitivity of the test was 100%. This patient was found to have IgG class EMA and anti-TG2 antibodies in her serum. From the prospectively evaluated cohort nine patients were diagnosed with Crohn's disease and all had negative POCT results.

κ-statistics indicated excellent agreement of POCT results with either serum EMA or TG2 antibody detection ($\kappa = 0.96$, 95% CI: 0.91–1.0). The results obtained in laboratory vs. onsite testing did not differ statistically (Table 1), there was no difference between the results of children ($n = 184$) and adults ($n = 100$); the overall sensitivity of the rapid test was 97.1% and the specificity 97.3% in the 137 untreated coeliac disease patients and 147 biopsied controls having different gastrointestinal diseases (Table 1). Interobserver agreement between POC evaluators and laboratory personnel was 96.7% ($\kappa = 0.90$, 95% CI: 0.72–1.0).

Use of POCT to monitor dietary compliance

During the evaluation of known coeliac patients on diet, POCT identified all nine IgA-deficient blood samples by the absence of the IgA-positive control line. These nine patients thus yielded invalid POCT results and were excluded from the evaluation of dietary compliance. In the case of the 254 IgA-competent patients, coeliac autoantibodies were detected in 52 patients by POCT (20.5%), 50 by the EMA test (19.7%) and 47 by measuring serum anti-TG2 antibodies (18.5%).

All three tests gave either negative or positive results in 91% of all diet samples ($n = 263$). The POCT results agreed with EMA in 93.9% ($\kappa = 0.81$, 95% CI: 0.71–0.90), and with serum TG2 antibody in 92.0% ($\kappa = 0.74$, 95% CI: 0.63–0.85) of the samples, and the EMA and serum TG2 antibody results were concordant in 95.8% ($\kappa = 0.86$, 95% CI: 0.78–0.94). POCT was similarly sensitive as EMA in finding samples with low TG2 antibody positivity and around the cut-off of ELISA (Table 3).

Table 3. Comparison of positive point-of-care (POCT) test results and positive endomysial antibody (EMA) results at low serum transglutaminase (TG2) antibody levels in coeliac patients on diet

TG2 antibody level (U/mL)	Number of samples	POCT+ (%)	EMA+ (%)
>8	31	28 (90.3)	31 (100)
5–8*	15	11 (73.3)	11 (73.3)
3–5†	24	8 (33.3)	8 (33.3)
<3	184	5 (2.7)	0
Total	254	52	50

* Equivocal range according to the manufacturer.
† Cut-off of positivity suggested by the manufacturer: 5 U/mL.

The rates for coeliac antibody positivity declined in all three tests with time on diet, and after 6 months on diet, 95% of compliant patients were antibody-negative (Table 4). However, both POCT and serum antibody tests detected serological activity indicating gluten consumption in a high percentage of patients with clinically suspected or admitted dietary transgressions (Table 4).

POCT and serum antibody results after intensified dietary advice

Of the long-term treated patients receiving intensified dietary instructions onsite after a positive POCT result, 16 were evaluated by POCT a second time, 3–6 months after the initial testing. In 12 of these patients (75%), POCT and EMA became negative and the whole group showed a significant reduction ($P < 0.001$) in serum TG2 antibody levels (Figure 2a). There was also a clear improvement in weight gain and iron status (data not shown). In contrast, out of a control group of 14 EMA-positive coeliac patients who did not participate in POCT and received only written advice to improve the diet, only four (28.6%) reverted to negative EMA results on a second examination after 3–6 months, and there was no significant change in TG2 antibody levels ($P = 0.57$; Figure 2b).

DISCUSSION

Increasing application of serology, i.e. the use of coeliac disease-specific autoantibody tests, has in recent years substantially contributed to our current understanding

Table 4. Positivity in point-of-care testing by self-transglutaminase-based whole blood rapid test, serum IgA endomysial antibody (EMA) and serum IgA transglutaminase antibody (TG2-Ab) tests in treated coeliac patients according to clinically estimated compliance with a gluten-free diet (IgA-deficient patients were excluded)

	Number of patients	Rapid test+ (%)	EMA+ (%)	TG2-Ab ELISA+ (%)
Admitted diet transgressions*	17	15 (88.2, 72.5–100)	16 (94.1, 82.6–100)	16 (94.1, 82.6–100)
Clinically suspected but not admitted diet transgressions†	20	14 (70.0, 49.4–90.6)	11 (55.0, 32.6–77.4)	9 (45.0, 22.6–67.4)
Strict gluten-free diet for ≤ 6 months	31	12 (38.7, 21.3–56.1)	14 (45.2, 27.4–63.0)	12 (38.7, 21.3–56.1)
Strict gluten-free diet for >6 months	186	11 (5.9‡, 3.4–9.3)	9 (4.8‡, 1.7–7.9)	10 (5.4‡, 2.1–8.6)
Total	254	52 (20.5, 15.5–25.4)	50 (19.7, 14.8–24.6)	47 (18.5, 13.7–23.3)

Percentages with 95% confidence intervals are shown in parentheses.
* Median time on diet 6.3 years (range: 1–13).
† Median time on diet 4 years (range: 0.8–12).
‡ $P < 0.001$ vs. all other diet groups by Fisher's exact test.
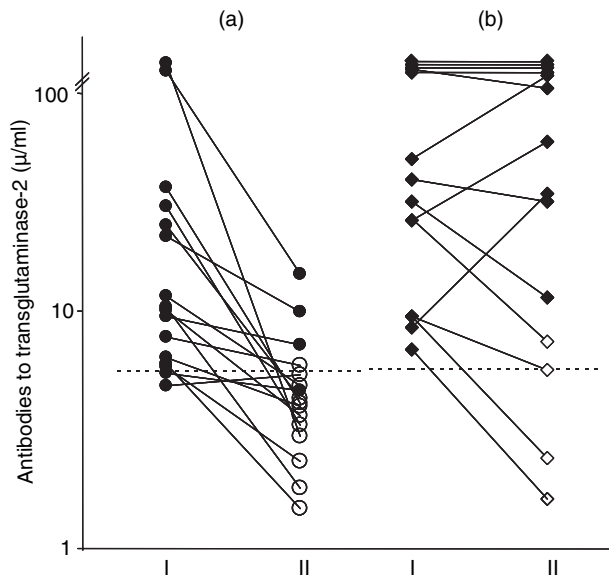IgA, immunoglobulin A; ELISA, enzyme-linked immunosorbent assay.

Figure 2. Serum immunoglobulin A transglutaminase antibody (TG2 Ab) concentrations at two consecutive evaluations in treated coeliac patients. (a) Patients ($n = 16$) receiving intensified dietary advice on site upon a positive rapid whole blood point-of-care test. (b) Patients ($n = 14$) receiving dietary advice by ordinary mail upon proving serum antibody-positive. I, initial testing; and II, follow-up evaluation after 3–6 months. Filled circles indicate positive, open circles negative results in the rapid point-of-care test. Filled diamonds indicate positive, open diamonds negative results in the serum endomysial antibody test. The dotted line represents the cut-off for serum TG2 Ab positivity (5 U/mL).

that the disorder may present with a variety of symptoms and organ manifestations which make recognition of patients on solely clinical grounds difficult.[1, 2, 17] These serological tests have acquired a central position in algorithms for diagnosing coeliac disease.[17, 18] We have now developed a novel, easy-to-perform and rapid detection method for coeliac disease case finding, a whole blood test with the same sensitivity and specificity in detecting the disease as the laborious traditional serum-based EMA and TG2 antibody tests. We further evaluated this new test prospectively in a POC setting and showed POCT to be a valid approach to detect coeliac disease-related antibodies directly by doctors or staff in office or ward. In addition, dietary intervention in cases tested positive while on diet improved dietary compliance.

The new test, based on the novel principle[11] whereby an autoantigen, the patients' own TG2 liberated by haemolysis from red blood cells, complexes itself with the autoantibody present in the same whole blood

sample and is then captured to enable the detection of the bound antibody, is the first of its kind in medicine. This is a simple POC procedure yielding immediate information on coeliac antibody status applicable in the selection of patients for more invasive diagnostic tests. The present academically developed 'proof-of-concept' test, showing 97% sensitivity and specificity in both laboratory and POC settings for biopsy-proven coeliac disease might be used as such in doctors' offices or further developed by the industry to furnish even more rapid and user-friendly test kits. We wish to emphasize that even if this onsite rapid whole blood test is highly accurate and did not show positivity in other disease groups, such as e.g. Crohn's disease, the diagnosis of coeliac disease today relies on histological demonstration of villous atrophy, and a small intestinal biopsy therefore remains a requisite.[17, 19] Screening studies based on serum anti-TG2 antibody measurements have shown that the disease affects nearly 1% of the population in European countries,[6, 7] and similar figures are now available also from the USA.[8, 20] Given the high prevalence and diverse clinical problems, general practitioners and doctors in many other fields have thus a key role in case finding and referral to the appropriate specialists.[5, 6] Two TG2-based immunochromatographic rapid assays and one dot-blot assay have hitherto been published, and these tests use laboratory serum samples.[21–23] The present study is to our knowledge the first to report on the clinical application of a rapid coeliac test in a real POC setting.

Although POCT for coeliac disease has certain limitations, such as observer-dependency, the same holds for coeliac antibody detections in general.[17, 19] The results of EMA test are highly influenced by expert reading, whereas the simple rapid test used here yielded high agreement between untrained and trained personnel. In addition, the antibodies may be negative if the subject has adopted a gluten-free diet before testing. Furthermore, the serum autoantibody tests commonly used detect only IgA class antibodies and IgA-deficient coeliac patients may thus be missed.[24] This problem is vital for rapid tests, where results are interpreted immediately, precluding the use of laboratory serum IgA measurements. As information on both coeliac antibodies and IgA status is required for decision in the diagnostic algorithms most commonly recommended,[18] we chose total plasma IgA detection as the positive test control in our test kit. This strategy indeed enabled us to pick out IgA-deficient samples.

The currently available, serum-based coeliac antibody diagnostic kits use purified or human recombinant TG2 antigens and are reliable only in a controlled laboratory environment.[9] TG2 is a protein particularly sensitive to heat, storage and oxidation, which may influence its antigenic properties.[10, 25] Use of fresh self-TG2 antigen from the patient's own blood may overcome kit storage problems in warm climates or developing countries and makes testing economical, as neither industrially purified TG2 nor serum separation is required.

