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-CONFIDENTIAL-

-GastroPanel test and Type 1 Diabetes-

Biohit *GastroPanel® test in Screening of the Patients with Type 1 Diabetes Mellitus (DM1) for Autoimmune Atrophic Gastritis (AAG)

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*The new **Unified GastroPanel®** test: ELISA biomarker test for pepsinogen I (P-PGI), pepsinogen II (P-PGII), gastrin-17 (P-G-17), and *H.pylori* IgG antibodies (P-HpAb).

Summary

Background: Autoimmune gastritis and pernicious anemia (PA) are common autoimmune disorders, being present in up to 2% of the general population. However, in patients with **type 1 diabetes (DM1)** or autoimmune thyroid disease, the prevalence is 3- to 5-fold. Autoimmune atrophic gastritis (AAG) is characterized by: 1) atrophy of the corpus and fundus; 2) autoantibodies to the parietal cell (PC) and to the intrinsic factor (IF); 3) achlorhydria; 4) iron deficiency anemia; 5) hypergastrinemia; 6) vitamin-B12 deficiency leading to PA; 7) in up to 10% of the patients, AAG may predispose to gastric cancer (GC) or carcinoid tumours. In addition to autoimmune disease, *Helicobacter pylori* (HP)-infection is the other important causative agent of atrophic gastritis (AG), and now suspected to be **a trigger of AAG** as well.

The high prevalence of AAG in DM1 and its potentially serious consequences provide **a strong rationale for screening**, early diagnosis, periodic surveillance by gastroscopy, and treatment. Whether regular **gastroscopic** surveillance is needed in patients with AAG/PA is **controversial.** To obviate the need for invasive diagnostic methods (gastroscopy) for these conditions, Biohit Oyj (Helsinki, Finland) launched several years ago an ELISA-based assay designed to measure the concentrations of four stomach-specific biomarkers (Pepsinogen I, Pepsinogen II, Gastrin-17, *H. pylori* IgG antibodies) from a single blood sample. This GastroPanel® test is **the first non-invasive** diagnostic tool for stomach health (testing both the structure and function). The new-generation **Unified GastroPanel®** is a technically advanced version of this test, where the processing conditions of all biomarkers are uniform, making the test more versatile.

Objective: To conduct a systematic GastroPanel® screening of DM1 patients to establish the **prevalence of AAG** and its associated risks (PA included). Another objective is to assess the utility of this non-invasive serum biomarker test as **a substitute to gastroscopy** in the **systematic monitoring** of the DM1 patients for AAG and its severe clinical sequels.

Study Design: This study is a targeted screening trial for asymptomatic and symptomatic DM1 patients. A cohort of patients with prevalent DM1 (newly diagnosed cases excluded) are subjected to screening by the Unified GastroPanel® test. The result is classified as one of the optional diagnostic categories: 1) healthy stomach, 2) superficial HP-gastritis, 3) AG of the antrum; 4) AG of the corpus, and 5) AG of the antrum and corpus (pan-gastritis). AG patients testing HP-negative are likely to represent AAG. All patients with GastroPanel® result suggesting AG/AAG will be referred for gastroscopy and biopsy confirmation used as gold standard.

Methods: Study subjects (above 45 years of age, both genders) are enrolled among the DM1 patients controlled in the outpatient department for diabetics at Hospital X. All eligible patients who consent to participate will be invited for blood sampling for GastroPanel® testing, instructed to be compliant with the patient preparation. All blood samples will be stored at -70C until delivered for analysis in the laboratory of Biohit Oyj (Helsinki). On the occasion of blood sampling, all patients fill in questions from **validated questionnaires** exploring possible dyspeptic symptoms and potential sequels of AAG. Patients with suggested AG/AAG in the GastroPanel® test will be referred to gastroscopic examination, with directed gastric biopsies, following the protocol of the Updated Sydney System (USS). Statistical analyses include calculation of the performance indicators of the GastroPanel® test for individual study endpoints, including ROC analysis for the optimal sensitivity/specificity balance, using moderate/severe AG/AAG as the endpoint.

Specific Aims: The main goal of this screening trial is to establish **the prevalence of AGG** among the patients with prevalent DM1. Related to the assessment of the validity of GastroPanel in systematic surveillance of DM1 patients, three clinically relevant issues need to be addressed: 1) the **rate of unnecessary referrals** for gastroscopy (false positive rate; 1-PPV) following a positive GastroPanel® test; 2) the **rate of gastroscopies to be avoided** after a negative GastroPanel® examination (true negative rate; NPV), and 3) the rate of clinically **significant diseases** (conditions) that are **missed** by the GastroPanel® examination (i.e., false negative rate; 1-SE).

