-CONFIDENTIAL-

Celiac Quick Test CLINICAL TRIAL

Performance of the CELIAC Quick Test in Diagnosis

of Celiac Disease in a biopsy-controlled clinical trial.

Jointly Executed by:

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Research Team:

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Summary

Background: Celiac disease is an autoimmune disease caused by the ingestion of gluten. Classically, it presents with diarrhoea and failure to thrive within the first couple of years of life. The clinical manifestations of celiac disease that have been identified are extensive and varied and not necessarily confined to the gastrointestinal tract. Celiac disease is associated with many other autoimmune conditions, including autoimmune thyroid disease and type 1 diabetes. Conclusive diagnosis is based on abnormalities in small intestinal biopsy, and their recovery upon adherence with gluten-free diet (GFD). However, screening for celiac disease can be performed using serological markers with very high sensitivity and specificity, such as IgA antibodies to tissue transglutaminase (tTG). However, testing only for tTG IgA antibodies is hampered by the risk of missing IgA-deficient patients who have a 10-fold increased risk for celiac disease. This shortcoming is evaded by serological tests measuring all classes of tTG antibodies; e.g. the recently launched **CELIAC Quick Test** (Biohit Oyj, Helsinki), specific for tTG IgA-, IgG- and IgM-antibodies.

Objective: To test the clinical performance of the Biohit Celiac Quick Test in diagnosis of celiac disease.

Study Design: This clinical trial evaluates the performance of Celiac Quick Test among patients in whom the diagnostic algorithm for celiac disease remains to be completed by serological tTG testing and **endoscopy** with **duodenal biopsies**.

Methods: Study subjects (both genders) for the cohort are enrolled among the consecutive (adult and pediatric) patients with clinical suspicion of celiac disease who have been referred for completion of their diagnostic algorithm by serological testing and endoscopic duodenal biopsies at Hospital X (City Y, Country Z). CELIAC Quick test is performed following the manufacturer's instructions using the blood sample from the fingertip. All patients are subjected to endoscopic examination with directed biopsies from the duodenum, following the protocol of the American Gastroenterological Association (4 or more biopsies, including the duodenal bulb). Biopsies are examined at the Pathology laboratory of Hospital X, and interpreted using the modified **Marsh Classification** into one of the 5 stages (Marsh 0-4). Statistical analyses include calculation of the performance indicators (SE, SP, PPV, NPV) of the CELIAC Quick Test for the individual study endpoints (Marsh 2+, consistent with celiac disease), including ROC analysis (AUC) for the optimal sensitivity/specificity balance.

Specific Aims: The single most important goal of this study is to assess the overall performance of the CELIAC Quick Test in diagnosis of celiac disease in pediatric and adult population, using duodenal biopsies as **the gold standard**. Performance indicators are calculated separately for different study endpoints, based on modified Marsh Classification, where Score 2, Score 3 and Score 4 represent celiac disease, while Score 1 is considered non-diagnostic. Related to these specific aims, there are three clinically relevant measures to be addressed in this **100% biopsy-confirmed** study: 1) the rate of unnecessary referrals for endoscopy (false positive rate; 1-PPV) following a positive CELIAC Quick Test result; 2) the

rate of endoscopies to be avoided after a negative CELIAC Quick Test result (true negative rate; NPV), and 3) the rate of biopsy-confirmed celiac disease (Marsh 2+) that are missed by the CELIAC Quick Test(i.e., false negative rate; 1-SE).

Study execution and time table: The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached the agreement with Biohit Oyj. The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. Given that the subjects in the study will be enrolled among consecutive patients with clinically suspected celiac disease, attending the Outpatient Department of Endoscopy at Hospital X, it is estimated that 300 subjects (both genders, all age groups) need to be screened by CELIAC Quick Test to reach a cohort of 200 patents enriched with equal numbers (n=50) of all conditions of interest (Marsh 3+, Marsh 2, Marsh 1, March 0), needed to calculate the performance indicators for the study endpoints.

Impact of the study: State-of-art diagnosis of celiac disease is based on a diagnostic (5-score) algorithm, including 1) symptoms of celiac disease; 2) positive serology at high titer; 3) the presence of a DQ2 or DQ8 haplotype; 4) characteristic histological changes in duodenal biopsy; and 5) a serological or histological response to the GFD. Detection of IgA tTG antibodies is considered as the serological test of choice, but the downside of measuring only tTG IgA antibodies is the potential of missing the IgA-deficient subjects, with 10-fold higher risk of celiac disease. The CELIAC Quick Test detecting all classes (IgA, IgG, IgM) of tTG antibodies evades this risk. Being a quick test performed in a blood sample from the fingertip, this test is also user-friendly, particularly for pediatric patients. If confirmed to perform adequately in this clinical setting, Biohit CELIAC Quick Test should represent a major step forward towards a reliable and user-friendly **point-of-care diagnosis** of celiac disease, as well as in subsequent **monitoring** of the patients adhered to their gluten-free diet.

1. BACKGROUND

Celiac disease is an autoimmune disorder of the small intestine that occurs in genetically predisposed people of all ages from middle infancy onward (1,2). This condition has several other names, including celiac sprue, non-tropical sprue, endemic sprue, gluten enteropathy or gluten-sensitive enteropathy, and gluten intolerance. Symptoms include pain and discomfort in the digestive tract, chronic constipation and diarrhea, failure to thrive (in children), anemia and fatigue, but these may be absent, and symptoms in other organ systems have been described (3,4). Vitamin deficiencies are often noted in people with celiac disease owing to the reduced ability of the small intestine to properly absorb nutrients from food. Increasingly, diagnoses are being made in asymptomatic persons as a result of increased screening (5), and e.g. in the USA, the condition is thought to affect between 1 in 1,750 and 1 in 105 people (6).

