-CONFIDENTIAL-

GastroPanel SCREENING PROTOCOL

**Biohit *GastroPanel® Assay In Screening of Patients
at Risk** for Gastric Cancer.**

Jointly Executed by:

BIOHIT Oyj (Helsinki, Finland); Hospital X (City Y, Country Z)

Research Team:

First Name, Last Name, ...

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* ELISA biomarker test for pepsinogen I (P-PGI), pepsinogen II (P-PGII), gastrin-17 (P-G-17), and H.pylori antibodies (P-HpAb); **Patients with atrophic gastritis, gastric dysplasia, Helicobacter pylori infection

Summary

Background: Atrophic gastritis (AG) is the single most important precursor condition for gastric cancer (GC) known so far. On the other hand, *H. pylori* -infection is the most important causative agent of gastritis, and subsequent AG. *H. pylori* infection and AG in the antrum may lead to GC and peptic ulcer disease. To obviate the need for invasive diagnostic methods (gastroscopy) for these conditions, Biohit HealthCare (Helsinki, Finland) launched an ELISA-based assay designed to measure the concentrations of certain key stomach-specific biomarkers from a single plasma sample (www.biohithealthcare.com /additional-information).

The GastroPanel® test is the first non-invasive diagnostic tool providing possibilities for detecting the patients at risk for GC and peptic ulcer as well as malabsorption of vitamin B12, iron, magnesium, calcium and some drugs. A well designed clinical study is still missing to fully assess the performance of GastroPanel® examination in detecting the surrogate intermediate endpoints of GC.

Objective: To conduct a screening trial with the Biohit HealthCare's GastroPanel test for early detection of patients at risk for GC in country X.

Study Design: This study is a population-based screening trial using the GastroPanel® examination to diagnose all those specific gastric conditions that are known to be associated with an increased risk for GC among an adult population in country X.

Methods: A cohort of 1000 patients (45 years and older, both genders) will be enrolled among randomly selected patients attending the Hospital X (City Y, Country Z), with any indication, other than gastroscopy referral patients. For patient preparation, sampling and processing, the instructions for an adequate completion of GastroPanel® test are followed. The GastroPanel® test contains four biomarkers specific for the gastric mucosa: 1) Pepsinogen I (P-PGI), 2) Pepsinogen II (P-PGII), 3) Gastrin-17 (P-G-17) and 4) H. pylori antibody (P-HpAb). All four ELISA tests will be done in the clinical laboratory of Hospital X. Primarily, only the patients testing positive with the GastroPanel® test will be subjected to gastroscopic examination, with directed biopsies from the antrum and corpus, following the protocol of the Updated Sydney System (USS). **To correct for the verification bias, a random sample of 5% of the subjects testing negative with the GastroPanel® test, will be invited for gastroscopy.** Biopsies are examined at the Pathology laboratory of Hospital X, and interpreted using the USS for classification of gastritis. Statistical analyses include calculation of the performance indicators of the GastroPanel® test for individual study endpoints, including ROC analysis for cut-off values that give the optimal sensitivity/specificity balance.

Specific Aims: The single most important goal of this screening trial is to establish the performance indicators for the GastroPanel® examination in detecting the intermediate surrogate endpoints of GC. These study endpoints include the following: i) atrophic gastritis in the antrum, ii) atrophic gastritis in the corpus, iii) atrophic gastritis in both antrum and corpus (=atrophic pan-gastritis), and iv), biopsy-confirmed dysplasia (intestinal metaplasia) of the gastric mucosa. For all these endpoints, we will calculate sensitivity (SE), specificity (SP), negative predictive value (NPV), positive predictive value (PPV) and AUC (area under ROC curve) for GastroPanel® biomarkers, collectively and individually for each marker. ROC analysis can be used to estimate the best SE/SP balance for each single marker against each different endpoint. One of the aims is to assess, whether the test cut-offs for each endpoints can be further optimized to give the GastroPanel® test an optimal performance. Because only a minor

fraction (test positives +random 5% of test-negatives) are subject to examination by the gold standard (gastroscopy), the results must be corrected for verification bias, to obtain unbiased estimates of test performance in a screening setting. Combined analysis of the gastroscopy cohort (with test+ and test- subjects) will also enable assessment of 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 necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached the Agreement with Biohit HealthCare. 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 randomly selected adult patients attending Hospital X for reasons other than gastric symptoms, it can be estimated that the screening of a cohort of at least 1000 patients will take approximately 2 months (max). The laboratory arm of this study is expected to proceed almost online with the progress of patient enrolment and gastroscopies. Similarly, there will be only a minor delay (of days) due to the biopsy examination at the Department of Pathology, until the triple-test (GastroPanel® examination, gastroscopy, biopsy) results of each individual subject are available to be entered into the study database.

Impact of the study: Using the correction for verification bias, we expect to obtain unbiased estimates of the performance of each four markers in the GastroPanel® in detecting the different gastric conditions with increased risk of GC in this screening setting. These estimates can be compared with those obtained in a clinical setting among gastroscopy-referral patients (with 100% verification by the gold standard), translated to a hypothetical screening setting by simulation modelling. If this match is good, it can be concluded that both approaches are valid for evaluation of GastroPanel® test-based screening strategies for GC.

1. BACKGROUND

At present, the diagnosis of most gastric and esophageal diseases, requires an endoscopic examination which is an invasive, time-consuming and expensive procedure. A present, there are few non-invasive methods (e.g. tests for Helicobacter pylori) available for the diagnosis of the upper gastrointestinal tract diseases. Any of these tests do not, however, give possibilities for a comprehensive diagnosis of the different phenotypes of gastritis, i.e., whether superficial or atrophic, and located in the antrum or corpus (www.biohithealthcare.com/additional-information). Importantly, these tests do not give any clues about the severity (grade) of these lesions, as defined by the Updated Sydney Classification (USS).