Study execution and time table: The study plan necessitates a review by the regional review board (Hospital X, Ethical Committee) before start. Given that the subjects in the study will be enrolled among DM1 patients attending the outpatient department of Hospital X (other sites?), it is estimated that the screening of a cohort of 1000 patients will take approximately ? months. It is estimated that a cohort of 100-150 subjects (enriched by all grades of AG/AAG) should be enough to give this GastroPanel® study the necessary statistical power.

Impact of the study: The known high prevalence of AAG in DM1 and its potentially severe clinical sequels advocate 1) screening, 2) early diagnosis, 3) regular surveillance, and 4) treatment. However, it is still **controversial**, whether **regular gastroscopic surveillance** is needed for DM1 patients with AAG/PA. The present study will provide new insights i) in the true prevalence of AAG among the DM1 patients, as well as ii) on the utility of the **non-invasive biomarker** test (GastroPanel®) as a substitute of gastroscopy in the systematic **surveillance** of DM1 patients for AAG.

1.BACKGROUND

Autoimmune atrophic gastritis (AAG) and pernicious anemia (PA) are common autoimmune diseases with respective prevalence of 2% and 0.15–1% in the general population, increasing with age (1,2,3). In patients with type 1 diabetes (DM1) (4, 5) or autoimmune thyroid disease (6, 7), the prevalence is 3- to 5-fold increased. AAG is characterized by atrophy of the corpus and fundus, and the presence of circulating autoantibodies to the parietal cell (PCA) and to their secretory product, intrinsic factor (IF) (8). Chronic auto-aggression to the gastric proton pump, H+/K+ATPase, may result in decreased gastric acid secretion, hypergastrinemia, and iron deficiency anemia (9, 10). In a later stage of the disease, PA may result from vitamin-B12 deficiency, which is 10 times more common in DM1 patients than in non-diabetic subjects (5). Finally, in up to 10% of patients, AAG may predispose to gastric carcinoid tumors or adenocarcinomas (11-17). Determining demographic, immunological, and genetic risk factors, and early diagnosis of AAG are important to prevent and treat iron deficiency anemia, PA, and (pre)malignant gastric lesions: intestinal metaplasia (IM) and enterochromaffin-like cell (ECL) hyper/dysplasia (5).

1.1. Definition of autoimmune atrophic gastritis (AAG)

AAG affects the parietal cell-containing gastric corpus and fundus with sparing of the antrum (8, 18). PCA, targeted against gastric H+/K+ ATPase, are detected in 60–85% and IF antibodies in 30–50% of patients with AAG (5,19). Besides PA, iron deficiency anemia is frequently observed (9, 10). Furthermore, AAG is characterized by hypo- or achlorhydria, high serum gastrin, and low pepsinogen I concentrations (20,21). Chronic hypergastrinemia causes the ECL cells in the oxyntic mucosa to undergo hyperplasia (22), which may progress toward dysplasia and gastric carcinoid tumors (11, 21).

PCA are detected by immunofluorescence staining of the cytoplasm of gastric parietal cells (23). However, Karlsson et al. (24) showed that the ELISA to detect gastric H+/K+ ATPase antibodies is 10-fold more sensitive than the indirect immunofluorescence technique and has a high specificity. Current ELISAs have a sensitivity and specificity of respectively 85–93% and 80–85%. PCA are detected 60–90% of the patients with AAG and/or PA (1,8,23).

The recognition of antibodies to IF (AIF) derives from the work of Taylor et al. (25) and Schwartz (15). Two types of autoantibodies bind to IF. Type I AIFs block the binding of vitamin-

B12 to IF, thereby preventing the transport of vitamin-B12 from the stomach to its absorption site in the terminal ileum. Type I AIFs are demonstrable in 70% of the patients with PA (24). Type II AIFs do not interfere with vitamin-B12 transport. They can be found in 30–40% of patients with PA.

The destruction of H+/K+ATPase-containing parietal cells results in hypo- or achlorhydria. This can be measured using 24-h gastric pH-metry or after stimulation with pentagastrin. Hypochlorhydria is defined as a maximal acid output less than 15 mmol H+/h after injection of pentagastrin. A progressive decrease in acid secretion in the case of AAG with a decreased parietal cell mass has been found (21,26,27). Total achlorhydria is diagnostic of PA. Achlorhydria interrupts the negative feedback of somatostatin on antral gastrin-producing G cells causing hypergastrinemia (28). Fasting serum gastrin (G-17) levels correlate negatively with peak acid output, and positively with the degree of corpus atrophy (21, 29) and with PCA levels (21). Low serum pepsinogen I levels, resulting from destruction of chief cells or zymogenic cells, are also characteristic to AAG (20,30,31).