Celiac disease is caused by a reaction to gliadin, a prolamin (gluten protein) found in wheat, and similar proteins found in the crops of the tribe Triticeae (which includes other common grains such as barley and rye)(7). Upon exposure to gliadin, and specifically to three peptides found in prolamins, the enzyme **tissue transglutaminase (tTG)** modifies the protein, and the immune system cross-reacts with the small-bowel tissue, causing an inflammatory reaction. That leads to a truncating of the villi lining the small intestine (called villous atrophy). This interferes with the absorption of nutrients because the intestinal villi are responsible for absorption. The only known effective treatment is a lifelong gluten-free diet (7).

1.1. Epidemiology

Our understanding of the epidemiology of celiac disease has evolved over the last several decades with the advent of serologic tests as screening tools for celiac disease. Celiac disease was initially thought to be relatively rare with prevalence rates of approximately 1:5000 (5). These rates were based on the classic presentation of the disease and the classical presentation is now viewed only one form of celiac disease. It has become clear that these

prevalence rates represent the tip of the celiac disease iceberg and the majority of people with celiac disease present with a milder, more insidious onset of symptoms. Screening of blood donors for antibodies associated with celiac disease have shown rates of positive antibodies of approximately 1:133 among non-risk individuals (8), and certain populations as high as 1:100 (9,10). Other studies using celiac disease-related antibodies followed-up with small intestinal biopsy have also revealed an overall prevalence of 1% across many different populations. Coeliac disease is more prevalent in women than in men. Most of the subjects have mild if any symptoms and would not have been identified without screening. This suggests that for every case identified through symptoms, another eight exist undetected (5).

The clinical consequences of an untreated individual with subclinical celiac disease are unclear but may range from none to significant with osteoporosis, infertility and intestinal lymphomas as potential risks. Given the background prevalence of celiac disease of 1:100, there are populations that are at an increased risk for disease, including patients with type 1 diabetes (11), autoimmune thyroid disease (both hyper- and hypothyroidism) (12,13), relatives of patients with celiac disease (8) and type 1 diabetes as well as patients with Turner (14,15) and Down syndromes (16). Rates of celiac disease in these populations range from 5–10% (2,5).

1.2.Clinical presentation

Severe celiac disease leads to the characteristic symptoms of pale, loose and greasy stool (steatorrhea) and weight loss or failure to gain weight (in young children). People with milder celiac disease may have symptoms that are much more subtle and occur in other organs than the bowel itself. It is also possible to have celiac disease without any symptoms whatsoever. Many adults with subtle disease only have fatigue or anemia (5).

1.2.1.Gastrointestinal manifestations

The clinical presentation of celiac disease is remarkably varied and depends on age (2,17,18).

The classic presentation with failure to thrive, malnutrition, diarrhea, abdominal pain and distension within the first couple of years of life represents the tip of what is commonly referred to as the "celiac disease iceberg". In contrast to the dramatic presentation noted typically in younger children, many patients with celiac disease present at a later age with subtle symptoms and the diagnosis of celiac disease may be delayed. Gastrointestinal symptoms may include abdominal pain, diarrhea or constipation, bloating, and excessive gas. Avoidance of foods containing gluten may also occur and a careful diet history is necessary to identify this symptom. Vitamin deficiencies due to fat mal-absorption can also occur. With longer-standing disease, patients may present with profound vitamin D deficiency resulting in rickets or hypocalcemia and tetany or coagulopathy secondary to vitamin K deficiency. Anemia secondary to iron and/or folate deficiency is also observed (2,5).

Children and adolescents often present with short stature and constitutional delay of puberty. Two to 8% of children and adolescents presenting for evaluation of short stature have evidence of celiac disease (19). Once endocrine causes of short stature have been excluded, rates of celiac disease increase two- to four-fold depending upon the population and referral base studies (19). Children presenting with celiac disease often will experience a decline in both height and weight growth velocity resulting in a decrease in the growth percentiles. In contrast, children presenting with constitutional delay of puberty often have low-normal growth velocity and will have no change in their growth percentiles. In the setting of declining growth percentiles or where the data are not available, the diagnosis of celiac disease should be entertained and testing with autoantibodies performed (20).

Adults have diarrhea as a major symptom of celiac disease in approximately 50% of cases (21). They may also be diagnosed in the setting of anemia or osteoporosis (2,5). Adults may be symptomatic for years prior to their diagnosis or have short stature (suggesting long-standing celiac disease). They are often initially misdiagnosed with irritable bowel syndrome (IBS) and may have had multiple procedures and/or hospital admissions that can ultimately

be traced to their undiagnosed celiac disease (21). Patients identified by screening due to genetic risk factors are often asymptomatic or mildly symptomatic for celiac disease (9). This is the population of individuals with celiac disease that is rapidly growing due to increased screening efforts.

1.2.2.Extra-intestinal manifestations

The phrase extra-intestinal manifestations of celiac disease refer to conditions that are associated with celiac disease and are at least partially responsive to a gluten free diet (2,5,21). The distinction from conditions that are associated with celiac disease can be difficult and categorization is not necessarily exact.

Arthritis involving both the peripheral and axial skeletal was originally reported in as many as 25% of patients presenting with celiac disease, but more recent reports suggest a much lower proportion of subjects with celiac disease presenting with arthritis (1%) (8). The arthritis is described as acute and non-erosive and generally resolves with the institution of a gluten free diet. Abnormalities of the dental enamel including pitting and or grooving may be present in up to 20–70% of patients with celiac disease (22). The diagnosis of celiac disease may therefore first be raised by the dentist finding dental enamel hypoplasia. The recurrent apthous stomatitis associated with celiac disease has been attributed to nutritional deficiencies and generally resolves with a gluten free diet (23). Abnormalities of liver transaminases occur in up to 40% of patients presenting with celiac disease, resolution of these elevations occurs in the majority of these patients upon treatment (24).

