Department of Gastroenterology, Hepatology and Infectious Diseases

Otto-von-Guericke University, Magdeburg

INFLUENCE OF LABORATORY AND ENDOSCOPY-RELATED FACTORS ON THE ASSESSMENT OF SERUM PEPSINOGENS AND GASTRIN-17.

Dissertation

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Elisabetta Goni

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Summary

Background & aim: Serum pepsinogen (PG) I and PG II are non-invasive parameters in the detection of atrophic gastritis. The diagnostic add on value of serum gastrin-17 (G-17) remains uncertain. The aim of the study was to assess the stability at different time point and to evaluate the influence of clinical factors on of these serum parameters. **Methods:** For stability analyses the plasma and serum samples from 23 subjects were processed at different time points. Ten additional patients were included to evaluate the influence of upper GI endoscopy and 18 patients to evaluate the effect of bowel cleansing before colonoscopy. **Results:** PG I, PG II and G-17 levels were not statistically different in serum and plasma. PG I and PG II serum levels were stable overtime and their assessment is not influenced by laboratory factors. G-17 is associated with time-dependent degradation (p= 0.0001). Upper GI endoscopy and bowel preparation prior colonoscopy were associated with minimal variations in PG I, PG II, while G-17 showed subject-specific alterations. **Conclusions:** PG I and PG II serum levels are stable overtime. However, G-17 stability is closely dependent on time of processing and storage. Upper GI endoscopy and colonoscopy preparation lead to minimal non-significant changes in basal PG I, PG II and G-17 levels.

Keywords Pepsinogens, gastrin-17, pre-analytical factors, endoscopy-related factors, gastric biomarkers

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1. Abbreviations

AC: adenylate cyclase

ACh: acetylcholine

ANP: atrial natriuretic peptide

ATPase: adenosine triphosphatase

Ca++: calcium

CAG: chronic atropic gastritis

CCK: cholecystokinin

cAMP: Cyclic adenosine monophosphate

CGRP: calcitonin gene-related peptide

CRC: colorectal cancer

DU: duodenal ulcer

EC: enterochromaffin cell

ECL: enterochromaffin-like cell

EGF: epithelial growth factor

ELISA: Enzyme-linked immunosorbent assay analysis

G-17: gastrin-17

GC: gastric cancer

GI: gastrointestinal

GRP: gastrin-releasing peptide

GU: gastric ulcer

H+: hydrogen

HCl: Hydrochloric Acid

HpAb: anti-*Helicobacter pylori* antibodies

H. pylori: Helicobacter pylori

H_2RA: histamine-2 receptor antagonists

HRP: horseradish peroxidase

H2 receptor: Histamine receptor type 2

H3 receptor: Histamine receptor type 3

K+: Potassium

MAO: Maximal Acid Output

mM: millimolar

ml: milliliters

M3 receptor: muscarinic receptor type 3

µg/l**:** micrograms pro liter

Na+: Natrium

NV: normal value

O.L.G.A: Operative Link for Gastritis Assessment

PACAP: pituitary adenylate-cyclase activating peptide

PG: Pepsinogen

pH**:** potential of hydrogen, concentration of H+ ions

PPI: Proton pump inhibitor

pg/l: pictograms pro liter

PU: peptic ulcer

SST: somatostatin

TMB: 3,3',5,5'-tetramethylbenzidine

VIP: vasoactive intestinal peptide

2. Introduction

2.1 Physiology of gastric acid secretion and its regulation

The stomach consists of three anatomical regions: fundus, body, and antrum. Functionally, there are two glandular regions: oxyntic and pyloric mucosa. The oxyntic gland mucosa, the hallmark of which is the oxyntic or parietal cell, comprises 80% of the fundus and body. The pyloric gland mucosa, the hallmark of which is the G cell, comprises 20% of the antrum. The glandular mucosa is organized in vertical tubular units that consist of four regions: the pit region, consisting mainly of columnar surface mucous cells; the isthmus, where the multipotent progenitor cells reside; and the actual gland region, which forms the lower part of the unit [1, 2, 3]. The latter consists of a neck and a base. The mucous-producing pit cells migrate upward from the progenitor cell toward the gastric lumen. Acid-secreting parietal cells migrate downward to the middle and lower regions of the gland [4, 5, 6]. Chief cells predominate at the base and secrete pepsinogens (PG) and leptin [7]. A variety of distinct neuroendocrine cell types are contained within the gland, but only some of their products have been assigned physiologic functions. These cells include: a) enterochromaffin (EC) cells, contain atrial natriuretic peptide (ANP), contain somatostatin (SST), serotonin and adrenomedullin [8, 9, 10, 11, 12, 13, 14, 15, 16, 17]; b) enterochromaffin-like (ECL) cells, which contain histamine [18, 19]; c) D cells, which contain amylin [20, 21]; d) A-like or Gr cells, which contain ghrelin and obestatin [22, 23]. *Table 1* resumed the gastric cells type, substance secreted and its functions.

The G cells are found deep within the pyloric glands of the stomach antrum and they secrete gastrin, mostly gastrin-17 (G-17) *(Figure 1)*.

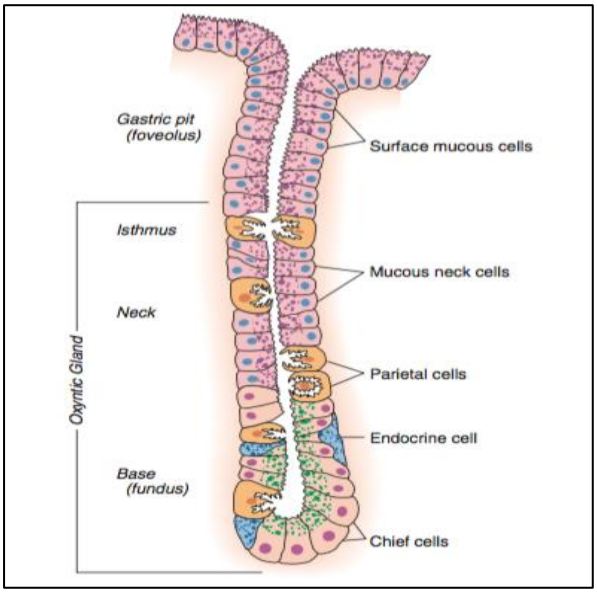

Figure 1. Diagrammatic representation of the oxintic gastric gland, cells type, substance secreted and its functions *(Adapted from Ito S. et al. [24])*

Source	Substance secreted	Stimulus for release	Function
Mucous neck cells	Mucus	Tonic secretion; Increased with irritation of mucosa	Physical barrier between lumen and epithelium
	Bicarbonate	Secreted with mucus	Buffers gastric acid to prevent damage to epithelium
Parietal cells	Gastric acid (HCl)	Acethylcholine, gastrin, histamine	Activates pepsin, kills bacteria
	Intrinsic factor		Complexes with vitamin B_{12} to permit adsorption
Enterochromaffin-like cells	Histamine	Acethylcholine gastrin	Stimulates gastric acid secretion
Chief cells	Pepsinogen	Acethylcholine acid, secretin	Digests proteins
	Gastric lipase		Digests fats
D cells	Somatostatin	Acid	Inhibits gastric acid secretion
G cells	Gastrin	Acethylcholine, peptides, amino acids	Stimulates gastric acid secretion

Table 1. Gastric cells type, substance secreted, stimulus for release and its functions. *(Adapted from Ito S. et al. [24])*

The vagus nerve innervates the G cells. Gastrin-releasing peptide is released by the post-ganglionic fibers of the vagus nerve onto G cells during

parasympathetic stimulation. Gastrin-releasing peptide, as well as the presence of amino acids in the stomach, stimulates the release of gastrin from the G cells. Gastrin stimulates ECL to secrete histamine. Gastrin also targets parietal cells by increasing the amount of histamine and the direct stimulation by gastrin, causing the parietal cells to increase hydrochloric acid (HCl) secretion in the stomach [2]. *(Figure 2 and Figure 3).*

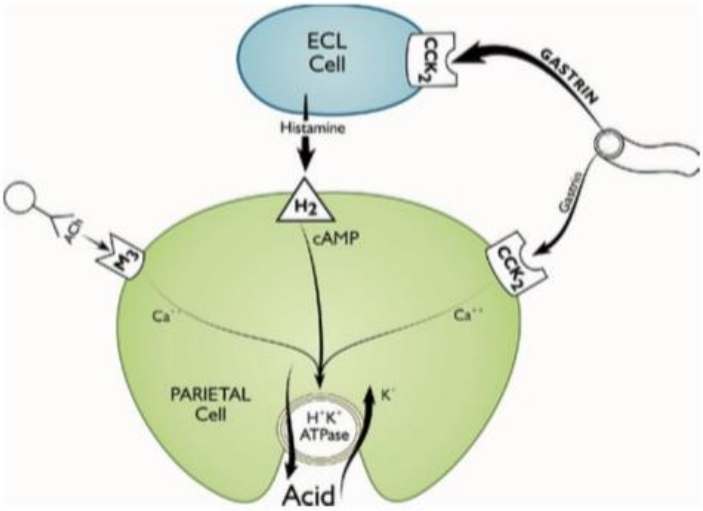

Figure 2. Model illustrating parietal cell receptors and transduction pathways. *The principal stimulants of acid secretion at the level of the parietal cell are histamine (paracrine), gastrin (hormonal), and acetycholine (ACh; neurocrine). Histamine, released from enterochromaffin-like (ECL) cells, binds to H2 receptors that activate adenylate cyclase (AC) and generate cAMP. Gastrin, released from G cells, binds to CCK2 receptors that activate phospholipase C to induce release of cytosolic calcium (Ca++). Gastrin stimulates the parietal cell directly and, more importantly, indirectly by releasing histamine from ECL cells. ACh, released from intramural neurons, bind to M3 receptors that are coupled to an increase in intracellular calcium. The intracellular cAMP- and calcium-dependent signaling systems activate downstream protein kinases ultimately leading to fusion and activation of H+K+-ATPase, the proton pump. (Adapted from Schubert ML et al. [2])*

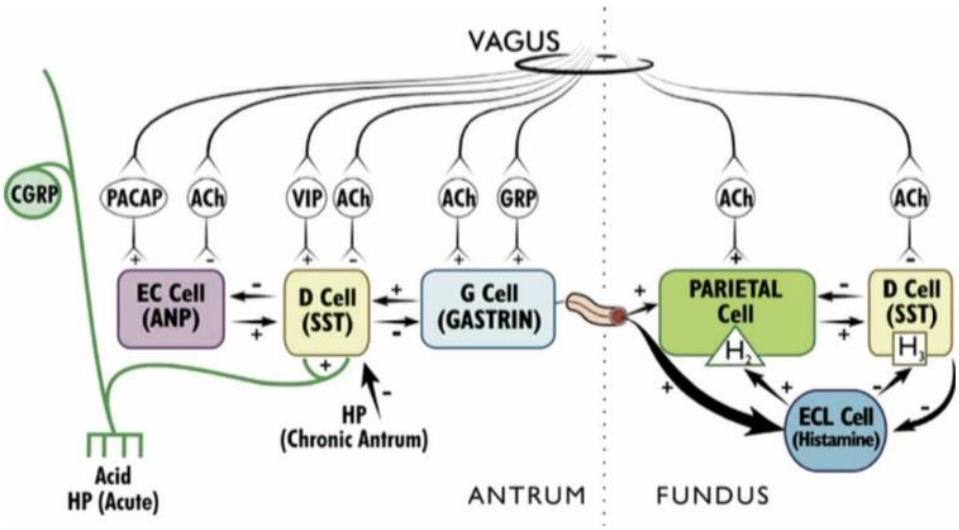

Figure 3. The neural, paracrine, and hormonal regulation of gastric acid secretion. *Efferent vagal fibers synapse with intramural gastric cholinergic (ACh) and peptidergic (gastrin-releasing peptide [GRP], vasoactive intestinal peptide [VIP], and pituitary adenylate-cyclase activating peptide [PACAP]) neurons. In the fundus, ACh neurons stimulate acid secretion directly via M3 receptors on the parietal cell and indirectly by inhibiting somatostatin (SST) secretion, thus eliminating its restraint on parietal cells and histamine-containing enterochromaffin-like (ECL) cells. In the antrum, ACh neurons stimulate gastrin secretion directly and indirectly by inhibiting SST secretion, the latter by a direct effect on the D cell and an indirect effect mediated by inhibition of atrial natriuretic peptide (ANP) secretion from enterochromaffm (EC) cells. GRP neurons, activated by intraluminal protein, also stimulate gastrin secretion. VIP neurons stimulate SST and thus inhibit gastrin secretion. PACAP neurons stimulate SST, via release of ANP, and thus also inhibit gastrin secretion. Dual paracrine pathways link SST-containing D cells to parietal cells and to ECL cells in the fundus. Histamine released from ECL cells acts via H3 receptors to inhibit SST secretion. In the antrum, dual paracrine pathways link SST-containing D cells to gastrin cells and to EC cells. Release of acid into the lumen of the stomach restores SST secretion in both the fundus and antrum; the latter is mediated via release of calcitonin gene-related peptide (CGRP) from extrinsic sensory neurons. Acute infection with H.pylori (HP) also activates CGRP neurons to stimulate SST and thus inhibit gastrin secretion. (Adapted from Schubert ML et al. [2])*

2.2 Gastric biomarkers in the detection of pathological consitions

In the last decades a large number of translational studies were performed in order to assess biomarkers employing in clinical practice. However, in plasma or serum biological molecules may be subject to proteolytic degradation caused by intrinsic peptidase activities [25].