1.2. Endoscopy and Pathology of AAG

On endoscopy, the affected corpus and fundus mucosa appears shiny and red because of the visibility of submucosal blood vessels. The stomach wall is thinned, and the rugal folds flatten or disappear. In biopsy specimens, lymphocytic infiltrates are present in the submucosa and lamina propria (19,21). In the next stage, there is a marked reduction in the number of oxyntic glands, parietal and zymogenic cells, followed by replacement of normal glands by glandular structures lined with mucus-containing cells resembling those of the small bowel mucosa (intestinal metaplasia, IM). A proliferation of ECL cells in the oxyntic mucosa (22), due to sustained hypergastrinemia, can be seen, which may progress in a small proportion of patients toward carcinoid tumors (11,32,33,34).

1.3. Epidemiology of AAG

In the general population, there is an age-related increase in the prevalence of parietal cell antibodies (PCA), from 2.5% in the third decade to 12% in the eighth decade (1,2). The prevalence is even higher in subjects affected by another autoimmune disorder. In DM1, PCAs are found in 10–15% of the children and in 15–25% of the adults (4,5,35,36,37). The respective prevalence of AAG and PA in the general population are 2% and 0.15–1% (2,3,38, 39),

compared with 5–10% and 2.6–4%, respectively, in DM1 patients (5,21,38,40,41). Iron deficiency anemia is present in 20–40% of patients with AAG (10, 42), whereas PA can be diagnosed in up to 15–25% of the patients (43). The progression of AAG to PA is likely to span 20–30 years (44).

Finally, gastric carcinoid tumors are observed in 4–9% of the patients with AAG/PA, which is 13 times more frequent than in controls (11,32,33,34,45). Patients with AAG/PA also have a 3- to 6-fold increased risk of GC, ranging from 0.9–9% (11,32,34,46,47,48).

1.4. Pathogenesis of AAG

The target autoantigens in AAG are the 100-kd catalytic α-subunit and the 60- to 90-kd glycoprotein β-subunit of the gastric H+/K+ATPase (49, 50). **Autoantibodies to parietal cells (PCA)** and to their secretory product, IF, are present in the serum and in gastric juice. The titer of PCAs correlates with the severity of corpus atrophy and is inversely proportional to the concentration of parietal cells (21, 29). CD4+T-cells recognizing parietal cell H+/K+ ATPase mediate autoimmune gastritis. During normal cell turnover, PCs release H+/K+ ATPase, which may result in its selective uptake and processing by antigen-presenting cells (51).

Alternatively, ***Helicobacter pylori*** (HP)-infection may play **an initiating role** in the pathogenesis of AAG and PA (52,53,54,55) by inducing autoreactive T-cells through gastric H+/K+ ATPase-HP molecular mimicry at the T-cell level (53,54), epitope spreading, and bystander activation. B-cells produce autoantibodies to gastric H+/K+ ATPase and to their secretory product, IF, with help from activated CD4+T-cells (50). Finally, PC loss from the gastric mucosa may result from CD4+ T-cells initiated perforin-mediated cytotoxicity or Fas-FasL apoptosis (55).

Regardless of whether PCAs are pathogenic or not, their presence provides a convenient diagnostic probe for AAG. A precise understanding of the pathogenesis of autoimmunity may lead to rational therapeutic strategies directed toward restoration of tolerance or impeding the progression of autoimmunity. Whether HP could trigger AAG or not remains controversial (5). However, should this be the case, HP eradication might be able to prevent AAG. Currently, it is recommended that HP-infection should be tested and treated in patients with gastric atrophy, intestinal metaplasia/dysplasia, and hypo- or achlorhydria (5).

1.5. Demographic factors

Advancing age is a risk factor that has been associated with PCA positivity (5). In the general population, PCA positivity increases from 2.5% in the third decade to 12% in the eighth decade (1,2). In DM1 patients, PCA are present in 10–15% of the children and 15–25% of the adults (41). Some authors (4,35) report a female preponderance for PCA positivity, although this has not been consistently confirmed in other studies (37,41).

