

-CONFIDENTIAL-

Research Project

**Effect of Acetium® Capsules* in Restoration of the Structure
and Function of Gastric Mucosa After *H. pylori*
Eradication in Patients with Atrophic Gastritis.
A randomized, controlled trial.**

Executed by:

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SYNOPSIS

Background: It is estimated that 50% of all gastric cancer (GC) cases develop through the “Correa cascade”, progressing from *Helicobacter pylori* (HP)-associated gastritis to mucosal atrophy, intestinal metaplasia (IM), dysplasia, to invasive adenocarcinoma. The concept on atrophic gastritis (AG) and IM as precancerous conditions is based on long-term prospective cohort studies, demonstrating that the risk of GC is significantly increased among patients with AG, which is currently considered as the single most powerful independent risk factor of GC. Gastroscopy with biopsies is the time-honored method to diagnose and grade these gastric precancer lesions, recently re-classified by WHO as intraepithelial neoplasia (IEN), to circumvent the unsatisfactory inter-rater agreement of previous classifications. Recently an ELISA-based assay (GastroPanel®) was designed to measure the serum concentrations of four stomach-specific biomarkers: pepsinogen I (PGI) and II (PGII), gastrin-17 (G-17) and HP IgG antibodies (IgG-HP), making it the first non-invasive diagnostic tool for detection of the subjects at risk for GC, i.e., those with AG and/or HP.

According to traditional thinking, both AG and IM are irreversible conditions, which if not properly monitored, inevitably lead to invasive GC, despite eradication of HP. It appears that once IM has become established, HP-eradication, although retarding the progression of IM, cannot completely prevent gastric cancer. This is not necessarily true for AG, however, for which there appears to be a discrepancy between the corpus and antrum; eradication of HP-infection results in significant improvement in atrophy of the corpus but not in the antrum.

In strict contradict to this conventional thinking, an Italian group represents a fundamentally novel idea suggesting that the function of an atrophic corpus mucosa could be restored by a simple treatment with a natural amino acid, L-cysteine (Acetium® capsules, Biohit Oyj). In this study, GastroPanel® test (for PGI and G-17) was used to demonstrate a markedly improved function of an atrophic corpus mucosa after a 3-month treatment of HP-eradicated AG patients. Unfortunately, the failure to have a biopsy confirmation after treatment makes it impossible to determine, where this apparent normalization of the serum levels of these stomach-specific biomarkers is accompanied by concomitant recovery of the AG. Similarly, because of the lack of placebo control, there is no means to rule out the possibility that the observed effect is due to HP-eradication only. Because of major conceptual and clinical importance, this concept needs to be assessed in a flawless study design.

Because Acetium® capsules have no systemic effects, we assume that their therapeutic effect on atrophic gastric mucosa must be local, mediated by the established efficacy of L-cysteine to inactivate free acetaldehyde in the stomach, irrespective of its origin (alcohol, smoking or foodstuffs). This would implicate that continuous exposure to acetaldehyde of the gastric mucosa is a prerequisite for sustaining the “Correa cascade” of gastric carcinogenesis.

Objective: To validate the novel hypothesis that L-cysteine administered by daily intake of Acetium® capsules (**200 mg x 3 a day**, and additional 1 caps with meals and alcohol intake) is an effective remedy to i) improve the function of the gastric mucosa among AG patients who have undergone eradication of *H. pylori* infection, and to ii) induce concomitant repair of the atrophic mucosa.

Study design: A double-blind, randomized placebo-controlled clinical trial (RCT) comparing Acetium® capsules (2 capsules 3 times a day, and additional ones) and placebo i) in restoring the physiological functions of stomach mucosa, and ii) to demonstrate whether such an eventual functional improvement is accompanied by concomitant recovery of the atrophic mucosa. Potentially

eligible AG patients are screened among the consecutive gastroscopy-referral patients by GastroPanel® test. All eligible patients are **incident cases** of **biopsy-confirmed AG** (in corpus, antrum or both), graded as **moderate** or **severe** in degree. All patients are tested *H. pylori* positive with GastroPanel® test, and active infection confirmed in biopsies. After **successful HP-eradication**, eligible patients (n=120) are randomized (1:1) to two study arms, receiving Acetium® (200 mg 3 x day, and additional 1 with meals and alcohol) and Placebo (2 capsules 3 x day) for **6 months**. After the 6-month trial period, the randomization code is opened, and all 120 patients will be administered daily Acetium® (the same dosage), followed-up for **two years** by repeated testing with GastroPanel® test (including stimulated G-17) at 6-month intervals. At study conclusion at **24 months**, all patients are subjected to gastroscopy and biopsies. In the final analysis, these follow-up biopsies are mixed with the baseline biopsies and read blinded by two expert pathologists, using the OLGA classification of gastritis. Before enrolment, all subjects are requested to sign a written consent.

Methods: GastroPanel® test in the initial screening and all follow-up visits will be performed for all four biomarkers, according to instructions. In classifying the phenotypes of gastritis in gastroscopy biopsies, the USS is used. Eradication of *H. pylori* infection and control of its efficacy is necessary. The study setting is “triple-blind” (participant-blind, investigator-blind, statistician blind). Placebo preparation with design and package identical to the test preparation will be used. Parallel group design instead of cross-over design is used. Randomization will be performed using a random number generator, with block size of 4, and stratified by severity, location and extent of their AG.

In statistical analysis, both conventional techniques (e.g. non-parametric paired-samples and non-paired samples t-test), and more sophisticated methods will be used. The **power of the study** is calculated grounding the effect size estimates on the reported increase of PGI marker levels after Acetium treatment.^{62,63} Using the two-sample mean test for paired samples, this study would be adequately powered (Type II error 0.80, type I error 0.05) to detect a true difference in PGI increase of the reported magnitude (3.5 µg/L), if there are 55 patients in the Acetium study arm. With the cohort of **120 subjects**, randomized (1:1) into Acetium and placebo arms, this study is adequately powered to detect a true **difference in effect** estimates as follows: **3.5 µg/L (effect of Acetium)** and **2.15 µg/L (effect of Placebo)**. Even a slightly smaller effect in the placebo arm would increase the study power close to 100%, and allow much wider SD.

Specific aims: The present study is designed to validate or invalidate the null hypothesis implicating that the intake of Acetium® capsules is no better than placebo in restoring the physiological functions of stomach mucosa, and there is no evidence whatsoever on the recovery of gastric atrophy (AG) as a specific result of this medication. Rejection or not of the null hypothesis is based on comparison of the two strata (Acetium and placebo) against **two primary study endpoints** (efficacy measures): 1) Changes in the serum levels of the relevant stomach-specific biomarkers (GastroPanel® test), from the baseline values of AG, towards the values (or falling) within the normal range; and 2) Biopsy-confirmed recovery of atrophic gastric mucosa by at least one histological (USS) grade, based on a blinded reading of the baseline and follow-up biopsies.

Study execution and time-table: This RCT is not an easy design to set up and sustain. Moderate and severe AG are uncommon conditions representing a small minority of patients even among consecutive gastroscopy referrals. HP-eradication is not always successful, and that might result in exclusion of some of the otherwise potentially eligible patients. The study should be ideally conducted as a joint project between 3 clinics. The **6-month treatment** period (randomized) is

followed by **2-year follow-up** with 6-month control visits to monitor with the GastroPanel® testing, and the final control by gastroscopy and biopsies. This lengthy period of monitoring may cause potential challenges for the compliance of the patients, and needs special attention not to compromise the power of the study. Due to these uncertainties, it is not possible to estimate the accurate time-table of the study execution. At this stage, the best estimates suggest that completion of the whole study protocol for the cohort of 120 subjects cannot be done in less than three years. However, after the initial 6-month period with randomized trial, we will have the preliminary results that will enable us to compare the Acetium and Placebo arms with regard to the first study endpoint (mucosal function assessed by the biomarkers). The estimation of the second study endpoint is possible only after conclusion of the 2-year follow-up.

Impact of the study: Given that GC precursor lesions (AG, IM) are traditionally considered as irreversible conditions which, at best, can only be halted but not reverted by an early HP-eradication, the concept on restoring the mucosal functionality and recovery of the atrophic mucosa by a simple treatment with L-cysteine is ground-breaking, both conceptually and clinically. L-cysteine, a natural (semi-essential) amino acid, converted to inert substance (MTCA) in the alimentary tract, would comprise an ideal means to conduct long-term treatment for years, without concern about systemic side effects. If the claimed efficacy is proven in this formal RCT, the practice of using Acetium capsules in the treatment of AG would represent a major step forward in the prophylaxis of GC, making it one among few human malignancies that are preventable.

1.BACKGROUND

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).

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.