In the present study, our novel rapid test found coeliac patients both in high risk and low risk patient groups including primary care, and worked similarly to EMA even in cases when the antibody levels were low. The gelatine-coated test surface binds blood TG2 via blood fibronectin in the same and oriented fashion as TG2 epitopes are exposed in natural tissue sections used in the EMA test.[4] Unlike to ELISA, where the plate-coated TG2 antigen may be exposed in distorted or non-physiological ways and may also attract some non-specific antibodies,[26] the EMA reaction is coeliac-specific[3, 4, 7, 9, 16] and basically on-off. The similar antigenic orientation in the rapid test ensured specificity and enabled us to adjust the plus/minus colour development to high sensitivity. The EMA test has already proved to be reliable also in low prevalence situations, e.g. population screening,[7] thus the POCT test equipped with the IgA control line could be equally efficient and even more convenient for such applications.

Based on the low interobserver variability and the experiments presented here, the POCT kit can satisfy quality assurance requirements. For quality assurance regarding the policy of POCT for finding coeliac disease, further education of all health care professionals will be important that after a positive POCT result a confirmatory biopsy is still needed and cases with severe gastrointestinal symptoms may require a referral to gastroenterologist despite a negative POCT result.

The setting where POCT, in addition to new case finding, may have a special role is the long-term surveillance of coeliac patients after adopting a gluten-free diet. In the present study, both POCT and serum antibody measurements detected coeliac antibody positivity in the majority of patients who admitted dietary transgressions, but also in a sizeable proportion of those who could not be identified by history alone. Being a test with positive/negative results, the rapid test is not suitable to show initial decrease in the antibody titres, but in the present study, it was able to correctly demonstrate negative seroconversion after a diet for 6 months or longer. Although occasional dietary lapses may not lead to measurable seropositivity,[27, 28] sustained detectability of coeliac autoantibodies is consistent with ongoing gluten consumption, in most cases accompanied by damaged villous structure.[28] Such patients are at risk of late complications, including osteoporosis and malignancy, even if they are currently clinically asymptomatic.[1, 2, 17] The long-term success of coeliac disease care is largely dependent on a good doctor–patient relationship reinforcing the diet,[29] and readily available antibody results may help to target non-compliant individuals already during the consultation. In the present study, better dietary adherence was achieved following this type of intervention.

In conclusion, we have shown that subjects with undetected coeliac disease and known patients with dietary failure can be picked out by POCT using a simple self-TG2-based rapid technique. This test can be applied already in its present form or might be further developed into commercial user-friendly kits.

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Alimentary Tract

Performance of a new rapid whole blood coeliac test in adult patients with low prevalence of endomysial antibodies

T. Raivio [a], I. Korponay-Szabó [a,g], P. Collin [b,d], K. Laurila [a], H. Huhtala [b,c], T. Kaartinen [f], J. Partanen [f], M. Mäki [a,e], K. Kaukinen [b,d,*]

[a] Paediatric Research Centre, University of Tampere, Tampere, Finland
[b] Medical School, University of Tampere, Tampere, Finland
[c] School of Public Health, University of Tampere, Tampere, Finland
[d] Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, Finland
[e] Department of Paediatrics, Tampere University Hospital, Tampere, Finland
[f] Department of Tissue Typing, Finnish Red Cross Blood Service, Helsinki, Finland
[g] Department of Paediatrics, Medical and Health Science Centre,
University of Debrecen, Debrecen, Hungary

Abstract

Background. In coeliac disease endomysial and transglutaminase autoantibodies are directed against the human autoantigen, transglutaminase. The conventional coeliac antibody tests are performed from serum samples in centralized laboratories.

Aims. To evaluate a rapid and easy immunoglobulin A-class whole blood point-of-care test and its commercial application, *the Biocard test*, in coeliac autoantibody detection.

Methods. In the whole blood point-of-care test transglutaminase is liberated from the red blood cells by haemolysis. Transglutaminase antibodies, if present, complex with the liberated antigen, and are visualized. Altogether 51 biopsy-proven untreated coeliac adult patients, 48 of the same patients after treatment, and 36 controls were tested. The point-of-care test results were compared with serum endomysial and transglutaminase antibody and *Biocard test* results and histology.

Results. The whole blood point-of-care test was as sensitive (82%) as the serum endomysium test (80%) in detecting untreated coeliac disease while the serum transglutaminase antibody test was superior (88%). The tests had 100% specificity. A positive point-of-care test result seroconverted or the test reaction weakened in 90% of the treated coeliac patients. *Biocard test*-positive were 22 of the 24 tested untreated coeliac patients. *Biocard test*-negative were 15 of 19 controls.

Conclusions. The whole blood rapid tests are as reliable as the conventional serological tests in detecting untreated coeliac disease and in coeliac disease diet monitoring.

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Keywords: Coeliac disease; Point-of-care test; Tissue transglutaminase; Whole blood

1. Background

Coeliac disease is a common autoimmune disorder in genetically susceptible individuals, in whom small-intestine mucosal damage and circulating coeliac autoantibodies against the autoantigen, tissue transglutaminase (tTG), are found to result from gluten ingestion [1–5]. Diagnosis of the disease requires endoscopy [6]. However, sensitive and specific coeliac disease antibody tests performed from serum samples, for example endomysial (EMA) and tissue transglutaminase antibody (tTG-ab) tests, are widely used in case finding and gluten-free diet monitoring [7]. These tests are performed in centralized laboratory facilities, and are often time-consuming.

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* Corresponding author at: Medical School, University of Tampere, FIN-33014 Tampere, Finland. Tel.: +358 3 3551 8403; fax: +358 3 3551 8402.
   E-mail address: katri.kaukinen@uta.fi (K. Kaukinen).
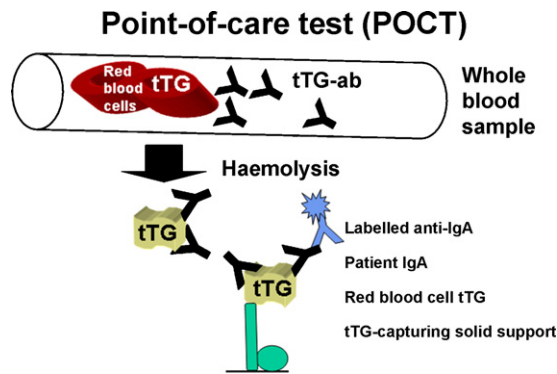
Point-of-care test (POCT)

Fig. 1. Principle of measuring IgA-class tissue transglutaminase antibodies (tTG-ab) from a whole blood sample using an in-house point-of-care test (POCT). First the coeliac disease autoantigen, tissue transglutaminase (tTG), is liberated from the red blood cells by haemolysing the whole blood sample. IgA-class tTG-ab form complexes with the liberated tTG if present in the sera. The formed complexes are bound to a solid surface with the help of tTG/fibronectin capture proteins. The IgA-class tTG-ab of the complexes is visualized in colour reaction by labeled anti-human IgA.

Recently we have developed a new coeliac disease antibody test, a rapid and easy point-of-care test (POCT), for immunoglobulin (Ig) A-class tTG-ab detection (Fig. 1) [8]. Testing involves neither serum separation, sample transport, laboratory equipment nor external autoantigen, tTG. In the POCT, the patient's own red blood cell tTG is liberated upon haemolysis [9]. If coeliac-specific antibodies are present in the circulating blood they form complexes with the liberated tTG and these can be captured and detected on the basis of a colour reaction. In our previous study the in-house POCT had a sensitivity and specificity of 97% for coeliac disease case finding when mainly children were studied. The test also proved reliable in dietary monitoring [8]. Furthermore, based on the same innovation as POCT, a more user-friendly commercial application (Biocard Celiac Disease[TM], AniBiotech, Vantaa, Finland), utilizing a lateral flow method, has been developed. The commercial test seems to find untreated coeliac disease satisfactorily, having a sensitivity of 97% and a specificity of 93% [10].

In the present study, we sought further to establish how the IgA-class in-house POCT recognizes untreated coeliac disease in adult patients investigated at tertiary clinics in series where serologic screening tests are known to give a lower sensitivity than in paediatric series [11,12], and how the test results behave in follow-up on a gluten-free diet in the same patients. Additionally, we compared the in-house POCT results with those obtained from intestinal small-bowel biopsy, IgA-class serum EMA and tTG-ab tests and the whole blood *Biocard test*.

2. Materials and methods

2.1. Subjects

In the first part of the study, the untreated coeliac disease patients' group comprised 51 consecutive adults referred to a tertiary clinic owing to diagnostic difficulties. The patients were known to be IgA-competent. They were examined at the Department of Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital, Finland, between the years 1998 and 2000. The diagnosis of coeliac disease was based on the finding of small-bowel mucosal severe partial or subtotal villous atrophy with crypt hyperplasia and on clinical or histological response to a gluten-free diet [6]. Patients' presenting symptoms are shown in Table 1. Altogether 48 of these 51 coeliac disease patients were also investigated after a 1-year gluten-free diet. The strictness of the diet was assessed by a dietary inquiry and a four-day record of food intake. The non-coeliac disease control group comprised 36 adults examined in a health care centre in Tampere due to dyspepsia or heartburn, and all had normal small-bowel villous morphology (Table 1). From all of the patients small-bowel mucosal biopsies were obtained by gastroscopy, the mucosal morphology was investigated and serum and whole blood samples obtained and stored at −20 °C until tested.

In the second part of the study samples from 24 of the above-mentioned untreated coeliac disease patients (median age 42 years, range 24–68), 11 treated coeliac disease patients

Table 1

Demographic data on untreated coeliac disease patients and non-coeliac controls and indications for endoscopy in the first part of the study when tested with in-house POCT and in the second part when tested using a commercial *Biocard test*

	Whole blood in-house POCT		Whole blood *Biocard test*	
	Coeliac disease, untreated $n = 51$	Controls $n = 36$	Coeliac disease, untreated $n = 24$	Controls $n = 19$
Age: median (range), years	48 (24–69)	56 (23–73)	42 (24–68)	62 (22–72)
Female, n (%)	38 (75)	23 (64)	18 (75)	11 (58)
Indication for endoscopy (%)				
Gastrointestinal complaints	67[a]	100[b]	14[a]	100[b]
Anaemia or malabsorbtion	14	0	6	0
Screening of associated conditions[c]	19	0	4	0

POCT: point-of-care test.

[a] Diarrhoea, loose stools, abdominal distention and pain.

[b] Heartburn, dyspepsia.

[c] Sjögren's syndrome, thyroid diseases, insulin-dependent diabetes mellitus, neurological symptoms, infertility, rash, arthritis, osteoporosis, family history.

(median age 38 years, range 28–68) and 19 controls (median age 62 years, range 22–72) were also available for commercial whole blood rapid testing (Biocard Celiac Disease™).