1.6. Endocrine factors

AAG is frequently accompanied by other autoimmune diseases, including DM1 (5) and autoimmune thyroid disease (Hashimoto's thyroiditis and Graves' disease) (6,38,56). AAG is also part of the **autoimmune polyglandular syndrome** type 3 (PGA3)(57). PA occurs in up to 4% of DM1 patients (5, 40), 2–12% of patients with autoimmune thyroid disease (6, 58), 6% of those with Addison's disease, 9% of those with primary hypoparathyroidism, and 3–8% of those with vitiligo (1,5).

In patients with DM1, immunological risk factors that have been associated with PCA positivity include persistent islet cell antibody positivity (35,36), glutamic acid decarboxylase-65 antibody positivity (41,59), and thyroid peroxidase autoantibody positivity (41,59). The association with glutamic acid decarboxylase-65 antibodies might be explained by the fact that glutamate decarboxylase-65 is not only present in the pancreas and brain but can also be found in the thyroid gland and stomach. PCA are more frequent in DM1 patients than in their first-degree relatives, even after HLA matching, suggesting that the diabetic condition itself plays an important role (60). The association of AAG/PA with the other autoimmune disease falls outside the scope of this text (5).

1.7. Environmental factors

Helicobacter pylori (HP) might be implicated in the induction of AAG (52,53,55). This hypothesis is supported by studies reporting a high prevalence of HP-seropositivity and a low prevalence of positive HP-staining in subjects with atrophic corpus gastritis (61,62,63,64). Furthermore, the finding of gastric autoantibodies in 20–50% of HP-infected patients and reports of a positive correlation between gastric autoantibodies and antibodies to HP in patients with AAG/PA (63,65,66,67,68) suggest that chronic HP-infection is linked with gastric autoimmunity.

However, a correlation between HP and PCAs has not been reported in all studies (39,69,70). Moreover, others found no or a negative link between HP and atrophic corpus gastritis (71). On the other hand, HP eradication in patients who have antigastric antibodies leads to the loss of those antibodies in some subjects (72). These data revisit the concept of possible reversibility of gastric mucosa atrophy (5).

1.8. Monitoring the patients at risk for AAG and its sequels

AG/AAG is the single most powerful independent risk factor for distal (non-cardia) GC (73,74,75,76). It is estimated that 50% of all GC cases develop through the "Correa cascade" (74,77,78,79), leading from HP-associated- or autoimmune gastritis to mucosal atrophy, intestinal metaplasia, dysplasia, and to invasive adenocarcinoma. There are some implications that early eradication of HP-infection can slow down or even revert this cascade (80,81). Because this process takes several decades, there should be good prospects for early detection of precancerous lesions (82), but the problem is lack of a suitable test for GC screening (83). Furthermore, most of the patients report only a short period of symptoms before the diagnosis of GC, and up to 40% report no dyspeptic symptoms at all (84).

It is **controversial** whether patients with AAG/PA should be placed under a surveillance program with **regular gastroscopies**, including multiple gastric biopsies (5). The gastric carcinoids that occur in these patients generally do not pose a great threat to life, whereas the risk of developing GC is real. Nevertheless, in many clinics, endoscopy with biopsies remains the gold standard diagnostic tool, disclosing HP-infection, mucosal atrophy, intestinal metaplasia or dysplasia, and their topography (73,85) However, this invasive method is uncomfortable, distressing and quite costly, emphasizing the need for rapid, reliable and inexpensive non-invasive tests for screening and monitoring these patients(83,84,86).

1.8.1. Non-invasive diagnostic tools for monitoring

Such non-invasive methods providing information on the structure and function of gastric mucosa were introduced in the early 1980's, when Miki et al. (87) and Samloff et al. (88) developed assays measuring pepsinogen (PG) concentrations in the blood. The latest development in this field represents the panel combining serum pepsinogen I (PGI) and II (PGII), gastrin-17 (G-17) and HP IgG antibodies (IgG-HP) using ELISA technique (GastroPanel® test, Biohit Oyj, Helsinki), proposed as the first-line diagnostic test for dyspeptic symptoms

(89,90,91). According to a recent meta-analysis, serum PGs are not suitable for GC screening, however, but they proved to be useful for detecting the patients at risk for GC (92). Recently, these stomach-specific biomarkers were recommended by an authoritative international group of experts for diagnosis and screening of AG (93).