1.1.Gastric carcinogenesis

Based on extensive documentation by a large number of cohort studies conducted during the past several decades, a relatively clear picture has emerged concerning the stepwise development of invasive gastric cancer.¹⁻²⁴ This increased understanding of the pathogenesis of GC has also prompted the scientific community to develop international recommendations for the management of the precancerous conditions of the stomach.²⁵ These management guidelines are based on three key statements, of which a relatively good consensus prevails among the leading gastroenterology experts in Europe.²⁵

1) Patients with chronic atrophic gastritis (AG) or intestinal metaplasia (IM) should be considered to be at higher risk for gastric adenocarcinoma; 2) High grade dysplasia and invasive carcinoma should be regarded as preventable outcomes, when the patients with chronic AG or IM are adequately managed; and 3) Patients with endoscopically detectable high grade dysplasia or carcinoma should undergo staging and adequate management.²⁵

In most instances, the development of so-called “intestinal” gastric adenocarcinoma represents the culmination of an inflammation–metaplasia–dysplasia–carcinoma sequence, known as the Correa cascade of multistep gastric carcinogenesis,^{16,19-21} where a progression may occur from normal mucosa through chronic non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia, to dysplasia, and finally to carcinoma, as has been consistently confirmed in different studies.^{26,27}

1.1.1. Precancerous conditions of the gastric mucosa

As already stated, gastric mucosal atrophy (AG) and IM confer a high risk for the development of GC as they constitute the background in which dysplasia and intestinal-type gastric adenocarcinoma develop.²⁸⁻³¹ Thus, chronic AG and IM are considered to be precancerous conditions.²⁵ Chronic AG should be diagnosed and graded on the basis of the presence of chronic inflammatory cells, including lymphocytes and plasma cells that infiltrate the connective tissue stroma, associated with the disappearance of the normal glands.^{8,14,32-34} The severity of gland loss (atrophy) should be graded, as mild, moderate or severe, although admittedly, the inter- and intra-observer agreement are not satisfactory.²⁵

Individuals may develop different phenotypes of chronic gastritis due to different genetic profiles and environmental exposure. Cases of inflammatory changes limited to the antrum and without gland atrophy and/or IM are defined as diffuse antrum gastritis.^{8,14} In contrast, cases with gland atrophy and/or IM distributed multi-focally (including the lesser curvature of the corpus and fundus), are best defined as multifocal AG. When associated with AG of the antrum, the condition is called atrophic pangastritis.⁸

Thus, the overall background changes in the stomach should be described in terms of the i) severity and ii) distribution of any premalignant conditions/lesions. Several classification schemes have been developed for chronic gastritis and pre-neoplastic changes. At present, the updated Sydney System (USS) is widely used both in clinical practice and in research, combining topographic, morphological, and etiological information in reporting systems designed to include both grading and staging of gastritis.^{35,36} More recently, the systems known as OLGA (operative link for gastritis assessment), and OLGIM (operative link on gastric intestinal metaplasia) assessment have been proposed for staging of gastritis.³⁷ Meritorious as they are, both classifications suffer from an unsatisfactory reproducibility measured as inter- and intra-observer variation.

1.1.2. Precancerous lesions of the gastric mucosa

Gastric dysplasia represents the penultimate stage in gastric carcinogenesis,^{16,19-21,38} defined as histologically unequivocal neoplastic epithelium without evidence of tissue invasion, i.e., a full-blown neoplastic precancerous lesion. It is characterized by cellular atypia reflective of abnormal differentiation, and disorganized glandular architecture.³⁹⁻⁴¹ Both the correct diagnosis and grading of dysplasia are critical, because they predict both the risk of malignant transformation and the risk of metachronous GC. Importantly, the reported progression rates of dysplasia to GC vary within a wide range, from 0% to 73% per year.^{8,14,25,42,43} Undoubtedly, these wide variations reflect the diverse factors, including differences in study design and populations under study and also differences in definitions and assessment of gastric dysplasia.²⁵

There are well-known differences between Japanese and European/North American pathologists in categorizing gastric dysplasia.^{25,35,36} While in Japan, non-invasive intra-mucosal neoplastic lesions with high grade cellular and architectural atypia are termed “non-invasive intra-mucosal carcinoma,” the same lesions are diagnosed as high grade dysplasia by most pathologists in the West.^{44,45} In an attempt to resolve this issue, the World Health Organization (WHO) recently launched its new classification of gastric precancer lesions.⁴⁶ In this new WHO classification of dysplasia/intraepithelial neoplasia, the widespread use of both dysplasia and **intraepithelial neoplasia (IEN)** is acknowledged, and these terms are used as synonymous. According to the current WHO classification, the following diagnostic categories should be recognized:

1. Negative for intraepithelial neoplasia/dysplasia
2. Indefinite for intraepithelial neoplasia/dysplasia
3. Low grade intraepithelial neoplasia/dysplasia
4. High grade intraepithelial neoplasia/dysplasia
5. Intra-mucosal invasive neoplasia/intra-mucosal carcinoma

In WHO classification, category 1 (negative for intraepithelial neoplasia/dysplasia), includes two important precancerous conditions (chronic AG and IM)(see 1.1.1). Whenever there is any doubt, whether a lesion is neoplastic or non-neoplastic (i.e., reactive or regenerative), particularly in small biopsies exhibiting inflammation, the WHO category 2 (indefinite for intraepithelial

neoplasia/dysplasia) should be used. In such cases, the issue can be usually solved by cutting more sections, by obtaining additional biopsies, or after controlling for possible etiologies.²⁵

Intraepithelial neoplasia/dysplasia comprises unequivocally neoplastic epithelial proliferations, characterized by variable cellular and architectural atypia, but without convincing evidence of invasion. Low-grade intraepithelial neoplasia/dysplasia shows minimal architectural disarray and only mild-to-moderate cytological atypia. In contrast, high-grade intraepithelial neoplasia/dysplasia encompasses neoplastic cells that depict a high nuclear/cytoplasm ratio, prominent nucleoli, more pronounced architectural disarray, and numerous mitoses, many of which are atypical. As repeatedly stated, most patients with high-grade dysplasia lesions are at high risk for either synchronous invasive GC or its rapid development.^{8,14,25,42,43}

Intra-mucosal invasive neoplasia/intra-mucosal carcinoma defines carcinomas that invade the lamina propria and are distinguished from intraepithelial neoplasia/dysplasia not only by desmoplastic changes that can be minimal or absent, but also by distinct structural anomalies, such as marked glandular crowding, excessive branching, budding, and fused or cribriforming glands.²⁵ The diagnosis of intra-mucosal carcinoma (also called carcinoma in situ) indicates that there is an increased risk of lymphatic invasion and lymph-node metastasis. However, endoscopic techniques allow treatment without open surgery, particularly for lesions ≤ 2 cm in size and for those that are well differentiated with no lymphatic invasion.²⁵

1.1.3. Atrophic gastritis and intestinal metaplasia - precursors of IEN/dysplasia

It is estimated that 50% of all GC cases develop through the "Correa cascade",^{16,19,20,21} leading from HP-associated gastritis to mucosal atrophy, intestinal metaplasia, dysplasia, to invasive adenocarcinoma. The concept of classifying AG and IM as precancerous conditions of the gastric mucosa is based on well documented data from long-term prospective cohort studies, demonstrating that the risk of GC is significantly increased among patients with AG, subjected to long enough follow-up.^{1-24,28-31} Indeed, based on this convincing evidence, AG is currently considered as the single most powerful independent risk factor for distal (non-cardia) GC.^{8,16,17,18} Because this process takes several decades, there should be good prospects for early detection of precancerous lesions,²² but the problem is a lack of a suitable test for GC screening.²³ 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.²⁴

In the pathway towards GC,^{16,19-21} the next step following AG (and frequently accompanying it) is the condition known as intestinal metaplasia (IM). Similar as with gastritis, different phenotypes have recently been recognized also for IM. Traditionally, IM has been classified as “complete” or “incomplete.” Complete intestinal metaplasia (“small-intestinal” or type I) displays goblet and absorptive cells, decreased expression of gastric mucins (MUC1, MUC5AC, and MUC6), and expression of MUC2 (an intestinal mucin). Incomplete intestinal metaplasia (“enterocolic” or type IIA/II, and “colonic” or type IIB/III), displays goblet and columnar non-absorptive cells, in which gastric mucins (MUC1, MUC5AC, and MUC6) are co-expressed with MUC2.

Recently, another pattern of metaplasia, termed spasmolytic polypeptide-expressing metaplasia (SPEM), has been described.⁴⁷ This is characterized by the expression of the TFF2 spasmolytic polypeptide that is associated with oxyntic atrophy. SPEM, which characteristically develops in the gastric body and fundus, appears to share some characteristics with pseudopyloric metaplasia, has a strong association with chronic *H. pylori* infection and gastric adenocarcinoma, and may represent another pathway to gastric neoplasia. At present, however, identification of SPEM is not yet among the diagnostic routine of gastric biopsies.

