1.0 TITLE PAGE

EXPERIMENTAL STUDY TO DETERMINE THE EFFECTS OF REFLUXATE (HUMAN: BILIARY, GASTRIC, DUODENOGASTRIC/MIXED) ON MACROPHAGE PHENOTYPE AND MACROPHAGE PLASTICITY AND ITS CORRELATION WITH DIFFERENT FORMS OF ACID REFLUX DISEASE (NON-EROSIVE AND EROSIVE) AND COMPLICATIONS THEREOF (BARRETT'S OESOPHAGUS): MOTILITY TALENT GROUP STUDY.

Product Name:	No investigational product
Type of Study:	IIS (Human, Animal (in vitro))
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This study will be conducted in compliance with the protocol, Good Clinical Practice, Good Laboratory Practice, International Guiding Principles for Biomedical Research Involving Animal and all other applicable regulatory requirements

2.0 LIST OF ABBREVIATIONS

APS	American Physiological Society
CD	Cluster of differentiation
GERD	Gastroesophageal reflux disease
GCP	Good clinical practice
GLP	Good laboratory practice
H2RA	H2 receptor antagonists
IBD	Inflammatory bowel disease
IL	Interleukin
LEC	Local Ethics Committee
M1	Macrophage type 1
M2	Macrophage type 2
NERD	Non-erosive reflux disease
pCRF	Paper Case Report Form
PPIs	Proton-pump inhibitors
Th1	Type 1 T-helper
Th2	Type 2 T-helper
TNFα	Tumor necrosis factor alpha
TNFβ	Tumor necrosis factor beta

3.0 INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common diseases and, according to recent epidemiological studies, clinical and endoscopic GERD symptoms can be detected in 8-25% of the population depending on country, race and gender [1]. Although GERD rarely is a cause of death, it is associated with prominent morbidity and complications, such as esophageal ulceration, peptic stricture, Barrett`s esophagus and therefore leading cause of esophageal adenocarcinoma [2].

In the Russian Federation, the prevalence of GERD reaches 11-15% [2]. The incidence of GERD in the past 15 years has increased significantly and is currently 5 per 1000 population per year [1].

Despite improvements in diagnostics and treatment of GERD, there are still many unresolved issues. An example of a diagnostic challenge is identifying patients with non-erosive reflux disease (NERD), which has been documented to occur in 50-60 % of GERD patients. These patients periodically or continuously complain on heartburn, while they do not have endoscopic evidence of esophagitis.

Another challenge concerns patients suffering from refractory GERD, who despite treatment do not improve clinically and/or endoscopically, but on the contrary, progress to more extensive erosion, ulceration and esophageal complications such as peptic esophageal stricture, bleeding, and Barrett's esophagus which constitutes a precancerous disease [4].

In GERD, damage of the esophageal mucosa may occur as a result of activity of different types of refluxate, i.e. gastric, biliary or duodenogastric/mixed. Along with acid and pepsin, duodenal contents with duodenogastric reflux may be harmful to the esophageal mucosa. Previous studies demonstrate that conjugated bile acids produce fewer harmful effects than deconjugated bile acids in a more neutral pH environment [24]. Although proton pump inhibitors may weaken both acid and duodenogastric reflux by decreasing its volume, their prescription not always adequately addresses or eliminates the cause of the disease.

In recent years, the study of refractory GERD at the tissue and cellular level, which is based on the study of esophageal mucosal injury depending on the nature of the refluxate (gastric, biliary or duodenogastric/mixed) has increasingly become an area of interest particularly also in the light of investigating the immune response in patients with GERD. For instance, it appears that patients with high levels of interleukin-8 (IL-8) have a higher risk of recurrent GERD [5], and patients with high levels of Il-1b have a higher risk of torpid course of GERD [6].

Cytokines, important for cell signaling, are mainly produced by macrophages. Macrophages may change their phenotype under influence of different factors (known as macrophage plasticity). Macrophages produce not only inflammatory and anti-inflammatory cytokines, but also growth factors and reactive oxygen species (superoxide radical anions, singlet oxygen, hydrogen peroxide), which define immune response of the whole body [7].

GERD is characterized by disorders in the immune response as presented by misbalanced cellular (Th1) and humoral (Th2) parts of immune response, which might be determined by different phenotypes of macrophages – M1 (pro-inflammatory) or M2 (anti-inflammatory). Previous studies have shown that the activation of Th1-immune response leads to the erosive form of GERD, whereas activation of Th2- immune response may lead to the development of Barrett`s esophagus [8, 9].

4.0 RATIONALE

According to the published data, type of esophageal injury and its mucosal response greatly depends on the nature/type of refluxate (gastric, biliary or duodenogastric/mixed) and patient immune profiles [6]. It has been shown that Th-2-predominant cytokine response leads to Barrett`s esophagus. Also it was shown that acid suppression and alkaline duodenal reflux may increase the risk of Barrett`s esophagus [23].

We hypothesize that the different types of refluxate may somehow differentiate macrophage phenotype and thereby the immune profile, determining the type of GERD disease and its progression.

This study is proposed to evaluate the role of motoric dysfunctions and burden of different kinds of refluxate on immune cells and the subsequent course of the disease. This study also aims to establish a bridge between instrumental findings (pH-impedance test) and clinical assessments (FSSG questionnaire), aiming to facilitate the use of simple questionnaire techniques in the clinical setting in the future.

This study will potentially also provide further insight and input into future uses and indications for prokinetics in GERD and may aid in improving the treatment concepts of GERD and complications thereof (Barrett's oesophagus).