2.2. POCT

An in-house POCT was performed in the laboratory from thawed whole blood samples in a blinded fashion, utilizing the Nunc-Immunostick pad (Nunc A/S, Roskilde, Denmark) as recently described [8]. In short, two wings of the pad are precoated with gelatin (0.05%), which serves as a capture protein for fibronectin and tTG. The uncoated part of the pad serves as a negative control. In the POCT first a drop (25 μl) of thawed, an anticoagulated citrated or ethylenediamine tetra-acetic acid (EDTA) whole blood sample is haemolysed with the help of 500 μl distilled water in order to liberate the patient's own tTG from the red blood cells. After haemolysis 500 μl of buffer (hypotonic saline with 0.05 M Tris, 0.01 M EDTA and 0.1% Tween 20) is added to preserve tTG antigenicity. Subsequently, a coated Nunc-Immunostick pad is incubated in the solution for fifteen minutes before washing under tap water. The pad is then incubated for another fifteen minutes in one ml of peroxidase-conjugated anti-human IgA solution (DAKO, Glostrup, Denmark, diluted 1:2000 in 0.05 M Tris, pH 7.4), washed again and inserted into a gel containing buffered tetramethylbenzide solution (Sigma–Aldrich, Steinheim, Germany) with Sephadex powder (Amersham Pharmacia Biotech, Uppsala, Sweden), which keeps the colour reaction around the coated surface of the stick. After five minutes' incubation the developed colour reaction can be evaluated onsite from the stick by inspection. If coeliac disease-specific IgA-class autoantibodies, tTG-ab, are present in the whole blood sample they form complexes with the liberated autoantigen, tTG (Fig. 1). These attach to the coated Nunc-immunostick surface and the IgA-class tTG-ab of the complexes is visualized in a blue colour reaction on the pad within 35 minutes from haemolysis with the help of labelled anti-IgA (Fig. 1). Blue on the coated surface is considered a positive test result, which is further semi-quantified by inspection as either strong (deep blue, ++) or weak positive (faint blue, +). When the pad remains completely blank the in-house POCT result is interpreted as negative (0).

Interobserver agreement (97%) between point-of-care evaluators was studied before testing the actual study group and the results were reproducible in 94% of cases as previously described [8]. Moreover, immunosticks coated with gelatin were effective even after nine months of storage at +4 °C [8].

2.3. Commercial whole blood rapid test by lateral flow method

The Biocard test is a commercial, more user-friendly immunochromatographic application of the same innovation as the in-house POCT. Testing was carried out from EDTA whole blood samples in laboratory facilities as instructed by the manufacturer and described elsewhere [10]. In brief, in *Biocard* testing the patient's own tTG from red blood cells is utilized as in the POCT in IgA-class tTG-ab detection. A drop of whole blood sample is first mixed with a haemolysing sample buffer. Three drops of the sample dilution are then placed on a round application field of the test card. The sample dilution moves by a lateral flow method in the test card. The test result can be seen within five minutes. The appearance of an integrated control line in the control field of the test card ensures proper test function and a line in the test field indicates a positive test result.

2.4. Serology

Serum IgA-class EMA was determined by an indirect immunofluorescence method using human umbilical cord as antigen, a titre 1:≥5 being considered positive [13,14]. Positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. Serum IgA-class tTG-ab were determined by enzyme-linked immunosorbent assay (Celikey®, Phadia GmbH, Freiburg, Germany), where human recombinant tTG is the antigen, a unit value (U) ≥5 being considered positive.

2.5. HLA DQ typing

The human leukocyte antigen (HLA) DQ2 and DQ8 alleles were determined in all the patients and controls using the Dynal SSP low-resolution DQ typing kit (Dynal AS, Oslo, Norway).

2.6. Statistics

Sensitivities, specificities, positive and negative predictive values and efficiencies of the tests were calculated for each coeliac disease antibody test and expressed as percentages [15]. A positive predictive value of a test was the ratio of true-positive (biopsy-proven untreated coeliac disease) results and all positive test results, a negative predictive value a ratio of true-negative (non-coeliac disease controls having normal duodenal mucosal morphology) and all negative test results, respectively. The efficiency of a test was the ratio of the sum of true-negative and true-positive tests results and the sum of all untreated coeliac disease and non-coeliac disease patients.

2.7. Ethical considerations

The study protocol was approved by the Ethical Committee of Tampere University Hospital. All subjects gave written informed consent.

3. Results

In this selected patient series the in-house POCT gave a sensitivity (82%) comparable with the serum EMA test (80%)

Table 2
Sensitivity, specificity, negative and positive predictive values and efficiencies of the IgA-class whole blood in-house POCT, serum EMA and serum tTG-ab tests in biopsy-proven untreated coeliac disease patients and non-coeliac controls on stored samples

	Whole blood in-house POCT		Serum EMA		Serum tTG-ab	
	Positive	Negative	Positive	Negative	Positive	Negative
Untreated coeliac disease, n = 51	42	9	41	10	45	6
Controls, n = 36	0	36	0	36	0	36
Treated coeliac disease, n = 48	7	41	6	42	7	41
Sensitivity (%)[a]		82		80		88
Specificity (%)[a]		100		100		100
Positive predictive value (%)[a]		100		100		100
Negative predictive value (%)[a]		80		78		86
Efficiency of the test (%)[a]		90		89		93

POCT: point-of-care test, EMA: endomysial antibody, tTG-ab: tissue transglutaminase antibody.

[a] Sensitivities, specificities, negative and positive predictive values and efficiencies of the tests calculated from the test results of the biopsy-proven untreated coeliac disease patients and the non-coeliac disease controls evincing normal small-bowel mucosal morphology.

while the serum tTG-ab ELISA test was superior to both (sensitivity 88%) (Table 2). One untreated coeliac disease patient was detected by POCT only. Furthermore, only one patient was negative for all three tests. The concordance in untreated coeliac disease between in-house POCT and serum EMA test (75%) was as good as that between the serological EMA and tTG-ab tests (76%). Moreover, the in-house POCT gave test results concordant with the serum tTG-ab test in 86% of cases in the untreated coeliac disease patient group. The nine in-house POCT-negative untreated coeliac disease patients had low serum EMA titres (range 0–1:5, median 1:5) and tTG-ab levels (range 3.1–9.9, U/ml, median 4.1). The relation between the in-house POCT and the serum tTG-ab test results in untreated and treated coeliac disease patients and non-coeliac disease controls is shown in Fig. 2. In the control group all antibody tests gave a negative result, thus giving a

specificity of 100% for each test and a concordance of 100% between the tests. In the coeliac disease group all patients had an HLA-type consistent with coeliac disease [16,17]: 43 patients (86%) had HLA DQ2, 4 (8%) DQ8 and 3 (6%) both DQ2 and DQ8. In the control group, 18 out of 35 (51%) had HLA DQ2 or HLA DQ8.

In-house point-of-care testing was revealed to be gluten dependent as positive test results seroconverted from positive to negative or the test reaction weakened in parallel with the duodenal villous structure recovery in 90% of the coeliac disease patients after a 1-year gluten-free diet (Fig. 3 and Table 2). All in-house POCT-positive treated patients still had partial villous atrophy in their duodenal biopsy, and two of them admitted dietary lapses. The concordance in treated coeliac disease patients was 90% between in-house POCT and serum EMA test results, 92% between in-house POCT and serum tTG-ab test and 94% between serum EMA and tTG-ab tests.

In the second part of the study, 22 out of 24 untreated coeliac disease patients were *Biocard test*-positive, 23 in-house POCT-positive, 20 serum EMA-positive and 23 serum
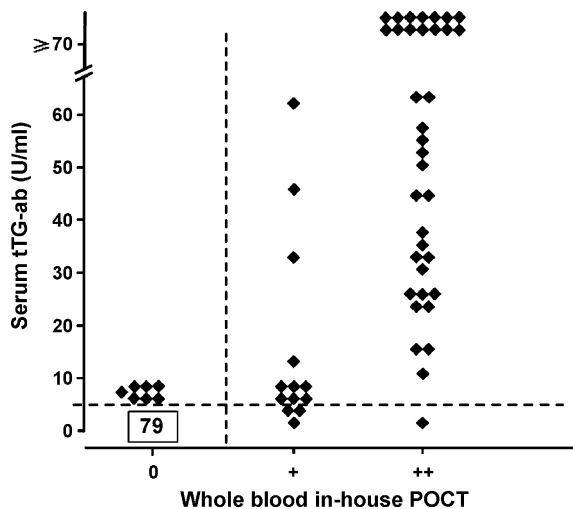

Fig. 2. Relation between IgA-class serum tissue transglutaminase antibody (tTG-ab) test and in-house point-of-care test (POCT) results in untreated and treated coeliac disease and control patients. The cut-off level for serum tTG-ab-positivity (5 U/ml) is shown by a horizontal dotted line and for the in-house POCT-positivity by a vertical line. POCT results were divided into negative (0), weak positive (+) and strong positive (++).
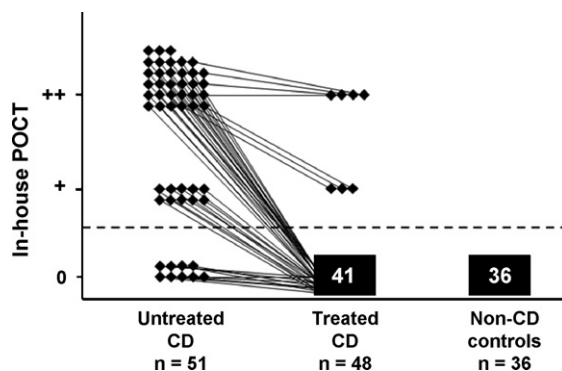

Fig. 3. In-house point-of-care test (POCT) results in 51 untreated coeliac disease (CD) patients, 48 patients after 1-year gluten free diet and 36 non-coeliac controls. POCT results were divided into negative (0), weak positive (+) and strong positive (++); the horizontal dotted line shows the cut-off level for POCT positivity.

tTG-ab-positive. Among 19 non-coeliac disease controls *the Biocard test* was negative in 15 patients, while the other tests were negative in all cases. Altogether nine of the 11 treated coeliac disease patients were *Biocard test*-negative. All these treated patients yielded negative test results in in-house POCT, serum EMA and tTG-ab tests. *Biocard test* results were concordant with those of in-house POCT in 85% of cases, serum EMA test results in 81% and serum tTG-ab test results in 83%.

4. Discussion

On the basis of our earlier studies the whole blood in-house POCT is sensitive and specific in detecting untreated coeliac disease in children [8]. In the present study, we further evaluated the test in adults investigated at tertiary clinics. It has previously been shown that coeliac antibody levels among adult untreated coeliac disease patients are more heterogeneous and the sensitivities of coeliac disease antibody tests may therefore be lower [11,12]. In this material the in-house POCT showed as good sensitivity as the serum EMA test, while the serum tTG-ab test was slightly better (Table 2). The specificity of POCT was 100%, as in the serological tests.