1.1.4. Non-invasive diagnosis of gastric precancer lesions

There is no disagreement that biopsy specimens of the stomach are essential to the establishment and grading of gastric precancer lesions.²⁵ The updated Sydney System (USS) is the most widely accepted classification and grading of gastritis.^{35,36} The system was primarily designed to provide standardization for reporting of gastric biopsies. The updated version recommended five biopsies, two from the antrum (3cm from the pylorus, greater and lesser curvatures), one from the incisura, and two from the corpus (one from the lesser curvature, 4cm proximal to the incisura, and one from the middle of the greater curvature). Although this biopsy protocol generally correctly establishes *H. pylori* status and chronic gastritis, the number of biopsies is controversial with regard to adequate staging of premalignant gastric lesions, mainly because of the multifocal nature of these lesions.^{48,49} This multifocal nature affects their detectability, in turn affecting decisions regarding the patient's therapy or future surveillance.^{19,21}

To obviate the excessive use of this invasive and expensive procedure (endoscopy), there has been 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.^{50,51} For this purpose, a Finnish biotechnology company (Biohit Oyj) recently launched an ELISA-based assay designed to measure the concentrations of four key stomach-specific biomarkers from a single blood sample. The test is known as GastroPanel®, combining serum pepsinogen I (PGI) and II (PGII), gastrin-17 (G-17) and HP IgG antibodies (IgG-HP). This test is proposed as the first-line diagnostic test for dyspeptic symptoms,⁵²⁻⁵⁴ capable of accurately diagnosing the phenotypes of gastritis, including AG in both the corpus and antrum. Thus, GastroPanel® examination is the first non-invasive diagnostic tool providing possibilities for detecting the patients at risk for GC and peptic ulcer diseases as well as the AG-accompanying conditions (malabsorption of vitamin B12, iron, magnesium, calcium and some drugs).⁵⁴ 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.⁵⁵ Consequently, these stomach-specific biomarkers are recommended by international experts for diagnosis and screening of AG.⁵⁶

1.1.5. AG and IM - are these conditions always irreversible?

Whether gastric carcinogenesis progressing through the stepwise manner from HP-infection, through AG and IM to invasive GC, once on-going, can be arrested or even reverted, is a contradictory subject.⁷ This issue is of crucial importance from the clinical point of view, because according to our current thinking, both AG and IM are irreversible conditions once established, and without adequate monitoring, inevitably lead to invasive GC.^{16,19-21}

However, there are some recent implications that an early eradication of HP-infection can slow down or even revert this cascade,^{7,13} suggesting that HP eradication has the potential to prevent gastric cancers.⁵⁷ In a recent study on the effect of HP eradication on patients with premalignant lesions, it was shown that eradication may prevent their progression.⁵⁸ It is thought that a so-called 'point of no return' may exist in the histological cascade from chronic gastritis to adenocarcinoma after which eradication is unlikely to prevent GC.⁷ It appears that once IM has become established, eradication, although retarding the progression of IM, cannot completely prevent gastric cancer.^{59,60} This is not necessarily true for gastric atrophy, however, for which there appears to be a discrepancy between

the effect of eradication in the corpus and in the antrum. Thus, according to a recent meta-analysis of 12 studies comprising 2.658 patients, eradication of HP-infection results in significant improvement in atrophy of the corpus but not in the antrum, and, importantly, has no effect on IM of the gastric mucosa.⁶¹

2.STUDY HYPOTHESIS

2.1.Atrophic gastritis might be reversible by simple treatment

Given the considerations in section 1.1.5., the recent two reports from an Italian group represent a fundamentally novel idea, while suggesting that the function of the gastric mucosa among (HP eradicated) AG patients could be recovered by a simple treatment with a natural (semi-essential) amino acid, L-cysteine (Acetium® capsules, Biohit Oyj, Helsinki, Finland).^{62,63} In their two separate reports, these authors used the GastroPanel® test to demonstrate a markedly improved function of the gastric mucosa during the follow-up of AG patients after treatment with Acetium capsules.

Thus, these authors assessed the alteration in gastric function after *H. pylori* eradication on moderate-severe body atrophic gastritis by determination of PGI levels and Gastrin 17 (G-17). A series of 74 dyspeptic patients, selected from 738 consecutive *H. pylori* positive patients, with histological features of moderate-severe AG of the corpus, underwent an upper gastrointestinal endoscopy with gastric biopsies and PGI and G-17 determination at baseline. All patients underwent HP-eradication therapy, and serum PGI and G-17 were measured again after 6 months, and at 1, 2, 3, 5 and 6 yrs after eradication therapy. In the first report, they had data on **5 patients** (4 female, mean age 56, range 49-66 yrs) out of the 74 AG patients, who underwent a 3-month treatment with Acetium 100 mg (3 capsules daily) before having a meal. In these subjects, the mean levels of sPGI increased from 5,3 µg/L at baseline to 7,8 µg/L after 90 days of Acetium intake, meanwhile sG-17 remained unchanged (74,6 and 79,8 pmol /L).⁶² Authors concluded that after *H. pylori* eradication, these subjects AG of the corpus showed long-lasting improvement of physiological gastric functions, reflected by significantly and stabile increasing of PGI levels.

In their second report, they had similar 3-month follow-up data from 21/74 of these AG patients, after 3 months of treatment with Acetium capsules, but 6-year follow-up data for all 74 subjects having undergone HP-eradication.⁶³ Mean PGI levels prior to HP-eradication were 13,4µg/L, but increased already at 6 months to 16,6 µg/L (p=0.05), and further to 27,3 µg/L at the completion of

the study 6 years later ($p=0.01$). Conversely, the G-17 dropped from 84,8 at baseline to 67,6pmol/L after the 6-year follow up period ($p<0.01$). Among the **21 patients** who received treatment with Acetium, the mean levels of PGI increased from 7,9 $\mu\text{g/L}$ at baseline to 11,4 $\mu\text{g/L}$ after 90 days of Acetium intake, with concomitant drop of G-17 from 30,3 to 25,5pmol/L.⁶³ These data clearly implicate that HP-eradication among patients with AG of the corpus results in a long-lasting improvement of the gastric mucosal functions as measured by the GastroPanel® test, with significant and stable increase of PGI levels and a parallel decreased of G-17 over a 6-year follow-up period.^{62,63}

Unfortunately, the study design has some inherent weaknesses that preclude making reliable conclusions about **the true effect of L-cysteine** in the recovery of gastric mucosal function. Most importantly, the failure to have a biopsy confirmation of the gastric mucosal structure after the 3-month L-cysteine intervention (or at study conclusion), makes it impossible to determine, where this apparent normalization of the serum levels of these stomach-specific biomarkers⁵²⁻⁵⁴ is, indeed, associated with concomitant recovery of the AG as well. Similarly, because of **the lack of placebo** control, there are no means to rule out the possibility that the observed effect is due to HP-eradication only. **Because of major conceptual and clinical importance, this concept needs to be assessed in a flawless study design, as described here.**

2.2.Components of study hypothesis

The striking novel hypothesis of this study is simply the following: **Prolonged L-cysteine administered by daily intake of Acetium® capsules (200 mg x 3 a day) is an effective remedy to i) improve the function of the gastric mucosa among AG patients who have undergone eradication of *H.pylori* infection, and to ii) induce concomitant recovery (reversal) of atrophic gastric mucosa in these subjects.**

As described in the subjacent sections, Acetium® capsules (and lozenges) were designed for inactivation of acetaldehyde in the stomach and in the saliva after alcohol intake and smoking, respectively. This novel concept on the potential therapeutic effect of regular L-cysteine intake on both the structure and function of gastric mucosa among subjects with AG, by eliminating acetaldehyde in the stomach contents, would be a definite **DIRECT proof of the concept** that **acetaldehyde is causally associated** with the development of GC.

2.2.1.L-cysteine eliminates acetaldehyde in the stomach

Cysteine is a non-essential amino acid, which was shown (almost 40 years ago) to be capable of eliminating the toxicity of acetaldehyde by reacting covalently with it to form a stable 2-methylthiazolidine-4-carboxylic acid (MTCA).⁶⁴ MTCA is an inert and non-toxic compound that is eliminated from the body through feces and urine, without being absorbed into the blood circulation. This simple principle was used in the recent innovation of Biohit Acetium® capsule, which contains 100mg L-cysteine.

In the proof-of-concept study, oral administration of Acetium was confirmed to effectively bind acetaldehyde originated from ethanol metabolism in achlorhydric stomach.⁶⁵ In that setting, the mean acetaldehyde level of gastric juice was 2.6 times higher with placebo than with L-cysteine (13 vs. 4.7 μ M, $p < 0.05$), implicating that L-cysteine can be used to decrease acetaldehyde concentration in non-acidic stomach during alcohol exposure.

This led the authors to examine the concept, whether it would be possible to eliminate alcohol-derived acetaldehyde also from the saliva, using L-cysteine slowly released from a special buccal (Acetium) tablet.⁶⁶ Indeed, this was shown to be the case in volunteers, in whom, up to two-thirds of acetaldehyde (after alcohol intake) could be removed from the saliva with a slow-releasing buccal L-cysteine formulation. This might have important implications e.g. in prevention of upper GI-tract cancers among individuals with high acetaldehyde exposure (heavy drinkers, smokers).⁶⁶

As the logical next step, Biohit Oyj also developed an Acetium sucking tablet (lozenge) that releases L-cysteine into the oral cavity during smoking, and tested this formulation as a potential chemopreventive agent against toxicity of tobacco smoke, i.e., in harm reduction.⁶⁷ Seven volunteers smoked five cigarettes, and during every smoking period, sucked a blinded tablet containing 0, 1.25, 2.5, 5, or 10 mg of L-cysteine, followed by acetaldehyde analysis of the saliva at 0, 5, and 10 minutes from the beginning of the smoking. L-cysteine reduced highly significantly the salivary acetaldehyde. In fact, carcinogenic acetaldehyde could be totally inactivated in the saliva during smoking by the sucking tablet containing 5 mg of L-cysteine.⁶⁷

2.2.2.Acetaldehyde: Group 1 carcinogen (IARC)

Tobacco smoke contains several classes of carcinogens that include among others polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines. Tobacco smoke contains also high

concentrations of toxic aldehydes,⁶⁸ of which the most abundant is acetaldehyde, its concentrations in tobacco smoke being >1,000 times greater than those of polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines.⁶⁹ Acetaldehyde is also the first metabolite of ethanol oxidation. It binds to DNA, forming stable DNA adducts that are observed in alcohol consumers. Numerous epidemiological studies among alcohol drinkers who have alcohol dehydrogenase (ADH2) deficiency or low aldehyde dehydrogenase (ALDH1B) activity provide the most compelling evidence for the carcinogenicity of acetaldehyde.⁷⁰ This deficiency results in accumulation of acetaldehyde locally in the saliva during ethanol metabolism and is a markedly increased risk for many upper gastrointestinal tract cancers.