Atrophic gastritis (AG) is a disease associated with a significantly increased risk of gastric cancer, being the single most important precursor condition for gastric cancer (GC) known so far. On the other hand, *H. pylori* -infection is the most important causative agent in the development of gastritis, and subsequent AG. *H. pylori* infection and AG in the antrum may lead to GC and peptic ulcer disease. It is well known that a minority of cases of AG in the corpus develop by autoimmune mechanisms. The risk of GC is 4-5 times higher among patients suffering from severe atrophy of the corpus mucosa as compared with their healthy counterparts. Among the patients with severe atrophy in the antrum, this risk is 18 fold higher than in healthy subjects, and the risk increases up to 90-fold if severe atrophy exists in both antrum and corpus (i.e., with severe pan-atrophy). AG in corpus mucosa also leads to malabsorption of vitamin B12 and, subsequently, the deficiency of this vitamin raises the levels homocysteine in blood and tissues. High homocysteine levels may increase the risk of heart and vascular diseases, as well as neurodegenerative disorders.

The prevalence of AG and GC increases with increasing age, and the risk for both diseases is highest among the subjects >45 years of age. The majority of GCs among the elderly are of the intestinal subtype, developing through the AG-to-GC sequence. Because of the high cancer risk among the elderly, the current consensus recommendations suggest endoscopy for all dyspeptic elderly people as well as for those aging above 45 (50) years.

To obviate the excessive use of this invasive and expensive procedure (endoscopy), there is an urgent need to develop non-invasive diagnostic tools capable of accurately detecting the patients at high risk for GC, i.e. the different phenotypes of gastritis as well as their related *H.*

pylori infections (www.biohithealthcare.com/ Scientific: State of the art GastroPanel and Acetium innovations of the unmet need).

For this purpose, a Finnish company (Biohit HealthCare) recently launched an ELISA-based assay designed to measure the concentrations of certain key stomach-specific biomarkers from a single blood sample. The test is known as GastroPanel®, being a cost-effective and user-friendly ELISA technique, intended both for research purposes and clinical practice. The GastroPanel® test contains four biomarkers specific for the gastric mucosa: 1) Pepsinogen I (P-PGI), 2) Pepsinogen II (P-PGII), 3) Gastrin-17 (P-G-17) and 4) H. pylori antibody (P-HpAb). This ELISA test is intended for diagnosis of gastritis and AG, comprehensively for both corpus and antrum. The GastroPanel® examination is the first non-invasive diagnostic tool providing possibilities for detecting the patients at risk for GC (and esophageal cancer, EC) and peptic ulcer diseases as well as malabsorption of vitamin B12, iron, magnesium, calcium and some drugs. (www.biohithealthcare.com/additional-information).

After ELISA-testing for P-PG I, p-PG II, P-G-17 and P-Hp-Ab in a plasma sample, an endoscopic examination can be preserved only for those patients whose GastroPanel® test results suggest AG, whereas an endoscopic examination can be avoided in subjects with negative GastroPanel® result, or in whom the test biomarkers indicate a non-atrophic gastritis or a healthy stomach. Gastroscopy is also recommended if the GastroPanel® examination reveals high acid output (P-G-17 below 1,0 pmol/l) or chronic *H. pylori* infection with symptoms. (www.gastropanel.com).

In addition of being the first non-invasive diagnostic test for gastric diseases related to and/or associated with an increased risk for GC, the state-of-art GastroPanel® examination is devoid of several serious medical problems (ANNEX 1). Most importantly, the serious medical and ethical problems of the "test and treat" strategy can be corrected simply and economically by replacing its ^{13}C-urea breath test or stool antigen test by the GastroPanel® examination. In many countries, the "test and treat" strategy alone is not considered sufficient, because the tests for *H. pylori* in the "test and treat" strategy does not find AG and related risks, such as GC and its precursor lesions, which should be confirmed by gastroscopy and biopsy. Importantly, the ^{13}C-urea breath test (UBT) or stool antigen test of the "test and treat" strategy do not indicate, whether the patient has AG of the corpus and/or antrum of the stomach. It remains to be seen, how many of these deaths could have been prevented by the GastroPanel screening, which

reveals the risk of peptic ulcer disease (www.biohithealthcare.com/ Scientific: State of the art GastroPanel and Acetium innovations for the unmet need).

Furthermore, it has been estimated that e.g. in Finland (the country of origin of the GastroPanel test), 250 to 300 annual GC deaths among persons >50 years of age could be avoided, simply by screening of all elderly people and especially all suspected *H. pylori* positive patients for AG using the GastroPanel® examination. In addition to the risk assessment for GC, the GastroPanel® screening, diagnosing and check-ups produce a lot of additional, reliable and valuable information. According to the recently published recommendations of leading gastroenterologists from 12 countries, the stomach-specific biomarkers included in GastroPanel® provide information about the stomach health and about the function of stomach mucosa and are a non-invasive tool for diagnosis and screening of AG and acid-free stomach (Agréus L, et al. Scand. J. Gastroenterol 2012;47:136–147).

The studies published on GastroPanel test have been subjected to two recent meta-analysis (Anticancer Res. 2016;36,5133-5144; Aliment. Pharmacol. Ther. 2017;1-11). The results of both meta-analyses were concordant, both confirming the high accuracy of GastroPanel biomarkers in diagnosis of both AGC and AGA. The authors concluded that GastroPanel® test appears to be a reliable tool for the diagnosis of AG, and applicable for both screening of the subjects or populations at high-risk of GC.

1.1.The GastroPanel® test

The GastroPanel® is a cost-effective test based on user-friendly ELISA technique, intended both for research purposes and for clinical practice. The GastroPanel® test contains four biomarkers specific for the gastric mucosa: 1) Pepsinogen I (P-PGI), 2) Pepsinogen II (P-PGII), 3) Gastrin-17 (P-G-17) and 4) H. pylori antibody (P-HpAb).

1.1.1.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. Atrophic corpus gastritis leads to a 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.

While not secreted by any other cells at any other anatomic sites, these two biomarkers are specific for gastric mucosa, i.e., stomach-specific biomarkers.

In the GastroPanel® test, P-PGI and P-PGII biomarkers are determined according to the instructions of the manufacturer from plasma samples. (www.gastropanel.com). Pepsinogen I ELISA kit (Biohit Cat. No. 601 010.01), Pepsinogen II ELISA kit (Biohit Cat. No. 601 020.01). Both P-PGI and P-PGII ELISA is based on a sandwich enzyme immunoassay technique with PGI- and PGII-specific capture antibody, adsorbed on a microplate and the detection antibody labeled with horseradish peroxidase (HRP). The monoclonal PGI antibody is applicable also for immunohistochemical (IHC) detection of PGI expression in formalin-fixed, paraffin-embedded specimens (dilutions 1/5000–1/10.000).   In IHC, this antibody stains specifically and exclusively the chief cells and mucous neck cells of the gastric corpus.