Neurologic and psychiatric disorders including depression, anxiety, irritability, peripheral neuropathy, cerebellar ataxia and migraines have all been reported (2). Hypotonia, developmental delay, epilepsy, headache, and ataxia were all reported in greater rates in subjects with celiac disease compared with controls. The majority of the reported associations did not improve with a gluten free diet (25).

Celiac disease is associated with a number of other medical conditions, many of which are autoimmune disorders: diabetes mellitus type 1, autoimmune thyroiditis, primary biliary cirrhosis, and microscopic colitis (2,6,21). In many other conditions ascribed to be associated with celiac disease, it is unclear whether the gluten-induced bowel disease is a causative factor or whether these conditions share a common predisposition. Such conditions include the following: 1) IgA deficiency; 2) Dermatitis herpetiformis; 3) Growth failure and/or pubertal delay in later childhood; 4) Recurrent miscarriage and unexplained infertility; 5) Hyposplenism (a small and underactive spleen)(2,5,6,21).

1.3.Pathogenesis

Celiac disease appears to be multi-factorial, both in that more than one genetic factor can cause the disease and in that more than one factor is necessary for the disease to manifest in an individual. Celiac disease is unique from other autoimmune diseases in that the there is a clearly identified environmental trigger (gluten), a dominant HLA contribution required for disease to occur (DQ2 or DQ8), and **autoantibodies against tTG** are detectable in over 95% of individuals (2,5,21).

1.3.1.Genetics

Almost all people with celiac disease have either the variant HLA-DQ2 allele or (less commonly) the HLA-DQ8 allele (5). However, about 20–30% of people without celiac disease have also inherited either of these alleles (26). This suggests additional factors are needed for celiac disease to develop. In other words, the predisposing HLA risk allele is necessary but not sufficient to develop celiac disease. Furthermore, around 5% of those people who do develop celiac disease do not have typical HLA-DQ2 or HLA-DQ8 alleles (2,3,4,5,21).

1.3.2.Prolamins

The majority of the proteins in food responsible for the immune reaction in celiac disease are prolamins. These are storage proteins rich in proline (prol-) and glutamine (-amin) that dissolve in alcohols and are resistant to proteases and peptidases of the gut (5). Prolamins

are found in cereal grains with different grains having different but related prolamins: wheat (**gliadin**), barley (hordein), rye (secalin), corn (zein) and as a minor protein, avenin in oats. Gliadin in wheat is the best-understood member of this family, but other prolamins e.g. hordein and secalin may contribute to celiac disease (8). However, not all prolamins will cause this immune reaction, and there is ongoing controversy on the ability of avenin to induce this response in celiac disease.

One region of α-gliadin stimulates membrane cells, enterocytes, of the intestine to allow larger molecules around the sealant between cells. Disruption of tight junctions allow peptides larger than three amino acids to enter circulation (27). Membrane leaking permits peptides of gliadin that stimulate two levels of immune response, the innate response and the adaptive (T-helper cell mediated) response. Gliadin is a glycoprotein extract from gluten that is felt to be directly toxic to the enterocytes of individuals with celiac disease, primarily through the overexpression of IL-15 in the intestine (28). This innate response to gliadin results in immune-system signaling that attracts inflammatory cells and increases the release of inflammatory chemicals (8). In addition, gliadin peptides have been shown to up-regulate both the stress molecule MIC-A on the surface of enterocytes and also the NKG2D receptor on the infiltrating intraepithelial lymphocytes, to promote a lymphocyte-mediated cytotoxic response against enterocytes that is also IL-15 dependent (28).

1.3.3.Transglutaminase (tTG)

TG is important in the pathogenesis of celiac disease in that the enzyme cross-links ingested gliadin and causes specific deamidation of glutamine into glutamic acid in gliadin peptides. When such deamidation occurs, the gliadin peptides are able to be more efficiently presented (in the context of MHC DQ2 molecule) to gliadin-reactive CD-4 T cells, therefore increasing its immunogenicity. Without TG, it is believed that gliadin is less immunogenic, and may not stimulate T cells as effectively (29).

Finally, since gliadin is unusually rich in proline residues, there is an intrinsic resistance to digestion in the intestines along with a preference for binding to DQ2 molecules. An example is a 33-AA residue of gliadin identified to be stable despite digestion with gastric, pancreatic, and intestinal brush border membrane proteases, with preserved immunogenicity (30). It is believed that the absorption of such larger intact peptides of gliadin allows the immunogenic response to occur.

Accordingly, TG appears to play a primary molecular role in cross-linking and deamidation of gliadin, with little evidence to support a direct immunological role. TG autoantibodies are proposed to occur by antigen presenting cells initially targeting the toxic gliadin peptides "inadvertently" take up TG-gliadin complexes, resulting in an immune reaction against **both gliadin and TG** (31). It has been proposed that TG autoantibodies play a role in disease pathogenesis, but lacks sufficient supportive evidence. Therefore, there is a combination of activity by the innate and adaptive immune system in the generation of gliadin-reactive T cells, a cytotoxic response, and autoantibody formation (2,5,21).

2.DIAGNOSIS

There are several tests that can be used to assist in diagnosis of celiac disease. The level of symptoms may determine the order of the tests, but all tests lose their usefulness if the person is already eating a gluten-free diet. Intestinal damage begins to heal within weeks of gluten being removed from the diet, and antibody levels decline over months. For those who have already started on a gluten-free diet, it may be necessary to perform a re-challenge with some gluten-containing food in one meal a day over 6 weeks before repeating the investigations (2,5,8,21).

2.1.Serological testing

Serologic testing can be performed in subjects in whom 1) the diagnosis of celiac disease is pending, such as those with malabsorption and vitamin- or mineral deficiencies, osteoporosis/osteopenia, infertility or other clinical symptoms. It can also be used to 2)

screen individuals considered to be at high risk for celiac disease, such as those with type 1 diabetes or first-degree relatives of an affected individual (2,32). Finally, serologic testing can also be used 3) to monitor the efficacy of celiac disease therapy, because antibody levels are expected to decline with successful treatment.