The GastroPanel test is based on combined analysis of PG-I, PG-II, amidated G-17 and HP-antibodies, designed to give information on both the structure and function of the stomach mucosa. Most importantly, this panel gives accurate estimates of the capacity of the corpus and antrum mucosa to secrete acid and G-17, respectively, as well as of important gastric pathologies like inflammation, grade and topography of AG (91,99,100,101). Normal plasma levels of these biomarkers indicate that the stomach mucosa has normal structure and function, whereas the abnormal levels are signs of a non-healthy stomach, reflecting the disturbances in the feedback mechanisms between the acid output, PGs and G-17 (102).

GastroPanel test has been on the market for several years by now, and during this time, it has been validated in clinical studies in Finland and elsewhere (95,96,103,104). Due to the inherent characteristics of the natural history of AG/AAG, the PGI values (and PGI/PGII ratio) remain within normal range as long as AG of the corpus is graded only as mild by the USS. However, mild AG/AAG is a poorly reproducible diagnostic category even among experienced pathologists, and because of this, mild AG of the corpus should never be used as the study endpoint in calculating the performance indicators of the PGI, PGI/PGII, as repeatedly emphasized (103,104,105). The only appropriate way of calculating the predictive indicators of PGI and PGI/PGII ratio for AG of the corpus is to use the combined moderate/severe AG as the study endpoint. Using this approach in an adequately powered study with validated USS classification gives ROC (AUC) values above 0.970 for PGI and >0.950 for the PGI/PGII ratio as predictors of moderate/severe AG of the corpus (103,105).

2. THE GASTROPANEL® TEST

The GastroPanel® is a user-friendly ELISA technique, intended both for research purposes and for clinical practice. The GastroPanel® test contains four biomarkers specific for the stomach mucosa: 1) Pepsinogen I (P-PGI), 2) Pepsinogen II (P-PGII), 3) Gastrin-17 (P-G-17) and 4) H. pylori antibody (P-HpAb) (89,90,91).(www.gastropanel.com)

2.1. ELISA test for Pepsinogen I and Pepsinogen II

P-PGI is secreted solely by the chief cells (chief cell/mucous neck cells) of the corpus mucosa. AG of the corpus leads to loss of these cells and, as a result, the P-PGI level in circulation decreases. P-PGII is produced by the chief cells and mucous neck cells of the gastric mucosa, by pyloric glands in the gastric antrum and by Brunner's glands in the proximal duodenum. The ratio of PGI to PGII concentration in the plasma of normal subjects is above 3.0.

2.2. ELISA test for Gastrin-17

The P-G-17 ELISA method in the GastroPanel® is specific to amidated G-17 molecule. G-17 peptide is the most important member of the gastrin/cholecystokinin-family which regulates the physiology of the upper gastrointestinal tract. This peptide is the biologically most active gastrin peptide, stimulating gastric acid secretion with 6-times higher potency than the biologically next most active gastrin, G-34. The G-17 ELISA in GastroPanel® assay allows estimation of the number and function of antral G-cells, without background noise and cross-reactivity with other gastrin fragments. G-17 is secreted exclusively by the gastrin-cells (G-cells) in the antrum, representing a fraction of the total gastrin concentration in the circulation. When dormant, the G-cells in antrum secrete only small amounts of G-17 hormone. The maximal secretion is achieved after physiological protein stimulation, or when the acid secretion in the stomach decreases, is low or absent (www.gastropanel.com/GastroPanel Sample Collection Instructions: Stimulated Gastrin-17s). As a result of antral atrophy (i.e., loss of glands), the amount of G-cells decreases and, consequently, both the basal and post-prandial secretion of G-17 will decrease.

2.3. ELISA test for Helicobacter pylori (HpAb ELISA)

The *H. pylori* infection is the most important cause of chronic gastritis. Another well-known cause for severe AG is the autoimmune mechanism, which can also be activated by *H. pylori* infection (52,53,54,55). GastroPanel® test for *H. pylori* is performed from the plasma samples. The test is based on an ELISA technique, with purified *H. pylori* bacterial antigen, adsorbed on a microplate, and a detection antibody labeled with horseradish peroxidase (HRP).

2.4. The new unified GastroPanel® test

Prompted by the design of the original GastroPanel itself, where all four biomarkers are being processed under different laboratory (incubation) conditions, Biohit R&D Department started

a project towards unified GastroPanel® test in 2013. The concept was to develop a new assay, where all four markers are being processed under the same conditions using an automatic ELISA instrument or manual processing. The new Unified GastroPanel® test contains the same four markers as the current version (1.1), maintaining its basic design as an ELISA test. Because of the crucial modifications in the key test components, the unified test will be treated as a novel test by the registration authorities. Because of this fact, the new version must undergo all the necessary steps needed for CE registration, including clinical validation in a cohort of study subjects with relevant gastric pathologies.