1.1.2.ELISA test for Gastrin-17

Simple blood tests have not been available for diagnosing atrophic gastritis of the antrum. In this respect, the Gastrin-17 (P-G-17) biomarker of the GastroPanel® test offers a unique opportunity. P-G-17 is one of the most important peptide hormones of the gastrointestinal tract, playing a role in a wide variety of functions. Amidated 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. The G-17 fraction of the total gastrin can be measured with high specificity by Gastrin-17 Advanced ELISA Test Kit (Biohit Cat. no 601 035).

When dormant, the G-cells in antrum secrete only small amounts of G-17 hormone. The maximal secretion is achieved after physiological protein stimulation ("steak stimulus" or protein stimulation, www.gastropanel.com/GastroPanel Sample Collection Instructions: Stimulated Gastrin-17s), or when the acid secretion in the stomach decreases, is low or absent. 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 gastrin decreases.

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 does not react with G-34 or other gastrin fragments. Thus, this G-17-specific test allows estimation of the number and function of antral G-cells, without cross-reactivity with G-34 peptide or other gastrin fragments, which are also derived from sources other than the G-cells.

1.1.3.ELISA test for Helicobacter pylori (HpAb ELISA)

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

2. STUDY DESIGN

The present study is a population-based screening trial with the Biohit HealthCare's GastroPanel® test for early detection of patients at risk for GC in Country Z. The screening "population" in this case denotes the adult patients attending a hospital due to different referral indications, except those referred for gastroscopy consultation. The conditions representing the study endpoints (outcome measures) in these analyses include the following: i) atrophic gastritis in the antrum, ii) atrophic gastritis in the corpus, iii) atrophic gastritis in both antrum and corpus (=atrophic pan-gastritis), and iv), biopsy-confirmed dysplasia and intestinal metaplasia of the gastric mucosa. As an additional endpoint, down-stream in the path to AG, is the detection of *H. pylori* infection (in antrum or in corpus). The other endpoints not related to increased risk of GC include: i) superficial antrum gastritis, and ii) superficial corpus gastritis, also detectable by the GastroPanel® examination.

2.1. Aims of the Study

The single most important goal of this study is to establish the performance indicators for the GastroPanel® examination in detecting the intermediate surrogate endpoints of GC, i.e., various conditions that confer an increased risk for GC in **a random adult population**. This goal is reached through several specific aims in this study.

1. Sensitivity (SE), specificity (SP), negative predictive value (NPV), positive predictive value (PPV) and AUC (area under ROC curve) for GastroPanel® biomarkers (all four) in detecting **atrophic antrum gastritis**.

2. SE, SP, NPV, PPV and AUC for GastroPanel® biomarkers (all four) in detecting **atrophic corpus gastritis**.

3. SE, SP, NPV, PPV and AUC for GastroPanel® biomarkers (all four) in detecting **atrophic pangastritis** (AG in the antrum and corpus).

4. SE, SP, NPV, PPV and AUC for the individual biomarkers in the GastroPanel® in detecting the four endpoints listed above.

5. Given that the four GastroPanel® biomarkers are a quantitative test, ROC analysis can be used to estimate the best SE/SP balance for each single marker against each different endpoint. One of the aims is to assess, whether the test cut-offs for each endpoints can be adjusted to give the GastroPanel® test an optimal performance.

6. In this context, we will also assess: 1) the number needed to screen (NNS), separately for all study endpoints, 2) the rate of unnecessary referrals for gastroscopy (false positive rate; 1-PPV) following a positive GastroPanel® test; 3) the rate of gastroscopies to be avoided after a negative GastroPanel® examination (true negative rate; NPV), and 4) the rate of clinically significant diseases (conditions) that are missed by the GastroPanel® examination (i.e., false negative rate; 1-SE).

2.2. Patients

This population-based screening trial is conducted as collaboration between Biohit HealthCare (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 randomly allocated patients (over 45 years of age), attending the Hospital with different referral indications, except those with appointment to gastroscopy. As to their upper gastrointestinal tract, the eligible patients can be asymptomatic or symptomatic, but those referred for gastroscopy will be excluded from this screening trial. The preliminary cohort to be screened is 1000 subjects (both genders).

Patient enrollment is taking place in two steps. In brief, the potentially eligible patients are identified among the outpatient clinic attendants 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. Because none of the patients are enrolled among the subjects attending the Endoscopy clinic due to an appointment to gastroscopy, their preparation is not necessarily compliant with the preparatory steps needed for the GastroPanel® examination (details to follow).

Eligible patients are all adult females and males (over 45 years of age), irrespective whether symptomatic or asymptomatic as to their upper gastrointestinal tract. However, the following patients should be considered <u>**non-eligible**</u>: 1) the patients who are referred to hospital for gastroscopy; 2) the patients whose treatment requires surgery, or immediate follow-up treatment for major symptoms, as well as 3) those who refuse to participate.

2.2.1.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 (ANNEX 3), because these medicines

interfere with the output of GastroPanel® biomarkers (www.gastropanel.com/GastroPanel® Sample collection Instructions).

2.3.Methods

2.3.1.Sample Collection for GastroPanel® Examination

The person taking the blood sample shall fill the TEST REQUIST FORM (**ANNEX 3**) 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.

2.3.2.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).

2.3.3.Stimulation of Gastrin-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).

2.3.4.Evaluation of GastroPanel® Test 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®). A model Report of test results is enclosed (**ANNEX 4**). The principles and algorithm used by the GastroSoft® software is based on the Updated Sydney System (USS) for classification of gastritis, as schematically presented in **ANNEX 5**. This ANNEX also illustrates the most important clinical conditions (disease states) associated with each of the gastritis phenotypes, including the risk of GC.