A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease. Biomarkers can be classified into markers of exposure, effect and susceptibility. If biomarkers are to contribute to environmental and occupational health risk assessments, they have to be relevant and valid. Relevance refers to the appropriateness of biomarkers to provide information on questions of interest and importance to public and environmental health authorities and other decision-makers. The second characteristic of potentially useful biomarkers is validity. Validity of biomarkers has been widely discussed. It includes both laboratory and epidemiological aspects. Validity refers to a range of characteristics that is the best approximation of the truth or falsehood of a biomarker. The validity of a biomarker is a function of intrinsic qualities of the biomarker and characteristics of the analytic procedures. Additionally, three broad categories of validity can be distinguished: measurement validity, internal study validity and external validity. Measurement validity is the degree to which a biomarker indicates what it purports to indicate. Internal study validity is the degree to which inferences drawn from a study actually pertain to study subjects and are true. External validity is the extent to which findings of a study can be generalized to apply to other populations. The use of invalid biomarkers can lead to invalid inferences and generalizations and ultimately to erroneous risk assessments [26].

The recent guidelines proposed PG I, PG II and PG I/PG II ratio as the best non-invasive test in order to detect chronic atrophic gastritis (CAG) [27, 28]. The correlation of serum pepsinogens with histology was strengthened in a twelve-year clinico-pathological follow-up study in which gastritis staging along a scale of progressively severe atrophic gastritis, from the lowest (O.L.G.A stage 0) to the highest (O.L.G.A stage IV) and serology. This led to the proposal of serum pepsinogens as risk assessment markers for gastric cancer [29, 30, 31, 32]. However, a recent multicenter-blinded study discouraged the use of PG I, PG II, G-17 and *H.pylori*-IgG in the serological diagnosis of CAG [33].

The diagnostic benefit of serum G-17 as well as the stability of these serum parameters are still a matter of debate.

2.2.1 Pepsinogen I (PG I), pepsinogen II (PG II) and PG I/II ratio

PG I encompasses five of the eight fractions of pepsinogens found in the bloodstream. PG I is the inactive precursor of the proteolytic enzyme pepsin and it is produced by the chief cells of the gastric glands. PG I secretion is stimulated by the vagus nerve as well as hormonal activity of gastrin, secretin and cholecystokinin (CCK). When gastric pH is acidic, PG I is converted to pepsin, which acts on aminoacids in the first step of protein digestion [25].

In clinical practice, serum PG I levels are significantly correlated with gastric acid production; *Table 2* gives a résumé of the main clinical conditions leading to an increase or decrease of serum PG I levels.

The role of serum PG I in screening patients with CAG and gastric cancer (GC), and in detecting peptic ulcer (PU) patients with high relapse risk, was ascertained in 276 subjects. Although not diagnostic per se, PG I was found to be under 20 μg/l in patients with CAG and in some GC or partially gastrectomized patients. In patients presenting with relapsing duodenal ulcer (DU), PG I values were significantly higher than in the non-relapsing ones, but a satisfactory identification of all the duodenal ulcer patients with high relapse risk was not possible on this basis. Even the correlation between

PG I and maximal acid output (MAO) was not accurate in every subject considered *(Figure 4)* [34]. These results suggest that the value of PG I is limited to assessing patients with upper gastrointestinal diseases in which a reduction of peptic secretion, and therefore of PG I in serum, is present.

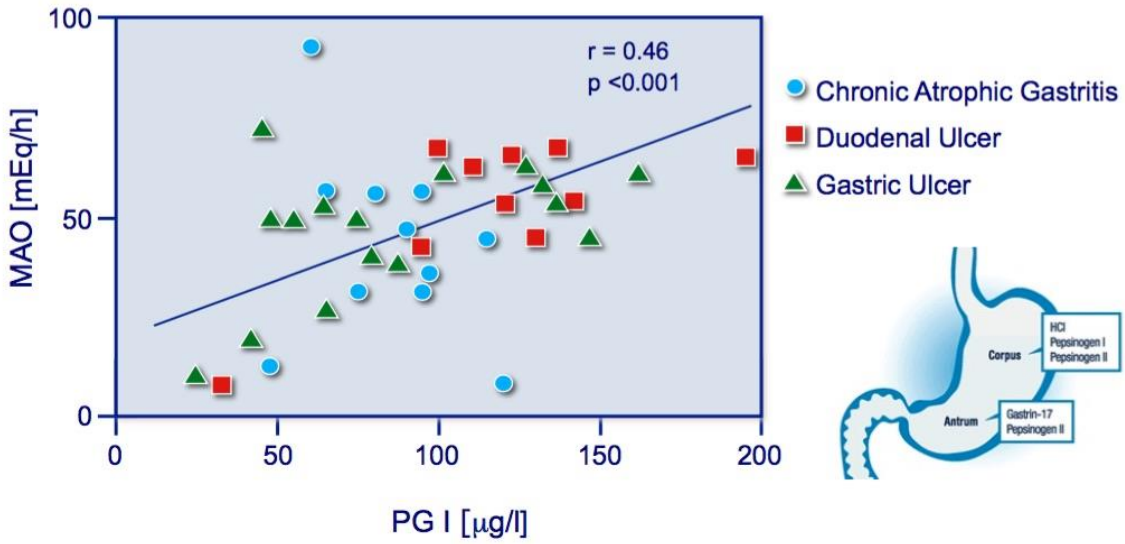

Figure 4. Direct correlation between PG I and MAO in chronic atrophic gastritis (CAG), duodenal ulcer (DU) and gastric ulcer (GU). *(Adapted from Plebani et al. [34])*

The remaining related pepsinogens are collectively termed "PG II". PG II groups are related to PG I group but there are released from the Brunner's gland and pyloric glands in the gastric antrum and proximal portion of duodenum. In clinical practice, serum PG II levels are markers of inflammation [35, 36]. *Table 2* sums up the main clinical conditions leading to an increase or decrease of serum PG II levels.

PG I/II ratio decreases linearly with the severity of atrophic gastritis, a condition related with an increased risk of gastric cancer development.

A small amount of PGs (1%) are absorbed into the bloodstream and can be assayed [25]. Normal values (NV) are considered PG I 30 - 160 µg/l, PG II 3-15 µg/l.

Normal value (NV)	Decrease	Increase
PG I NV 30 - 160 µg/l	CAG Gastric cancer PPI Intake Gastrectomy	Peptic ulcer disease Ménétrier disease
PG II NV 3-15 µg/l	CAG Gastric cancer Gastrectomy	Gastritis

Table 2. Main clinical conditions leading to an increase or decrease of serum PG I and PG II levels [25, 34, 35, 36].

2.2.2 Gastrin-17 (G-17) and the gastrin-acid feedback

G-17 is a heptadecapeptide secreted in the gastric antrum and the isoform G-17 I (non-sulfated form) and G-17 II (sulfated) appear equipotent. Their biological effects are chiefly associated with the amidated isoforms and consist of promotion of gastric epithelial cell proliferation and differentiation to acid-secreting cells, direct promotion of acid secretion and indirect stimulation of acid production through histamine release. In addition, gastrin stimulates gastric motility and release of pepsin and intrinsic factor. Most gastrin isoforms with atypical posttranslational modifications and most small gastrin fragments display reduced or absent bioactivity [25].

Intraluminal stomach pH is the main factor regulating gastrin production and secretion. Rising gastric pH levels result in increasing serum gastrin levels, while falling pH levels are associated with mounting somatostatin production in gastric D cells *(Figure 5)* [37].

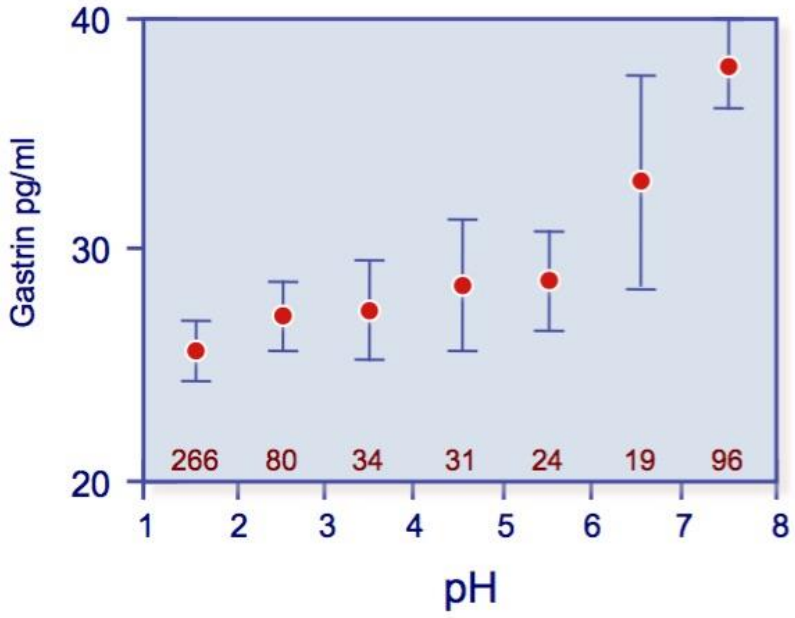

Figure 5. The relationship between basal gastric pH and serum gastrin levels. *(Adapted from Bins M et al. [37])*

The somatostatin downregulates the G-17 synthesis and release. Other, weaker factors that stimulate gastrin secretion are gastric distention, protein-rich foods, and elevated secretin or serum calcium levels.

G-17 is predominantly secreted by the antral mucosa of the stomach, where endocrine G cells secrete the peptide in response to the presence of amino acids, dietary amines, and calcium in the stomach, for the purpose of stimulating gastric acid secretion [38, 39]. Neutralization of acid or inhibition of acid secretion also stimulates gastrin release [40, 41, 42, 43, 44]. In addition to luminal stimuli, basolateral stimuli of the G cells by GRP or acetylcholine (ACh) from nerve fibers, or by humoral factors such as EGF, also cause the expression and secretion of gastrin in some species [24]. In response to increased acid levels or vasoactive intestinal peptide (VIP), endocrine D cells, in turn, secrete somatostatin, which acts to inhibit the secretion of gastrin [24].

G-17 has two major roles in the GI tract, the first of which is the well-known stimulation of gastric acid secretion in the stomach. The mature gastrins (as well as a small percentage of non-classical gastrins) are secreted via Ca^{2+}-

dependent release in the regulated secretory pathway by G cells in the gastric antrum and, to a lesser extent, the duodenum [54, 55]. These mature gastrins act upon ECL cells of the gastric fundus, stimulating the release of histamine, again dependent on intracellular calcium from stores or from the activation of calcium channels [47]. Histamine then, via paracrine diffusion, interacts with parietal cells, stimulating the upregulation of surface H^+/K^+ proton pumps and thus the gastric acid secretion. Gastrin itself and acetylcholine also act directly on parietal cells through surface receptors to stimulate acid secretion; in this case, histamine potentiates the activity of gastrin, but this effect is still secondary to the stimulation of acid secretion by histamine [48, 49]. Gastrin may also sensitize parietal cells to other acid secretagogues [50]. As acid secretion increases, D endocrine cells release somatostatin, which acts on parietal cells to inhibit acid secretion, and on ECL cells to inhibit histamine secretion, providing a negative feedback control mechanism [51, 52, 53, 54] *(Figure 6)*.