Celiac disease is characterized by the presence of diverse antibodies in the serum that are made against 1) (a component of gluten) gliadin: conventional gliadin antibodies and deamidated gliadin peptide (DGP) antibodies; and 2) connective tissue components: **tissue transglutaminase (tTG)** antibodies and endomysial antibodies (EMA). Overall, these tests are useful in the diagnosis of celiac disease, although their performance may be different.

2.1.1.Anti-gliadin antibodies

Conventional gliadin antibodies are no longer recommended because of the lower sensitivity and specificity compared with other available serologic tests (32). However, there is considerable interest on the use of new-generation **deamidated gliadin peptide (DGP)** antibodies because these novel tests have improved diagnostic accuracy in comparison with conventional gliadin antibodies (33) In a recent review, the pooled sensitivity for IgA DGP antibodies was 88%, with specificity of 95% (32).

In children, the present evidence suggests that tTG IgA and DGP IgA and IgG have superb and similar abilities to detect celiac disease, and that DGP IgG is also as good as tTG IgG in detecting IgA-deficient celiac disease patients (32). In addition, DGP appears to have a unique and superior role in screening for very young children with celiac disease, and may be superior to tTG in carefully monitoring dietary compliance in diagnosed patients (32).

2.1.2.Tissue transglutaminase (tTG) antibodies

The enzyme tissue transglutaminase (tTG) was recognized as the celiac disease autoantigen (34). This enzyme has many functions, including deamidation of gliadin peptides (35). A wide range of kits with different characteristics measure tTG antibodies, most often by

quantitative enzyme-linked immunosorbent assay (ELISA)(36). The substrates could be guinea pig liver (first-generation assays), human red-cell derived, and human recombinant. In general, specificity tends to be higher with human-based assays than with first-generation assays (37). The pooled sensitivity and specificity for human-based IgA tTG are both 98%. However, sensitivity (and to a lesser degree specificity) may vary among laboratories (37). Because of its simplicity and overall good diagnostic accuracy, detection of **IgA tTG antibodies** is **the serologic test of choice** for the diagnosis of celiac disease (38,39). False-positive tests are unusual with human substrates, especially at high titers.

However, it is well known that **IgA-deficiency** is more common in subjects with celiac disease (1 in 40) than among general population (1 in 400), which could make serological detection of celiac disease challenging. Therefore, quantitative measurement of total IgA levels should be considered in individuals in whom celiac disease is suspected, either in conjunction with tTG IgA autoantibody measurement, or following a negative test (2,32). IgG antibodies to tTG and DGP can be used, but with questionable diagnostic accuracy (40). Although the presence of IgA-deficiency may affect the utility of these screening tests, some patients with celiac disease and IgA-deficiency will still have positive celiac disease related antibodies.

When celiac disease is suspected clinically in patients with IgA-deficiency, upper intestinal endoscopy with biopsy is usually recommended, regardless of the autoantibody results (32). A recent modification of the tTG assay is the **CELIAC Quick Test** developed by Biohit Oyj (Helsinki), based on the principle of immunochromatography (lateral flow). This quick test detects **anti-tTG IgA, IgG and IgM** antibodies, thus minimizing the risk of missing the IgA-deficient patients, who have 10-fold higher risk of celiac disease (41).

2.1.3.Endomysial antibodies

Endomysial antibodies (EMA) have been available for diagnosis of celiac disease for almost 30 years (32). The antibodies have been measured using an indirect immunofluorescence

technique using monkey esophagus, human jejunum, or human umbilical cord as substrate. The target antigen is tTG. The pooled sensitivity and specificity for IgA EMA were found to be 95% and 99%, respectively. Despite the high specificity of this antibody, there are several test-related issues that may limit its use in clinical practice. It is semi-quantitative, time consuming, operator dependent, and expensive. However, IgA EMA testing can be clinically useful if the result of the IgA tTG test is equivocal. Thus, a positive IgA EMA test is strong evidence for celiac disease among patients with non-atrophic intestinal lesions (32).

2.2.Genetic testing

Almost all patients with celiac disease are positive for either HLA-DQ2 (heterodimer DQA1*05/DQB1*02) or HLA-DQ8 (heterodimer DQA1*03/DQB1*0302). HLA-DQ2 is carried in approximately 95% of patients with celiac disease, and thus, the absence of these heterodimers has a high negative predictive value (NPV)(32). On the other way round, some 25% to 30% of persons of European ancestry have one of these genotypes, so a positive result is of little diagnostic value. Routine addition of genetic testing (HLA-DQ testing) to tTG and EMA (or vice versa) does not increase diagnostic performance compared with either testing strategy alone (42). Thus, HLA-DQ genotyping is **not indicated** in the initial evaluation of celiac disease.

2.3.Biopsy of small intestine

Despite the diagnostic advances afforded by the availability of serological testing, the histological finding on small intestinal biopsy remains **the gold standard** for the diagnosis of celiac disease. In certain scenarios, this may no longer be the case in pediatric patients, however (43). Duodenal biopsy for the diagnosis of celiac disease is most commonly performed after a patient is found to have a positive serological test. However, diagnostic biopsy may also be performed in seronegative individuals with signs and symptoms highly suspicious for celiac disease, as none of the available serological tests has a sensitivity of 100%. Biopsy can also be useful in the common (but less than ideal) scenario where a patient has already commenced a gluten-free diet (GFD) before seeking medical care. Serological

tests in most patients with celiac disease normalize after 6 to 12 months of adherence to GFD, but the histological changes can persist far longer, up to several years (44). Even if mucosal healing is established (as normal crypt-to-villous ratio), intraepithelial lymphocytosis can persist (45). Therefore, a patient with celiac disease who is already adherent to the diet may have normal serology but persistent morphological evidence of the disease. Starting a GFD is not recommended before confirmation of the diagnosis.