2.4.Gastroscopy and Biopsy Procedures

In this study, all patients are examined with the GastroPanel® test, and ONLY the GP-positive patients will be subjected to gastroscopy, providing the histological confirmation to be used as the gold standard in calculating the performance indicators for the test. Because of the fact that

the GastroPanel® test results are reported by the GastroSoft® software based on the algorithm of the USS for classification of gastritis (ANNEX 5), it is important that also the taking of gastric biopsies follows the same system.

All patients who undergo gastroscopic examination will be complemented by biopsy sampling from the antrum and corpus, according to the principles of the USS. In endoscopy, all observed abnormal mucosal lesions are noted and photographed, and if necessary (e.g. suspicion of malignancy) subjected to additional biopsy.

2.4.1.Biopsy Protocols

The optimal biopsy protocol following the USS is illustrated in **ANNEX 6.** 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**).

2.4.2.Preparation of the Microscopy Slides

The biopsies from formalin bottles/tubes are embedded in paraffin using the routine procedures at the Pathology Laboratory of BCH. The blocks are cut into 4-μ-sections, and stained with hematoxylin eosin (HE) for routine diagnosis and with modified Giemsa for identification of *H.pylori* in the specimens.

2.4.3.Confirmation of *H.pylori* with an Antibody Test (optional)

In case of doubt, it is recommended that the presence or absence of the *H. pylori* infection is confirmed with the endoscopic *H. pylori* Quick Test (*Helicobacter pylori* Quick Test, or UFT300 test, Biohit). For this testing, additional biopsies are needed, one from the antrum and another one from the corpus, immersed into physiological saline solution, or used immediately for the

test. Both biopsies are used in the same test, i.e., set together to the same test plate. The feasibility of this procedure remains in discretion by the gastroscopist in each individual case.

2.4.4. Interpretation of the Biopsies

All gastroscopy biopsies are examined by the expert pathologists at BCH among the daily routine samples. The diagnoses are reported using the USS for classification of gastritis, and diagnosed into different "phenotypes" of gastritis, as schematically presented in ANNEX 4 for the GastroPanel® examination and in **ANNEX 7** for histopathological examination.

In brief, the topography of the gastritis is considered the core of the classification. Accordingly, the gastritis restricted to the antrum, restricted to the corpus, or a pan-gastritis should be reported separately. The etiology of gastritis, if known, is to be added to the diagnosis as **a prefix** (e.g. *H. pylori* antrum gastritis; autoimmune corpus gastritis, etc.). As **a suffix**, one should give a grading foe each five morphological variables shown in ANNEX 7. These are; 1) chronic inflammation (chronic gastritis); 2) the activity of the gastritis measured by the presence of polymorphonuclear leucocytes alongside the mononuclear inflammatory infiltrate; 3) intestinal metaplasia (IM); 4) atrophy manifested by the loss of the normal mucosal glands; and 5) the presence of *H. pylori* organisms. The guidelines recommend these five parameters to be recorded separately for both antrum and corpus, with at least two random biopsies to be taken from each site. Furthermore, it is recommended that these parameters should be semi-quantitatively graded as absent, mild, moderate or severe, each successive grade to represent an increase in severity of approximately one third. Examples of valid diagnosis are found in the Figure legend of ANNEX 7.

2.5.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. For both tests, the number needed to screen (NNS), will be calculated separately for all study endpoints, all age groups, and for women and men. The study design represents a typical screening setting with verification (work-up) bias inherent to the use of the gold standard (gastroscopy) for verification of disease states ONLY for subjects testing positive for GastroPanel. To be able to calculate the performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of

the GastroPanel test, **AND TO CORRECT FOR the VERIFICATION BIAS, a random sample of 5% of GastroPanel® test-negative subjects must be invited for gastroscopy and biopsy confirmation.**

In this setting, with GastroPanel® test+ and test- patients verified by gastroscopy, the performance indicators can 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 calculates the area under ROC (Receiver Operating Characteristics) called AUC [(SE+SP)/2], for each study endpoint. Because GastroPanel® test consists of two components, these performance indicators can be calculated separately for each biomarker in the test panel, increasing the flexibility of this assay. Significance of the difference between AUC values is estimated using the roccomb test (STATA) with 95%CI. These crude (non-corrected) results will then be subjected to correction for verification bias, which is done by a commonly used method described by Reichenheim et al. (2001), and of which an algorithm (validesi) is available is STATA. In this procedure, the 95%CIs are derived using the parametric bootstrap method, with the simulation of 10,000 replications.

3.ETHICAL ISSUES

The study design and its execution do 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 Institutional Ethical Committee of Hospital X, and the study is conducted in accordance with the Declaration of Helsinki. All patients must sign the informed consent for their participation. When the results of the GastroPanel examination, gastroscopy and biopsies are available, the patients will be informed about the results, following the usual hospital practices, including an explanation of the meaning of these test results, and the appropriate measures for further conduct.

4.TIME TABLE

The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached the Agreement with Biohit HealthCare. 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 random population (hospital patients) attending Hospital X, it is estimated that screening of a minimum of 1000 subjects will

take approximately ? months. More detailed time frame is impossible to predict in advance, because the exact annual numbers of AG are not available.

The execution of the work in the laboratory can be started in parallel with the patient enrollment. The laboratory has all the necessary facilities to run the ELISA tests for the GastroPanel® biomarkers, and the instruments will be calibrated for these tests using the optimized protocols available in Biohit HealthCare for each specific type of ELISA instrument. The laboratory arm of this study is expected to proceed almost online with the progress of patient enrollment and performed gastroscopies. Similarly, there will be only a minor delay (of days) due to the biopsy examination at the Department of Pathology, until the triple-test (GastroPanel® examination, gastroscopy, biopsy) results of each individual subject are available to be entered into the study database. Accordingly, the full database of the patients will be ready for statistical analysis practically in real-time after completion of the enrollment of the cohort and examination of their blood and biopsy samples.