Similarly, it was recently shown that acetaldehyde from the tobacco smoke is easily dissolved into the saliva during smoking.⁷¹ Thus, toxic aldehydes could mediate the carcinogenic effect of tobacco smoke through saliva to oral cavity and further down to the larynx, esophagus, and stomach. Based on firm epidemiological and toxicological documentation, IARC proclaimed (in 2009) acetaldehyde as Group I carcinogen, equivalent to asbestos, formaldehyde and others.⁷²

2.2.3.L-cysteine interferes with the “Correa cascade” by eliminating acetaldehyde in atrophic stomach

Acetium® capsule is classified as a medical device, and is not a medicine, because of its inherent design; L-cysteine in the capsule is (slowly) released with regulated speed inside the stomach, free L-cysteine reacts with acetaldehyde in the gastric contents, and is NOT absorbed from the duodenum. This makes the crucial difference to L-cysteine obtained from the foodstuffs as a natural (semi-essential) amino acid, which is liberated only in the duodenum (by pancreatic enzymes) and readily absorbed into blood circulation. This precludes the possibility of Acetium® capsules having any systemic effects, which is a prerequisite to be classified as a medicine.

At the same time, this fact also restricts the framework of the new hypothesis to be tested in this study, while excluding the assumptions that the eventual therapeutic effect of Acetium® capsules on atrophic stomach mucosa and restoration of its functionality could be mediated by some systemic effects. This leaves us less room for building up complex pathways on these mechanisms. Instead, we assume that **the therapeutic effects** (if established) of Acetium® capsules on atrophic stomach mucosa **must be entirely local**, in other words mediated by the established efficacy of L-cysteine

to inactivate free acetaldehyde in the stomach, irrespective of its origin (alcohol, smoking or foodstuffs). Given this would necessitate that continuous exposure to acetaldehyde of the gastric mucosa is a prerequisite for sustaining the so called "Correa cascade" in the stomach,^{16,19-21,38,58} Once initiated by the infection of *H. pylori*, this cascade would continue through the progressing steps of AG and IM to dysplasia (IEN) and GC, unless interrupted by close monitoring of the precursor lesions.

As discussed in section 1.1.5., there is also a chance that an early eradication of HP-infection can slow down or even revert this cascade,^{7,13} suggesting that HP-eradication has the potential to prevent gastric cancers.⁵⁷⁻⁶¹ Whether this interruption of the Correa cascade is also associated with marked improvement of the gastric mucosal atrophy, remains a controversial issue.⁶¹ At the current level of the presented evidence suggesting that such an effect would be mediated by L-cysteine,^{62,63} however, we are unable to exclude the possibility that i) the suggested improvement in gastric mucosal functions after L-cysteine intake are, in fact, merely due to HP-eradication therapy, and ii) that this eventual improvement in physiological functions is not necessarily accompanied by any significant improvement in mucosal morphology, i.e., recovery of severely atrophic mucosa towards less severe disease or even restoration of morphologically normal gastric mucosa.

The present study is designed to test the null hypothesis implicating that the intake of Acetium® capsules (2 capsules 3 times a day, and additional one with meals and alcohol) is no better than placebo in restoring the physiological functions of stomach mucosa, and there is no evidence on the recovery of gastric atrophy (AG) as a specific result of this intervention.

3.STUDY DESIGN

This double-blind, placebo-controlled randomized trial (RCT) is designed i) to test the claimed efficacy of intervention by Acetium capsules (three times a day, for 3 months) in restoring the physiological functions of stomach mucosa, and ii) to demonstrate whether such an eventual functional improvement is accompanied by concomitant recovery of the atrophic mucosa, confirmed by gastric biopsies before and after intervention.

To avoid the flaws inherent to the published two studies,^{62,63} great care has been taken in the study design, with special emphasis on the following issues. 1) A flawless study design necessitates a double-blind, placebo-controlled randomized trial (RCT); 2) A cohort must be adequately powered

to demonstrate minor differences between the two study arms; 3) All eligible patients are incident cases of biopsy-confirmed AG (in corpus, antrum or both), graded as moderate or severe in degree; 4) All patients must be tested *H. pylori* positive with GastroPanel® test, and an ongoing infection confirmed in biopsies and/or *H. pylori* quick test (HPQT); 5) All subjects must undergo *H. pylori* eradication, controlled for efficacy following the European guidelines;⁷ 6) Patients are randomized to two study arms, receiving Acetium (100 mg three times a day) and Placebo (capsule 3 times a day) for 3 months; 7) Patients are followed-up for at least one year by repeated testing with GastroPanel (at 3-month intervals; including stimulated G-17) and pentagastrin stimulation; 8) At study conclusion after one year follow-up, all patients are subjected to gastroscopy and biopsies; 9) Follow-up biopsies are mixed with the baseline biopsies and read blinded by two expert pathologists, using the USS for classification of gastritis.

3.1. Aims of the study

The single most important goal of this study is to establish whether Acetium® capsule is an effective treatment **in restoring both the structure and function** of the gastric mucosa among patients with AG, who underwent eradication of *H. pylori* infection. The null hypothesis of the study implicates that the intake of Acetium® capsules is no better than placebo in restoring the physiological functions of stomach mucosa, and there is no evidence on the recovery of gastric atrophy (AG) as a specific result of this medication.

Rejection or not of the null hypothesis is based on comparison of the two strata (Acetium and placebo) against **two primary study endpoints** (efficacy measures): 1) Changes in the serum levels of the relevant stomach-specific biomarkers (GastroPanel test) from the baseline values consistent with AG, towards the values (or falling) within the normal range; and 2) Biopsy-confirmed recovery of atrophic gastric mucosa by at least one histological (USS) grade (e.g. from severe to moderate AG; moderate to mild AG), based on a blinded reading of the baseline and follow-up biopsies.

In addition to these primary efficacy endpoints, **secondary endpoint** in this study includes the calculation of the performance indicators (sensitivity, specificity, negative- and positive predictive value, AUC) for the GastroPanel® test, separately for the different histological endpoints: AG in the antrum, AG in the corpus, and atrophic pangastritis. In case that enough cases of intestinal metaplasia

(IM) and dysplasia (IEN) will be included, these indicators can be calculated also for these two conditions, although not specifically diagnosed by GastroPanel®.

3.2. Patient selection

This intervention trial is designed and conducted in conformity with the current European guidelines of both management of *H. pylori* infections⁷ and those of management of gastric cancer precursor lesions,²⁵ by Biohit Oyj (Helsinki, Finland) and X Hospital (Y City, Z country). The study is supervised by a steering committee consisting of the members of the Company's Scientific Advisory Board and representatives of the partner clinic. These two guidelines give detailed recommendations for patient selection (diagnosis), trial design, as well as evaluation of the results, to be described in the following.

3.2.1. Definition of atrophic gastritis

Enrolment of the patients in the study will take place at X Hospital. Only patients who have a biopsy-confirmed **AG** of the corpus or antrum or both will be enrolled, classified as **moderate or severe**, using the Updated Sydney System (USS) for classification of gastritis.^{35,36} To be eligible, the patient must give a written consent before enrollment in the cohort. The FlowChart of the patient enrolment in the cohort is illustrated in **ANNEX 1**.

3.2.2. Duration of disease

Only the patients who are **incident cases of AG** will be eligible. This is because of the strictly defined criteria of diagnosis and the initial management procedures of the patients before randomization into the two study arms, which are impossible to standardize in a cohort of prevalent AG patients.

3.2.3. Age at study entry

In principle, there is no specific age limit for the subject to be eligible for this study. Given that AG is a rare condition before age 45-50 years, it is anticipated that the vast majority of the subjects to be enrolled will be **above 50 years** of age.

3.2.4. Gender

Both male and female participants will be eligible in the cohort. In this study, every effort is done to minimize any gender selection bias by encouraging both women and men to participate, except those (obviously rare) women who are pregnant, and excluded due to this reason.

3.2.5. Concomitant medication

Because Acetium capsules have no known interactions with other drugs, the subjects are generally allowed to continue their regular medication for ailments not related to AG. However, other (eventual) medication targeted to AG should be discontinued prior to the study entry.

Excluded are the following subjects: patients who meet the criteria for medication overuse; patients who have taken anti-psychotics or anti-depressant medications during the previous month; patients who abuse alcohol or other drugs; and potentially fertile and sexually active women who do not practice contraception.

3.2.6. Co-morbidity

The subjects will be enrolled among the patients who are referred for gastroscopy due to specific symptoms or with other indications (e.g. risk-group subjects), which means that eligible patients can be either symptomatic or asymptomatic. The intention is to enroll a cohort of subjects with minimum co-morbidity. Specific co-morbid medical conditions that exclude participation in this trial include the following categories of patients: severe psychiatric disease, infections, malignancy, short life expectancy, cardiovascular disease, cerebrovascular disease, uncontrolled hypertension, degenerative central nervous system diseases, as well as pregnant and lactating women. In addition, the following patients should be considered **non-eligible**: 1) the patients whose stomach condition requires surgical treatment, or immediate follow-up treatment for major symptoms, as well as 2) those who refuse to sign the written consent.

3.3. Trial design

In the design of this RCT, the recommendations discussed in the two consensus guidelines^{7,25} for gastric precancer conditions and management of *H. pylori* infections have been carefully considered as far as pertinent to the design of this RCT.