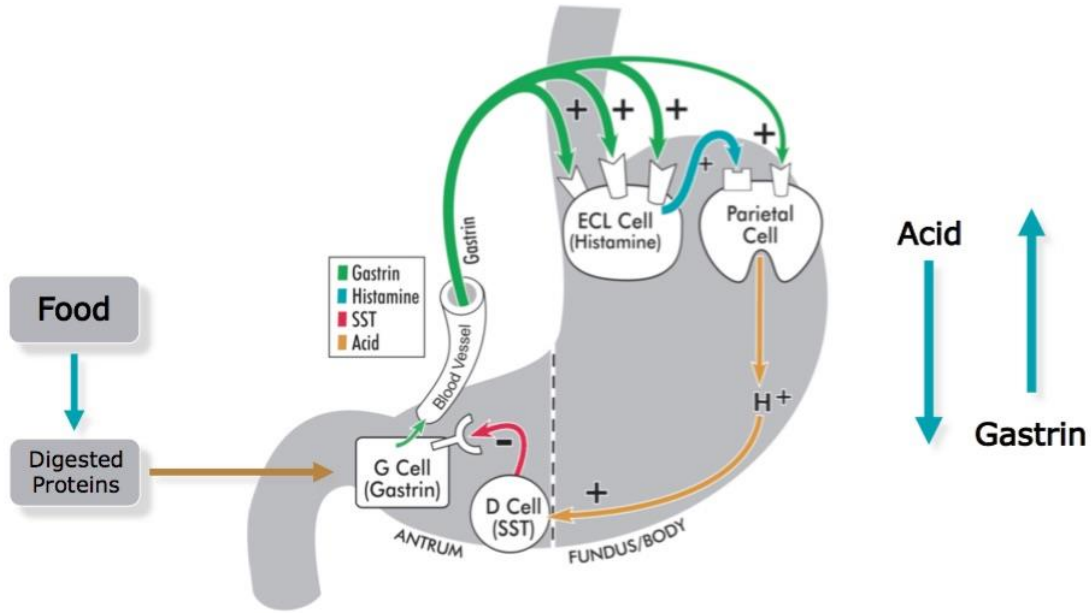

Figure 6. The negative feedback of G-17 on gastric acid secretion. *(Adapted from Goni E et al. [51])*

In the duodenum, secretin (through bicarbonate release) and CCK (through the potentiation of secretin) also inhibit HCl secretion as stomach contents pass into the small intestine. Additionally, gastrin promotes the maintenance and proliferation of the gastric epithelium [55, 56].

It has been suggested that gastrin itself is not a factor in tumor formation, but merely a marker of achlorhydria, which is the actual factor in causing infection and inflammation that leads to carcinogenesis [57, 58]. Thus, increased serum G-17 is a marker for hypochlorhydria or achlorhydria [59]. Normal value for G-17 is considered 1-7 pmol/l [60]. *Table 3* resumed the main clinical conditions leading to an increase or decrease of serum G-17 serum levels.

G-17	**Decrease**	**Increase**
NV 1-7 pmol/l	Pangastritis Gastrectomy	Hypo-/achlorhydria: a) PPI Intake b) Atrophic gastritis with or without pernicious anemia c) Dumping syndrome Gastrinoma Zollinger-Ellison syndrome

Table 3. Main clinical conditions leading to an increase or decrease of serum G-17 levels. G-17 normal value (NV) 1-7 pmol/L. [2, 51, 59].

2.3 Influence of PPI on pepsinogens and gastrin-17 assessment

PPIs are the most potent inhibitors of gastric acid secretion available. It has been reported in literature that serum PG I and G-17 levels rise during proton pump inhibitor (PPI) administration.

Di Mario F. *et al.* performed a study on 126 consecutive patients with upper gastrointestinal symptoms at baseline condition and after 2 months of PPI treatment. A significant increase in serum PG I levels was found after a 2-month treatment for all five different PPIs: omeprazole, pantoprazole, esomeprazole, lansoprazole and rabeprazole ($p < 0.05$). The effect of rabeprazole on PG I was less pronounced as compared with other PPIs, whereas esomeprazole achieved superior serum PG I levels, with no overall statistically significant difference among the five groups ($p > 0.05$). However,

a comparison within a single group of PPIs showed a statistical significance when the esomeprazole group was compared with the rabeprazole group (p = 0.007). PG I levels are significantly influenced by antisecretory therapy, rising under PPI treatment. Moreover, a statistically significant difference in PG I levels between the rabeprazole and esomeprazole groups has been demonstrated [61] *(Figure 7)*.

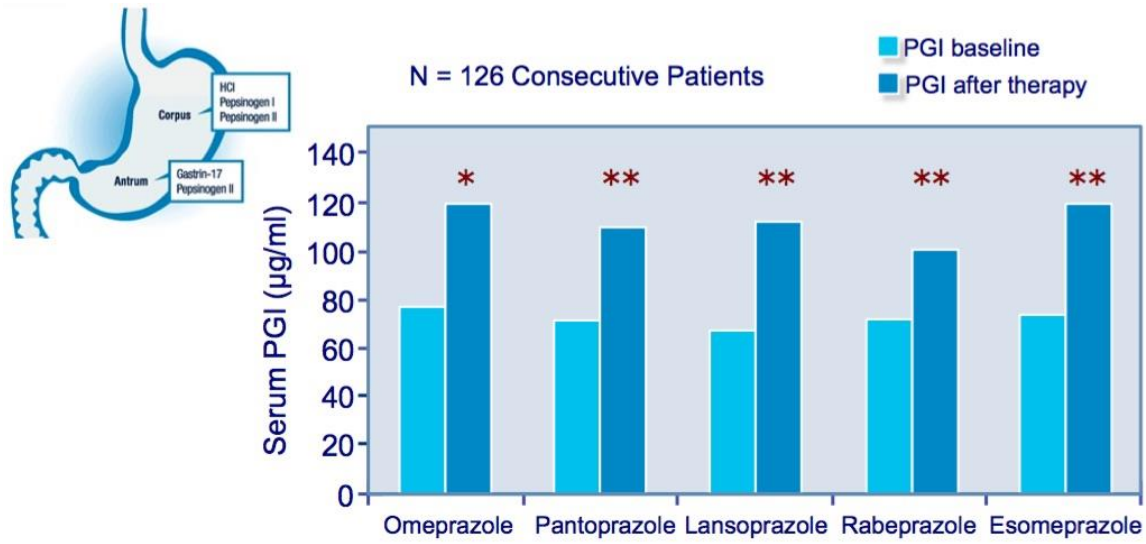

Figure 7. The influence of PPI intake on PG I at baseline and after therapy *(Adapted Di Mario F et al. [61])*

There is a significant inverse relationship between intragastric acidity and G-17 serum levels. All generally available gastric acid antisecretory drugs induce a release of G-17 into the circulation. As a consequence, patients receiving long-term treatment with PPIs show an increase in serum G-17 levels, which are however associated with considerable inter- and intra-individual variations [62]. Agreus L. *et al.* performed a study on 1000 Swedish subjects with uninfected stomach mucosa in order to examine the effect of PPIs, H_2 receptor antagonists (H_2RAs) and antacids/alginates on serum levels of G-17 and PGs. The results showed that PPIs but not antacids/alginates or H_2RAs markedly increase the fasting levels of serum G-17 and pepsinogens. Indeed, serum levels of G-17 or pepsinogens in the subjects who reported use of H_2RAs or

antacid/alginates during the previous 3 months did not differ from those in non-users. However, the median levels of G-17 and pepsinogens were significantly ($p < 0.001$) higher among the PPI users than among non-users: the levels were approximately doubled. The PG I/II ratio was, however, similar between PPI users and non-users, or those using antacids/alginates or H_2RAs. Among subjects using PPIs, the serum levels of pepsinogens correlated positively ($p < 0.01$) with the serum levels of G-17 [63, 64]. Recently, Martín-Alcolea M. *et al.* confirmed on 112 subjects that PPI chronic treatment decreased the diagnostic accuracy of PGs and particularly of G-17 [65].

Hypergastrinemia alone is unable to induce carcinoid formation in humans, as outlined by several long-term clinical trials with different members of this class of drugs. However, PPI-induced hypergastrinemia does stimulate hyperplasia of ECL cells, without provoking any neoplastic change. This effect on ECL cells has been more frequently observed in *H. pylori* positive patients, particularly in those with gastric atrophy and severe inflammation of gastric mucosa [62] *(Figure 8).*

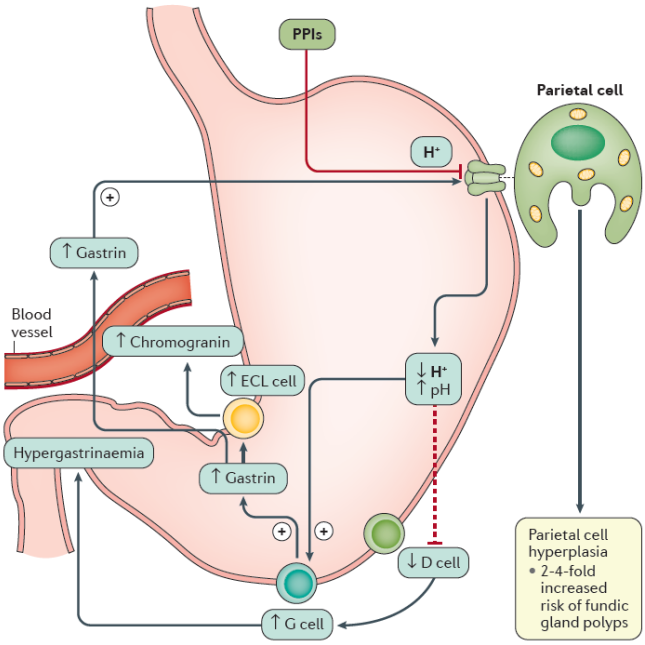

Figure 8. G-17-induced hyperplasia of ECL and parietal cells due to chronic PPI intake or hypo-achloridic conditions. *Increased gastrin levels can result from achlorhydria, chronic use of PPIs, or H. pylori infection. Gastric cancer epithelial cells that express CCK-B receptors also produce their own gastrin de novo, which in turn stimulates growth and metastases of gastric cancer by an autocrine mechanism. (Adapted from Malfertheiner P et al. [66]).*

2.4 Influence of clinical factors on pepsinogens and gastrin-17 assessment

H. pylori serology was a major source of variation in serum PGs levels. Independent of these effects, serum PGs levels may also vary with age, height and weight. In *H. pylori* seronegative participants, both PG I and PG II levels rose with increasing age; PG I levels increased with increasing height and were higher in smokers, but decreased with increasing weight. The effect of smoking on PG I levels was also detectable in seropositive individuals, but was considerably less marked [67].

Nakamura K. *et al.* studied 47 patients without upper GI disorders; acid secretion, pepsin secretion, serum pepsinogen level, and serum *H. pylori* antibody titer were measured. Dietary habits were investigated by questionnaire. Gastric secretion did not differ among the young, middle, and elderly age groups. Compared with the group without atrophy (PG I/II ratio > 3.0, 32 subjects), the group with atrophy (PG I/II ratio < or = 2.5, 11 subjects) showed significantly decreased gastric secretion and a significantly high *H. pylori* seropositive rate. In the group without atrophy, acid and pepsin secretion was significantly correlated with energy intake, pepsin secretion was significantly correlated with glucose intake, and gastric secretion tended to increase with age [68].

Tanaka M. *et al.* performed a cross-sectional study in 1,985 subjects who underwent a health screening test. Subjects had in medical history no medications for hyperuricemia, dyslipidemia, diabetes mellitus, or hypertension. All subjects were classified into two groups. Subjects with a pepsinogen I/II ratio below 3 were defined as having atrophic gastritis. Results underlined that PG I/II ratio was related to glucose, triacylglycerol, and uric acid levels. Indeed, a multiple linear regression analysis demonstrated that the PG I/II ratio was an independent determinant of glucose level ($\beta = 0.104$, $p < 0.0001$), triacylglycerol level ($\beta = 0.072$, $p = 0.0014$), uric acid level ($\beta = 0.048$, $p = 0.0138$), and hemoglobin ($\beta = 0.037$, $p = 0.0429$) after adjustments for age, sex, smoking status, alcohol consumption, and body mass index [69].