5. PROJECTED COSTS
This part to be agreed upon joint discussions.

ANNEX 1:

MEDICAL PROBLEMS POTENTIAL AVOIDABLE BY THE GastroPanel® EXAMINATION

The i) **¹³C urea breath test** (UBT), ii) **stool antigen test,** and iii) **antibody tests** do not detect atrophic gastritis which is caused by *H. pylori* infection or an autoimmune disease. The diagnosis of AG is important because of the associated risks: GC, EC, malabsorption of vitamin B12, iron, magnesium, calcium and some drugs. Calcium deficiency causes osteoporosis, and vitamin B12 deficiency can cause Alzheimer's disease, dementia, depression and polyneuropathy, as well as high homocysteine content in the body, which in turn is thought to be an independent risk factor for atherosclerosis, heart attacks and strokes.

The absorption of dipyridamole, some iron products and antifungals (fluconazole, itraconazole), thyroxine and atazanavir is considerably impaired in an anacidic stomach. Atrophic gastritis in the gastric corpus and PPI therapy cause anacidity (aclorhydria) of the stomach. The risk of pneumonias and, in senior citizens, even the risk of fatal intestinal infections (such as giardiasis, malaria, *Clostridium difficile* and *E. coli* EHEC) may increase significantly in an anacidic stomach. *H. pylori* gastritis may also develop into antral atrophic gastritis, which increases the risk of peptic ulcer disease and GC. If both antrum and corpus mucosa are atrophic, this condition has the highest risk for GC known to date.

Furthermore, none of the aforementioned three *H. pylori* tests provides any information on excessive gastric acid secretion (high acid output), which in patients with gastro-oesophageal reflux disease may cause complications of this disease in esophagus. Such complications are often asymptomatic and include ulcerative oesophagitis and Barrett's oesophagus, which may lead to EC if left untreated. In addition, the ¹³C urea breath test and stool antigen test may give up to 50 % false negative results, if the patient has a) AG b) MALT lymphoma or c) bleeding peptic ulcer disease or d) if the patient is currently receiving antibiotics or PPIs.

ANNEX 2:

GastroPanel® and Acetium®

The GastroPanel® and Acetium® innovations are together a unique combination that may help preventing GC and EC. GastroPanel® detects AG and related gastric and oesophageal cancer risks while the conditions are still treatable. AG of the corpus, which is usually irreversible, leads to permanent achlorhydria. In an achlorhydric stomach, microbes from the mouth can survive and produce acetaldehyde from sugars and alcohol present in food. In the new cancer classification issued by WHO in October 2009, acetaldehyde is classified as Group I carcinogen, together with the known carcinogens such as asbestos, tobacco and benzene. Globally, acetaldehyde exposure is linked to approximately four million new cases of cancer each year, nearly 40% of all cancers. Biohit has developed products and a method to reduce physical and nutritional exposure to acetaldehyde.

The same ethical and legislative principle concerns all Group I carcinogens, regardless of their source. Physical and nutritional exposure to them should be reduced by all possible means. Biohit's Acetium® capsule is the only means of inactivating carcinogenic acetaldehyde in the stomach, thus helping to prevent gastric and oesophageal cancers. Acetium® capsules are currently available in pharmacies without prescription. They are recommended at meals and when consuming alcohol for the subjects who have:

1. achlorhydria caused by AG (diagnosed by GastroPanel)
2. a chronic *H. pylori* infection (diagnosed by GastroPanel)
3. a long-term use of antacids (PPIs, H2-receptor antagonists)
4. a resected stomach
5. ALDH2-deficiency or high active ADH genotype

Obviously, randomized intervention studies with Acetium® are not possible for ethical reasons, because of the Group I carcinogenicity classification of acetaldehyde. It is feasible to anticipate that a systemic reduction of acetaldehyde may protect against GC and EC at high-risk groups. Studies are in the pipeline to estimate the efficacy of Acetium® not only in reducing the levels of acetaldehyde in the gastric fluid, but also in reducing the addition to cigarette smoking, while reducing acetaldehyde level in saliva (orodispensible Acetium® tablet or ODT).

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ANNEX 3.

THE TEST REQUEST FORM

BIOHIT HealthCare
Innovating for Health

TEST REQUEST FORM

SAMPLE RECEIVED TO LABORATORY (DATE)

PATIENT'S LAST NAME

PATIENT'S FIRST NAME

SOCIAL SECURITY NUMBER

TELEPHONE NO

NAME AND ADDRESS OF SENDER

☐ Send results to doctor   ☐ Send results to patient

NAME OF DOCTOR REQUESTING THE TEST (when appropriate)

BILLING ADDRESS *

ADDRESS WHERE RESULTS REPORT SHOULD BE SENT *

* If different from address of sender

PATIENT CONSENT

By signing I give my consent to the use of my sample results and the information given on this form in medical research, with the exception of my name and social security number, which will be kept confidential.

Patient's signature

PATIENT INFORMATION

Have the patient previously been in GastroPanel test? ○ Yes   ○ No

Has the patient received *H. pylori* eradication treatment? ○ Yes, over 1 y   ○ Yes, less than 1 y   ○ No   ○ Not known

Is the patient currently using anti-acid (PPI) medication? ○ Yes, continuously ○ Yes, occasionally   ○ No   ○ Not known

Pause in PPI medication [   ] days before blood test

Does the patient experience heartburn and/or acidic taste in the mouth? ○ Yes, continuously   ○ No   ○ Not known

Is the patient currently using NSAID (pain killers) medication? ○ Yes, continuously   ○ No   ○ Not known

I HAVE CHECKED INFORMATION GIVEN BY PATIENT

Signature of person collecting the sample   **SAMPLE TAKEN (DATE)**

TESTS

☐ **GastroPanel:** Pepsinogens I and II, *Helicobacter pylori* IgG Ab and Gastrin-17b (fasting)

☐ **GastroPanel, stimulated:** Pepsinogens I and II, *Helicobacter pylori* IgG Ab, Gastrin-17b (fasting) and Gastrin-17s (stimulated)

☐ **Single Tests**
  ☐ Pepsinogen I (fP/S-Pepsin 1)
  ☐ Pepsinogen II (fP/S-Pepsin 2)
  ☐ *Helicobacter pylori* IgG Ab (fP/S-HepyAbG)
  ☐ Gastrin-17b fasting (fP/S-Gastr17b)
  ☐ Gastrin-17s stimulated (P/S-Gastr17s)

SAMPLE INFORMATION (a minimum of 1,0 ml) :

SAMPLE ID: [   ]

☐ EDTA PLASMA

☐ EDTA PLASMA + Gastrin-17-STABILIZATOR

DELIVERING TO LABORATORY:

☐ FASTING SAMPLE   ☐ STIMULATED SAMPLE

☐ ROOM TEMPERATURE   ☐ FROZEN

Biohit Oyj Service Laboratory, Laippatie 1, 00880 Helsinki, www.gastropanel.net

gpform_en_ver_06

ANNEX 4.