As coeliac disease has revealed itself to be more common than previously thought and the clinical picture of the untreated condition is protean, the use of coeliac disease antibody tests has increased significantly [1,2,18]. The widely used serum EMA and tTG-ab tests are available only in centralized laboratories and testing requires expert personnel in ideal conditions and therefore a long testing time. Here, we present an innovation, an in-house POCT method to measure tTG-ab rapidly and economically from a whole blood sample using the patient's own red blood cell tTG as coeliac disease autoantigen. The principle of the in-house POCT method is further utilized in a more user-friendly test application, a commercial whole blood *Biocard test*, which can also be used in physician's office, domestic circumstances or non-centralized laboratories, for example in a developing country. The main focus here was on evaluating the in-house POCT, as whole blood samples from all patients were not available for Biocard testing done subsequently. Nonetheless, in some of the patients *Biocard testing* was also carried out and the test results in this sample were as those in our previous study of *the Biocard test* [10]. The test recognized the untreated condition as well as the other tests. On the other hand, *the Biocard test*, unlike the in-house POCT and the serological tests, gave some positive test results among non-coeliac controls in the study. However, a false-positive test result should not be considered a problem, as untreated coeliac disease can be excluded by using other laboratory tests and examining small-bowel mucosal morphology.

Treatment of coeliac disease with a gluten-free diet and follow-up of the patients to ensure strict dieting is essential to avoid the known complications of the condition [1,4,19–23]. Application of serology has been recommended and used widely in the dietary assessment of coeliac disease [24]. In this study, 48 untreated coeliac disease patients were followed up and tested after a 1-year gluten-free diet. Similar to the serological antibody tests, the in-house POCT also proved satisfactory in dietary assessment of the disease, as a positive POCT result seroconverted or the reaction at least weakened in 90% of coeliac patients after adopting a gluten-free diet (Fig. 3). Moreover, the duodenal villous structure of these patients recovered in parallel with the seroconversion in POCT. Four patients remained strongly positive in the test despite the diet and two of them had admitted dietary lapses, and all the in-house POCT-positive treated patients still had partial villous atrophy in their duodenal biopsy. It has to be remembered, however, that the coeliac disease antibody tests may remain negative despite occasional dietary transgressions. Furthermore, a coeliac disease test result can seroconvert from positive to negative despite an ongoing small-bowel mucosal damage [25,26]. Yet, a seroconversion from positive to negative test result suggests a favourable response to the diet. Afterwards, if the test result seroconverts from negative to positive during the diet it is an indication of dietary lapses, which is a subject that should be discussed with the patient.

In the present study, the patient group consisted of a high-risk population, in which the prevalence of coeliac disease differs greatly from the prevalence of coeliac disease in the general population (approximately 1%) [2,3,18]. As the whole blood self tTG-based rapid test proved accurate in coeliac disease antibody detection the value of the test should next be examined on a population level in different centres. Also the cost-effectiveness evaluations are needed about the whole blood rapid testing in the future. On the basis of this study it can be foreseen that the whole blood rapid test is a good tool in coeliac disease screening as it can easily be performed straight from a whole blood sample without any need for laboratory equipment or expert personnel. These advantages enable minimal workload, immediate decision-making and sending a patient straight to intestinal biopsy. A prompt diagnosis of coeliac disease by screening might further reduce costs related to avoidance of unnecessary medical examinations and laboratory testing, not to mention personal suffering of a coeliac disease patient. Nevertheless, it has to be noted that coeliac disease antibody testing is a first step in the diagnostic work-up of coeliac disease and the diagnosis of the condition is still based on intestinal biopsy, after which an action has to be taken to set a patient on a strict gluten-free diet.

None of the present patients was suffering from selective IgA deficiency. Thus, detection of total IgA in the patient's serum was not included in the in-house POCT as in the pilot study of POCT previously reported [8]. When a patient is known to be IgA-deficient IgG-class coeliac antibodies should measured instead [27,28], as the in-house POCT remains negative.

To conclude, the rapid and easy in-house POCT offers a novel means of measuring coeliac-specific autoantibodies directly from a whole blood sample, which makes coeliac disease antibody testing possible on site even in a local health care centre. Like the conventional serological coeliac disease antibody tests the in-house POCT is sensitive and specific in finding untreated coeliac disease and helps in the dietary monitoring of a coeliac disease patient.

Practice points

- Coeliac disease is often underdiagnosed due to the protean clinical picture of the condition.
- Serum autoantibodies against tTG (tTG-ab and EMA) are valuable in coeliac disease case finding and selecting patients to undergo diagnostic intestinal biopsy.
- Coeliac disease autoantibodies can now be detected reliably without a need for serum separation, external antigen or centralized laboratory facilities by a novel whole blood self tTG-based rapid test.

Research agenda

- The value of the whole blood rapid coeliac disease test should next be evaluated on a population level.
- After obtaining results from screening studies cost-effectiveness of the whole blood rapid testing can be calculated.

Conflict of interest statement

None declared.

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Comparison of a novel whole blood transglutaminase-based ELISA with a whole blood rapid antibody test and established conventional serological coeliac disease assays

*Tiina Raivio, M.D., * **Ilma R. Korponay-Szabó, M.D., *Tuula Paajanen, BSc, * §Merja Ashorn, M.D., *Sari Iltanen, M.D., †‡Pekka Collin, M.D., *Kaija Laurila, MSc, **Éva Nemes, M.D., ††Judit B. Kovács, M.D., ¶Géraldine Carrard, PhD, ‖Mika Saramäki, MSc, * §Markku Mäki, M.D., *†‡ Katri Kaukinen, M.D.

*Paediatric Research Centre and †Medical School, University of Tampere, Departments of ‡Gastroenterology and Alimentary Tract Surgery and §Paediatrics, Tampere University Hospital, Tampere, ¶Research and Development Department, Ani Labsytems Ltd. Oy and ‖Research and Development Department, Ani Biotech Oy, Vantaa, Finland, and **Department of Paediatrics, Medical and Health Science Centre, University of Debrecen, Debrecen and ††Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest, Hungary

Short title: Whole blood self-tTG antibody testing in coeliac disease

Correspondence: Katri Kaukinen, M.D,

Medical School FIN-33014 University of Tampere, Finland

Tel: +358 3 3551 8403, fax: +358 3 3551 8402, email: katri.kaukinen@uta.fi

ABSTRACT

Objectives: Serum IgA-class tissue transglutaminase (tTG-ab) and endomysial antibody (EMA) tests play a key role in the diagnostic work-up of coeliac disease. Recently, a novel self tissue transglutaminase (tTG)-based whole blood rapid test was developed for coeliac disease case finding. Based on the same principle, a whole blood self-tTG enzyme linked immunosorbent assay (ELISA), especially applicable for large-scale screening of coeliac disease, have been produced. We assessed the value of this new test in coeliac disease antibody detection.

Methods: The new test utilizes endogenous tTG found in red blood cells of whole blood in IgA-class tTG-ab measurement by detecting tTG-tTG-ab complexes formed after haemolysing the whole blood sample. Stored whole blood samples from 150 untreated coeliac disease patients and 107 non-coeliac disease controls were evaluated and the test results compared with those of the whole blood rapid test, two conventional serum-based tTG-ab ELISA and two EMA tests.

Results: Altogether 15 whole blood samples were found to be clotted or dried after storage and were excluded from further evaluations. The whole blood ELISA test had a specificity (98%) comparable to that of the conventional serological tests, the sensitivity (91%) being slightly lower. The test was concordant with the whole blood rapid test in 92% of cases, with two serological ELISA tests in 91% and 94% and with EMA tests in 94% and 93%.

Conclusions: Whole blood self tTG-based testing is accurate in coeliac antibody detection, also when an ELISA method is applied. The testing requires no serum separation or external tTG.

INTRODUCTION

Coeliac disease is a gluten-induced genetically determined lifelong autoimmune disorder presenting with a variety of symptoms ranging from gastrointestinal problems to extraintestinal manifestations, or patients can also be totally asymptomatic (1-3). The condition affects approximately 1% of the European population and high prevalences are reported widely around the world (4-9). However, as revealed by screening studies, 85-90% of sufferers still remain undiagnosed (3, 10). Diagnosis is based on histological finding of small-bowel mucosal villous atrophy with crypt hyperplasia (11). Gluten-triggered tissue autoantibodies, endomysial (EMA) and tissue transglutaminase antibodies (tTG-ab), can be used as a first-step non-invasive coeliac disease screening method among subjects evincing symptoms indicative of the disease and in coeliac disease risk groups such as first-degree family members or patients having other autoimmune diseases (5, 6, 12-14).

IgA-class EMA, measured from serum samples by an indirect immunofluorescence (IF) method, are highly specific for coeliac disease (15). However, the IF method is laborious and requires visual interpretation, which is subjective (16). Since the recognition of tissue transglutaminase (tTG) as the major coeliac disease autoantigen, serum tTG-ab enzyme-linked immunosorbent assays (ELISAs) have been produced for easier and objective coeliac disease antibody detection (17-21). Many of the ELISA tests today utilize human recombinant tTG for the detection of antibodies. Recognition that the enzyme, tTG, is also present in human red blood cells (22) led to the introduction of serological tTG ELISA tests with human native red blood cell-derived antigen (23, 24). Recently, a whole blood-based rapid self tTG method was developed to detect IgA-class tTG antibodies directed to the patient's own red blood cell tTG, self tTG (25). In self-tTG testing a whole blood sample is haemolysed, resulting in liberation of the enzyme from the red blood cells and in complexing with autoantibodies if present in the serum of the sample (26).

This novel means of detecting tTG-ab from whole blood (25, 27-29) is also suitable for coeliac antibody detection using a recently developed self tTG-based ELISA method applicable for large-scale screening of coeliac disease. We now assessed the value of the self tTG-based tTG-ab ELISA in detecting untreated coeliac disease patients and non-coeliac disease controls, and compared the test results with those of the rapid test, two widely used serum tTG-ab ELISA and two EMA tests. Small-bowel mucosal histology was used as reference.

MATERIALS AND METHODS

Patients

The study group consisted of consecutive 150 untreated coeliac disease patients (96 female, median age 9 years, range 1.4-40) and 107 non-coeliac disease controls (41 female, median age 12 years, range 1-55). The patients were examined between the years 2000-2005 at the Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest, Hungary and at the Department of Peadiatrics in Tampere University Hospital, Finland. The coeliac disease diagnosis was based on severe partial, subtotal or total villous atrophy with crypt hyperplasia, also defined as Marsh IIIA-IIIC in the Marsh classification, in the small bowel and on the clinical and/or histological response to a gluten-free diet (11, 30). Non-coeliac disease controls with normal small-bowel mucosal morphology comprised subjects with dyspepsia (n = 44), autoimmune condition such as Crohn's disease (n = 20) or colitis ulcerosa (n = 7), gastro-oesophageal reflux disease (n = 12), intestinal polyposis (n = 8), non-infectious unspecific gastroenteritis or colitis (n = 2), retarded growth (n = 2), unspecific abdominal pain (n = 3), melaena (n = 1), constipation (n = 1), vomiting (n = 1), abscess of the buttocks (n = 1), haematochezia (n = 1), cystic fibrosis (n = 1), congenital sucrase-isomaltase deficiency (n = 1), intestinal lymphangiectasia (n = 1) and Shwachman Diamond

syndrome (n = 1). Serum and ethylene diaminetetraacetic acid (EDTA) or sodium citrate whole

blood samples were obtained at the time of biopsy and stored at –20 C until tested.