The data on the possible influence of preanalytical factors as well as on endoscopy-related effect on the measurement of PG I, PG II and G-17 in serum are lacking.

2.5 Aim of the study

The aim of the present study was to test the stability of PG I, PG II and G-17 over time and to evaluate the influence of endoscopy-related conditions on serum PG I, PG II and G-17 assessment.

3. Subjects and methods

3.1 Ethical statement

The study was approved by the local Ethics Committee (Number 80/11, *Anlage 1)*. All investigations were performed at the Department of Gastroenterology, Hepatology and Infectious Diseases at Otto-von-Guericke University Magdeburg (Germany) in 2015. All study participants provided a written informed consent *(Anlage 2).*

3.2 Study design

3.2.1 Enrollment period, inclusion and exclusion criteria

We designed a prospective study with an enrollment period from July 2015 to January 2016. Subjects over 18 years of age who had an indication for endoscopy (dyspeptic symptoms, screening colonoscopy) were included.

Exclusion criteria in the present study were as follows: age < 18 years, chronic PPI therapy in medical history and a positive pregnancy status.

3.2.2 Evaluation of stability of PG I, PG II and G-17 *(Part 1)*

The stability of serum biomarkers (PG I, PG II, and G-17) was evaluated in serum and plasma of 23 individuals (M:F=10:13), healthy individuals and patients, undergoing upper gastrointestinal (GI) endoscopy for dyspeptic symptoms.

All participants provided 20 ml of peripheral blood samples divided into one plasma and three serum aliquots, 5 ml each.

Serum and plasma samples were centrifuged (2000g at 4°C for 10 minutes) at different time points (T0=within 30 minutes, T6=6 hours and T24=24 hours, overnight) following the sample collection, with and without the addition of G-17 stabilizer (Biohit Oyj, Helsinki, Finland). *Figure 9a* summarizes the study design (Part 1).

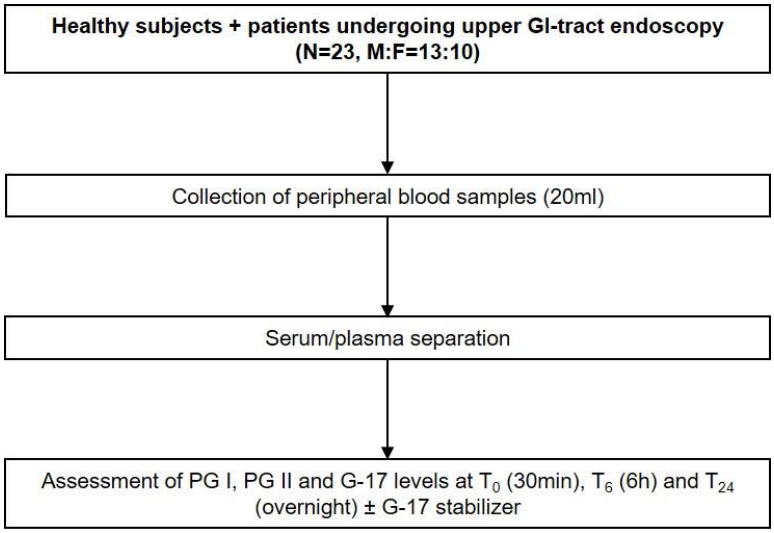

Figure 9a. Study design, Part 1: Evaluation of stability of PG I, PG II and G-17

3.2.3 Endoscopy-related factors on PG I, PG II and G-17 *(Part 2)*

In a second step, the influence of endoscopy-related factors on serum biomarkers was investigated.

3.2.3.1 Influence of upper GI endoscopy

We prospectively enrolled additionally 10 patients (M:F=4:10) undergoing upper GI endoscopy for dyspeptic symptoms.

PG I, PG II and G-17 were determined in blood samples (5 ml each) obtained within 30 minutes before and 30 minutes after the endoscopic procedure of patients requiring upper GI endoscopy for dyspeptic symptoms. Upper GI endoscopy was performed between 08.00 and 12.00 h in the morning, after an overnight fast using Olympus (Olympus Europe, Hamburg, Germany) and Fujifilm (Fujifilm Europe, Düsseldorf, Germany) endoscopes. Sedation was performed either with Propofol and/or Midazolam, according to the German Guidelines [70].

3.2.3.2 Influence of bowel cleansing

Eighteen patients (M:F=14:4) undergoing bowel cleansing for colonoscopy were included.

To evaluate the impact of colonoscopy preparation on serum PG I, PG II, and G-17 values, we determined these biomarkers in blood samples of patients admitted in our department and undergoing colonoscopy for different

indications. Blood samples were obtained after an overnight fast at two different time points, that is 1 day before and the day on which colonoscopy was scheduled before endoscopic investigation was performed. All patients underwent colonoscopy preparation with a solution containing Macrogol 3350, sodium sulfate, sodium bicarbonate, sodium chloride, and potassium chloride in split doses of 2 liters in the evening and 2 liters in the morning. *Figure 9b* gives an overview of the study design (Part 2).

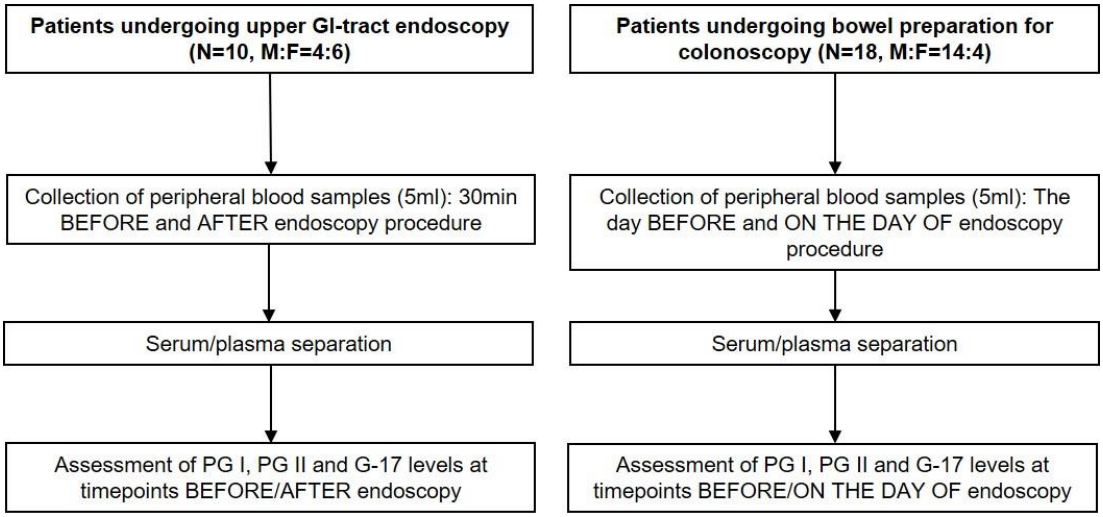

Figure 9b. Study design, Part 2: Evaluation of the role of endoscopy-related factors on PG I, PG II and G-17

3.3 Assessment of serum parameters

3.3.1 Enzyme-linked immunosorbent assay analysis

Blood samples were centrifuged (2000g at 4°C for 10 minutes) and stored at -80°C, with and without the addition of 25µl of G-17 stabilizer (Biohit Oyj, Helsinki, Finland) at different time points (T_0=within 30 minutes, T_6=6 hours and T_{24}=24 hours, overnight) by means of enzyme-linked immunosorbent assay (ELISA) (Biohit Oyj, Helsinki, Finland) according to manufacturers' instructions.

This PG I ELISA is based on a sandwich enzyme immunoassay technique with a PG I specific capture antibody adsorbed on a microplate and a detection antibody labeled with horseradish peroxidase (HRP).

The assay proceeds according to the following instructions:

1. A monoclonal antibody, specific to human PG I, PG II and G-17, on the polystyrene surface of the wells binds PG I, PG II and G-17 molecules present in the sample.
2. Wells are washed to remove the residual sample.
3. An HRP-conjugated monoclonal detection antibody is added to the wells and it binds to the PG I molecules.
4. The wells are washed and TMB-substrate is added. The substrate is oxygenized by the enzyme and a blue colored end product is produced.
5. The enzyme reaction is terminated with stop solution. The solution in the microwells should turn yellow. The intensity of the yellowish color developed is directly related to the PG I concentration of the sample.

3.4 Statistical analysis

All statistical analyses were performed using Graph Pad Prism Pro Version 6.0 (Graph Pad Software, San Diego, CA, USA). For groupwise comparison of parametrical data we used student's t-test, Wilcoxon-test for comparison of two groups and the Friedman’s test for non-parametrical comparisons of three or more groups with Dunn´s multiple comparisons post-test. For all tests a two-sided $p < 0.05$ was considered to be statistically significant.

4. Results

4.1 Stability of PG I, PG II and G-17

Overall, serum and plasma samples obtained of 23 subjects (M:F= 10:13; mean age ± SD 53.3 ± 19.4) were recruited for the stability analysis of PG I, PG II, G-17.

Overall, PG I and PG II were stable over a period of 24 hours (PG I mean values ± SD T_0 166.9±114.1µg/l, T_6 164.6±108.8 µg/l, T_{24} 166.6±113.3 µg/l, Friedman's test *p* value > 0.9; PG II mean values ± SD T_0 14.1±7.9 µg/l, T_6 14.3±8.2 µg/l, T_{24} 14.0±8.1 µg/l, Friedman's test *p* value= 0.8) whereas serum values of G-17 showed a statistical significant decrease over time (G-17 mean values ± SD T_0 12.0±19.5 pmol/l, T_6 10.7±17.8 pmol/l, T_{24} 8.2±14.8 pmol/l, Friedman's test *p* value = 0.0001). The relative decrease in G-17 value was of 20.9% and 60.7% at 6 and 24 hours after blood collection, respectively *(Figure 10).*

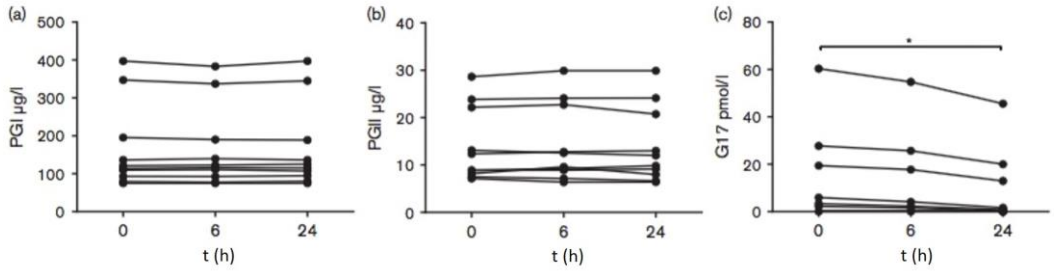

Figure 10. Stability over time of serum PG I, PG II and G-17 levels
*a) PG I (µg/l) b) PG II (µg/l) c) G-17 (pmol/l); T_0=within 30 minutes, T_6= 6 hours, T_{24}= 24 hours (overnight). Friedman's test for non-parametrical comparisons of three or more groups with Dunn´s multiple comparisons post-test *p < 0.05*

Having shown the G-17 time-dependent degradation, we questioned if some pre-analytical optimization such as using plasma or serum samples or adding stabilizer or the time point of centrifugation could have an influence on the biomarkers assessment.