Because the pathologic findings in celiac disease can be patchy and can affect different regions of the duodenum with varying degrees of severity, multiple biopsies from different sites of the duodenum should be submitted for examination. Guidelines issued by the American Gastroenterological Association state that 4 to 6 specimens be submitted during duodenal biopsy (39). This recommendation is based on the concept of the patchy (=multifocal) nature of the disease, and it has been supported by the study, in which the sensitivity of biopsy for the diagnosis of celiac disease declined when fewer than 4 specimens were submitted (46). Not unexpectedly, adherence to these standards of submitting 4 to 6 duodenal biopsies appears to vary from country to country (32). Other factors that may interfere with the correct diagnosis of celiac disease during esophagogastroduodenoscopy (EGD) include failure to biopsy the duodenal bulb (47), and misinterpretation of subtle histopathological alterations (48). Thus, it seems feasible that adequate sampling for diagnosing celiac disease should include 4 or more specimens of the duodenum and should include **the duodenal bulb**.

2.4.New diagnostic algorithm for celiac disease

Because of these limitations associated with duodenal biopsy, some authorities have recently proposed novel diagnostic criteria of celiac disease that include, but do **not entirely depend on**, biopsy results. Thus, Catassi and Fasano (49) proposed a 5-point scoring system that incorporates 1) symptoms of celiac disease (such as diarrhea, weight loss, and iron-deficiency anemia); 2) positive serology at high titer; 3) the presence of a DQ2 or DQ8 haplotype; 4) characteristic histopathological findings in duodenal biopsy; and 5) a serological or

histological response to the GFD. The presence of 4 out of 5 criteria (or 3 out of 4, if gene testing is not included) would meet the diagnostic criteria of celiac disease (32,49).

This algorithm would allow for patients who have signs and symptoms of celiac disease but have i) borderline histology, or ii) who refuse biopsy, to be classified as having celiac disease (32,49). Importantly, patients meeting the 4 non-histological criteria of celiac disease would not necessarily require a biopsy. However, it can be reasoned that any gain in sensitivity using this non-invasive scoring system is achieved at the expense of specificity (32). The algorithm would consider patients to have celiac disease if they are not HLA-tested but have symptoms and serological results that improve on a GFD. Given the imperfect specificity of currently available serological tests (32-36), and the known phenomenon of gluten sensitivity in the absence of celiac disease (50), a significant proportion of such patients may not have a positive HLA-DQ2 or HLA-DQ8, and thus will likely not have celiac disease by definition. Thus, an algorithm described here that allows for a diagnosis of celiac disease in adults without histological evidence has not been widely adopted yet by clinicians, and additional validation is prospective settings is needed.

To obviate the excessive use of invasive and expensive endoscopic procedures (EGD) particularly among children, there is an urgent need to develop **non-invasive diagnostic** tools capable of accurately diagnosing the patients with celiac disease. These non-invasive (serological) diagnostic tests have three well accepted applications: 1) to test the individuals in whom the diagnosis of celiac disease is pending, such as those with malabsorption and vitamin- or mineral deficiencies, osteoporosis/osteopenia, infertility or other clinical symptoms; 2) for screening of individuals considered to be at high risk for celiac disease, such as those with type 1 diabetes or first-degree relatives of an affected individual; and finally 3) in monitoring the efficacy of celiac disease therapy, because antibody levels are expected to decline with successful treatment.

There are two special groups that need particular attention while diagnosing celiac disease: i) children, and ii) individuals with IgA-deficiency. In children, the present evidence suggests that tTG IgA and DGP IgA and IgG are equivalent in detecting celiac disease, and DGP IgG is also as good as tTG IgG in detecting IgA-deficient celiac disease patients (32). The superiority of DGP or tTG in screening of very young children for celiac disease, as well as in monitoring the dietary compliance in diagnosed patients remains more controversial (32). When celiac disease is suspected in patients with IgA-deficiency, endoscopy with biopsies is usually recommended, regardless of the autoantibody results (32). A recent modification of the tTG assay is the **CELIAC Quick Test** developed by Biohit Oyj (Helsinki), based on the principle of immunochromatography. This quick test detects anti-tTG IgA, IgG and IgM antibodies, thus **minimizing the risk of missing the IgA-deficient** patients, who have 10-fold higher risk of celiac disease (41). Because of the fact that the test can be performed in a blood sample taken from a fingertip, CELIAC Quick Test is very user-friendly, particularly for small children.

3.THE CELIAC QUICK TEST

The CELIAC Quick Test is an immunochromatographic test designed for the qualitative detection of antibodies (IgA/IgG/IgM) against human tissue transglutaminase (tTG) in whole human blood. Transglutaminase (ref. 2.1.2) is the principal auto-antigen recognized by the anti-endomysial antibodies (ref 2.1.3). It is particularly useful for testing pediatric patients (up to 16 years-old).

3.1.Test principle

In CELIAC Quit Test, anti-tTG antibodies present in a blood sample react with latex particles covered by human recombinant tTG. These latex particle-tTG-anti-tTG complexes reach the reaction zone through **a chromatographic** process, where immobilised human tTG captures the complex, forming a red/pink line (Section 4.3.3). The CELIAC Quick Test is a qualitative test, and no quantitative interpretation on the magnitude of the result can be made by visual reading of the positive test line intensity. The Celiac Quick Test result must be

interpreted as part of the diagnostic algorithm (Section 2.4), i.e., together with the patient's clinical presentation and other information available to the physician (32,49).

4.STUDY DESIGN

The present study is a 100% biopsy-controlled clinical trial testing the performance of Biohit CELIAC Quick Test as part of the diagnostic algorithm for celiac disease among the patients with clinical suspicion of the disease. The conditions representing the **two study endpoints** (outcome measures) include the biopsy-confirmed duodenal pathology classified as i) **Marsh score 3**, or ii) **Marsh score 2** considered as diagnostic hallmarks of celiac disease (51,52). As an additional study endpoint, down-stream in the path to celiac disease is the detection of **Marsh score 1** pathology (presence of intraepithelial lymphocytes alone), which is not considered diagnostic for celiac disease (51,52).