Whole blood self-tTG antibody testing

In self tTG-based coeliac antibody testing the patient's own tTG found in red blood

cells in whole blood is used as an antigen for IgA-class tTG-ab detection by haemolysing a whole

blood sample and liberating tTG from the red blood cells (26). If tTG-ab is present in the sample it

will complex with its liberated autoantigen. The complexes are captured from the haemolysed

sample by tTG binding protein to a solid support. IgA-class tTG-ab is futher visualized by a

solution conjugated with horseradish peroxidase (HRP)-labelled anti-human IgA. This innovative

test principle to detect tTG-ab without the need for external tTG and serum separation was first

investigated in point-of-care fashion (25, 29) and subsequently by a commercial rapid lateral flow

immunochromatographic test (27, 28). As the testing proved accurate in IgA-class tTG-ab

detection, the pinciple was further developed into a commercial ELISA method suitable for large-

scale coeliac disease antibody screening.

In the self tTG-based whole blood tTG-ab ELISA (Celiac IgA EIA, catalog number

6300100, Ani Labsystems Ltd. Oy, Vantaa, Finland, abbreviated as self-tTG ELISA in this report)

IgA-class tTG-ab is detected by binding the patient's own tTG immunocomplexed by its

autoantibodies to a specific antigen attached to the polystyrene surface of a 96-well Microstip®

(Table 1). Autoantibodies, if present, are further discerned as a colour reaction by using solutions

containing HRP-labelled anti-human IgA and chromogen. In this study the whole blood tTG-ab

ELISA was carried out in the laboratory in blinded fashion according to the manufacturer's

instructions (31). A value ≥ 5.0 U/ml was considered positive, as suggested by the manufacturer.

Additionally, self-tTG testing was carried out with the whole blood self-tTG rapid test (Biocard™ Celiac Test, catalog number 3-027-000, Ani Biotech, Vantaa, Finland) according to manufacturer's instructions (Table 1). The whole blood rapid test, utilizing the same principle as the whole blood self-tTG ELISA, is a lateral flow immunochromatographic test measuring IgA-class tTG-ab within five minutes (27). In the test used in this study, the signal generator, gold-labelled mouse antibodies to human IgA, was bound to the filter tip of the tube containing the haemolysing sample buffer. Otherwise, the test functioned in the same manner as previously described (27).

Conventional serum-based antibody testing

The methods used here to measure coeliac disease antibodies are presented in Table 1. IgA-class tTG-ab was determined by a commercial ELISA using native human tTG isolated from red blood cells as antigen (QUANTA Lite™ h-tTG IgA, INOVA Diagnostics, San Diego, USA, abbreviated as nh-tTG ELISA in this report), the suggested cut-off value being ≥ 20 units. tTG-ab were also determined by another commercial ELISA using human recombinant tTG as antigen (Celikey™, Phadia GmbH, Freiburg, Germany, abbreviated as hr-tTG ELISA in this report) with concentrations ≥ 5.0 U/ml being considered positive.

Serum IgA-class EMA was determined by an indirect IF method using human umbilical cord as antigen (abbreviated as in-house EMA) (19, 32). A titre of 1: ≥ 5 was considered positive. Additionally, EMA was also measured by a commercial indirect IF assay using primate smooth-muscle tissue as a substrate and IgA/IgG conjugate (The ImmuGlo™ Anti-Endomysial Antibody Test System, IMMCO Diagnostics, Buffalo, USA, abbreviated as commercial EMA in this study), a titre of 1: ≥ 2.5 being considered positive.

Statistical Analysis

Sensitivities, specificities, negative and positive predictive values and efficiencies of the tests were calculated for the six coeliac disease antibody tests (33).

Ethical considerations

The study protocol was approved by the local ethical committees in Hungary and Finland. All subjects gave informed consent.

RESULTS

The results obtained from 242 intact samples are shown in Table 2 for each antibody test; whole blood samples from 11 untreated coeliac disease patients and four non-coeliac disease controls were found to be clotted or dried after storage and were excluded from further evaluations. The whole blood self-tTG ELISA had a specificity (98%) similar to that of the serum tests, but the sensitivity (91%) was slightly lower compared to that of 99% of all serological tests. The corresponding figures for the rapid test were 94% and 93%, respectively. The positive predictive values of the self-tTG ELISA and of the rapid test were 98% and 96%. Table 3 shows the false-negative and false-positive self-tTG ELISA results as compared to those of the other tests.

In untreated coeliac disease and non-coeliac disease controls the concordance between the self-tTG ELISA and the whole blood self-tTG rapid test was 92%, the self-tTG ELISA and the serum nh-tTG ELISA 91%, the self-tTG ELISA and hr-tTG ELISA 94%, the self-tTG ELISA and the in-house EMA test 94% and the self-tTG ELISA and the commercial EMA test 93%. Whole blood and serum coeliac disease antibody test results for each of the six tests in untreated coeliac disease patients and non-coeliac disease controls as well as the discordances between the different

tests are illustrated in Figure 1. The six antibody test results were concordant throughout in 86% of all cases. The whole blood self-tTG ELISA agreed with at least one other coeliac disease antibody test in 97% of cases.

DISCUSSION

It has previously been shown that patients' own red blood cell tTG, self tTG, -based whole blood coeliac disease antibody tests utilizing either Nunc-Immunostick or the lateral flow immunochromatographic strip system are reliable in case finding and dietary monitoring of coeliac disease (25, 27-29). In the present study we further showed that the same principle might also be utilized in an ELISA format suitable for large-scale screening purposes. In our series, the specificity of the IgA-class whole blood self-tTG ELISA was comparable to those of the IgA-class serological coeliac disease antibody tests, while the sensitivity of the test was slightly lower. Additionally, self tTG-based whole blood rapid testing was accurate in detecting coeliac disease, as previously shown (27, 28) and was extremely easy and quick to carry out compared to the other tests.

In this paper we present a novel method, the whole blood self-tTG ELISA, for IgA-class tTG-ab detection and selection of patients to undergo diagnostic endoscopy without need for serum separation or external antigen. As the sensitive antigen tTG is not included in the test kit, the shelf life of the test is long. The test might thus also preserve its functionality better in demanding conditions such as exceptional storage temperatures and humidity, than external tTG-utilizing serum tTG-ab ELISAs (22). Additionally, the whole blood self-tTG ELISA, like the serum tTG-ab ELISA tests, is objective in interpretation and easier and faster to carry out compared to serum indirect IF EMA tests (16). Moreover, theoretically the new whole blood self-tTG ELISA test could be more economic in the future compared to the conventional serum-based coeliac disease antibody tests as labour costs can be reduced when serum separation is not needed and external tTG antigen

is not utilized in the self-tTG ELISA testing. When the self tTG-based whole blood rapid testing is applied even more cost savings might be generated as the testing can be easily and quickly carried out without sample transportation, expert personnel and laboratory facilities (34).

As previously shown, we as well here detected discordance between results obtained from different tests even though they measure the same antibodies (20, 35-39) (Figure 1). Some discrepant test results were also detected between the two self-tTG whole blood antibody tests. This might partly be explained by the use of different kinds of test formats and reagents although the test principle is the same. Likewise, discordance was seen not only between the two whole blood self-tTG tests but also between two different well-established serum ELISA and EMA tests (Figure 1). This has been the case in all serological tests so far developed (20, 35-39), and no ideal test exists.

The current study showed, however, that in whole blood self-tTG testing the sample should be of good quality to guarantee effective exploitation of its red blood cell tTG. tTG is a sensitive protein (22) and if damaged might prove incompetent to form immunocomplexes with its serum autoantibodies. To ensure proper function of the antigen and the test, multiple freezing, thawing or prolonged storage of samples should be avoided. Moreover, clotted whole blood samples should not be used and where possible fresh whole blood samples should be preferred. In our series 15 of the whole blood samples were discarded because as being clearly damaged after storage due to clotting or drying. When we carried out self-tTG ELISA with damaged whole blood samples we often obtained negative test results. In contrast, when the counterpart serum samples were mixed with fresh coeliac antibody-negative whole blood containing undamaged red blood cells, all but one false-negative test result was again positive (data not shown). This finding suggests that the sensitivity of the whole blood self-tTG tests might be improved when whole blood samples with functional tTG able to form immunocomlexes are applied.

IgA-class serum tTG-ab and EMA, as well as the whole blood self-tTG rapid or ELISA tests, are not suitable for determination of coeliac disease autoantibodies in patients

suffering from selective IgA deficiency, which is found more frequently in coeliac disease patients compared to the general population (40). In such cases coeliac disease antibodies should be measured in IgG-class with the conventional serum tTG-ab and EMA tests (32, 41, 42), or untreated coeliac disease should be excluded by intestinal biopsy. In this study one coeliac disease patient suffered from selective IgA deficiency and was thus positive only in the commercial EMA test using IgA/IgG conjugate. Furthermore, it is known that sero-negative coeliac disease exist, but mainly among adults (43). It has been shown earlier that the self tTG-based whole blood testing is working equally in this kind of patient material as the conventional serological coeliac disease antibody tests (29).

In conclusion, whole blood self tTG-based testing is suitable for large-scale IgA-class coeliac disease antibody screening using the ELISA method. In order to obtain a reliable test result good whole blood sample quality is required. The self-tTG-based ELISA and rapid antibody tests offer health care professionals an alternative to the known serological IgA-class EMA and tTG-ab tests, and when using such tests there is no need for serum separation or external recombinant or purified tTG antigen.

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TABLE 1. *Methods used in the study to measure coeliac disease antibodies.*

Abbreviation	Commercial name and producer	Antigen	Test principle	Cut-off level
self-tTG ELISA	Celiac IgA EIA, Ani Labsystems, Vantaa, Finland	Whole blood sample red blood cell self tTG	Whole blood-based ELISA	5.0 U/ml
self-tTG rapid test	Biocard™ Celiac Test, Ani Biotech, Vantaa, Finland	Whole blood sample red blood cell self tTG	Whole blood-based immunochromatographic test	Visible colour formation
nh-tTG ELISA	QUANTA Lite™ h-tTG IgA, INOVA Diagnostics, San Diego, USA	Native human red blood cell-derived tTG	Serum-based ELISA	20 U
hr-tTG ELISA	Celikey™, Phadia GmbH, Freiburg, Germany	Human recombinant tTG	Serum-based ELISA	5.0 U/ml
in-house EMA	S-EMAbA, Service Laboratory, Coeliac Disease Study Group, University of Tampere, Finland	Human umbilical cord	Serum-based indirect IF	1:5
commercial EMA	The ImmuGlo™ Anti-Endomysial Antibody Test System, IMMCO Diagnostics, Buffalo, USA	Primate smooth-muscle tissue	Serum-based indirect IF	1:2.5

ELISA indicates enzyme-linked immunosorbent assay; tTG, tissue transglutaminase; EMA, endomysial antibody; IF, immunofluorescence.