Furthermore, we evaluated the difference between serum and plasma samples. PG I, PG II and G-17 levels were comparable between serum and plasma (PG I mean values ± SD in serum *versus* plasma: 191.5±163.4 µg/l vs 192.7±163.9 µg/l; Wilcoxon-test *p* value = 0.6; PG II mean values ± SD serum 33.4±58.5 µg/l *versus* plasma 31.5±52.3 µg/l; Wilcoxon-test *p* value= 0.4; G-17 mean values ± SD serum 25.1±36.1 pmol/l *versus* plasma 25.1±37.3 pmol/l; Wilcoxon-test *p* value= 0.2) *(Table 4).*

	Serum (mean ± SD)	**Serum + Stabilizer** (mean ± SD)	**Plasma** (mean ± SD)	**Plasma + Stabilizer** (mean ± SD)	***p* value**
PG I (µg/l)	191.5 ± 163.4	191.6 ± 158.5	192.7 ± 163.9	187.4 ± 149.5	0.4
PG II (µg/l)	33.4 ± 58.5	32.6 ± 54.4	31.5 ± 52.3	30.4 ± 50.3	0.1
G-17 (pmol/l)	25.1 ± 36.1	26.5 ± 38.7	25.1 ± 37.3	27.5 ± 41.4	0.03

Table 4. Impact of stabilizer on stability of PG I, PG II, G-17 at T_0 (within 30 minutes from blood samples collection) *Friedman's test for non-parametrical comparisons of three or more groups with Dunn´s multiple comparisons post-test *p < 0.05*

Finally, we evaluated whether the addition of a stabilizer or the time point of centrifugation may influence biomarker assessment. The use of G-17 stabilizer has been proposed by the manufacturer to improve G-17 stability. Addition of G-17 stabilizer had no effect on the serum and plasma stability of PG I, PG II (PG I Friedman's test p value= 0.4; PG II Friedman's test p value= 0.1). However, the addition of G-17 stabilizer improve the G-17 stability in plasma (G-17 mean values ± SD plasma without stabilizer 25.0±37.3 pmol/l versus plasma with stabilizer 27.4±41.4; Wilcoxon-test p value = 0.007) but not in serum (G-17 mean values ± SD serum without stabilizer 25.1±36.1 pmol/l versus serum with stabilizer 26.5±38.7 pmol/L; Wilcoxon-test p value = 0.5) (*Figure 11).*

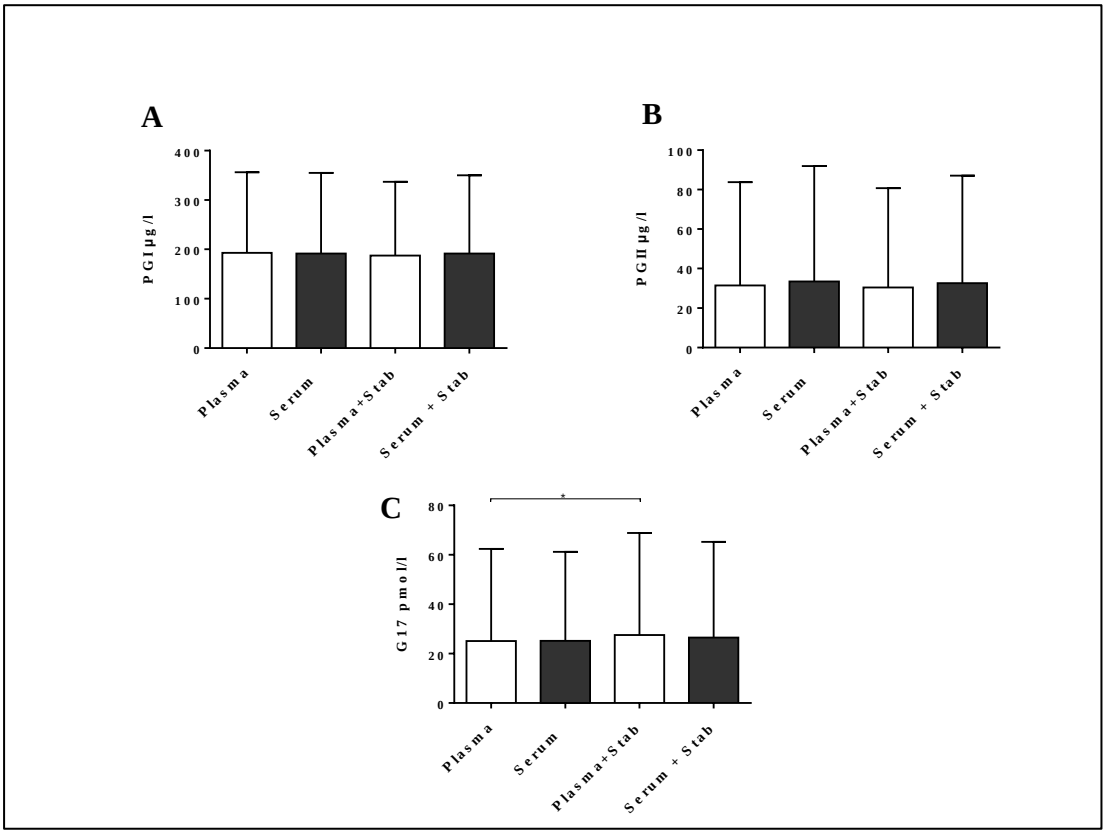

Figure 11. PG I, PG II and G-17 levels in plasma and serum with and without stabilizer. *Assessment of plasma and serum for (A) PG I (µg/l) (B) PG II (µg/l) (C) G-17 (pmol/l). Friedman’s test for non-parametrical comparisons of three or more groups with Dunn´s multiple comparisons post-test. * $p < 0.05$*

G-17 stabilizer did not improve the stability in serum of G-17 over time even in samples with immediate centrifugation (within 30 minutes after sample collection) and storage. Our results confirmed that G-17 assessment depends on time to processing and storage *(Figure 12).* Indeed, G-17 levels were decreased by 20.9% and 60.7% in serum samples that were centrifuged and stored 6 and 24 hours after blood collection, respectively and this was independent from the addition of the stabilizer. For samples that were centrifuged immediately but further stored at room temperature, the addition of the stabilizer was associated with improved stability (95.8% versus 82.8% after 6 hours and 78.9% versus 54.0% after 24 hours) compared to samples without G-17 stabilizer, respectively *(Figure 12).*

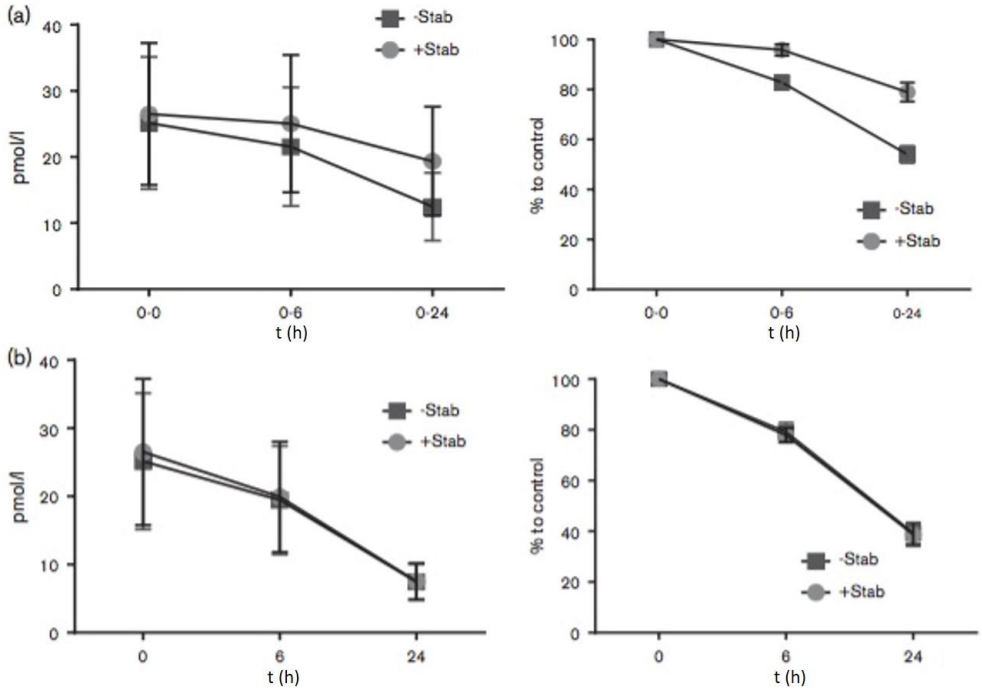

Figure 12. The role of G-17 stabilizer addition, before and after samples processing ***A)*** *Centrifugation and storage, with or without the addition of stabilizer addition immediately before sample processing; T_0= within 30 minutes, T_6= 6 hours, T_{24}= 24 hours (overnight); % to control= percentage of variation in G-17 serum levels; Stab: G-17 stabilizer.* ***B)*** *Addition of stabilizer after samples processing and storage; Stab: G-17 stabilizer. T_0=within 30 minutes, T_6= 6 hours, T_{24}= 24 hours (overnight); % to control= percentage of variation in G-17 serum levels; Stab: G-17 stabilizer.*

4.2 Influence of endoscopy-related factors

4.2.1 Influence of upper GI endoscopy

The influence of endoscopy-related factors on serum PG I, PG II, G-17 was analyzed in the 10 patients undergoing upper GI endoscopy (M:F= 4:6, mean age ± SD 58.3±19.4).

Upper GI endoscopy did not did not influence serum PG I (mean values ± SD before 213.6±181.8 µg/l versus after 212.8±189.5 µg/l; Wilcoxon-test p value= 0.16), PG II (mean values ± SD before 40.3±65.8 µg/l versus after 38.8±65.8 µg/l; Wilcoxon-test p value= 0.01) and G-17 (mean values ± SD before 32.2±38.9 pmol/l versus after 29.7±34.0 pmol/l; Wilcoxon-test p value >0.99) assessment *(Table 5).*

Overall no changes were observed for PG I and PG II serum levels before and after upper GI endoscopy. However, substantial patients-specific variations in

serum G-17 levels were observed with respect to endoscopic procedures. For instance, 30% patients showed an increase and 30% a decrease in G-17 levels after upper GI endoscopy.

4.2.2 Bowel preparation for colonoscopy

Eighteen patients (N=18, M:F= 14:4, mean age ± SD 66.6±13.3) undergoing colonoscopy were prospectively enrolled in order to assess the potential influence of bowel cleansing on the assessment of gastric biomarkers. Serum values of the analyzed biomarkers one day before and at the day in which colonoscopy was scheduled were as follows: PG I (mean values ± SD 154.6±83.0 µg/l *versus* 147.4±83.3 µg/l; Wilcoxon-test *p value*= 0.9), PG II (mean values ± SD 10.9±6.0 µg/l *versus* 10.5.4±6.1 µg/l; Wilcoxon-test *p value*= 0.9), G-17 (mean values ± SD 13.3±17.2 pmol/l *versus* 16.6±14.8 pmol/l; Wilcoxon-test *p value*= 0.2) *(Table 4)*. Bowel preparation did not influence serum PG I and PG II values. With respect to serum G-17, individual variations between the two time points of blood collection were observed. In particular, serum G-17 values increased in 4 patients (22.2%), decreased in 4 patients (22.2%) and remained approximate similar in the remaining 10 patients (55.5%) *(Table 5).*

	Upper GI endoscopy (N=10)			**Bowel cleansing** (N=18)		
	Before (mean ± SD)	**After** (mean ± SD)	***p* value**	**Before** (mean ± SD)	**After** (mean ± SD)	***p* value**
PG I (µg/l)	213.6±118.8	212.8±189.5	0.16	154.6±83.0	147.4±83.3	0.9
PG II (µg/l)	40.3±65.9	38.8±65.3	0.01	10.9±6.0	10.5±6.1	0.9
G-17 (pmol/l)	32.2±38.7	29.8±34.0	> 0.99	13.3±17.2	16.6±14.9	0.2

Table 5. The influence of upper GI endoscopy and bowel cleansing on PG I, PG II, G-17 assessment. *Wilcoxon-test *p<0.05*

5. Discussion

Serological tests for the diagnosis of chronic gastritis and gastric atrophy have been in use for more than 25 years. These include *H. pylori* serology (crude antigen with or without additional determination of anti-CagA antibodies) for the diagnosis of gastritis, and serum pepsinogen I and II and gastrin for the diagnosis of gland loss resulting in hypoacidity. These tests are usually applied in panels of tests and have been shown to be useful as non-invasive diagnostic tool in the management of individual patient and as population screening and surveillance tool [26, 27, 28].