4.1.Aims of the study

The single most important goal of this study is to assess the overall performance of the CELIAC Quick Test in diagnosis of celiac disease in pediatric and adult population. This goal is reached through the following specific aims.

1. Sensitivity (SE), specificity (SP), negative predictive value (NPV), positive predictive value (PPV) and area under ROC curve (AUC) for CELIAC Quick Test in detecting **Marsh Score 3+** (Marsh 3 and Marsh 4) duodenal pathology.

2. SE, SP, NPV, PPV and AUC for CELIAC Quick Test in detecting **Marsh Score 2** duodenal pathology.

3. SE, SP, NPV, PPV and AUC for CELIAC Quick Test in detecting **Marsh Score 1** duodenal pathology.

4. Related to the above aims, there are three clinically relevant issues, also to be addressed in this **100% biopsy-confirmed** study, devoid of any verification bias: 1) the rate of unnecessary referrals for endoscopy (false positive rate; 1-PPV) following a positive CELIAC Quick Test result; 2) the rate of endoscopies to be avoided after a negative CELIAC Quick Test result (true negative rate; NPV), and 3) the rate of biopsy-confirmed celiac disease (Marsh 2+) that are missed by the CELIAC Quick Test (i.e., false negative rate; 1-SE).

4.2.Patients

This clinical trial is conducted as collaboration between Biohit Oyj (Helsinki, Finland) and Hospital X (City Y, Country Z)(hereafter called "the Partners"). The study is performed exclusively in Hospital X, supervised by a steering committee consisting of members from both research partners.

Enrolment of the patients in the study will take place at Hospital X, including consecutive patients (at any age), referred for esophagogastroduodenoscopy (EGD) at the Outpatient Department of Endoscopy, because of a **clinical suspicion of celiac disease**. The eligible patients can be of any age (pediatric or adult), in whom the diagnostic algorithm for celiac disease remains to be completed by **serological testing** and **EGD**. Thus, they have 1) symptoms of celiac disease (diarrhea, weight loss, and iron-deficiency anemia); and 2) have tested positive for DQ2 or DQ8 haplotype. Thus, they are scheduled for serological testing (DGP, tTG) and endoscopy to confirm the diagnosis, before starting GFD.

The estimated cohort to be screened by CELIAC Quick Test would be **at least 300 subjects** (both genders, all age groups), to reach a **cohort of 200 patents** enriched with **equal numbers (n=50) of all conditions** (Marsh 3+, Marsh 2, Marsh 1, March 0), needed to calculate the performance indicators for the study endpoints.

Patient enrollment is taking place **in a single step**. In brief, the potentially eligible patients are identified among the endoscopy-referral subjects with pending diagnosis of celiac disease, by the members of the research team. At this stage, every patient will be asked to consent the study and sign a written consent to participate. For pediatric patients, a written consent should be obtained from both parents (national regulations may differ!).

Eligible patients are all pediatric and adult subjects, scheduled for completion of their diagnostic algorithm to diagnose/exclude celiac disease. However, the following patients should be considered __non-eligible__: 1) those who refuse to sign written consent; 2) those who refuse duodenal biopsy (the gold standard).

4.2.1.Patient preparation

Proper conduction of and reliable results from the CELIAC Quick Test does not necessitate any preparatory measures of the patient. The preparatory measures required for EGD for which the subjects are referred to the hospital are fully compatible with the sampling for CELIAC Quick Test.

4.3.Methods

4.3.1.Sample collection for CELIAC Quick Test

Test performance in the laboratory, including the sampling procedure, is illustrated in **ANNEX 1.** Importantly, collection and application of the sample must be rapid, to avoid sample coagulation in the micropipette. A whole blood sample is collected by finger prick; index, middle or ring finger can be used. Clean fingertip with alcohol and allow to air dry. Position the hand palm-side up and with a NEW sterile lancet, prick the fingertip.

To operate the lancet, one needs to i) push the yellow cap into the body of the lancet until it clicks; ii) twist off the lancet cap until you feel it separate from the device, this activates the lancet. Do not pull the cap, just twist; finally, iii) press the open end of the lancet on the patient's fingertip and press the release button. Discard the lancet immediately into an

appropriate container. Hold the finger lower than the elbow and gently apply intermittent pressure to the base of the pricked finger several times. Wipe away the first drop of blood (using a sterile gauze pad or cotton)(ANNEX 1; Figure 1).

To collect the blood sample, hold the blood collection micropipette horizontally, with the air vent free, touch the blood sample with the tip of the tube. Do not touch or scrape the skin. Fill the micropipette to the fill line, avoiding air bubbles. Do not squeeze the micropipette during collection (ANNEX 1; Figure 2).

4.3.2.Sample processing

Test the sample immediately to avoid coagulation of the blood. Slowly dispense the blood sample onto the test cassette. Carefully, place the pipette into the round window (indicated by an arrow), then cover the pipette ventilation hole with a finger and gently press the upper part of the pipette and squeeze the bulb of the micropipette gently (ANNEX 1; Figure 2).

Wait 30-60 seconds until the blood has been properly absorbed, and add two drops of the dilution buffer in the same window. Add one drop at a time, holding the dropper bottle in a vertical position. Avoid contamination of the dispenser with the sample. If the dispenser is not used, then add 100 microliters of dilution buffer (ANNEX 1; Figure 3).

4.3.3.Interpretation of the CELIAC Quick Test results

Visually read the results 10 minutes after adding the dilution buffer. Examples of the test results are illustrated in ANNEX 1; Figure 4. Test is NEGATIVE if only one BLUE band appears across the result window close to the "C" letter (Control line) of the test cassette. This positive control band must be always present. In POSITIVE test, a clearly distinguishable PINK-RED band must appear across the result window close to the letter "T" (Test line), in addition to the BLUE control band. The intensity of the line depends on the concentration of antibodies in the sample (Figure 4), but as said, this test is only qualitative when interpreted by visual inspection.