3.3.1. Pre-trial period

As explained in Section 3 (ANNEX 1), special measures must be taken before randomization of the study subjects into the two treatment arms (Acetium and placebo). All patients represent **incident cases of AG**, are testing *H. pylori* positive in GastroPanel test (serology), and their ongoing HP-

infections must be confirmed by biopsies and/or other HP tests. **Gastroscopy and biopsies** are needed for confirmation of the diagnosis of **moderate to severe AG** (the eligibility criteria). ***H. pylori*** infection shall be **eradicated** using the recommended standard treatment protocols.⁷ Importantly, the efficacy of the *H. pylori* eradication therapy must be controlled, using any of the tests that accurately measure ongoing (active) HP-infection. For this purpose, GastroPanel® test is not an adequate means, because serum antibodies may persist for several months (up to years) after successful eradication of *H. pylori*. To make the cohort as homogeneous as possible, only those patients should be enrolled in whom an ongoing HP-infection has been successfully eradicated as established by an adequate post-treatment control.⁷ One of the measures to increase this homogeneity (related to *H. pylori*) is to accept only patients with documented eradication after the first-line or second-line treatment regimens,⁷ and exclude those who fail to respond, i.e. show resistance to these two tested regimens.

All these procedures that are necessary before randomization into the study arms will easily require a **pre-trial period of at least one month**, and possibly longer. This is inevitable, however, because the study subjects need to be standardized with respect to their *H. pylori* status prior to the treatment trial. Selecting only baseline HP-negative subjects will not be valid, because *H. pylori* is considered as the necessary prerequisite for initiation of the Correa Cascade, and AG without evidence of *H. pylori* is considered another entity. In this RCT, we are not interested in assessing the effect of HP-eradication on stomach function and structure, but instead in validating the concept, whether L-cysteine administration (to inactive acetaldehyde) is capable of restoring the function and structure of atrophic gastric mucosa, controlled for confounding by their HP-eradication.

3.3.2. Blinding

Following the most stringent recommendations of the guidelines, this company-sponsored trial with Acetium capsules will be conducted in **triple-blind** fashion; i.e., 1) participant-blind, and 2) investigator-blind. In addition, this RCT will be 3) sponsor-blind, i.e., the statistician evaluating the study results is blinded, to exclude the possibility of undue bias caused by analysis of the results.

3.3.3. Placebo control

Placebo preparation with design and package identical to the test preparation (Acetium capsule, 100mg) will be used in this trial, received by one half of the randomly allocated study subjects.

3.3.4.Parallel-group design

Based on careful weighting of the advantages and drawbacks between the parallel design and the cross-over design, the current trial will be conducted as a **parallel group** design. Despite the undeniable advantages of the cross-over design in study power issues, its several important drawbacks contributed to the decision in favour of the parallel group design. Importantly, the extension of the 3-month trial period by another 3 months would likely increase the prop-outs (censoring) that would compromise the power of the study.

3.3.5.Randomization

Because the subjects into this RCT will be recruited over an extended time (including the pre-trial period), the subjects will be randomized in relatively small blocks, to avoid the potential bias due to varying the selection criteria over time. In this trial, randomization will be performed using the random number generator (<https://www.sealedenvelope.com/simple-randomiser/v1/lists>) with block size of 4, and creating unique randomization codes for each study subject. The latter will be used as the identifier of each subject in all datasets. Printed list (CSV Excel) is sealed in an envelope and stored in the company safety box, until opened at the completion of the study and all data analysis.

3.3.6.Stratification

Randomization alone may not ensure full comparability between participants in the two treatment arms, and stratified randomization is needed to remedy this potential imbalance between the two arms. Of the baseline characteristics of AG that potentially affect the efficacy outcomes in the trials, the most obvious include the following: 1) severity of AG (moderate/severe), 2) localization of AG (antrum/corpus), and 3) extent of AG (antrum-only, corpus-only vs. pan-gastritis).

The rational of stratified randomization is straightforward. Stratification is intended to create two study groups that are matched by the key characteristics of the disease that might influence on the efficacy measures. These three covariates impacting the natural history of atrophic gastritis in this trial are most likely too powerful to be remedied only by statistical treatment without stratification. According to the recommended practice, however, these baseline stratification variables will be entered as covariates in the final multivariate models to control for their potential confounding of the efficacy endpoints.

3.3.7. Duration of the treatment period

There is no firmly established documentation about the appropriate duration of the treatment period that could be used as the basis for the current RCT. In the only study raising the entire issue as the study hypothesis, the patients have received Acetium® capsules (100mg three times a day) for a period of three months.^{62,63} The rationale behind this treatment regimen is not clear. L-cysteine administered in this format (Acetium® capsule) is a natural amino acid, with no systemic effects, no known toxicity, and thus no upper limit of daily usage. When used in its original indication (inactivation of acetaldehyde in the stomach after alcohol intake), however, the recommended maximum daily dosage is 1000mg (10 capsules/day).

Similarly, there are no solid arguments to substantiate the selected 3-month period as the duration of the Acetium® capsule treatment.^{62,63} One could equally well argue that because gastric mucosa has a very short renewal time (under normal conditions), one could anticipate that removing the noxious agent (acetaldehyde) by Acetium® capsules could result in interruption of the Correa cascade and mucosal healing within a much shorter time than 3 months. Equally valid, however, could be the claim that gastric carcinogenesis once initiated by *H. pylori* and advanced to the stage of AG is a long process, and even after elimination of *H. pylori* and the second trigger (acetaldehyde) would, indeed, evoke a mucosal recovery that is far less effective than e.g. after early eradication of active HP-infection, and in addition, would necessitate a more prolonged use of Acetium® capsules than just 3 months.

In the present trial, we selected the **trial period of 6 months**, however, which is a kind of compromise between the two hypothetical extremes. In addition, the advantage of reproducing the setting of the original study,^{62,63} we can confirm (or disprove) the observations reported in the two recent communications. Accordingly, the patients are **randomly allocated** to two study arms, one administered **Acetium® capsules** (2 capsules, 3 times a day, and additional one with meals and alcohol intake), and another arm receiving identical administration of **Placebo** (2 caps 3 x day), for **6 months**. After the 6-month randomized trial, all patients start receiving Acetium® capsules (2 capsules 3 x a day, and additional ones), and the use of placebo is discontinued.

3.3.8. Follow-up visits

The total **follow-up** period of the subjects in the cohort will be **two years after completion** of their 6-month **treatment** period. During the follow-up, the subjects are monitored by the GastroPanel® test at 6-month intervals, starting from the time point of completion of their treatment; altogether 4 times (6-, 12-, 18- and 24-months, since the study onset). **At the last follow-up visit**, each patient will be examined (in addition to GastroPanel®) by **gastroscopy and biopsies**. The latter are compared with the baseline biopsies in a random-reading setting, where the two pathologists are blinded by the biopsy origin (baseline biopsy or follow-up biopsy).

3.3.9. Compliance

Evidence of poor compliance in the prospective cohort studies on patients with chronic HP-infection and/or AG, is not unusual, but long-term cohort studies are still possible to complete.^{14,15,17,18,25,42,43} Therefore, it is crucial to monitor patients' compliance with the treatment and follow-up procedures during the entire study period. One such approach is the drug or pill count at every follow-up visit, and repeated emphasis on the values of adherence to the protocol requirements.

4. METHODS

The general outline of the study and the trial design have been detailed in Sections 3.-3.3.9. The methods described here are used to examine the patients and their samples at the baseline visit and follow-up visits, following these study protocols.

4.1. GastroPanel® screening for eligibility and follow-up of mucosal functions

GastroPanel® test is the first-line diagnostic test in this RCT, used for the **initial screening** of the potential study subjects attending the clinic. Due to its extremely high negative predictive value (NPV),⁵²⁻⁵⁶ a normal result in GastroPanel® tests excludes the possibility of AG, making these subjects non-eligible for this trial. In contrast, any patient whose GastroPanel® test results suggests AG (antrum, corpus or both), is potentially eligible for the trial and will be referred for gastroscopy and biopsy confirmation of the diagnosis. During the **follow-up visits** of the study subjects, GastroPanel® is used to monitor the functionality of the gastric mucosa, to disclose the eventual efficacy measures (one of the two primary study endpoints).

GastroPanel® is a user-friendly ELISA technique, consisting of a panel of four biomarkers specific for the gastric mucosa: 1) Pepsinogen I (PGI), 2) Pepsinogen II (PGII), 3) Gastrin-17 (G-17) and 4) *H. pylori*

antibody (HpAb). Each are performed as a separate ELISA test either manually or using a suitable ELISA automation.

4.1.1.ELISA test for Pepsinogen I and Pepsinogen II

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 PGI level in circulation decreases. 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, PGI and PGII biomarkers are determined according to the instructions of the manufacturer from a plasma samples. Pepsinogen I ELISA kit (Biohit Cat. No. 601 010.01), Pepsinogen II ELISA kit (Biohit Cat. No. 601 020.01). Both PGI and 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).

4.1.2.ELISA test for Gastrin-17

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 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 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 G-17 ELISA method in the GastroPanel® is specific to "amidated" G-17 molecule, which is the most important member of the gastrin/cholecystokinin-family, regulating the physiology of the upper gastrointestinal tract.

4.1.3.Stimulation of Gastrin-17

If the GastroSoft® report from the fasting sample implicates AG in the antrum, it is recommended to repeat Gastrin-17 test in a post-prandial blood sample. 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 (postprandial) blood sample must be taken 20 minutes after the intake of the protein juice.

4.1.4.ELISA test for Helicobacter pylori (HpAb ELISA)

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).