A MODEL REPORT OF GastroPanel® TEST RESULTS BY GastroSoft®

GastroPanel report				3.4.2012

Patient Data

Name	Patient Name (#1)
Date of birth	121254-1213
Age	57
Eradicated	No
Use of PPI	No
Acidic symptoms	No
Use of NSAIDs	No

Assay Data

Collected	12.2.2012		
Analyzed	12.2.2012		
			reference range:
Pepsinogen I (PGI)	20,0 µg/l	*	30 - 165 µg/l
Pepsinogen II (PGII)	15,0 µg/l		3 - 15 µg/l
PGI/PGII	1,3	*	3 - 20
Gastrin 17B	40,0 pmol/l	*	< 5 pmol/l
H. pylori antibodies (HPAB)	145,0 EIU	*	< 30 EIU

Interpretation

The results indicate atrophic corpus gastritis due to *Helicobacter pylori* infection. Acid secretion in the stomach is decreased.

Ask a doctor about the possibility of undergoing gastroscopy.

Information about the interpretation

Helicobacter pylori antibodies (IgG). *Helicobacter pylori* (*H. pylori*) colonizes the stomach mucosa of infected individuals. Infection is usually obtained in childhood, and it becomes chronic and life-long if not eradicated. *H. pylori* infection is particularly common among the elderly in particular. In some infected individuals, the stomach mucosa undergoes atrophy with time (decades); a process which may increase the risk of certain diseases (stomach cancer, peptic ulcer) and risk of malabsorptions (deficiencies) of certain vitamins, micronutrients and medicines (vitamin B12, iron, calcium, magnesium). *H. pylori* infection is probable when the level of antibodies is above 30 EIU.

Pepsinogen I level in the blood reflects the structure and function of the gastric corpus mucosa. When the corpus stomach mucosa undergoes moderate-severe atrophic gastritis, the level of pepsinogen I in the blood is below 30 µg/l.

Pepsinogen II level in the blood reflects the structure and function of the whole stomach mucosa. Its level in the blood rises during inflammation of stomach mucosa (cut-off 10 µg/l), which is usually caused by *H. pylori* infection, or sometimes by another factors (e.g. taking pain killers, strong alcohol etc.).
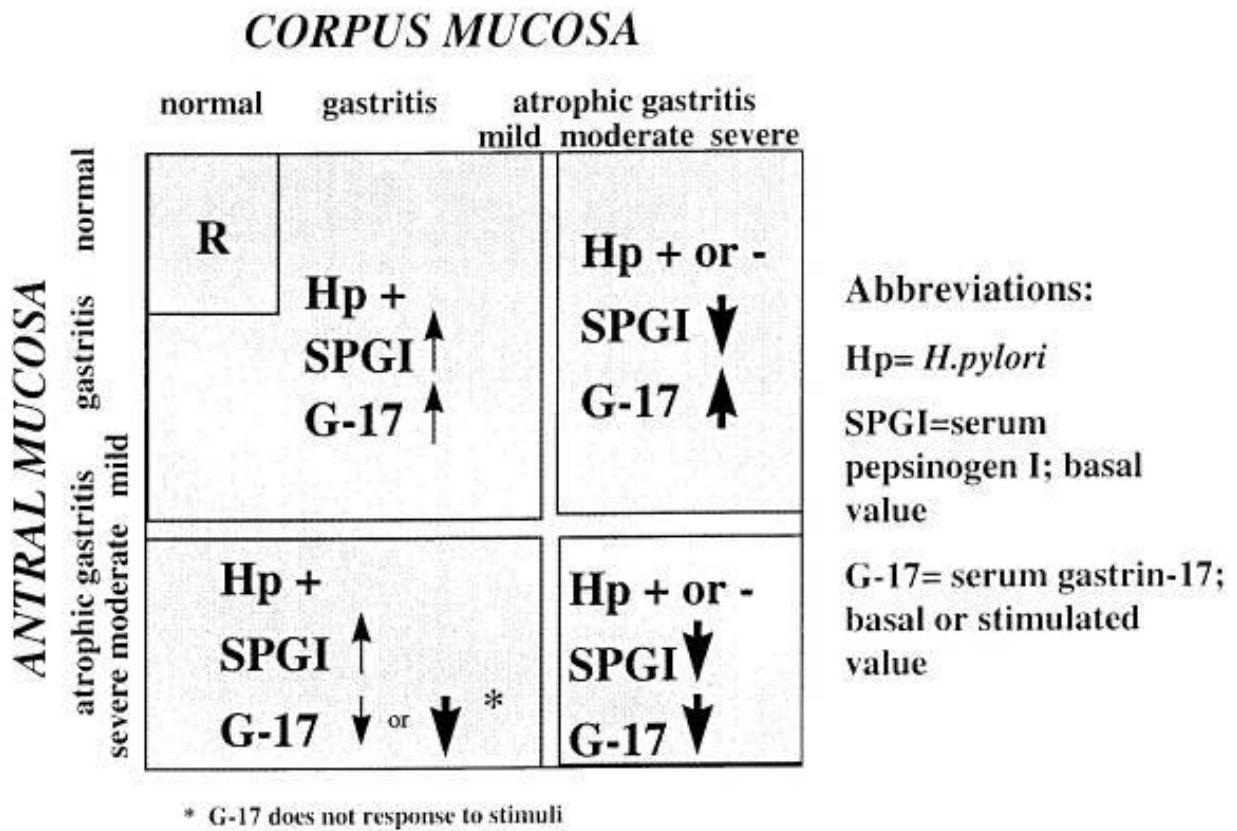
Pepsinogen I/Pepsinogen II ratio falls to below 3 in atrophy of the gastric corpus mucosa.