If no BLUE band appears in the control area, the test is INVALID and should be repeated with a new test cassette. Any line or color that appears later than after 15 minutes has no diagnostic value. It is very important to add the correct quantity of sample. Insufficient sample volume may not reach the reaction (C and T) area, and the test may not run correctly. If the sample volume is too large, the reaction mix in the device will be diluted, which may result in a false negative result. It is also very important to **control the reaction time**. If the reaction time is shorter than the recommended 10 min, this may lead to false negative result. If the reaction time is longer than recommended, this may lead to a false positive result.

4.4.Gastroduodenoscopy (EGD) and biopsy procedures

In this study, all patients examined with the CELIAC Quick Test will be subjected to gastroduodenoscopy (EGD), providing the histological confirmation to be used as the gold standard in calculating the performance indicators for the test. In endoscopy, all observed abnormal mucosal lesions are noted and photographed, and if necessary (e.g. suspicion of malignancy) subjected to additional biopsy. Because the pathologic findings in celiac disease can be patchy and can affect different regions of the duodenum with varying degrees of severity, **multiple biopsies** from different sites of the duodenum should be submitted for examination. Guidelines issued by the American Gastroenterological Association state that 4 to 6 specimens be submitted during duodenal biopsy (39). Thus, adequate sampling for diagnosing celiac disease should include **4 or more** specimens of the duodenum and should **include the duodenal bulb.**

4.4.1.Preparation of the microscopy slides

The biopsies from formalin bottles/tubes are embedded in paraffin using the routine procedures at the Pathology Laboratory of Hospital X. The blocks are cut into 4-μ-sections, and stained with hematoxylin eosin (HE) for routine diagnosis and with Alcian Blue PAS staining for different intestinal mucins.

4.4.2. Interpretation of the biopsies

All duodenal biopsies are examined by the expert pathologists at Hospital X among the daily routine samples. The diagnoses are reported using the **Marsh classification** of duodenal histology in celiac disease, classified into one of the 5 categories. The original Marsh's classification, introduced in 1992 (52), was subsequently modified, where the previous stage 3 was split into three sub-stages (53).

Stage 0
The mucosa is normal, so celiac disease is unlikely. Stage 0 is known as the "pre-infiltrative stage."

Stage 1
The cells on the surface of the intestinal lining (the epithelial cells) are being infiltrated by lymphocytes.

Stage 2
The changes of Stage 1 are present (increased lymphocytes), and the crypts are hyperplastic.

Stage 3
The changes of Stage 2 are present (increased lymphocytes and hyperplastic crypts), and the villi are atrophic. There are three subsets of Stage 3:

-Partial villous atrophy   (Stage 3a)
-Subtotal villous atrophy   (Stage 3b)
-Total villous atrophy   (Stage 3c)

Stage 4
The villi are totally atrophic and also the crypts are shrunken, making the mucosa appear as hypoplastic.

In true celiac disease, histology will show some degree of villous atrophy and crypt hyperplasia. Intraepithelial lymphocytes are typically seen in celiac disease lesion, but their presence alone is insufficient to make the diagnosis. In Marsh scoring, normal intestinal histology is scored a Marsh 0. The presence of intraepithelial lymphocytes alone is a Marsh 1. A biopsy specimen with crypt hyperplasia and increased numbers of intraepithelial lymphocytes is a Marsh 2. A specimen with any degree of villous atrophy is a Marsh 3 (51,52). **A Marsh score of 2 or 3 is consistent with celiac disease**. Score 4 represents the end stage of a full-blown (undiagnosed) celiac disease with no intervention by GFD.

Since other conditions can yield similar histological findings, the presence of infiltrative changes alone (Marsh type 1) on intestinal biopsy is not specific for celiac disease in children. Likewise, positive celiac disease serology (EMA or tTG autoantibodies) increases the likelihood that such an individual has celiac disease. A **diagnosis** of celiac disease is considered **definitive** when there is i) a complete resolution of symptoms on a gluten-free diet (GFD), or ii) when there is histological improvement on a follow-up intestinal biopsy. In addition, a positive serological test that resolves on a GFD is helpful in confirming the diagnosis (32,51,52).

4.5. Statistical analyses

All statistical analyses will be performed using the SPSS 25.0.0.1 for Windows (IBM, NY, USA) and STATA/SE 15.1 software (STATA Corp., Texas, USA). The descriptive statistics will be calculated according to routine procedures. Performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of CELIAC Quick test will be calculated separately for each study endpoint, using the STATA/SE software and the *diagti* algorithm introduced by Seed et al. (2001). This algorithm also enables calculation of the area under ROC (Receiver Operating Characteristics) called AUC, for each study endpoint. Significance of the difference between AUC values can be estimated using STATA's roccomb test with 95%CI.

5. ETHICAL ISSUES

The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. The study design and its execution do not involve any significant ethical issues except those in other clinical studies of similar type. The study is conducted in accordance with the Declaration of Helsinki. Special reference should be made to the regulations on pediatric patients enrolled in this trial.

Patients are enrolled among consecutive patients with clinically suspected celiac disease who attend the outpatient Department of Endoscopy to complete their diagnostic algorithm for

celiac disease by serological testing and EGD. Thus, they represent regular outpatients who have a reasonable clinical suspicion of celiac disease and who are scheduled for confirmatory clinical examinations. The only additional operation carried out to these patients is the blood sampling from a fingertip. All patients (and in case of pediatric patients, both parents) must sign the informed consent for their participation. When the results of the CELIAC Quick Test endoscopy and biopsies are available, the patients will be informed about the results, following the usual hospital practices. This includes an explanation of the meaning of these test results, and the appropriate measures for further conduct, i.e., adherence to gluten-free diet.