TABLE 2. *Results of the different antibody tests in untreated biopsy-proven coeliac disease patients and non-coeliac disease controls.*

	Whole blood testing						Serum testing					
	self-tTG ELISA		self-tTG rapid test		nh-tTG ELISA		hr-tTG ELISA		in-house EMA		commercial EMA	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
CD, n=139*	126	13	129	10	138	1	137	2	138	1	138	1
Controls, n =103*	2	101	6	97	7	96	1	102	0	103	0	103
Sensitivity (%)	91		93		99		99		99		99	
Specificity (%)	98		94		93		99		100		100	
PPV (%)	98		96		95		99		100		100	
NPV (%)	89		91		99		98		99		99	
ET (%)	94		93		97		99		100		100	

CD indicates coeliac disease; PPV, positive predictive value; NPV, negative predictive value; ET, efficiency of the test; self-tTG, self tissue tranglutaminase-based; ELISA, enzyme linked-immunosorbent assay; nh-tTG, native human red blood cell-derived tissue transglutaminase-based; hr-tTG, human recombinant tissue transglutaminase-based; EMA, endomysial antibody.

*Altogether 15 damaged samples excluded from evaluation.

TABLE 3. *False-negative and false-positive results obtained with the whole blood self-tTG antibody ELISA in biopsy-proven untreated coeliac disease patients and non-coeliac disease controls having normal mucosal morphology, respectively.*

Patient number	Whole blood testing		Serum testing			
	self-tTG ELISA	Self-tTG rapid test	nh-tTG ELISA	hr-tTG ELISA	in-house EMA	Commercial EMA
	(cut-off 5.0 U/ml)	(test result –ve or +ve)	(cut-off 20 U)	(cut-off 5.0 U/ml)	(test result -ve or +ve)	(test result -ve or +ve)
Untreated coeliac disease patients, false-negative						
1*	0	-	4	0.0	-	+
2	1.0	-	103	82.3	+	+
3	1.5	+	80	20.3	+	+
4	2.0	+	30	2.6	+	+
5	2.0	+	145	68.8	+	+
6	2.0	+	190	118.5	+	+
7	3.0	+	94	19.7	+	+
8	3.0	-	53	6.5	+	+
9	3.5	-	148	80.5	+	+

10	3.5	+	152	97.5	+	+
11	4.5	+	208	109.6	+	+
12	4.5	+	250	118.3	+	+
13	4.5	+	47	12.0	+	+

Non-coeliac disease controls, false-positive

14	5.5	+	8	1.4	-	-
15	8.5	+	19	3.0	-	-

Self-tTG indicates self tissue tranglutaminase-based; ELISA, enzyme linked-immunosorbent assay; nh-tTG, native human red blood cell-derived tissue transglutaminase-based; hr-tTG, human recombinant tissue transglutaminase-based; EMA, endomysial antibody.

*This subject was subsequently revealed to suffer from selective IgA deficiency and was thus posive only in the commercial EMA test using IgA/IgG conjugate.

FIGURE LEGEND

FIG. 1. Comparison between the IgA-class whole blood self tissue transglutaminase
ELISA (self-tTG ELISA), the IgA-class whole blood self-tTG rapid test, IgA-class
serum native human red blood cell-derived tTG ELISA (nh-tTG ELISA) and serum
human recombinant tTG ELISA (hr-tTG ELISA), serum in-house endomysial
antibody (EMA) and commercial EMA test results obtained in 242 cases (139
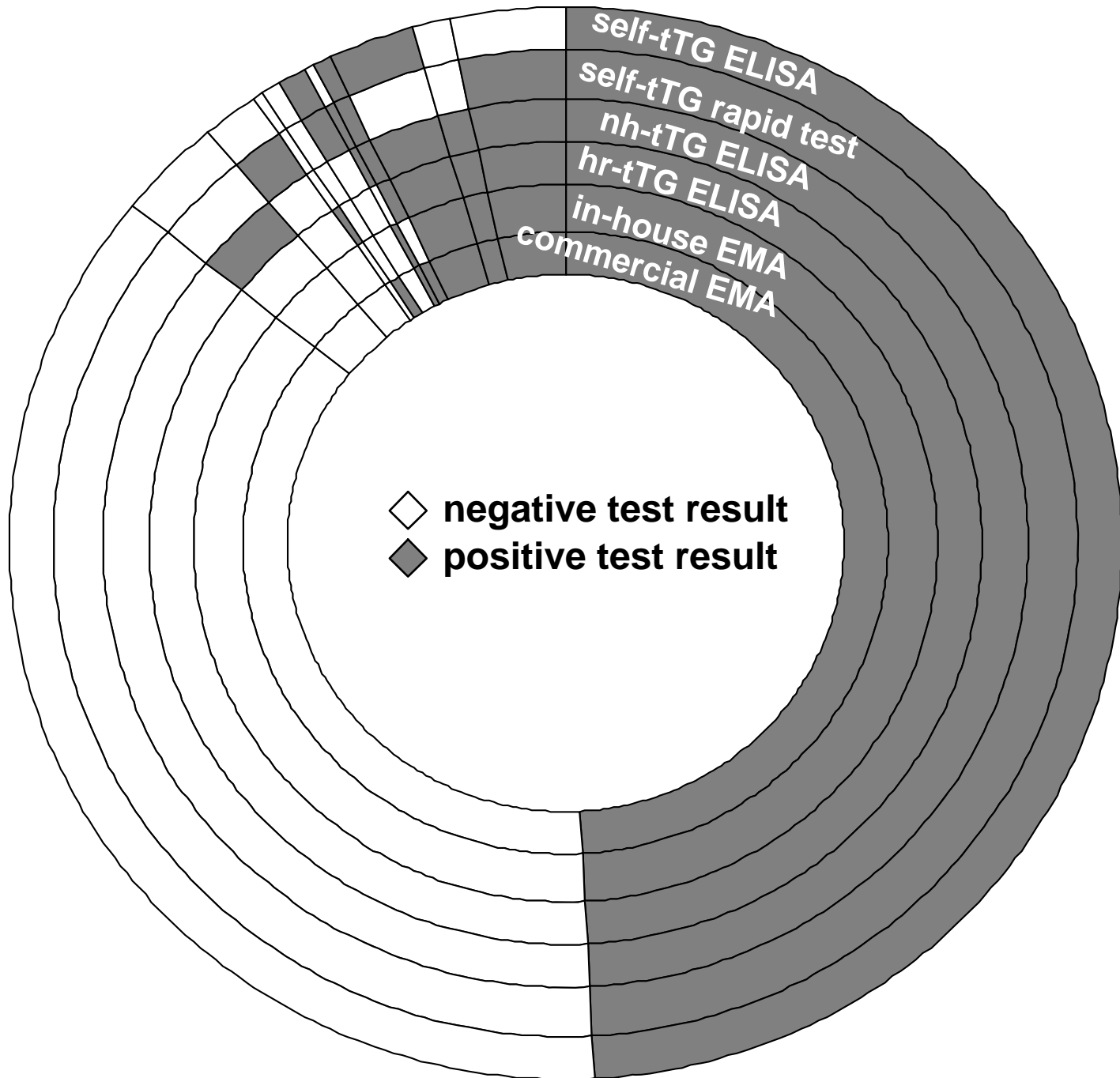untreated coeliac disease patients and 103 non-coeliac disease controls).

self-tTG ELISA
self-tTG rapid test
nh-tTG ELISA
hr-tTG ELISA
in-house EMA
commercial EMA

◇ negative test result
◆ positive test result

Figure 1

Self transglutaminase–based rapid coeliac disease antibody detection by a lateral flow method

T. RAIVIO*, K. KAUKINEN†,‡, É. NEMES¶, K. LAURILA*, P. COLLIN†,‡, J. B. KOVÁCS**, M. MÄKI*,§ & I. R. KORPONAY-SZABÓ*,¶

*Paediatric Research Centre and †Medical School, University of Tampere; ‡Departments of Gastroenterology and Alimentary Tract Surgery and §Paediatrics, Tampere University Hospital, Finland; ¶Department of Paediatrics, Medical and Health Science Centre, University of Debrecen, Debrecen; **Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest, Hungary

Correspondence to:
Dr M. Mäki, Medical School FIN-33014 University of Tampere, Finland.
E-mail markku.maki@uta.fi

SUMMARY

Background
The conventional coeliac disease antibody tests require patient's sera, and are laborious and time-consuming.

Aim
To evaluate a newly developed rapid whole blood test in coeliac disease antibody detection, and its suitability for office use.

Methods
Endogenous tissue transglutaminase found in red blood cells in a whole blood fingertip or venous sample is liberated upon haemolysis and complexes with tissue transglutaminase antibodies, if present. The complexes, captured by a lateral flow system, are visualized within 5 min. Stored samples from 121 untreated, 106 treated coeliac disease patients and 107 controls were evaluated and compared with serum endomysium and tissue transglutaminase antibody tests and histology; 150 patients were prospectively tested on site in the doctor's office.

Results
The rapid test showed sensitivity (96.7%) comparable with the serum endomysium and tissue transglutaminase antibody tests from stored samples; specificity was slightly lower (93.5%). When tested on site the results were concordant in 96.7% of cases compared with endomysium and tissue transglutaminase antibody results. The test recognized the disappearance of tissue transglutaminase antibodies on a gluten-free diet.

Conclusions
The self tissue transglutaminase-based rapid test can be easily carried out from a fingertip blood sample on site in the physician's office for both coeliac disease case finding and dietary monitoring purposes.

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INTRODUCTION

In untreated coeliac disease the clinical picture can range from the classic abdominal symptoms to extra-intestinal manifestations, or the disease may even be clinically silent.[1] The prevalence of the disease is as high as 1 in 100,[2, 3] but because of its protean picture, it frequently remains undiagnosed. General practitioners are in a crucial position in detecting the condition and therefore a non-invasive test which is also easy to use in a general practitioner's office would be helpful in selecting patients to undergo diagnostic small-intestinal biopsy.[4]

The conventional immunoglobulin (Ig) A-class endomysial antibody (EMA) test based on an indirect immunofluorescent (IIF) method is highly specific (97–100%) and sensitive (90–100%) in coeliac disease case finding,[5] but is subjective in interpretation.[6] Since the identification of tissue transglutaminase (tTG) as the endomysial autoantigen in coeliac disease,[7] it has been possible to develop easier and less expensive enzyme-linked immunosorbent assay (ELISA)-based screening tests.[8, 9] Both of these conventional screening tests require patient's sera and special laboratory facilities and test results are available only after a time lag.