4.1.5.Patient preparation for GastroPanel® test

Reliable results from the GastroPanel® examination necessitate some preparatory measures of the patient. Detailed instructions are given to each test subject at the time of his/her consenting to participate in the **GastroPanel screening phase** (ANNEX 1).

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 2), because these medicines interfere with the output of GastroPanel® biomarkers (www.gastropanel.com/ GastroPanel® Sample collection Instructions).

4.1.6.Sample collection for GastroPanel® test

The person taking the blood sample shall fill the TEST REQUIST FORM (**ANNEX 2**) as complete as possible. For each patient, 2 plasma tubes (XXX) will be taken for GastroPanel® test. Additionally, Gastrin-17 can be determined also after stimulation (see 4.1.3.). 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.

4.1.7. 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).

4.1.8. 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® software. A model Report of test results is enclosed (**ANNEX 3**). 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 4**. This ANNEX also illustrates the most important clinical conditions (disease states) associated with each of the gastritis phenotypes, including the risk of GC.

4.2. Gastroscopy and biopsy procedures

According to the Flowchart (**ANNEX 1**), all patients testing positive with the GastroPanel® test (**with AG cut-off**) are **potentially eligible** for the study, and will be confirmed by gastroscopy and biopsies to provide the histological confirmation of the diagnosis. GastroPanel® test results are interpreted by the GastroSoft® software based on the algorithm of the USS for classification of gastritis (ANNEX 4), and it is important that also the biopsy procedures follow the same USS.

All patients participating in this study shall undergo a routine gastroscopic examination, which 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 biopsies. Only the subjects with biopsy-confirmed **moderate to severe AG** are potentially eligible (ANNEX 1), the others being subjected to management by the routine procedures in the clinic.

4.2.1. Biopsy protocols

The optimal biopsy protocol following the USS is illustrated in **ANNEX 5**. 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 1 and 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 put into another formalin tube, and embedded into the same paraffin block (Block No. 2; labeled **CORPUS**).

4.2.2. Preparation of microscopy slides

The biopsies from formalin bottles/tubes are embedded in paraffin using the routine procedures at the Pathology Laboratory of the Hospital. 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.

4.2.3. Interpretation of the biopsies

All gastroscopy biopsies are examined by the expert pathologists at Hospital X, 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 6** for histopathological examination.

Following the routine diagnosis of all gastroscopy biopsies at the Department of Pathology, all pathological findings will be reviewed by the two pathologists in the study group (PS, KS), at the final stage of the study, blinded with the sample timing, i.e., whether a baseline biopsy or a follow-up biopsy (see Section 3.3.8).

4.3. Eradication of *H. pylori* infection

As described, to be eligible for the study, each subject must have a biopsy-confirmed moderate or severe AG, associated with ***H. pylori*** infection. This is to make sure that only the patients with HP-associated AG are included, while those having AG associated with other etiologies (autoimmune gastritis) will be excluded. In addition, all subjects must undergo a **successful eradication** of *H. pylori*, before randomization. The rationale is to control for the potential confounding by HP-eradication of the primary study endpoints (efficacy measures) following the Acetium treatment.

In the eradication of *H. pylori*, this trial will follow the recommendations of the recently published Maastricht IV consensus conference.⁷ Accordingly, **the first-line** treatment should consist of the triple treatment including **PPI-clarithromycin and amoxicillin** (or metronidazole), now being universally accepted for this purpose by all the consensus conferences. The efficacy of the treatment must be controlled (see 4.3.1.), and in case of failure, the treatment must be **repeated**, but **only once**. If failed again, these patients will be excluded from the study, and subjected to treatment and/or control following the routine practices of the clinic (ANNEX 1).

According to the Maastricht IV recommendations,⁷ the **second-line** therapy after failure of a PPI-clarithromycin-containing protocol should include **a bismuth-containing quadruple therapy** or **levofloxacin-containing triple therapy**. The rationale is to abandon clarithromycin in an empirical second-line treatment, because there is a likelihood that selection of a clarithromycin-resistant strain

has occurred. Use of 10-day PPI-levofloxacin-amoxicillin is the other alternative for a second-line treatment, based on evidence obtained during the past few years.⁷

4.3.1. Confirmation of the success of *H. pylori* eradication

To be eligible for the trial, each subject must have his/her *H. pylori* infection successfully eradicated before randomization into the study arms (ANNEX 1). This necessitates a strict control of the HP-eradication efficacy by the tests measuring an ongoing (active) *H. pylori* infection. For this purpose, there are different options available. If GastroPanel® is used for this purpose, it should be noted that high HP-antibody (IgG) titers may persist for months after a successful HP-eradication. To obviate this potential source of error, it is recommended that the control GastroPanel® test is NOT performed sooner than 3 months after the termination of HP treatment. Other optional tests to diagnose active HP-infection include the UBT (urea breath test) and stool antigen test (SAT). In this trial, these tests are NOT ACCEPTED, because of their well-established severe limitations.^{73,74} Indeed, both UBT and SAT give substantial proportion of false negative or false positive results, particularly in patients with AG, which precludes their use in the control of HP eradication in these patients.^{73,74}

Another solution for a reliable and rapid detection of an ongoing HP-infection is an endoscopic ***H.pylori* Quick Test** (HPQT; Biohit Oyj). For this test, additional biopsies are needed, one from the antrum and one from the corpus, used immediately for the test. If HPQT is preferred as the control test for HP eradication, the study Flowchart (ANNEX 1) needs to be modified in that the HP **eradication** is performed **before gastroscopy**. The advantage of this approach is the possibility of using histological biopsy as an additional tool to confirm HP-eradication. Moreover, this approach also speeds up the enrollment process, while avoiding the lag-time (3 months minimum) needed for HP-serology to stabilize after the eradication therapy, to make GastroPanel test a feasible option.

4.4. Statistical analyses

This study is a randomized controlled trial (RCT) testing the efficacy of Acetium capsules (L-cysteine) in restoring the structure and function of gastric mucosa among patients with HP-associated AG, administered after a radical eradication of their *H. pylori* infection. The null hypothesis of the study implicates that the intake of Acetium® capsules is no better than placebo in restoring the physiological functions of stomach mucosa, and there is no evidence whatsoever on the recovery of gastric atrophy (AG) as a specific result of this medication.

4.4.1. Study endpoints

Rejection or not of the null hypothesis is based on comparison of the two strata (Acetium and placebo) against **two primary study endpoints** (efficacy measures): 1) Changes in the serum levels of the relevant stomach-specific biomarkers (GastroPanel® test) from the baseline values (diagnostic to AG), towards (or falling within) their reference (normal) values; and 2) Biopsy-confirmed recovery of atrophic gastric mucosa by at least one histological (USS) grade (e.g. from severe to moderate AG; moderate to mild AG), based on a blinded reading of the baseline and follow-up biopsies.

In addition to these primary efficacy endpoints, **secondary endpoint** in this study includes the calculation of the performance indicators (sensitivity, specificity, negative- and positive predictive value, AUC) for the GastroPanel® test, separately for the different histological endpoints: AG in the antrum, AG in the corpus, and atrophic pangastritis. In case that enough cases of intestinal metaplasia (IM) and dysplasia (IEN) will be included, these indicators can be calculated also for these two conditions, although not specifically diagnosed by GastroPanel®.

4.4.2. Conventional statistical techniques

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 conducted according to routine procedures. Frequency tables will be analyzed using the χ^2 -test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. Standard statistics are used to compare **the efficacy of the two study arms** on the observed changes in the serum levels of the GastroPanel® biomarkers before and after Acetium treatment. The effects of test preparation and placebo can be analyzed separately in non-parametric paired samples t-test (Wilcoxon signed ranks test) by comparing the pairs of the baseline- and post-trial values. Another approach is to calculate the effect size in both arms (i.e., increase/decrease of the biomarker levels by treatment as compared with the baseline) and to compare these effects between the two study arms. For categorical outcomes (recovery; Y/N), conventional regression models can be used, where the results are expressed as crude OR (odds ratio), and their 95% confidence intervals (95% CI).

4.4.3. Multivariate logistic regression models

The independent effect of Acetium (adjusted for potential confounders) can be analysed using the

multivariate logistic regression model, where all recorded baseline characteristics (gender, age) can be entered as covariates, including the 3 stratification variables: i) severity, ii) localization, and iii) extent of AG. For categorical endpoints (recovery; Y/N), the results of these multivariate models are expressed as adjusted OR (odds ratio), and their 95% confidence intervals (95% CI).

4.4.4. Test performance indicators

Of secondary importance in this RCT are the performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of individual markers and whole GastroPanel® test. These are calculated 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, for each biomarker. 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 detection of the study endpoint (AG grades, localization, extent). Significance of the difference between AUC values can be estimated using STATA's *roccomb* test with 95%CI.

4.5. Power analysis

Because of the fact that GastroPanel® is composed of stomach-specific biomarkers, of which 3 are relevant for this purpose (others except HP-antibody assessment), and the combination of which is the key for appropriate diagnosis of the gastric mucosal structure and function, calculating the study power is not straightforward. In principle, it should be calculated separately using the biomarkers signifying AG of the corpus (PGI, PGII and PGI/PGII ratio) and those that implicate AG of the antrum (G-17).