3. STUDY DESIGN

The present study is designed as a targeted screening of patients with prevalent DM1, with or without signs of other autoimmune disease (I.E., PGA3), enrolled from the primary- and occupational health care. The cohort is screened by the Unified GastroPanel® test distinguishing 5 diagnostic categories: 1) healthy stomach, 2) HP-gastritis, 3) AG of the antrum, 4) AG of the corpus and 5) AG of both antrum and corpus. The test performance indicators will be calculated using moderate/severe AG/AAG as the endpoint, separately for markers of the antrum (G-17) and corpus (PGI, PGI/PGII ratio), respectively. As an additional endpoint, down-stream in the path to AG, is the detection of *H. pylori* infection in the antrum or in corpus.

3.1. Aims of the study

With the aid of a target screening, to establish the **true prevalence of AAG** among the patients with prevalent DM1, irrespective whether symptomatic or asymptomatic. Among DM1 patients, AAG can develop as a part of PGA3 syndrome or as solitary manifestation of autoimmune disease. Disclosing AAG as early as possible is important to be able to adopt adequate surveillance measures to prevent the serious clinical sequels of AAG in these patients.

Another aim is to demonstrate whether the currently **validated cut-off values** for the four biomarkers of GastroPanel (95,96,103,104,105) are applicable also for the atrophic gastritis developed through an autoimmune mechanism (AAG), apart from HP-associated AG. Given that GastroPanel® is a quantitative test, ROC analysis can be used to estimate the best SE/SP balance for each single marker against the relevant endpoint to adjust the optimal clinical performance of the new GastroPanel® test.

Related to the applicability of GastroPanel test in the **systematic surveillance** of DM1 patients, three clinically relevant issues will be addressed in this **biopsy-confirmed** study: 1) the rate of unnecessary referrals for gastroscopy (false positive rate; 1-PPV) following a positive GastroPanel® test; 2) the rate of gastroscopies to be avoided after a negative GastroPanel® examination (true negative rate; NPV), and 3) the proportion of clinically significant diseases (conditions) that are missed by the GastroPanel® examination (i.e., false negative rate; 1-SE).

3.2. Patients

This targeted trial is conducted in collaboration between Hospital X (City Y) and the Clinical Research Department of Biohit Oyj (Helsinki). The clinical arm is performed exclusively by the Hospital, while the laboratory analyses will be done in the service laboratory of Biohit Oyj.

Enrolment of the patients in the study will take place exclusively at Hospital X, including consecutive patients over 45 years of age, attending their regular monitoring visit for DM1 at the outpatient department (ambulatory). The eligible patients can be asymptomatic or symptomatic (=dyspepsia), all having prevalent DM1 diagnosed years before. The estimated cohort to be screened for GastroPanel is at least 1000 subjects (both genders), to reach a cohort of 100-150 patents enriched with roughly **equal numbers of the relevant study endpoints** (different grades of AAG).

Patient enrollment is taking place in a single step. In brief, the potentially eligible patients are identified among the DM1 outpatients 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. All consented patients will be interviewed using previously validated questionnaires (by the nurse administrator). They will be scheduled for an appointment to GastroPanel testing at the laboratory, to ensure compliance with the preparatory steps needed before the GastroPanel® sampling. Most importantly, apart from the recommended 10h fasting (overnight), the use of PPI-medication should be discontinued preferably one week before GastroPanel® sampling.

Eligible patients are all adults aging 45 years and above, irrespective whether symptomatic or asymptomatic as to their upper gastrointestinal tract**.** However, the following patients should be considered **non-eligible**: 1) the patients whose treatment requires surgery, or immediate

follow-up treatment for major symptoms, as well as 2) those who refuse to sign the written consent.

3.2.1. Patient preparation

Proper conduction of and reliable results from the GastroPanel® examination necessitate some preparatory measures of the patient. Detailed instructions are usually given to each test subject at the time of his/her consenting to participate, but this does not apply here, because all subjects already complete the preparation for gastroscopy. Their compliance with the taking of medicines listed below will be controlled before taking the blood sample.

The patient should not drink, eat or smoke for at least 4 hours before the sample collection, e.g., 10-hour fasting overnight is perfect. The patients are allowed to take their prescribed, regular medication. However, it is necessary to report any use of proton pump inhibitors (PPIs, such as Esomeprazole, Lanzoprazole, Omeprazole and Rabeprazole), and the time of discontinuation in PPI use) on the Request Form, because these medicines interfere with the output of GastroPanel® biomarkers (www.gastropanel.com/GastroPanel® Sample collection Instructions).