The coeliac disease autoantigen, tTG, is an intracellular enzyme found for example in fibroblasts, endothelial, mononuclear and also red blood cells.[10] We recently established that the patient's own tTG can be used in coeliac disease antibody detection by haemolysing the whole blood sample and thus liberating the enzyme from the red blood cells.[11] The liberated tTG complexes with circulating coeliac-specific autoantibodies, if present. In this method there is thus no need for purified or recombinant tTG or for serum separation. We also showed the rapid point-of-care test, based on this new innovation, to have a sensitivity of 97% and a specificity of 98% in detecting untreated coeliac disease.[12] As the proof-of-concept test proved to be highly predictive for the disease, a more user-friendly rapid whole blood coeliac disease test utilizing a lateral flow method and the patient's self tTG was developed. The test can be performed from a finger tip or venous whole blood sample in a few minutes and interpreted visually on site.

Our aim was to evaluate the new self tTG-based rapid whole blood test in detecting coeliac disease and in monitoring treatment. We first assessed stored samples from coeliac disease patients and non-coeliac controls in a laboratory setting and secondly, sought

to establish whether the new test works on site in the doctor's office in selecting patients for confirmatory small-bowel biopsy. The results of the rapid whole blood test were compared with those in conventional serum EMA and tissue transglutaminase antibody (tTG-ab) tests and to small-bowel mucosal histology.

PATIENTS AND METHODS

Subjects

The patients were investigated at the Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest, Hungary, at the Department of Paediatrics, University of Debrecen, Hungary and at the Department Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital, Finland.

In the first part of the study the rapid test was performed in the laboratory on stored whole blood samples. The study group comprised 121 consecutive untreated coeliac disease patients and 107 non-coeliac disease controls. The diagnosis of coeliac disease was based on severe partial or subtotal villous atrophy with crypt hyperplasia in the small-bowel and on the clinical or histological response to a gluten-free diet.[13] Patients evincing normal villous morphology served as non-coeliac controls. Demographic data on the patients and controls and the main indication for serological coeliac disease testing are shown in Table 1. None of the patients suffered from IgA deficiency. Follow-up results were available in 15 of the above-mentioned newly detected coeliac disease patients (median age 34 years, range 9–68 years) after 1 year on a gluten-free diet. Moreover, samples from 91 long-term treated (median duration of a strict gluten-free diet 9 years, range 1–24 years) coeliac disease patients (61 female; median age 58 years; range 23–82 years) were tested in laboratory. Small-bowel mucosal biopsy, serum and whole blood samples with ethylenediamine-teraacetic acid (EDTA) or sodium citrate were obtained from all patients before and after the gluten-free diet and stored at −20 °C until tested.

To assess the rapid whole blood testing onsite, 150 patients with suspicion of coeliac disease were studied prospectively in a tertiary gastroenterology centre (Table 1). Altogether 78 of these patients were referred to special health care due to symptoms suggestive of coeliac disease and the remaining 72 were first-degree family members of coeliac disease patients. The rapid test was performed from a fresh fingertip sample and

Table 1. Demographic data on untreated coeliac disease patients, non-coeliac disease controls and prospectively tested patients with coeliac disease suspicion

	Laboratory testing		On site testing
	Untreated coeliac disease ($n = 121$)	Non-coeliac disease controls ($n = 107$)	Prospectively tested patients ($n = 150$)
Age: median (range), years	12 (1.6–68)	15 (0.9–72)	9 (0.9–72)
Patients under 16 years, n (%)	81 (67)	59 (55)	95 (63)
Female, n (%)	85 (70)	46 (43)	86 (57)
Indication for coeliac disease antibody testing, n (%)			
Gastrointestinal complaints	85 (70)*	93 (87)†	58 (37)*
Anaemia or malabsorption	12 (10)	8 (7)	6 (4)
Screening of associated diseases or extraintestinal manifestations known to carry an increased risk of coeliac disease	24 (20)‡	6 (6)§	86 (57)¶

* Diarrhoea, abdominal distension and pain.
† Dyspepsia ($n = 60$), gastro-oesophageal reflux disease ($n = 15$), inflammatory bowel disease ($n = 14$), irritable bowel syndrome ($n = 2$), recurrent abdominal pain ($n = 2$).
‡ Insulin-dependent diabetes mellitus ($n = 2$), family history of coeliac disease ($n = 11$), retarded growth ($n = 5$), eating disorder ($n = 3$), arthritis ($n = 2$), rash ($n = 1$).
§ Familial adenomatosus polyposis ($n = 4$), intestinal lymphangiectasia ($n = 1$), rash ($n = 1$).
¶ Family history of coeliac disease ($n = 72$), rash ($n = 6$), retarded growth ($n = 6$), Sjögren's syndrome ($n = 1$), autism ($n = 1$).

the test result was interpreted immediately on site in the doctor's office and venous samples for serological EMA and tTG-ab testing were collected simultaneously. When patients yielded positive coeliac disease antibody test results they were also invited to undergo diagnostic small-intestinal endoscopy and biopsy.

Self tissue transglutaminase-based rapid coeliac antibody detection

The self tTG-based coeliac antibody detection was based on our innovation utilizing endogenous tTG found in the red blood cells.[11] The basic concept is to liberate the patient's own tTG from the red blood cells by haemolysing an anticoagulated citrated or EDTA whole blood sample. When tTG-specific antibodies are present in the sera they recognize and form complexes with the liberated self tTG. The complexes can be detected by binding tTG to a solid surface coated with tTG-capturing proteins. The bound antigen-antibody complexes can be seen in colour reaction with the help of labelled anti-human IgA solution.[12]

In the present study we evaluated a commercial application (Biocard Celiac disease™, AniBiotech, Vantaa, Finland) based on the above-mentioned innovation. This test utilizes lateral flow immunochromatographic strip system and colloidal gold-labelled mouse antibodies to human IgA as signal generator. In short, the testing was performed from thawed venous or fresh fingertip whole blood samples. Using a capillary supplied with the test, 10 μL of EDTA, citrate or capillary whole blood is added to the tube containing 0.5 mL of haemolysing sample buffer, thus achieving a sample dilution of 1:50. Three drops of the haemolysed sample dilution are then added to the round application field of the test card. In the card the diluted blood sample migrates by capillary diffusion through the conjugate pad, redydrating the gold conjugate.[14] If tTG-ab are present in the sample, they complex with tTG. These complexes bind with colloidal gold-labelled anti-IgA antibodies and are captured by tTG binding protein[12] linked to the nitrocellulose test membrane, forming a visible red test line (Figure 1). In addition, an integrated control system ensures the proper function of the test. The reaction in
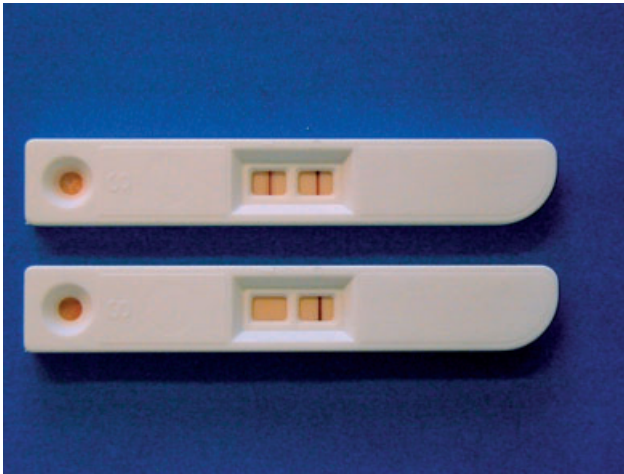
Figure 1. The rapid whole blood test card for coeliac-specific immunoglobulin A class tissue transglutaminase antibody detection. The haemolysed blood dilution is dropped onto the round application field. In a positive test result both the control line (right) and the line in the test field (left) can be seen (upper test card). When the result is negative (lower test card), only the control line is seen.

the control line happens between the colloidal gold-labelled anti-IgA mouse antibodies which passed the test line without binding and anti-mouse IgG antibodies, and shows that both the sample and reagents had moved over the test line and reached the control point. The manufacturer has suggested to interpret the results after 5 min but not later than 10 min. However, a positive test result may appear already within 1–2 min. The test result is positive when both the control line and the line in the test field can be seen; in negative cases only the control line forms.

Interobserver variability in the rapid test was assessed with 20 randomly selected EDTA or sodium citrate whole blood samples from the study cohort between two investigators in blinded fashion. Furthermore, intraobserver variation was evaluated blindly with the corresponding samples at different time points.

Serology

Serum IgA-class EMA was determined by an IIF method as previously described.[9, 15] Serum IgA-class tTG-ab were determined by ELISA using the Celikey[TM] test (Pharmacia Diagnostics, GmbH, Freiburg, Germany) according to the manufacturer's instructions.

Small-bowel mucosal morphology

Small-bowel mucosal biopsies were taken either by upper gastrointestinal endoscopy from the distal part of the duodenum or by Watson capsule from the proximal jejunum. Haematoxylin-eosin-stained biopsy specimens were studied under light microscopy and the villous height/crypt depth ratio calculated as previously described;[16] a ratio of <2 was considered to be abnormal and indicative of untreated coeliac disease.

Statistics

The sensitivities were calculated from the equation $a/(a + c) \times 100$, specificities $d/(b + d) \times 100$, positive predictive values $a/(a + b) \times 100$, negative predictive values $d/(d + c) \times 100$ and efficiencies of the tests $(a + d)/(a + d + c + b) \times 100$ respectively. In the equations, a stands for the number of untreated biopsy-proven coeliac disease patients recognized by the test; b for number of biopsy-proven non-coeliac disease controls with a positive test result; c for the number of untreated patients misclassified by the test and d for non-coeliac disease controls negative for the test.

Ethical considerations

The study protocol was approved by the local ethical committees in Hungary and Finland. All subjects gave informed consent.

RESULTS

In stored samples analysed in the laboratory the rapid whole blood test gave sensitivity results comparable with those of the serum EMA and tTG-ab tests (Table 2). The specificity of the rapid test was lower compared to the conventional serum tests. The test recognized untreated coeliac disease in children aged <16 years (sensitivity 99%, specificity 97%) better than patients aged over 16 years (sensitivity 93%, specificity 90%, respectively) (Table 3). The rapid test results were concordant with serum EMA test results in 215 of 228 cases (94.3%) and with serum tTG-ab test results in 216 (94.7%) respectively. In the laboratory, both the interobserver agreement between two investigators and the intraobserver agreement for the rapid whole blood test was 100%.