Given that there are no figures available for biomarker level fluctuations following placebo treatment, we must base the power calculations for the changes in biomarker values (after Acetium treatment) that are reported in the preliminary study from Italy.^{62,63} Importantly, however, this study only included cases with AG of the corpus (with low PGI & PGI/II ratio and elevated G-17), but not patients with AG in the antrum (where G-17 is low and PGI within normal range). Thus, we calculated the power of this study on the basis of these corpus-specific biomarkers only, and used these to estimate the appropriate cohort size needed in both the Acetium and placebo arms.

Accordingly, the Italian study reported a post-treatment (3-month) increase of PGI levels from 7.9 $\mu\text{g/L}$ to 11.4 $\mu\text{g/L}$ (difference of 3.5 $\mu\text{g/L}$). Unfortunately, the SDs for the two values were not reported, but based on estimates from another cohort ($n=42$) of AG (corpus) patients, with similar baseline PGI values, these SD values are estimated as 7.5 and 10 $\mu\text{g/L}$, respectively. Using the two-sample mean test for paired samples, this study would be adequately powered (Type II error 0.80, type I error 0.05) to detect a true difference in PGI increase of this magnitude (3.5 $\mu\text{g/L}$), if there are **55 patients in the Acetium study arm**. This estimate is sensitive to SDs as well as correlation between the samples. If the SD increases, a larger cohort size is needed, but if the correlation is higher than the default 0.5, a markedly smaller cohort size is needed.

To be on the safe side, a cohort of **60 patients** would give the power of 84% to detect this difference in PGI increase after Acetium treatment, and because of this, the present RCT is designed to include **60 subjects** in both the **Acetium** and **the placebo** arm. With the cohort of 120 subjects, randomized 1:1 into Acetium and placebo arms, and using two-sample mean test (for independent samples) for the effect differences, we enter up with the estimates that this study is adequately powered (Type II error 0.80, type I error 0.05) to detect a true difference in effect estimates as follows: **3.5 $\mu\text{g/L}$ (Acetium)** and **2.15 $\mu\text{g/L}$ (Placebo)**, with assumed SD of 3.0 and 2.0, respectively. Even a slightly smaller effect in the placebo arm would increase the study power close to 100%, allowing also a much wider range of SD values.

5. ETHICAL ISSUES

The study design and its execution do not involve any particular ethical issues except those in other clinical studies of similar type. The study protocol will be submitted for approval to the Institutional (Regional?) Ethical Committee of Hospital X (City Y, Country Z), and the whole study is conducted in accordance with the Declaration of Helsinki.

Patients are enrolled among the consecutive patients attending the outpatient Department of Endoscopy (Hospital X), because of referral to gastroscopy. Thus, all eligible patients represent regular outpatients with approved clinical indications for gastroscopy. The only additional procedure (outside normal routine) carried out to these patients is the blood sampling for GastroPanel® test. The maximum amount of venous blood taken is 10 ml. All patients must sign the informed consent for their participation even in this **initial GastroPanel screening** phase. Following the flowchart

(**ANNEX 1**), only the patients testing *H. pylori* positive in the GastroPanel® test which simultaneously indicates AG (antrum, corpus or both), will be potentially eligible for the study. All others (HP- cases with or without AG) will be managed following the clinical routines of the hospital.

In the next step, gastroscopy is performed (for all), with biopsies to confirm the diagnosis of AG, including its grade, topography and extent, as well as to confirm the presence of an active *H. pylori* infection. As soon as verified, all these patients will receive a **treatment for HP-eradication**, following the Maastricht IV recommendations.⁷ The efficacy of this eradication will be controlled following these same guidelines, now accepted almost universally as the state-of-art management of HP-infections. Finally, only the patients with biopsy-confirmed moderate or severe AG and clinically verified **successful eradication** of *H. pylori* infection, will be eligible for randomization into the two study arms (ANNEX 1).

Thus, the entire procedure undergone in the enrolment of the eligible patients into this RCT is based on **generally accepted ethical principles** that are **universally followed** in the routine clinical management of similar patients in all specialized gastroenterology clinics worldwide.

6.TIME-TABLE

This study design is based on the fact that the study execution necessitates a large and well equipped gastroenterology unit, specialized in the diagnosis, management and follow-up of patients with different types gastritis and gastric cancer precursors. With no affiliation to such an own clinic, Biohit Oyj must rely on its international partners to find a suitable clinic as the site of execution of this study.

This RCT is not an easy design to set up and sustain. Moderate and severe AG are uncommon conditions representing a small minority of patients who complain dyspeptic symptoms, and even among consecutive gastroscopy referrals. Furthermore, HP-eradication is not always successful, and that might result in exclusion of some of the otherwise potentially eligible patients. The 6-month randomized treatment period is followed by two-year follow-up period (with Acetium only) with 6-monthly control visits to monitor the gastric mucosal functionality with the GastroPanel® testing, and the final control by gastroscopy and biopsies. This lengthy period of monitoring may cause potential challenges for the compliance of the patents, and needs special attention not to compromise the power of the study.

Due to these uncertainties, it is not possible to estimate the accurate time table of the study execution. Of key importance is the selection of the clinic which agrees to make the commitment to this challenging study design. Once found and contracted, however, the study execution should be relatively straightforward. Given that the **120 subjects** (with HP-eradicated- or autoimmune-type moderate/severe AG) in the study will be enrolled among consecutive patients attending the Outpatient Department of Endoscopy (Hospitals X, Y and Z), it can be estimated that this initial screening phase necessitates GastroPanel® examination of at least 600-700 subjects. In a large gastroenterology clinic, however, examination of this number of gastroscopy patients will be a matter of months. The best estimates suggest that completion of the whole study protocol (including the **2-year follow-up**), for the cohort of 120 subjects will take around **four years**.

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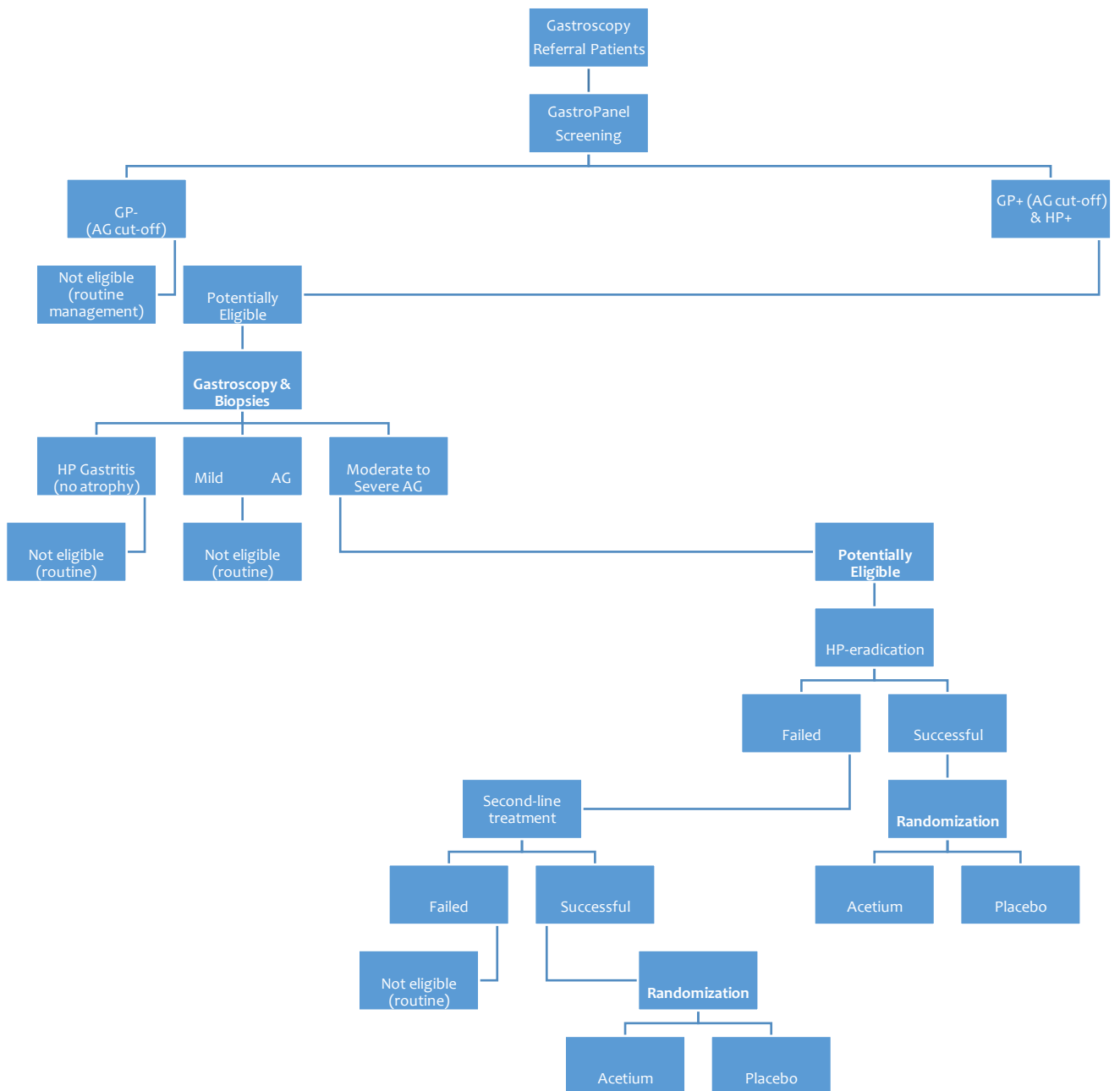
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ANNEX 1. FLOWCHART OF THE PATIENT ENROLMENT IN THE COHORT



ANNEX 2.