Gastrin-17* level in the blood reflects the structure and function of the mucosa in gastric antrum. Biohit's monoclonal antibody detects only amidated gastrin-17 peptide, which has a specific receptor only on parietal cells. Gastrin-17 is secreted only by antrum G-cells and it increases the secretion of acid into the stomach by parietal cells of the corpus mucosa. The level of gastrin-17 in blood (fasting sample) falls when the acidity of the stomach increases (pH below 2.5). A fasting gastrin-17 level below 1 pmol/l means that acid secretion is very high. A fasting level of gastrin-17 remains low also if there is atrophy of the antrum mucosa, along with loss of antral G cells. A fasting level of gastrin-17 may thus mean either atrophy of the antrum mucosa, or increased secretion of stomach acid. If the level of gastrin-17 increases to more than 3 mol/l following protein stimulation, the patient has an acidic stomach but not antrum atrophy. If the level of gastrin-17 does not increase following protein stimulation, the patient has atrophy of the antrum mucosa. If the fasting level of gastrin-17 is above10 pmol/l, it usually means the stomach is hypoacidic (low acidity due to PPI medication, or atrophy limited to corpus mucosa alone).
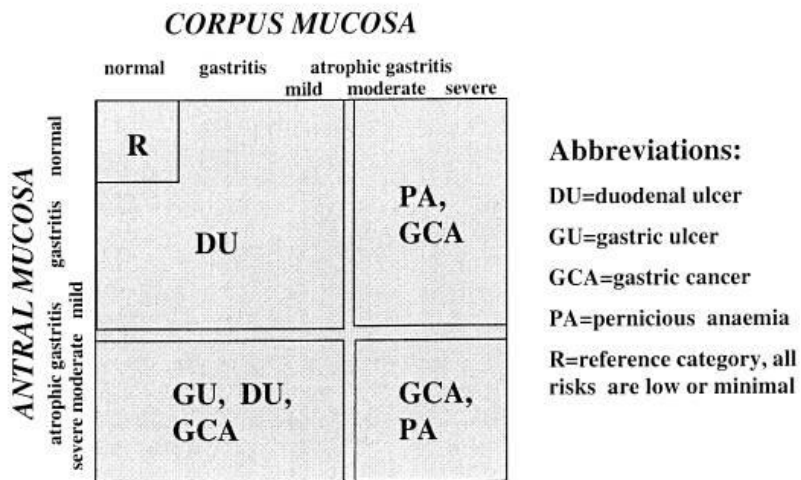*) Included only in GastroPanel.

ANNEX 5.

PHENOTYPING* OF GASTRITIS BY GastroPanel® TEST RESULTS

CORPUS MUCOSA

normal   gastritis   atrophic gastritis
mild moderate severe

ANTRAL MUCOSA

normal (gastritis)	
R, Hp + SPGI↑ G-17↑	Hp + or - SPGI↓ G-17↑
Hp + SPGI↑ G-17↓ or ↓↓ *	Hp + or - SPGI↓ G-17↓

Abbreviations:

Hp= *H.pylori*

SPGI=serum pepsinogen I; basal value

G-17= serum gastrin-17; basal or stimulated value

* G-17 does not response to stimuli

*Classification of gastritis by GastroSoft® is based on Updated Sydney Classification

The disease states and risks associated with different phenotypes of gastritis

CORPUS MUCOSA

normal   gastritis   atrophic gastritis
mild   moderate   severe

ANTRAL MUCOSA

R, DU	PA, GCA
GU, DU, GCA	GCA, PA

Abbreviations:

DU=duodenal ulcer
GU=gastric ulcer
GCA=gastric cancer
PA=pernicious anaemia
R=reference category, all risks are low or minimal

ANNEX 6.

BIOPSY PROTOCOL ACCORDING TO THE UPDATED SYDNEY SYSTEM

1. biopsy from antrum

2. biopsy from angulus (incisura)

3. biopsy between angulus and Z-line
   (incisura)

4. biopsy from antrum

5. biopsy in the middle of the main curvature

6. biopsy between body and fundus
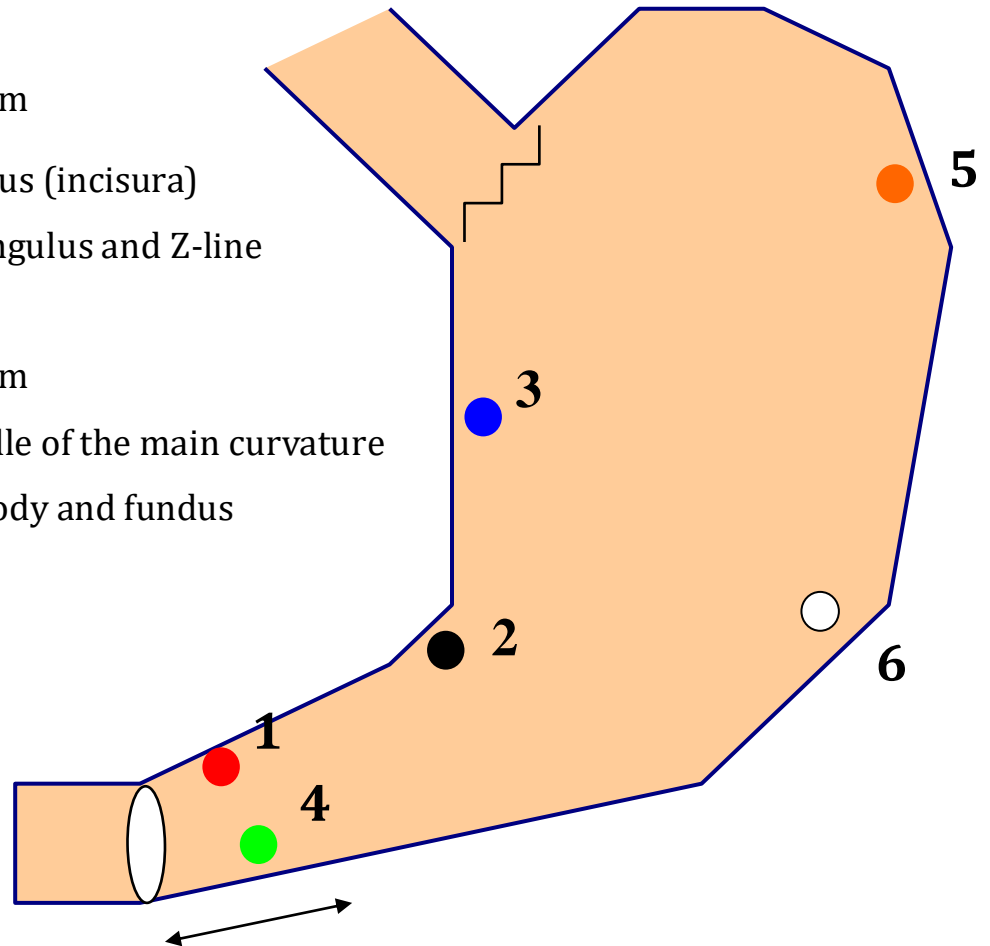
Routine biopsies are taken from the antrum **2 cm** least two biopsies from each. These biopsies are taken from the large and small curvatu... ...trum (biopsies 1 and 4) and from the large curvature of corpus (**biopsies 5 and 6**). In addition, two extra biopsies are taken from the incisura angularis (**biopsies 2 and 3**).