3.3. Methods

3.3.1. A GI-symptom questionnaire on i) functional dyspepsia and IBS according to the Rome III criteria and on ii) reflux disease symptoms according to the Montreal classification will be completed prior to blood sampling (106).

3.3.2. Sample collection for GastroPanel® test

The person taking the blood sample shall fill the TEST REQUIST FORM as complete as possible. For each patient, 1 EDTA tube (4ml tube) for ancestry determination and 2 plasma tubes for GastroPanel®, will be taken. Plasma sample for GastroPanel® examination is taken after fasting (see above). Additionally, Gastrin-17 can be determined also after stimulation (see below). A minimum of 2 ml EDTA plasma from a fasting blood sample is taken into an EDTA tube (e.g. Biohit Cat. no. 454235 Vacuette 4ml tube containing K2EDTA). Use of Gastrin-17 stabilizer 100µl/2ml plasma (Biohit Cat. No. 601 050 or 601 051) allows the sample transfer at room temperature (20-25°C), and permits the ELISA tests within 4 days from the sample collection.

3.3.3. Sample processing

The blood sample needs to be centrifuged within 30 minutes, at 1800-2000 g for 10 minutes (e.g. Vacuette, Biohit Cat. no. 454235) or as prescribed by tube manufacturer or centrifuge manufacturer (e.g. StatsSpin Express 2, at 4000 g for 2 minutes). Unless immediately used for testing, the EDTA plasma needs to be frozen instantly (-70°C). Preferable storage temperature of the sample with the Gastrin-17 stabilizer is in the refrigerator at 2-8°C, for up to 4 days. If the sample cannot be analysed within 4 days, it should be stored frozen at -15 to -20°C, but for any storage of over 2 weeks, the temperature should be -70°C.

The samples should be mixed thoroughly after thawing. Multiple freezing and thawing cycles should be avoided. Lipemic or cloudy specimens must not be used. If a postprandial blood sample is needed, it should be taken into an EDTA tube after 20 minutes upon the intake of the protein drink. For further details, refer to the section describing Gastrin-17 stimulation (see www.biohithealthcare.com /GastroPanel Sample Collection Instructions and below).

3.3.4. Stimulated G-17

If basal Gastrin-17b concentration is low (below 1.0 pmol/l) and the patient has no *H.pylori* infection (antibodies below 30 EIU, with no eradication history), the result suggests high acid output (with no AG in the antrum). In this case, there is no need for testing of Gastrin-17s.

If GastroPanel reveals *H. pylori* infection (antibodies over 30 EIU) and low Gastrin-17b (below 1.0 pmol/l), the result can indicate either high acid output of the corpus or AG in the antrum. Distinction between these two conditions is clinically relevant by measuring protein drink-stimulated Gastrin-17s. If stimulated Gastrin-17s concentration is over 3.0 pmol/l, AG in the antrum is excluded. If, however, Gastrin-17s remains below 3.0 pmol/l after protein stimulation, AG in the antrum is likely, advocating further examinations, e.g., gastroscopy.

The secretion of G-17 can be stimulated by the intake of a protein drink having average protein content of 77% [Biohit Cat. No. 601038 (50x20 g), Cat. No. 601037 (5x20 g)]. This stimulation should not be performed for patients who are sensitive to lactose (i.e., lactose intolerance or hypolactasia). To prepare the protein juice, 20 g of protein (one foil bag of protein powder) is mixed to 150 ml of water. The stimulated (post-prandial) blood sample must be taken 20

minutes after the intake of the protein juice (www.gastropanel.com /GastroPanel Sample Collection Instructions: Stimulated Gastrin-17s).

3.3.5. Evaluation of GastroPanel® results

Prerequisite for reliable results is an adequate EDTA plasma sample, taken following the manufacturer´s instructions for sampling (above) and for conducting the ELISA tests. The results of the GastroPanel® examination are evaluated using the GastroSoft® interpretation software (www.gastropanel.com/Interpretation of GastroPanel® by GastroSoft®). The principles and algorithm used by the GastroSoft® software is based on the Updated Sydney System (USS) for classification of gastritis. This also illustrates the most important clinical conditions (disease states) associated with each of the gastritis phenotypes, including the risk of GC.