THE TEST REQUEST FORM

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) :

EDTA PLASMA

EDTA PLASMA + Gastrin-17-STABILIZATOR

FASTING SAMPLE STIMULATED SAMPLE

SAMPLE ID:

DELIVERING TO LABORATORY:

ROOM TEMPERATURE FROZEN

ANNEX 3.

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
<i>H. pylori</i> antibodies (HPAB)	145,0 EIU	*	< 30 EIU
Interpretation			
The results indicate atrophic corpus gastritis due to <i>Helicobacter pylori</i> 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 pmol/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 above 10 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 4.

PHENOTYPING* OF GASTRITIS BY GastroPanel® TEST RESULTS

CORPUS MUCOSA

		normal	gastritis	atrophic gastritis		
				mild	moderate	severe
ANTRAL MUCOSA	normal	R				
	gastritis		Hp + SPGI ↑ G-17 ↑			
	atrophic gastritis mild			Hp + or - SPGI ↓ G-17 ↑		
	atrophic gastritis moderate severe		Hp + SPGI ↑ G-17 ↓ or ↓ *	Hp + or - SPGI ↓ G-17 ↓		

* G-17 does not response to stimuli

Abbreviations:
 Hp= *H.pylori*
 SPGI=serum pepsinogen I; basal value
 G-17= serum gastrin-17; basal or stimulated value

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

The disease states and risks associated with different phenotypes of gastritis

CORPUS MUCOSA

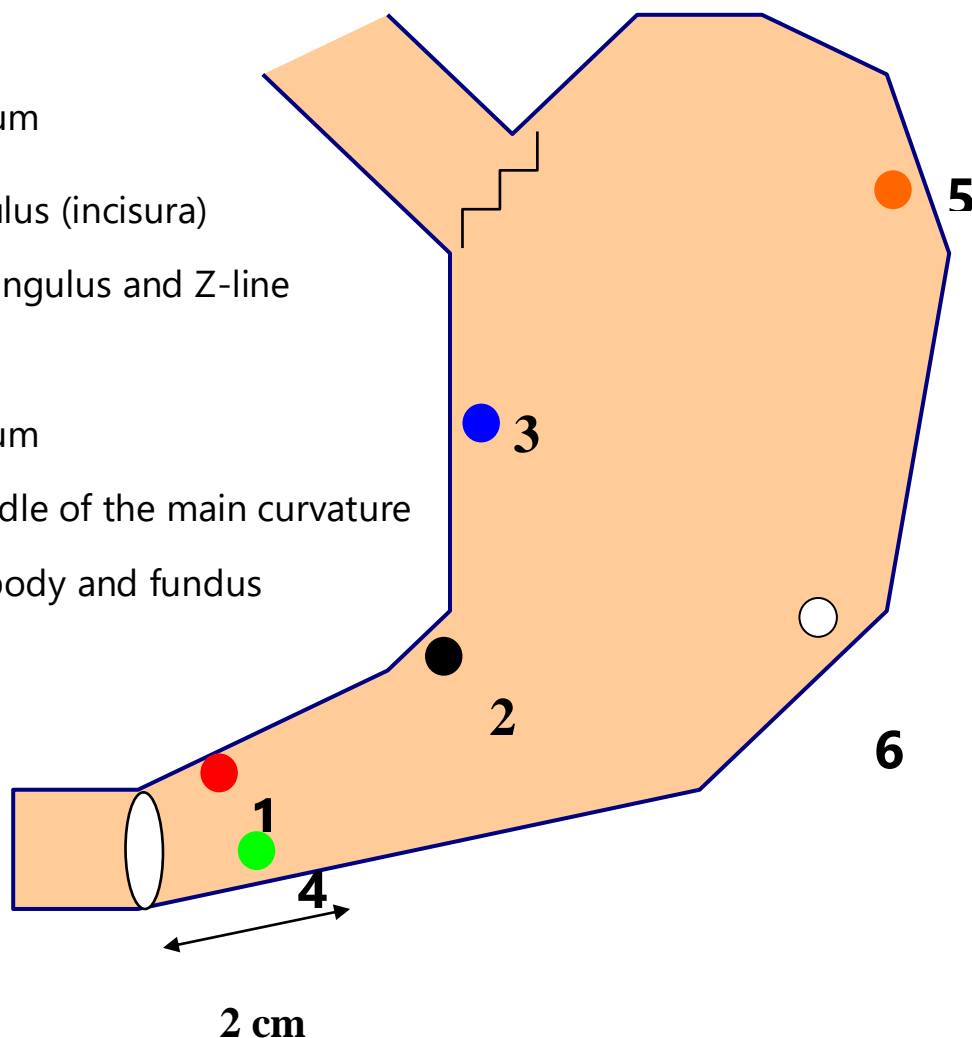
		normal	gastritis	atrophic gastritis		
				mild	moderate	severe
ANTRAL MUCOSA	normal	R				
	gastritis		DU			
	atrophic gastritis mild			PA, GCA		
	atrophic gastritis moderate severe		GU, DU, GCA	GCA, PA		

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

ANNEX 5.

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 and corpus; at least two biopsies from each. These biopsies are taken from the large and small curvature of middle antrum (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**).

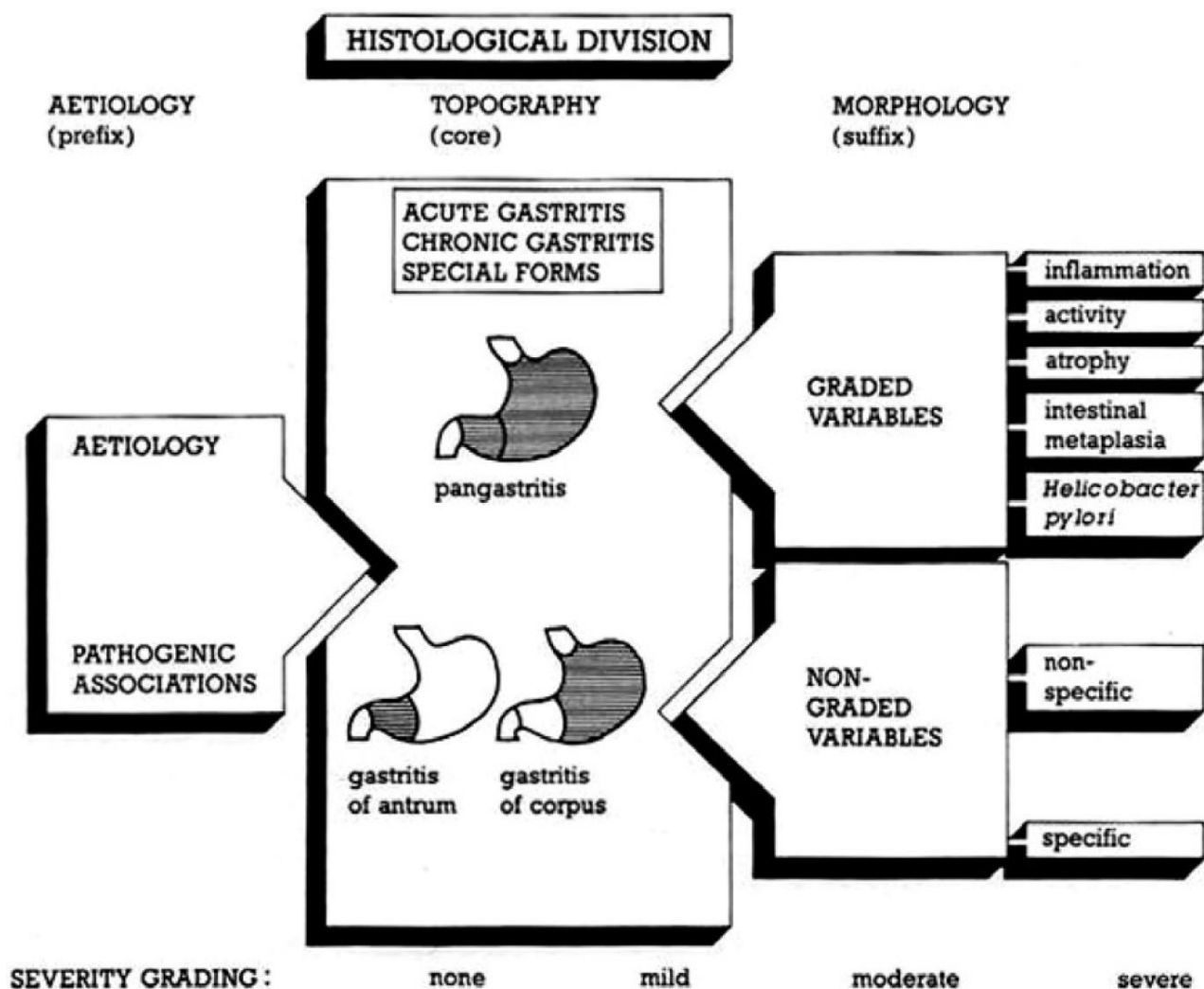
Biopsies from the antrum and incisura (biopsies 1, 2, 3, 4) are set into one and the same formalin bottle/tube (**tube No. 1**) and embedded into one and the same paraffin block. These can be labeled as "**antrum**". The biopsies from the corpus (No 5 and 6) set into one and the same formalin bottle/tube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

Price AB. The Sydney System: histological division. J Gastroenterol Hepatol. 1991;6:209-22.

Sipponen P, Kekki M, Siurala M. The Sydney System: epidemiology and natural history of chronic gastritis. J Gastroenterol Hepatol. 1991;6:244-51.

ANNEX 6.

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.