In clinical practice, serum PG I and PG II are established as non-invasive biomarkers for the detection of gastric atrophy. Patients with CAG with or without intestinal metaplasia have a risk up to twenty times to develop gastric cancer [71]. The risk is correlated with atrophic gastritis severity assessed by the updated Sydney classification. To the individual cancer risk, this is integrated into the operative link on gastritis (O.L.G.A.) staging system. Severe atrophic gastritis (O.L.G.A. III and IV) is associated with low serum PG I and low serum PG I/II ratio [73, 74]. International guidelines recommend endoscopic follow-up and gastric biopsies for subjects with atrophic gastritis, even after *H. pylori* eradication, to early detect gastric cancer and reduce mortality [27, 75]. However, identifying subjects with an underlying atrophic gastritis is still an issue. Gastroscopy and histology are the reference standard, but the use of endoscopy as a screening test is costly, uncomfortable and does not have good patient's compliance [75]. In countries with high gastric cancer incidence, non-invasive testing for severe atrophic gastritis by determination of serum pepsinogens with anti-*Helicobacter pylori* antibodies (HpAb) is a validated gastric cancer screening strategy. However, this strategy is little used in Europe, which has low to intermediate risk of gastric cancer. Furthermore, inconsistent results in the performance of serological testing for atrophic gastritis in Europe have discouraged its use. However, there is justification for non-invasive gastric cancer screening tools in Europe, where the disease is too often detected at an advanced stage [76, 77].

Recently, the Maastricht V Consensus Report recommended PG serology as the most useful non-invasive test to explore the gastric mucosa status (non-atrophic vs atrophic), however the PG I/PG II ratio can never be assumed as a biomarker of gastric neoplasia and the predictive value of PG testing is limited in patients with antrum-restricted atrophy [27, 78]. Moreover, as observed by Shiotani *et al.*, the reliability of PG testing “clearly depends on the cut-off of serum PG levels as well as the definition used to identify atrophy” [79]. A panel of serological tests (GastroPanel®) including serum PG I and PG II, G-17 and anti-*H. pylori* antibodies has recently been proposed as ‘serological biopsy’ in dyspeptic patients [75, 80]. In populations with a low prevalence of atrophic gastritis, the negative predictive value of the GastroPanel® in identifying atrophic gastritis is as high as 97% (95% CI 95% to 99%) [33].

An important milestone for PG’s as markers of atrophic gastritis has been the Kyoto Global Consensus Conference where the experts unequivocally agreed on serological tests (PG I and PG II and anti-*H. pylori* antibody) are useful for identifying patients at increased risk for gastric cancer” (Grade of recommendation: strong, Evidence level: high, Consensus level: 91.9%) [28]. The evidences for this statement are based on several large trials.

A Japanese cohort of 9,293 screenees underwent serological assessment by means of *H. pylori* serology and PG I and PG II measurement [99]. The annual progression to gastric cancer was very low in subjects with normal pepsinogens, irrespective of *H. pylori* status. The annual progression to gastric cancer was substantially higher (3.5–6 per 1000 per year) in individuals with low serum pepsinogen levels, compatible with presence of atrophic gastritis [81].

Miki K. *et al.* studied the feasibility of measuring serum PGs and PG I/II ratio levels for detection of gastric cancers in asymptomatic middle-aged Japanese between 1991 and 2005. The total number of subjects was 101,892 (mean age of 48.7 years). Subjects with a PG I ≤ 70 ng/ml and PG I/II ≤ 3 were defined as having a positive PG test. According to the obtained results of serum PG levels and previous individual records, those with a positive PG test and those

with a negative PG test took upper GI endoscopy every 2 and 5 years, respectively. In a total of 21,178 planned gastroendoscopies (20.8%), 13,789 (65.1%) underwent gastroendoscopy and 125 gastric cancers were detected (0.12% of all participants and to 0.91% of those with gastroendoscopy). Early-stage cancers and intestinal-type intramucosal cancers accounted for 80% and 39% of all the detected cancers, respectively. In conclusion, this study underlined that mass screening for gastric cancer by means of serum PG assessment led to high detection rates of mostly early-stage gastric cancer among the screened individuals [82]. Moreover, the same authors suggested a possible difference in the secretion and assessment of serum PGs between East and West population [83]. From the same research group, Osami H. *et al.* enrolled 650 patients received *H.pylori* eradication therapy from October 2008 to March 2013 and the evaluated the relationship between *H. pylori* eradication and percentage changes in serum PG I/II ratios before and 3 months after treatment. They found that an increasing percentage in the serum levels of the PG I/II ratios after treatment compared with the values before treatment clearly distinguished success from failure of eradication (108.2±57.2 vs. 6.8±30.7, $p<0.05$) [84].

The role of G-17 as non-invasive tool in the diagnostic of CAG as well as as marker to identify patients at increased risk for gastric cancer is still a matter of debate.

Zagari RM et *al.* performed a systematic review with meta-analysis of 20 studies with a total of 4,241 subjects (mostly from Europe) assessing the performance of serum panel test (PG I, PG II, G-17 and anti-H. pylori antibodies) for the diagnosis of atrophic gastritis regardless of the site in the stomach. The results showed that the combination of PG I, PG II, G-17 and anti-*H. pylori* antibodies assays appears to be a reliable tool for the diagnosis of CAG. The summary sensitivity was 74.7% (95%CI, 62.0% to 84.3%), specificity 95.6% (95%CI, 92.6% to 97.4%), positive likelihood ratio 16.9 (95%CI, 9.5 to 30.1) and negative likelihood ratio 0.26 (95%CI, .17 to .41). Using the median prevalence of atrophic gastritis across the studies of 27%,

the negative predictive value of the panel test was 91% and the positive predictive value was 86%. Authors concluded that the combination of pepsinogens, G-17 and anti-*H. pylori* antibodies serological assays appears to be a reliable tool for the diagnosis of atrophic gastritis. This test may be used for screening subjects or populations at high risk of gastric cancer for atrophic gastritis; however, a cost-effectiveness analysis is still missing. [85]. On the contrary, McNicholl AG and Colleagues demonstrated on ninentyone patients that the G-17 accuracy in the detection of CAG was only acceptable in the case of corpus localization [area under the receiver operating characteristic curve (AUC), 74%] [33].

The role of serum PGs and G-17 in the gastric pathophysiology and pathology has been investigated in several studies, however there are controversies regarding the translation of the results into the clinical practice.
This can be attributed to differences in study methodology, control group selection and different grading of gastric atrophy.
Furthermore, there continue to be uncertainties related to the methodology, the stability and reproducibility of these parameters depending on timing, sampling and processing of samples.

In the prospective study, we evaluated factors and conditions that might have an influence on the determination of these parameters in order to provide reproducible results.

5.1 Stability of PG I, PG II and G-17

The stability over time of the biomarker, reproducibility and simplicity of the method are crucial factors for the biomarker determination and translation into the clinical practice [25, 26]. Our results confirmed that PG I, PG II and G-17 levels showed no differences in serum versus plasma assessment. Moreover, we demonstrated that PG I and PG II levels are not influenced by pre-analytical factors including storage time at different time points, centrifugation and therefore can be considered reliable biomarkers.

Recently, a study including 91 patients analyzed the value of PG I, PG II, G-17 as non-invasive biomarkers in the diagnosis of atrophic gastritis [33]. In particular G-17, according to the authors, was not providing any additional diagnostic value. One of the potential explanations may be related to the stability of G-17 in serum samples over time. Indeed, we show that G-17 serum and plasma levels undergo substantial degradation depending on sample processing and storage. Compared with the samples immediately processed with best possible time/efficiency following blood collection, a serum level variation of 20.9 % is observed when samples were processed and stored no later than 6 hours after blood sampling. The variation of G-17 in serum reached up to 60.7% for samples centrifuged and stored within 24 hours after blood collection independently of the addition of the stabilizer. Therefore, we suggest that in real-life settings, documentation and processing should ideally be carried out within 30-60 minutes of sample recovery. While this time interval may be potentially difficult to attain in the clinical routine, a window of 6 hours should be observed.

G-17 degradation is one of the most important limitations of the biomarker in clinical practice. The addition of G-17 stabilizer did not improve of G-17 stability over time also in samples with immediate centrifugation (within 30 minutes after sample collection) and storage. The majority of previous studies available do not specify the time point of centrifugation, processing and storage time after samples collection. Our findings suggest that time of samples collection and processing should be carefully documented and specified for obtaining reliable results. Since also the addition of a stabilizer to the serum sample was not effective in antagonizing G-17 degradation over time the degradation dynamics would allow for normalizing the G-17 levels by a formula that considers time point of sampling and degradation in percentage per hour.

5.2 Influence of endoscopy-related factors

Several investigations report on a positive association between *H. pylori* infection and increased risk for the development of colonic neoplasms [86, 87, 88, 89, 90, 91]. This has led to the proposal of combining colorectal cancer

(CRC) screening with PG I, PG II and G-17 assessment. Our study demonstrated that upper GI endoscopy and bowel preparation did not influence serum PG I, PG II values. However, endoscopy procedures and bowel cleansing lead to slight but not significant changes in G-17 levels, associated with an intra-individual variation in 30% of the subjects. G-17 variations may be due mechanical stimulation of the gastric mucosa resulting from bowel cleansing, or gastric air insufflation, pH-changes or even biopsy sampling during upper GI endoscopy. Therefore, due to variations in gastric serum parameters, especially G-17 following endoscopy or bowel cleansing preparation, we stress that blood sampling should be performed only during the fasting state before endoscopic or other interventional procedures on the stomach and bowel.

5.3 Translation into the clinical practice

Our study showed that stability and reproducibility of these parameters depending on timing, sampling and processing of samples.

In future clinical studies, it is advised to take blood samples in the fasting state and prior to endoscopical procedures and bowel cleansing. Timing of samples collection and determination should be registered and specified in order to provide reproducible results.

5.4 Study limitations

The essential limitations of our study are the small size of the cohort; the heterogenity of the population (e.g medications, comorbilities) could limit the reproducibility in a study population of healthy subjects or patients.

6. Conclusions

In summary, PG I and PG II serum levels are stable overtime and their assessment is not influenced by pre-analytical factors such as time of centrifugation, storage or addition of stabilizer. On the contrary, G-17 stability was closely dependent on time to processing and storage and addition of G-17 stabilizer does not provide an additional benefit if the samples is frozen following centrifugation.

In future clinical studies, it is advised to take blood samples in the fasting state and prior to endoscopical procedures and bowel cleansing. Timing of samples collection and determination should be registered and specified in order to provide reproducible results.

7. Abstract

7.1 English Version

Serum pepsinogen I (PG I), pepsinogen II (PG II) are non-invasive parameters in the detection of atrophic gastritis. The diagnostic add on value of serum gastrin-17 (G-17) remains uncertain. The aim of the study was to assess the stability of these serum parameters over time and to evaluate the influence of clinical factors, such as upper GI endoscopy and bowel cleansing, on serum PG I, PG II and G-17 assessment.

A prospective study was conducted in healthy subjects and patients. For stability analyses the plasma and serum samples from 23 subjects were processed at different time points (T_0=30 min, T_1=6 hours, T_2=24 hours) with and without the addition of a stabilizer (Biohit Oyj, Helsinki, Finland). Ten patients were included to evaluate the influence of upper GI endoscopy and 18 patients to evaluate the effect of bowel cleansing before colonoscopy. Serum and plasma PG I, PG II and G-17 levels were assessed by means of enzyme-linked immunosorbent assay (ELISA).

PG I, PG II and G-17 levels were not statistically different in serum and plasma. PG I and PG II serum levels were stable overtime and their assessment is not influenced by laboratory factors including time of centrifugation and storage and addiction of stabilizer. G-17 is associated with time-dependent degradation (p= 0.0001) with an overall decrease of G-17 level of ~20% and ~60% in serum samples that were centrifuged and stored 6 hours or 24 hours after blood collection, respectively. The addition of the G-17 stabilizer showed no improvement of stability compared to samples with immediate centrifugation and storage. Upper GI endoscopy and bowel preparation prior colonoscopy were associated with minimal variations in PG I, PG II, while G-17 showed subject-specific alterations. In conclusion, PG I and PG II serum levels are stable overtime. However, G-17 stability is closely dependent on time of processing and storage; therefore samples for G-17 analysis need to be processed no later than 6 hours after blood collection. Upper GI endoscopy

and colonoscopy preparation lead to minimal non-significant changes in basal PG I, PG II and G-17 levels.

7.2 German Version *(Zusammenfassung auf Deutsch)*

Der Einfluss von laborchemischen und klinischen Faktoren bei der Bestimmung von Pepsinogen I, Pepsinogen II und Gastrin-17.