3.4. Gastroscopy and biopsy procedures

In this screening study, all patients are examined with the GastroPanel® test first. Only those who test GastroPanel-positive, i.e., **the result is classified as AG/AAG**, will be subjected to gastroscopy. This provides the histological confirmation to be used as the gold standard in calculating the performance indicators for the test. Gastroscopy referrals will be made to the outpatient department of Gastroenterology, Hospital X (City Y). It is important that also the taking of gastric biopsies follows the same USS system, including biopsy sampling from the antrum and corpus as specified by USS (97,100,103). In endoscopy, all observed abnormal mucosal lesions are noted and photographed, and if necessary (e.g. suspicion of malignancy) subjected to additional biopsy. Endoscopic findings from the esophagus, stomach, duodenal bulb and the second part of the duodenum will be recorded according to a predefined protocol to improve consistency between the endoscopists. At the time of endoscopy, the endoscopists will be blinded to the questionnaires. The future management and surveillance of the patients will be arranged according to the normal practices of the clinic.

3.4.1. Biopsy protocols

The optimal use of the USS system necessitates that the biopsy protocol follows an agreed systematic. In each patient, routine biopsy specimens are taken from the antrum and corpus, at least two biopsies from each. These biopsies are taken from the large and small curvature of the middle antrum (biopsies 1and 4) and from the large curvature of the corpus (biopsies 5 and

6). In addition, two extra biopsies are recommended to be taken from the incisura angularis (biopsies 2 and 3). Importantly, to facilitate the pathology reading, the biopsies from the antrum and incisura (Biopsies 1, 2, 3 and 4) must be immersed into one and the same formalin bottle, and embedded into the same paraffin block (Block No. 1; labeled **ANTRUM**). The two biopsies from the corpus are set into one and the same formalin tube, and embedded into the same paraffin block (Block No. 2; labeled **CORPUS**). In addition, biopsies from the bulb and the second part of the duodenum, distal and mid esophagus will be taken.

3.4.2. Interpretation of the biopsies

All gastroscopy biopsies are examined by the expert pathologists as part of their daily routine at the Department of Pathology, Hospital X. The diagnoses are reported using the USS for classification of gastritis, and diagnosed into different "phenotypes" of gastritis (97,100,101,102,103).

3.5. 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 done according to routine procedures. Performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of individual markers and whole GastroPanel® test will be calculated separately for each study endpoint, using the STATA/SE software and the *diagti* algorithm introduced by Seed et al. (2001)(107). This algorithm also calculates the area under ROC (Receiver Operating Characteristics) called AUC, for each biomarker at each endpoint. Because GastroPanel® is a quantitative ELISA test, these ROC curves can be used to identify the optimal sensitivity/specificity balance that gives each biomarker an optimal threshold for detection of each study endpoint. Significance of the difference between AUC values can be estimated using STATA's roccomb test with 95%CI.

4. ETHICAL ISSUES

The study design and its execution does not involve any significant ethical issues except those in other clinical studies of similar type. The study protocol will be submitted for approval to the Regional Ethical Committee (Hospital X), and the study is conducted in accordance with the Declaration of Helsinki.

Patients are enrolled among consecutive DM1 patients attending the outpatient department of Hospital X for the scheduled appointment to control their disease. Thus, they represent regular DM1 outpatients controlled in the hospital as part of their routine clinical surveillance for DM1. The only additional procedures carried out to the patients include filling of questionnaires and the blood sampling. The maximum amount of venous blood taken is 10 ml. All patients must sign the informed consent for their participation. When the result of the GastroPanel® test is available, clinical judgement is used to make the referral for gastroscopy. Based on existing clinical practice, however, any patient with suspected AG/AAG needs gastroscopic confirmation of the disease severity (5,97). In this respect, the study protocol does not include any diagnostic procedure additional to the existing clinical practices. Once all results are available, the patients will be informed about the results, following the usual clinical practices, including an explanation of the test results and the appropriate measures for further conduct.

5. TIME FRAME

Given that the subjects in the study will be enrolled among consecutive DM1 patients with estimated 10-15% prevalence of AAG/PA, attending the ambulatory of Hospital X, (with attendance rate of **X? patients/week**), and a cohort of patients enriched with sufficient numbers of all study endpoints will be needed, it is estimated that GastroPanel screening of a minimum of 1000 subjects will take **approximately ? months** of clinical work. The laboratory arm of this study is expected to proceed online with the progress of patient enrollment and gastroscopies. Despite a minor delay (of days) due to the biopsy examination by the pathologists, 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 blood and biopsy samples.

6. PROJECTED COSTS TO BE COVERED by Biohit Oyj

The company will compensate the extra effort put in the project by Hospital X. The details are subject to discussions and contractual agreement.

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