After adherence to a strict gluten-free diet for 1 year the rapid test result converted from positive to negat-

Table 2. Sensitivity, specificity, positive and negative predictive value and efficiency of the IgA-class rapid whole blood test, serum tissue transglutaminase antibody (tTG-ab) and serum endomysium (EMA) tests on stored samples in the laboratory. The sensitivities and specificities have been calculated from the untreated biopsy-proven coeliac disease patients and biopsied non-coeliac disease controls

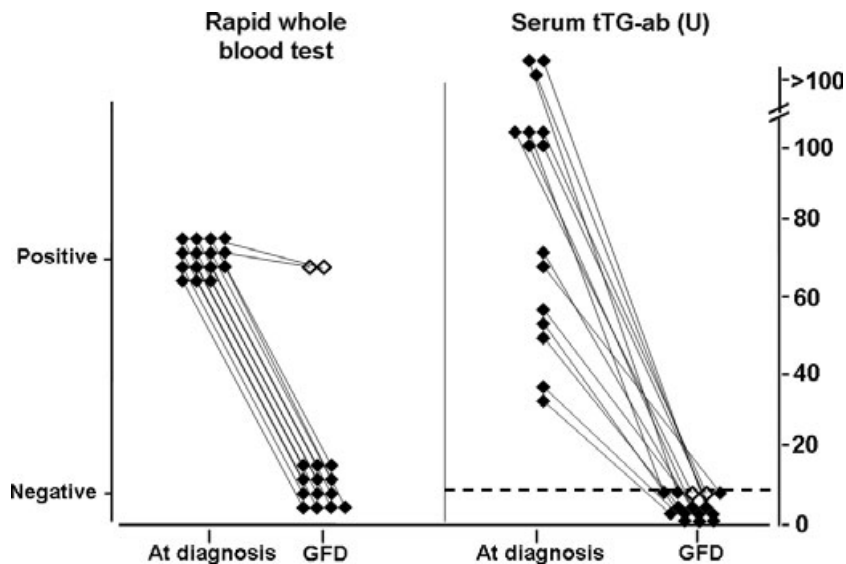|	Rapid whole blood test		Serum EMA		Serum tTG-ab	
	Positive	Negative	Positive	Negative	Positive	Negative
Untreated coeliac disease, $n = 121$	117	4	117	4	120	1
Controls, $n = 107$	7	100	0	107	0	107
Sensitivity (%)	96.7		96.7		99.2	
Specificity (%)	93.5		100.0		100.0	
Positive predictive value (%)	94.4		100.0		100.0	
Negative predictive value (%)	96.2		96.4		99.1	
Efficiency of the test (%)	95.2		98.2		99.6	

Table 3. Demographic data and the results of the immunoglobulin A-class rapid whole blood test, serum tissue transglutaminase antibody (tTG-ab) and serum endomysium (EMA) tests on patients under and over 16 years when tested on stored samples in the laboratory

	Patients under 16 years ($n = 140$)	Patients over 16 years ($n = 88$)
Female, n (%)	77 (55)	54 (61)
Indication for coeliac disease antibody testing, n (%)		
Gastrointestinal complaints	110 (79)	68 (77)
Anaemia or malabsorption	9 (6)	11 (13)
Screening of associated diseases or extra intestinal manifestations	21 (15)	9 (10)
Untreated coeliac disease patients, $n = 121$ (%)	81 (67)	40 (33)
Rapid test positive, n (%)	80 (99)	37 (93)
EMA positive, n (%)	81 (100)	36 (90)
tTG-ab positive, n (%)	81 (100)	39 (98)
Controls, $n = 107$ (%)	59 (55)	48 (45)
Rapid test positive, n (%)	2 (2)	5 (5)
EMA positive, n (%)	0 (0)	0 (0)
tTG-ab positive, n (%)	0 (0)	0 (0)

ive in 13 (87%) coeliac disease patients and the test result remained positive in two (Figure 2). Initially the two rapid test-positive treated patients had highly positive serum tTG-ab values before starting a gluten-free diet and they also had borderline serum tTG-ab results (4.2 and 4.6 U/mL) while adhering to the diet. In addition, from the 91 long-term treated coeliac disease patients 88 (96.7%) were negative in the rapid test, 88 (96.7%) in the serum EMA test and 90 (98.9%) in the serum tTG-ab test. Three of the 91 treated patients had small-bowel mucosal villous atrophy with crypt hyperplasia, in the rest villous mucosal morphology was normal. The rapid and serum EMA tests recognized two of the three patients with abnormal mucosa and the serum tTG-ab test one, respectively.

The rapid test, performed on site prospectively in the doctor's office, yielded concordant results with serologic EMA and tTG-ab tests in 145 of the 150 patients (96.7%) (Table 4). The rapid test achieved a sensitivity of 95.5% and a specificity of 97.1% in relation to serum EMA and tTG-ab results. Altogether 47 of the 150 patients (36 symptomatic patients and 11 first-degree relatives) were rapid test-positive. Forty-four of them agreed to undergo intestinal biopsy and they all had small-bowel mucosal lesion typical of coeliac disease (positive predictive value 100%). This high positivity rate was because of the fact that the setting of the testing was a tertiary centre with frequent referral of patients having a high probability of coeliac disease. The rapid test was negative in 103

Figure 2. The rapid whole blood test results and serum tissue transglutaminase antibody (tTG-ab) results in 15 coeliac disease patients at the time of diagnosis and after a one-year gluten-free diet. Diamonds connected with a line represent the values of the same patient before and after a gluten-free diet. Two coeliac disease patients (open diamonds) were still positive in the rapid test after a gluten-free diet and had also borderline tTG-ab values. The cut-off level for serum tTG-ab positivity (5 U/mL) is shown in the horizontal dotted line. GFD, gluten-free diet.

Table 4. Comparison of on site rapid whole blood test results and serum endomysial antibody (EMA) and tissue transglutaminase antibody (tTG-ab) test results when patients under coeliac disease suspicion were investigated prospectively

	Serum EMA		Serum tTG-ab	
	Positive	Negative	Positive	Negative
Rapid test positive	44	3	44	3
Rapid test negative	2*	101	2*	101

* One patient was positive in serum EMA and negative in tTG-ab test, the other negative in EMA and positive in tTG-ab test.

patients. Three of them had either positive serum EMA or tTG-ab test result and the small-bowel mucosal morphology showed villous atrophy with crypt hyperplasia.

DISCUSSION

The rapid self tTG-based whole blood test showed comparable sensitivity to detect untreated coeliac disease as the currently widely employed serological EMA and tTG-ab tests. The test result was easy to interpret visually on site and the test turned out to be highly repeatable and reproducible. This method speeds up and facilitates the diagnostic work-up of coeliac disease, as test-positive individuals can be sent for endoscopy without any time lag. The slightly lower specificity of the test in laboratory testing of stored samples is of no major importance, as the positive test results can be verified with serum EMA, tTG-ab or small-intestinal mucosal biopsy. Interestingly, three of the seven rapid test-positive controls without villous atrophy from the series tested in the laboratory showed signs of early developing coeliac disease upon further investigation beyond this study; they had coeliac-type HLA DQ2, an increased density of $\gamma\delta+$ intra-epithelial lymphocytes or tTG-specific IgA-deposits in their small-bowel mucosa.[17, 18] Furthermore, when the rapid testing was performed on site from fingertip blood, the test results were more concordant with the serum EMA and tTG-ab test results and had 100% positive predictive value for a coeliac-type histology finding. These results suggest that the rapid test might be more specific when used with fresh blood samples.

Currently, the only effective treatment of coeliac disease is a strict gluten-free diet.[1] It is known that the

coeliac-specific autoantibodies disappear from the blood during the diet parallel with the recovery of the small-intestinal mucosal damage.[19, 20] Similarly to the conventional serum tests, the rapid test result also seroconverted from positive to negative in coeliac disease patients after 1 year on a gluten-free diet and the test result was negative in 97% of long-term treated coeliac disease patients. The test might be thus suitable, in addition to coeliac disease case finding, for the detection of tTG-ab seroconversion from positive to negative after adoption of a long-term gluten-free diet. Subsequently, the test can be used again in coeliac disease patient's dietary monitoring, as a test result reconverted from negative to positive indicates dietary lapses. As noted in our earlier study, a rapid coeliac disease antibody test done on site in the doctor's office enables immediate feedback to encourage coeliac disease patients to strengthen their diet.[12] The commercial rapid test might also become available to coeliac patients themselves for diet monitoring at home as the manufacturer has taken care of the quality control issues outside laboratory situations and the test documentation has been evaluated and accepted for home testing and CE-marking (Communautée européenne) by Notified Body (RWTÜV Systems GmbH, the manufacturer number CE 0044).

The serological tTG-ab tests and also the previously reported coeliac disease rapid tests utilize external tTG,[21–23] which is sensitive to storage problems.[10] In the rapid test fresh tTG is liberated from the red blood cells of a whole blood sample on site. Furthermore, all the equipment needed in testing comes with the test kit and the test result can be read visually immediately. For these reasons, the coeliac disease rapid whole blood test seems to be useful in a wide range of circumstances, for example in developing countries or in remote areas, where are no centralized laboratories and sample storing possibilities.

The current rapid test was developed to uncover IgA-class tTG antibodies as do the frequently used serum IgA-class EMA and tTG-ab tests. However, clinicians must be aware of the limitation of IgA-class antibody detection in coeliac disease case finding among patients with selective IgA deficiency, which is found more often in coeliac disease patients.[24, 25] Further research is needed to attain a coeliac disease rapid test which can uncover, in addition to IgA-class tTG antibodies, also IgG-class tTG antibodies or deficiency of IgA. In addition, clinicians should be also aware of the variable prevalence of coeliac disease in different populations. In the study the prevalence of coeliac disease was high because the testing was done among coeliac disease risk groups. In general population the prevalence of coeliac disease is no more than 1 in 100.[2, 3] Instead, among first-degree relatives of coeliac patients the prevalence is clearly higher, around 10%.[1] Further investigations are still needed to see how the rapid test functions on a population level.

In conclusion, the present study showed that the self tTG-based commercial rapid whole blood test is as sensitive in detecting untreated coeliac disease as the conventional serum-based tTG-ab and EMA tests and thus in pinpointing patients for confirmatory endoscopy. The test also showed visually the seroconversion to negative during a gluten-free diet and it was easy to carry out onsite without any need for laboratory facilities. The test can therefore offer a useful tool in the general practitioner's office in coeliac disease case finding and coeliac disease diet monitoring.

ACKNOWLEDGEMENTS

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