Biopsies from the antrum and incisura (biopsies 1, 2, 3, 4) are set into one and the same formalin bottle/tube (**tube No. 1**) and embedded into one and the same paraffin block. These can be labeled as "**antrum**". The biopsies from the corpus (No 5 and 6) set into one and the same formalin bottle/tube (**tube No. 2**) and embedded into one and same paraffin block. These can be labeled as "**corpus**".

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ANNEX 7.

HISTOPATHOLOGICAL CLASSIFICATION OF GASTRITIS by UPDATED SYDNEY SYSTEM
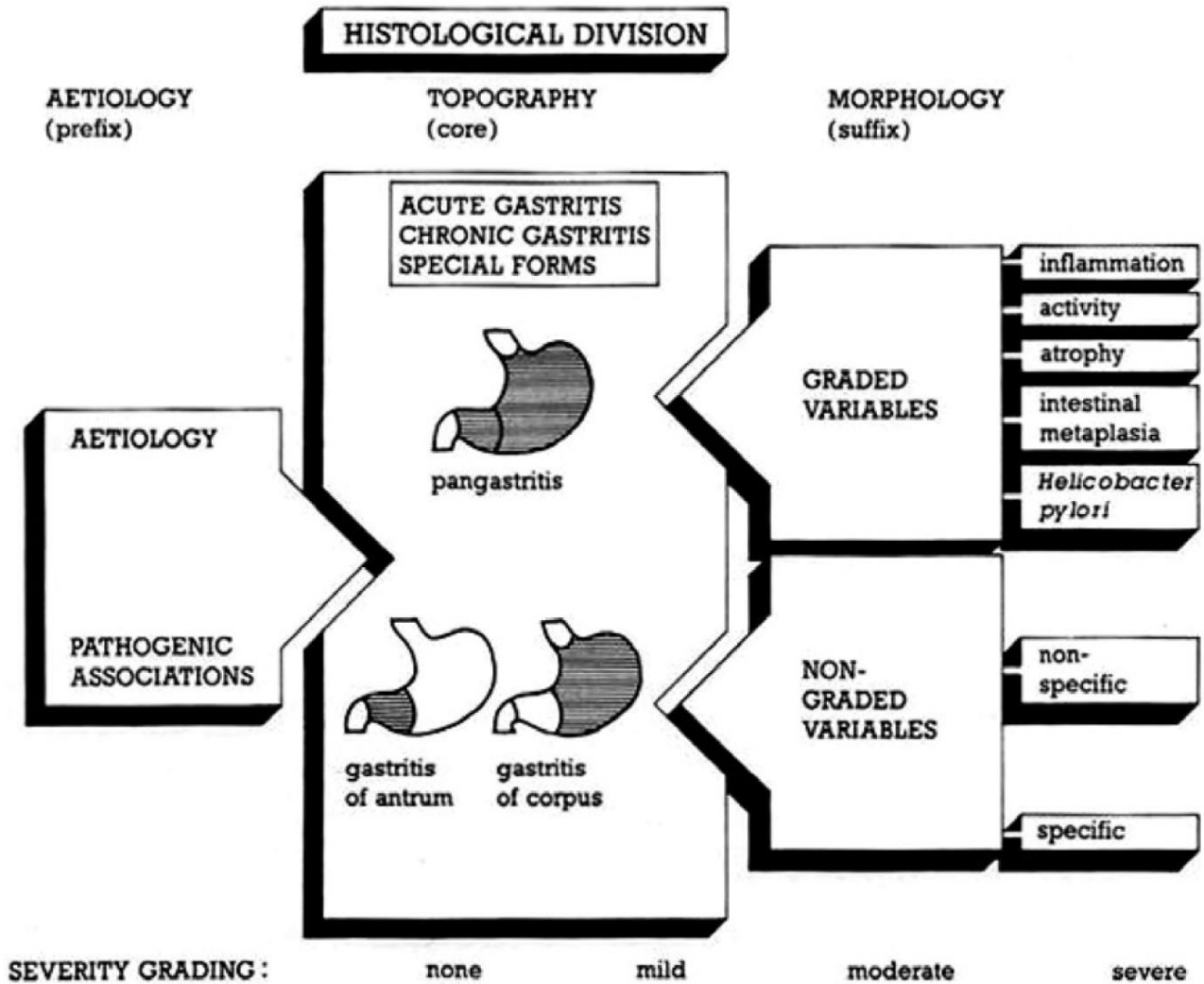
The chart designed for the histological division of the original Sydney System as presented to the Sydney World Congress of Gastroenterology in 1990, and published in the *Journal of Gastroenterology and Hepatology* in 1991. It incorporates etiology, topography and the morphological features to be documented when reading and reporting endoscopic gastric biopsies. The topography of gastritis is the core of the classification. Etiological hints can be added as a prefix and the graded variables as suffixes. Typical examples would be: "*H. pylori* pangastritis, severely active with mild panatrophy", "Autoimmune corpus gastritis with severe atrophy and intestinal metaplasia"; "Reactive mild antral gastritis; inactive; no *H. pylori*", etc.