Serum-Pepsinogen I (PG I), Serum-Pepsinogen II (PG II) und Serum Gastrin-17 stellen nicht-invasive Parameter zur Diagnostik der atrophischen Gastritis dar. Ziel dieser Studie war die Untersuchung der zeitabhängigen Stabilität von Serum PG I, PG II und G-17, sowie die Evaluation des Einflusses endoskopischer Prozeduren wie Ösophagogastroduodenoskopie (ÖGD) mit Biopsieentnahme und die Koloskopievorbereitung.

Die prospektive Studie wurde bei gesunden Probanden (n= 3) und Patienten (n= 28) durchgeführt. Es flossen 10 ÖGDs und 18 Koloskopien in die Auswertung ein. Für die Stabilitätsanalyse wurden Plasma-und Serumproben zu unterschiedlichen Zeitpunkten (T_0 = 30min, T_1 = 6h, T_2 =24h) vor und nach der endoskopischen Intervention mit und ohne Zusatz von Stabilisatoren (Biohit Oyj, Helsinki, Finland) analysiert. Serum und Plasma PG I, PG II und G-17 Konzentrationen wurden mittels enzyme-linked immunosorbent assay (ELISA) untersucht.

PG I, PG II und G-17 wiesen keine Konzentrationsunterschiede im Serum und Plasma auf. In beiden Medien wurden zu jedem Zeitpunkt die PG I, PG II Konzentrationen in vergleichbarer Höhe gemessen (PG I p> 0.9; PG II p= 0.8) und laborchemischen Faktoren inklusive Zentrifugationszeit, Lagerungszeit und Zusatz von Stabilisatoren beeinflussten diese Messergebnisse nicht. Die G-17 Serumkonzentrationen unterlagen einer zeitabhängigen Degradation (p= 0.0001). Nach Zentrifugation und einer Aufbewahrungszeit von 6h und 24h sank die Serumkonzentration um 20% respektive 60% ab. PG I und PG II zeigten vor ÖGD und Koloskopievorbereitung nur minimale Spiegelunterschiede.

PG I und PG II Serumspiegel zeigen sich zeitlich stabil. Die Stabilität von G-17 hängt von der Verarbeitungs- und Lagerungsdauer ab; die Proben sollten aufgrund der Instabilität von G-17 innerhalb von 6 Stunden nach Entnahme weiterverarbeitet werden.

ÖGD und Koloskopievorbereitung haben keinen Effekt auf die basalen PG I, PG II und G-17 Konzentrationen.

8. References

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9. Annexes

9.1 Ethics Committee

OTTO-VON-GUERICKE-UNIVERSITÄT MAGDEBURG
Ethik-Kommission
der Otto-von-Guericke-Universität an der Medizinischen Fakultät
und am Universitätsklinikum Magdeburg A.ö.R.
Vorsitzender: Univ.-Prof. Dr. med. C. Huth

Universitätsklinikum • Leipziger Straße 44 • D-39120 Magdeburg

Herrn Prof. Dr. med. P. Malfertheiner
Universitätsklinik für Gastroenterologie, Hepatologie und Infektiologie
Universitätsklinikum Magdeburg A.ö.R.
Leipziger Str. 44
39120 Magdeburg

Tel.	Fax	eMail	Datum
(0391) 67/14314 67/14344	(0391) 67/14354 67/290185	norbert.beck@med.ovgu.de ethikkommission@ovgu.de	

13.07.2011

Unser Zeichen: **80/11**

Adaptive Helicobacter pylori assoziierte genetische Marker und Wirtssuszeptibilitätsfaktoren in der Magenkarzinogenese

Sehr geehrter Herr Prof. Malfertheiner,
sehr geehrte Kolleginnen und Kollegen,

die Ethik-Kommission der Otto-von-Guericke-Universität an der Medizinischen Fakultät und am Universitätsklinikum Magdeburg hat die übergebenen Unterlagen zur o. g. Studie überprüft, in der letzten Kommissionssitzung eingehend erörtert und ist zu der Auffassung gekommen, dass gegen die Durchführung keine ethischen Bedenken bestehen.
Diese **zustimmende Bewertung** ergeht unter dem Vorbehalt gleichbleibender Gegebenheiten.

Die Verantwortlichkeit des jeweiligen Prüfwissenschaftlers / behandelnden Prüfarztes bleibt in vollem Umfang erhalten und wird durch diese Entscheidung nicht berührt. Alle zivil- oder haftungsrechtlichen Folgen, die sich ergeben könnten, verbleiben uneingeschränkt beim Projektleiter und seinen Mitarbeitern.

Beim Monitoring sind die Bestimmungen des Bundes- und Landesdatenschutzgesetzes sowie die sich aus der ärztlichen Schweigepflicht ergebenden Einschränkungen zu beachten, was eine Aushändigung kompletter Patientenakten zum Monitoring ausschließt.
Ein Monitoring personen- und studienbezogener Daten wird dadurch nicht beeinträchtigt.

Um die Übersendung von studienbezogenen Jahresberichten / Abschlussberichten / Publikationen wird unter Nennung unserer Registraturnummer gebeten.

Mit freundlichen Grüßen

(i. A. Dr. med. Norbert Beck, Geschäftsführer)
Prof. Dr. med. C. Huth
Vorsitzender der Ethik-Kommission

Ethik-Kommission
der Otto-von-Guericke-Universität an der Medizinischen Fakultät
und am Universitätsklinikum Magdeburg A.ö.R.
Vorsitzender: Univ.-Prof. Dr. med. C. Huth

Anlage zum Votum der Studie 80/11 vom 13.07.2011

Zum Zeitpunkt der Bewertung der vorstehenden Studie waren folgende Damen und Herren Mitglied der Ethik-Kommission der Otto-von-Guericke-Universität an der Medizinischen Fakultät und am Universitätsklinikum Magdeburg:

Herr Prof. Dr. med. Norbert Bannert	Medizinische Fakultät / Universitätsklinikum, Pädiater
Frau Prof. Dr. phil. Eva Brinkschulte	Medizinische Fakultät / Universitätsklinikum, Bereich Geschichte, Ethik und Theorie der Medizin
Herr Prof. Dr.-Ing. Rolf Findeisen	Fakultät für Elektrotechnik und Informationstechnik, Institut für Automatisierungstechnik
Herr Prof. Dr. med. Thomas Fischer	Medizinische Fakultät / Universitätsklinikum, Universitätsklinik für Hämatologie und Onkologie
Herr Prof. Dr. med. Christof Huth	Medizinische Fakultät / Universitätsklinikum, Universitätsklinik für Herz- und Thoraxchirurgie
Frau Assessorin Ute Klanten	Medizinische Fakultät / Universitätsklinikum, Stabsstelle Recht
Herr OA Dr. med. Werner Kuchheuser	Medizinische Fakultät / Universitätsklinikum, Institut für Rechtsmedizin
Herr Prof. Dr. rer. nat. Jürgen Läuter	Medizinische Fakultät / Universitätsklinikum, Mathematiker, Biometriker
Herr Prof. Dr. phil. Georg Lohmann	Fakultät Geistes-, Sozial- und Erziehungswissenschaften, Institut für Philosophie
Herr Prof. Dr. med. Frank Peter Meyer	Medizinische Fakultät / Universitätsklinikum, Klinischer Pharmakologe

Mitglieder der Ethik-Kommission, die in eine Studie eingebunden sind, haben für die Votierung der betreffenden Studie kein Stimmrecht.

Die Ethik-Kommission der Otto-von-Guericke-Universität an der Medizinischen Fakultät und am Universitätsklinikum Magdeburg ist unter Beachtung entsprechender internationaler Richtlinien (ICH, GCP) und nationaler Richtlinien (AMG, GCP-V, MPG, MPKPV) tätig, nach Landesrecht (Hochschulmedizingesetz des Landes Sachsen-Anhalt § 1 Abs. 4, Verordnung über Ethik-Kommissionen zur Bewertung klinischer Prüfungen von Arzneimitteln - Ethik-Kom-VO LSA - i. d. akt. Fassung) legitimiert. Weiterhin besteht eine Registrierung der Ethik-Kommission beim Bundesamt für Strahlenschutz nach § 28g Röntgenverordnung (EK-043/R) und § 92 Strahlenschutzverordnung (EK-046/S) sowie beim Office for Human Research Protections, reg. no. IRB00006099, Rockville, MD, U.S.A..

Dr. med. Norbert Beck
Geschäftsführer der Ethik-Kommission

9.2 Informed Consent

OTTO VON GUERICKE UNIVERSITÄT MAGDEBURG
MEDIZINISCHE FAKULTÄT

Klinik für Gastroenterologie, Hepatologie und Infektiologie
Prof. Dr. med. Peter Malfertheiner
Direktor

Otto-von-Guericke-Universität
Medizinische Fakultät
Leipziger Str. 44
39120 Magdeburg

Telefon: +49 391 67-13100
Telefax: +49 391 67-13105

peter.malfertheiner@med.ovgu.de
www.med.uni-magdeburg.de

Patienteninformation

„Adaptive *Helicobacter pylori*-assoziierte bakterielle und Wirtssuszeptibilitätsfaktoren in der Magenkarzinogenese“

Sehr geehrte/r Frau/Herr _____ ,

Sie haben die Möglichkeit an einer klinischen Studie teilzunehmen, die an unserer Klinik derzeitig durchgeführt wird. Um sich für eine Teilnahme zu entscheiden, ist es wichtig die Hintergründe zu verstehen, warum und mit welchem Aufwand die Datenerhebung für diese Studie durchgeführt wird. Bitte lesen Sie die folgenden Informationen sorgfältig und klären eventuell noch offene Fragen mit dem betreuenden Arzt.

Hintergründe und Ziele
Die Infektion mit dem Bakterium *Helicobacter pylori* verursacht bei allen Personen eine chronische Magenentzündung (Gastritis), die bei wenigen Menschen zur Entstehung von Magenkrebs führt. Wie das Bakterium eine bösartige Veränderung der Magenschleimhaut bewirkt, ist bis her nicht verstanden. Aus diesem Grund wollen wir die Veränderungen in der Magenschleimhaut detaillierter untersuchen und hierbei Virulenzfaktoren des Bakteriums als auch Ihr Erbmaterial und die Reaktion des Immunsystems gemeinsam studieren. Durch die gemeinsame Betrachtung dieser beiden Seiten, wollen wir spezifische Veränderungen identifizieren, die eine frühzeitige Erkennung von Personen ermöglicht, die ein erhöhtes Risiko für die Entwicklung des Magenkrebses besitzt.

Im Rahmen einer Blutentnahme ist es möglich zu untersuchen ob Sie in Kontakt mit dem Keim *Helicobacter pylori* gekommen sind. Weiterhin können Veränderungen im Erbmaterial aus dem Blut und Gewebe verglichen werden.

Während einer Magenspiegelung kann die Magenschleimhaut äußerlich beurteilt werden. Außerdem können Proben für die feingewebliche Routineuntersuchung, für die mikrobiologische Anzüchtung des Keims sowie für Forschungszwecke gewonnen werden.

Notwendigkeit der Teilnahme an der Studie
Ihre Teilnahme an der Studie ist freiwillig. Sie können die Teilnahme verweigern oder sich auch jederzeit für eine Beendigung ohne Angabe von Gründen entscheiden. Dies wird den

Behandlungsstandard in der Zukunft nicht beeinflussen. Sie werden mit oder ohne Teilnahme die bestmögliche Therapie erhalten. Wenn Sie sich für die Teilnahme entscheiden, bitten wir Sie, das anliegende Patienteneinverständnisformular zu unterschreiben.

Möglicher Nutzen der Studienteilnahme
Durch die Analysen ist eine genauere Einschätzung ihres Risikoprofils möglich. Weiterhin dienen die Untersuchung als Basis zur Entwicklung neuer Früherkennungs- und auch Therapiemaßnahmen für Patienten mit Magenkrebs. Dies hat für Sie keinen direkten diagnostischen Nutzen.

Ablauf der Studie

☐ Im Rahmen dieser Studie ist **eine zusätzliche Blutentnahme** (ca. 40 ml [ca. 2,5 Esslöffel] Blut wird hierfür aus einer Vene entnommen) erforderlich. Sie dient der Bestimmung von bekannten Risikofaktoren, v.a. im Rahmen einer *Helicobacter pylori* Infektion, die mit dem Fortschreiten von bösartigen Schleimhautveränderungen des Magens assoziiert sind.