6.TIME TABLE

The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached an agreement with Biohit Oyj to conduct the study. Given that the subjects in the study will be enrolled among consecutive patients with clinically suspected celiac disease, attending the Outpatient Department of Endoscopy at Hospital X, it is estimated that at least 300 subjects (both genders, all age groups) need to be screened by CELIAC Quick Test to reach a cohort of 200 patents enriched with equal numbers (n=50) of all conditions of interest (Marsh 3+, Marsh 2, Marsh 1, March 0), needed to calculate the performance indicators for the study endpoints.

Because of the test characteristics (quick test), the laboratory arm of this study is completed in parallel with the patient enrollment and performed duodenoscopies. There will be only a minor delay (of days) due to the biopsy examination at the Department of Pathology, until the results of the triple-diagnosis (CELIAC Quick Test, duodenoscopy, biopsies) of each subjects are available. Accordingly, the full database of the patients will be ready for statistical analysis practically on real-time after completion of the enrollment of the cohort and examination of their celiac serology and biopsy samples.

7. PROJECTED COSTS TO BE COVERED by Biohit Oyj

This section will be completed as soon as an agreement has been reached in the cost estimates for the (eventually modified) study protocol.

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ANNEX 1.
SAMPLING AND PERFORMANCE OF CELIAC QUICK TEST

PRELIMINARY PREPARATION
Bring a test cassette and the diluent buffer to room temperature. Remove the test cassette from its sealed pouch just before use and place on a flat surface.

SAMPLES
The test has been designed for use with fresh, non-hemolysed samples of whole blood. Samples can be kept in the refrigerator for 1 day before testing.

STEP 1: Sample collection and application
ATTENTION: Collection and application of the sample must be swift, in order to avoid sample coagulation in the micropipette.
A whole blood sample is collected by finger prick; index, middle or ring finger can be used. Clean fingertip with alcohol and allow to air dry. Position the hand palm-side up and with a NEW sterile lancet, prick the fingertip. To operate the lancet (Figure 1.):
a) Push the yellow cap into the body of the lancet until it clicks.
b) Twist off the lancet cap until you feel it separate from the device, this activates the lancet. Do not pull the cap, just twist.
c) Press the open end of the lancet on the patient's fingertip and press the release button. Discard the lancet immediately into an appropriate container.
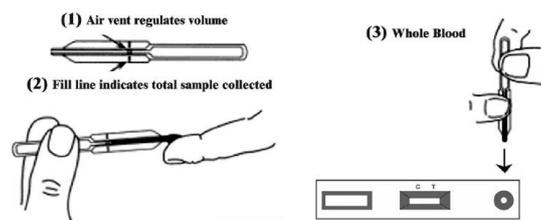
Figure 1.

Hold the finger lower than the elbow and gently apply intermittent pressure to the base of the pricked finger several times. Wipe away the first drop of blood (using a sterile gauze pad or cotton). To collect the blood sample (Figure 2.):
**(1)** Hold the blood collection micropipette horizontally, with the air vent free.
(2) Touch the blood sample with the tip of the tube. Do not touch or scrape the skin. **Fill the micropipette to the fill line, avoiding air bubbles. Do not squeeze the micropipette during collection.** Test the sample immediately to avoid coagulation of the blood.
(3) Slowly dispense the blood sample onto the test cassette. Carefully, place the pipette into the round window (indicated by an arrow), then cover the pipette ventilation hole with a finger and gently press the upper part of the pipette and squeeze the bulb of the micropipette gently.

Figure 2.

STEP 2: Dilution buffer (Figure 3.)
Wait 30-60 seconds until the blood has been absorbed, and add two drops of dilution buffer in the same window. Add one drop at a time, holding the dropper bottle in a vertical position. Avoid contamination of the dropper bottle with the sample. If the dropper is not used, then add 100 microliters of dilution buffer.

Figure 3.
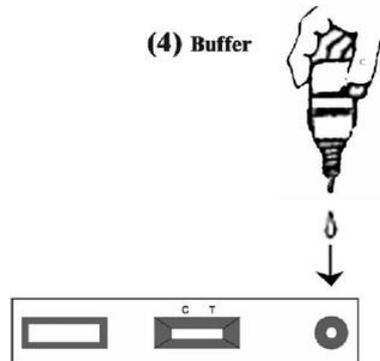
STEP 3: Reading of the results (Figure 4.)
Visually read the results 10 minutes after adding the dilution buffer.

Figure 4.
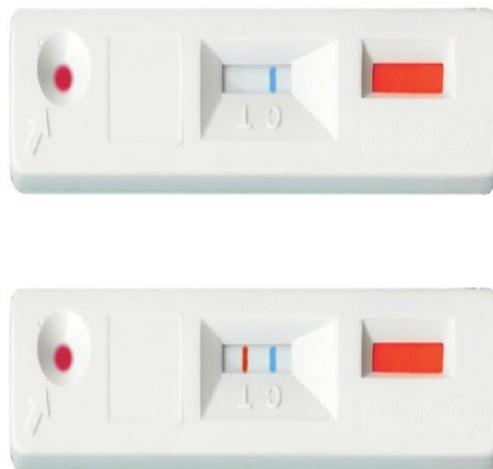
NEGATIVE: Only one BLUE band appears across the result window close to the "C" letter (Control line) of the test cassette. This band must always appear.
POSITIVE: In addition to the BLUE control band, a distinguishable PINK-RED band also appears across the result window close to the letter "T" (Test line) of the test cassette. The intensity of the line depends on the concentration of antibodies in the sample.
If no BLUE band appears in the control area, the test is INVALID and should be repeated with a new test cassette. Any line or color that appears after 15 minutes has no diagnostic value. The final diagnosis should not be based only on the result obtained with one test; it should be based on a correlation of test results with other appropriate data and with clinical symptoms.