Bei Notwendigkeit einer Magenspiegelung:

☐ Im Rahmen der geplanten Diagnostik wird bei Ihnen eine **Magenspiegelung** durchgeführt. Zur Routinediagnostik gehört die Entnahme von kleinen Schleimhautproben (etwa 2-3 mm) aus dem Magen und ggf. dem Zwölffingerdarm. Zur Durchführung dieser Studie ist die Entnahme von **16 zusätzlichen** Schleimhautproben aus unterschiedlichen Lokalisationen im Magen vorgesehen. Sollte ein Magenkarzinom vorliegen, so werden weitere 4 Biopsien aus dem Tumor entnommen.

Mögliche Komplikationen
Magenspiegelung: Die Magenspieglung ist ein risikoarmes Routineverfahren. Trotz größter Sorgfalt können in seltenen Fällen Komplikationen auftreten. Durch das Endoskop kann es zu Mißempfindungen im Rachen und zu Würgereiz kommen. Durch entsprechende Maßnahmen kann man dies jedoch verhindern bzw. lindern.
Sehr selten kann es zu Verletzungen der Wand des Verdauungstraktes oder des Kehlkopfes kommen. Ebenfalls sehr selten sind Herz-Kreislaufstörungen oder Infektionen bis hin zu Keimverschleppung in die Blutbahn. Sollte ein sedierendes Medikament (Midazolam 5 mg) intravenös verabreicht werden, so ist die aktive Teilnahme am Straßenverkehr für 24 Stunden untersagt.
Blutproben: Für die meisten Menschen stellt der Einstich mit der Nadel zur Entnahme einer Blutprobe kein ernsthaftes Problem dar. Es kann jedoch an der Einstichstelle zu einem Bluterguss, zu Blutungen, einer Entzündung und/oder Schmerzen kommen.
Gewebeproben: Die Gewinnung von Magenschleimhautproben birgt kein erhöhtes Risiko bei der Durchführung der Magenspiegelung.

Anonymität der erhobenen Daten
Die Daten werden zur weiteren statistischen Auswertung zur Beantwortung wissenschaftlicher Fragestellungen herangezogen. Wir sichern Ihnen auch bei der Auswertung der Daten zu, dass keinerlei personenbezogene Daten weitergegeben werden und dass die Analyse vollständig anonym erfolgt. Das heißt, dass eine Zuordnung von Untersuchungsbefund und Person später nicht mehr möglich ist. Somit haben nur Ärzte und Schwestern unserer Abteilung Zugang zu Ihren persönlichen Daten, die selbstverständlich der Schweigepflicht unterliegen. Auch im Rahmen der Erbgutuntersuchungen bleibt die Anonymität gewahrt. Im Fall der Identifikation von Erbgutveränderungen, welche mit potenziell erhöhtem Risiko für bestimmte Erkrankungen assoziiert sein könnten, erhalten Sie keine weitere Information. Im Rahmen von wissenschaftlichen Kooperationen werden Teile des Probenmaterials in faktisch anonymisierter Form in bestimmten

GASTROENTEROLOGISCHE ALLGEMEINSPRECHSTUNDE Montag - Freitag 08.00 - 13.00 [13139]
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spezialisierten Labors im In- und Ausland (Universitäten) untersucht. Die Weitergabe an Dritte einschließlich Publikation erfolgt ausschließlich in anonymisierter Form, d.h. kann nicht Ihrer Person zugeordnet werden. Die Studienergebnisse werden in der medizinischen Literatur veröffentlicht, jedoch ohne dass die Identität der Studienteilnehmer zu erkennen sein wird.

Ich verzichte darauf, dass mir die im Rahmen der wissenschaftlichen Untersuchung möglicherweise an meiner DNA-Probe identifizierten genetischen Risikofaktoren mitgeteilt werden. Dies betrifft genetische Risikofaktoren für das Magenkarzinom als auch andere genetische Risikofaktoren, die ursächlich für andere Erkrankungen sind und als Zufallsbefund bei den geplanten Analysen identifiziert werden könnten.

Kosten der Studienteilnahme

Für die zusätzlichen Untersuchungen im Rahmen der Studie (Blutentnahme, Biopsieentnahme) entstehen für Sie keine Kosten. Es sind keine zusätzlichen Visiten und Untersuchungen geplant, als die, die für die Behandlung Ihrer Erkrankung notwendig sind.

Schadensersatz

Sollte bei der Studienteilnahme ein Schaden für Sie entstehen, was nicht zu erwarten ist, werden eventuelle Entschädigungsansprüche im Rahmen der gesetzlichen Haftpflichtversicherung des Klinikums reguliert.

Kontakt

Sollten im Zusammenhang mit der Untersuchung Nebenwirkungen oder Unwohlsein beobachtet werden, sollten Sie sich umgehend an den behandelnden Prüfarzt wenden.

Name: Dr. med. A. Link / Dr. med. M. Venerito
Dr. med. Chr. Schulz / PD. Dr. med. A. Kandulski
Dr. med. J. Weigt/ Dr. R. Vasapolli
Klinik für Gastroenterologie, Hepatologie und Infektiologie
Otto-von-Guericke Universität Magdeburg
Leipziger Str. 44, 39120 Magdeburg
0391 – 6721511
0391 – 6713106

Vielen Dank, dass Sie sich diese Informationen sorgfältig durchgelesen haben. Sollten noch Fragen offen bleiben, wenden Sie sich bitte an Ihren Arzt.
Aus rechtlichen und ethischen Gründen benötigen wir für den Eingriff und für die Entnahme zusätzlicher Schleimhautproben für wissenschaftliche Untersuchungen vor der Untersuchung Ihr schriftliches Einverständnis und eine Erklärung darüber, dass Sie alle Informationen verstanden und über die Durchführung der Studie vollständig aufgeklärt wurden.

MEDIZINISCHE FAKULTÄT

Klinik für Gastroenterologie, Hepatologie und Infektiologie
Prof. Dr. med. Peter Malfertheiner
Direktor

Otto-von-Guericke-Universität
Medizinische Fakultät
Leipziger Str. 44
39120 Magdeburg

Telefon: +49 391 67-13100
Telefax: +49 391 67-13105

peter.malfertheiner@med.ovgu.de
www.med.uni-magdeburg.de

Zusätzliche Blut-/Schleimhautprobenentnahme zu wissenschaftlichen Zwecken
(Helicobacter pylori und Wirtsfaktoren in der Entstehung von Magenkrebs)

Patient (Etikett)

Name, Vorname _____

Geburtsdatum _____

Adresse _____

_____

Ich erkläre mich mit der Durchführung zusätzlicher Untersuchungen (Blutentnahme, Biopsien) einverstanden und bin mir darüber im Klaren, dass dieses Material nicht nur der klinischen Routinediagnostik, sondern auch wissenschaftlichen Zwecken (zum Beispiel Erbmaterialanalyse) dient. Ich wurde in einem ausführlichen Gespräch über Wesen, Bedeutung und Tragweite der klinischen Studie, an der ich teilnehmen möchte, unterrichtet und mir wurde die Gelegenheit gegeben, meine Entscheidung zu überdenken. Meine Fragen zu diesen Untersuchungen wurden umfassend beantwortet und ich fühle mich ausreichend informiert.

Für die Einwilligung zur Studienteilnahme danken wir Ihnen.

_____
Ort, Datum

_____ _____
Unterschrift/Stempel des aufklärenden Arztes Unterschrift des Patienten

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Name des aufklärenden Prüfarztes (in Druckbuchstaben)

10. Ehrenerklärung *(Declaration in German)*

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

“INFLUENCE OF LABORATORY AND ENDOSCOPY-RELATED FACTORS ON THE ASSESSMENT OF SERUM PEPSINOGENS AND GASTRIN-17.”

in der Klinik für Gastroenterologie, Hepatologie und Infektiologie mit Unterstützung durch Prof. Dr. med. Dr. h.c. Peter Malfertheiner

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 23.09.2019 Elisabetta Goni

_____

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Finally, I would like to express special thanks to my parents and my brother, who always encouraged me to concentrate on my study.

12. Curriculum vitae et studiorum

Name	Elisabetta Goni, MD
Current Position	Resident Internal Medicine and Gastroenterology
Professional Experience	**From June 2017 to date** Ludwig Maximilians University Hospital, Department of Internal Medicine II, Munich, Germany. (Prof. J. Mayerle) - Gastroenterology Unit - Intensive Care Unit (ICU) - Emergency department - Ultrasound - Pancreatic diseases (Ambulatory Care) **From June 2015 to May 2017** Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg, Germany. (Prof. P. Malfertheiner) - Gastroenterology Unit - Emergency Department **From January 2014 to May 2015** University Hospital of Parma Department of Clinical and Experimental Medicine (Prof. F. Di Mario) **From July 2013 to June 2015** University Hospital of Verona Department of Gastroenterology and Gastrointestinal Endoscopy Translational and basic science research on “ Epithelial Na+ channel (ENaC) mutations in patients with pancreatitis.” (Prof. L. Frulloni) **From February 2011 to April 2013** Vita-Salute San Raffaele University Hospital Gastroenterology and Gastrointestinal Endoscopy Unit Clinical and basic research on pancreatic diseases and other GI tract neoplasia. Clinical activities (Prof. PA Testoni and Prof. GM Cavestro) **From July 2010 to October 2012** University of Parma – Genetic Unit Basic science on HLA of autoimmune pancreatitis (DNA extraction from blood, polymerase chain reaction, PCR-

	sequencing, research projects and grant project) (Prof. TM Neri) **June 2011** Workshop on Pancreatic Cancer, Cold Spring Harbor Laboratory (NY) Tuveson D, Bar-Sagi D, Maitra A. Basic science applied to pancreatic cancer tissues
Qualification	- German National Qualification to practice as a Medical Doctor (08.2015) - Italian National Qualification to practice as a Medical Doctor (02.2013) - Medical Degree - University of Parma, Italy (07.2012) Final dissertation: ”The role of HLA in pathophysiology of autoimmune pancreatitis” (Prof. GM Cavestro, Prof. L. Frulloni)
Publications	1) Derikx MH, Kovacs P, Scholz M, Masson E, Chen JM, Ruffert C, Lichtner P, Te Morsche RH, Cavestro GM, Algül H, Berg T, Bödeker H, Blüher M, Bruno MJ, Buch S, Bugert P, Cichoz-Lach H, Dabrowski A, Farré A, Frank J, Gasiorowska A, Geisz A, ***Goni E***, Grothaus J, Grützmann R, Haas S, Hampe J, Hellerbrand C, Hegyi P, Huster D, Ioana M, Iordache S, Jurkowska G, Keim V, Landt O, Di Leo M, Lerch MM, Lévy P, Löhr MJ, Macek M, Malats N, Malecka-Panas E, Mariani A, Martorana D, Mayerle J, Mora J, Mössner J, Müller S, Ockenga J, Paderova J, Pedrazzoli S, Pereira SP, Pfützer R, Real FX, Rebours V, Ridinger M, Rietschel M, Rohde K, Sack S, Saftoiu A, Schneider A, Schulz HU, Soyka M, Simon P, Skipworth J, Stickel F, Stumvoll M, Testoni PA, Tönjes A, Treiber M, Weiss FU, Werner J, Wodarz N, Férec C, Drenth JP, Witt H, Rosendahl J.Polymorphisms at PRSS1-PRSS2 and CLDN2-MORC4 loci associate with alcoholic and non-alcoholic chronic pancreatitis in a European replication study. *Gut 2015;64:1426-33. doi: 10.1136/gutjnl-2014-307453.* 2) Ierardi E, **Goni E**, Losurdo G, Di Mario F. Helicobacter pylori and nonmalignant diseases. *Helicobacter 2014 Sep;19 Suppl 1:27-31.* 3) Di Mario F, **Goni E**. Misleading results in the diagnosis of atrophic gastritis. *Eur J Gastroenterol Hepatol 2014;26:1439-40.*

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Book chapter	"Recurrent pancreatitis: acute recurrent or chronic disease?" (Cavestro GM, **Goni E**, Testoni PA) in “Acute and chronic pancreatitis. New concepts and evidence-based approaches”, 2013 Edizioni Minerva Medica
Memberships	Italian Society for the Study of the Pancreas (AISP) – since 2011 Italian Society of Gastroenterology (SIGE) – since 2012 German Society of Gastroenterology (DGVS) – since 2016 Deutscher Pankreas Club (DPC) – since 2017 European Pancreatic Club (EPC) – since 2017