5.0 PROGRAM OBJECTIVE(S)

5. 1. Study Objectives:

Animal *(in vitro)*-part of the study:

1. To determine changes of macrophage phenotype and macrophage plasticity in mice, particularly C57/BL6 genetic strain after exposure to gastric refluxate (human origin)
2. To determine changes of macrophage phenotype and macrophage plasticity in mice, particularly C57/BL6 genetic strain after exposure to biliary refluxate (human origin)

3. To determine changes of macrophage phenotype and macrophage plasticity in mice, particularly C57/BL6 genetic strain after exposure to duodenogastric/mixed refluxate (human origin).

Clinical (patients) part of the study

1. To investigate blood and biopsy-origin monocyte/macrophage phenotype in patients with different forms of acid reflux disease (non-erosive GERD (NERD), erosive GERD and complications thereof: Barrett`s esophagus)

2. To investigate types of refluxate (gastric, biliary, duodenogastric/mixed) in patients with different forms of acid reflux disease (non-erosive GERD (NERD), erosive GERD and complications thereof: Barrett`s esophagus)

3. To investigate the relationship between clinical assessments (FSSG questionnaire) and measurements obtained from esophageal pH-impedance monitoring and esophageal high-resolution manometry.

5.2. Primary Endpoint

Animal *(in vitro*) part of the study:

To determine changes in macrophagal phenotype from baseline after exposure to three different forms of refluxate (gastric, biliary duodenogastric/mixed) by means of assessing:

a) Morphological characteristics (quantity of M1 (round forms) and M2 (fibroblasts-like) macrophages on 100 analyzing cells in 5 non-interfering sights).
b) Macrophagal phagocytosis activity (mean percentage of phagocytizing cells in all peritoneal macrophagal cells and mean number of bacteria ingested by macrophages)
c) Secretory activity (ratio of proinflammatory M1 cytokines IL-1β, IL-8, IL-12p70, IFNγ, TNFα and TNFβ to anti-inflammatory M2 cytokines IL-4, IL-5 и IL-10, as well as M1/M2 cytokines IL-2 и IL-6).

d) Receptor characteristics (ratio of superficial markers CD80 and CD 25 (M1-markers) and CD206 and CD163 (M2-markers). Changes in M1 and M2 ratios determined on the base of receptors will also be analyzed.

Clinical (patients) part of the study:

1. Characteristics of blood and biopsy-origin monocyte/macrophage phenotype (morphology, receptor characteristics) in patients with different types of acid reflux disease (NERD, erosive GERD) and complications thereof: Barrett's esophagus).

2. Number of patients with gastric, biliary, duodenogastric/mixed refluxate within each of the different patient groups (NERD, erosive GERD and complications thereof: Barrett's esophagus).

3. Mean FSSG scores (acid reflux related symptoms, dysmotility symptoms) in patients with gastric, duodenogastric/mixed reflux related to the three different patient subgroups (NERD, erosive GERD and complications thereof: Barrett's esophagus).

6.0 INVESTIGATIONAL PLAN

6.1. Selection of Program Population

Animal (in vitro) Part: Mice of genetic line C57/BL6 (90±15 mice)

Clinical (patients) part: 90 adult patients with different types of acid reflux disease (30±5 patients with non-erosive GERD, 30±5 patients with erosive GERD and 30±5 patients with complications thereof (Barrett's esophagus))

6.1.1 Inclusion Criteria

Animal (in vitro) Part of study:

Mice of genetic lines C57/BL6 (black) with mean weight 24 g, mean age 10 weeks, males

Clinical (patients) part of study:

1. Signed informed consent
2. Gender: Male or Female
3. Age: 18-65 years of age
3. Clinically and/or endoscopically confirmed diagnosis of GERD

6.1.2. Exclusion Criteria

Animal (in vitro) Part: None

Clinical: (patient)

1. Treatment with PPIs and/or H2RA 1 week prior to study inclusion.
2. Female patients who are pregnant, planning to become pregnant or lactating
3. Any acute diseases or conditions, exacerbations of concomitant chronic diseases (including but not limited to inflammatory bowel disease (IBD), ulcer disease etc.) at study start/inclusion and/or which are not resolved 14 days prior to study-enrolment.
4. Participation in a clinical trial in the past 3 months
5. Any condition which, in the opinion of investigator, makes the patient unsuitable for participation in the study

6.1.3. Patient completion and withdrawal

A patient who performed all visits will be considered to have completed the study.

A patient may voluntary discontinue participation in the study at any time and for any reason without explanation. Nevertheless, the investigator will make all reasonable effort to capture the reasons for withdrawal.

The investigator may also at his/her discretion discontinue the patient from participating in this study at any time.(e.g. if further participation in the study is not considered to be in the interest of the patient : if major complications of the procedures occur or in case of need to prescribe prokinetics 48 h or antacids 24h prior to esophageal pH-impedance test).

The reasons for withdrawal will be documented in the primary data source data file (patient records) and CRF.

6.2. Number of subjects to be enrolled

Animal (in vitro) Part of the study:

M1 macrophages from mice of genetic line C57/BL6 (90±15 mice) – 90±15 samples

i.e. number of laboratory sockets: 180±30 (90±15 samples in double)

Clinical (patients) Part of the study:

30±5 patients with non-erosive GERD (NERD, as confirmed by endoscopy)

30±5 patients with erosive GERD

30±5 patients with Barrett`s oesophagus

6.3. Investigator Selection Criteria: N/A

6.4. Program Duration

6.4.1. Clinical part (patients) of the study:

Program duration for each patient will be approximately 7(±3) days. Duration of recruitment is planned to be 14 months, 4 months is planned for Data Analysis, 6 months – for study report/ publication preparation.

6.4.2. Animal (in vitro) part of the study

Duration of in vitro part of the study for each sample will be defined by:

- period of macrophage culture – 48 hours,

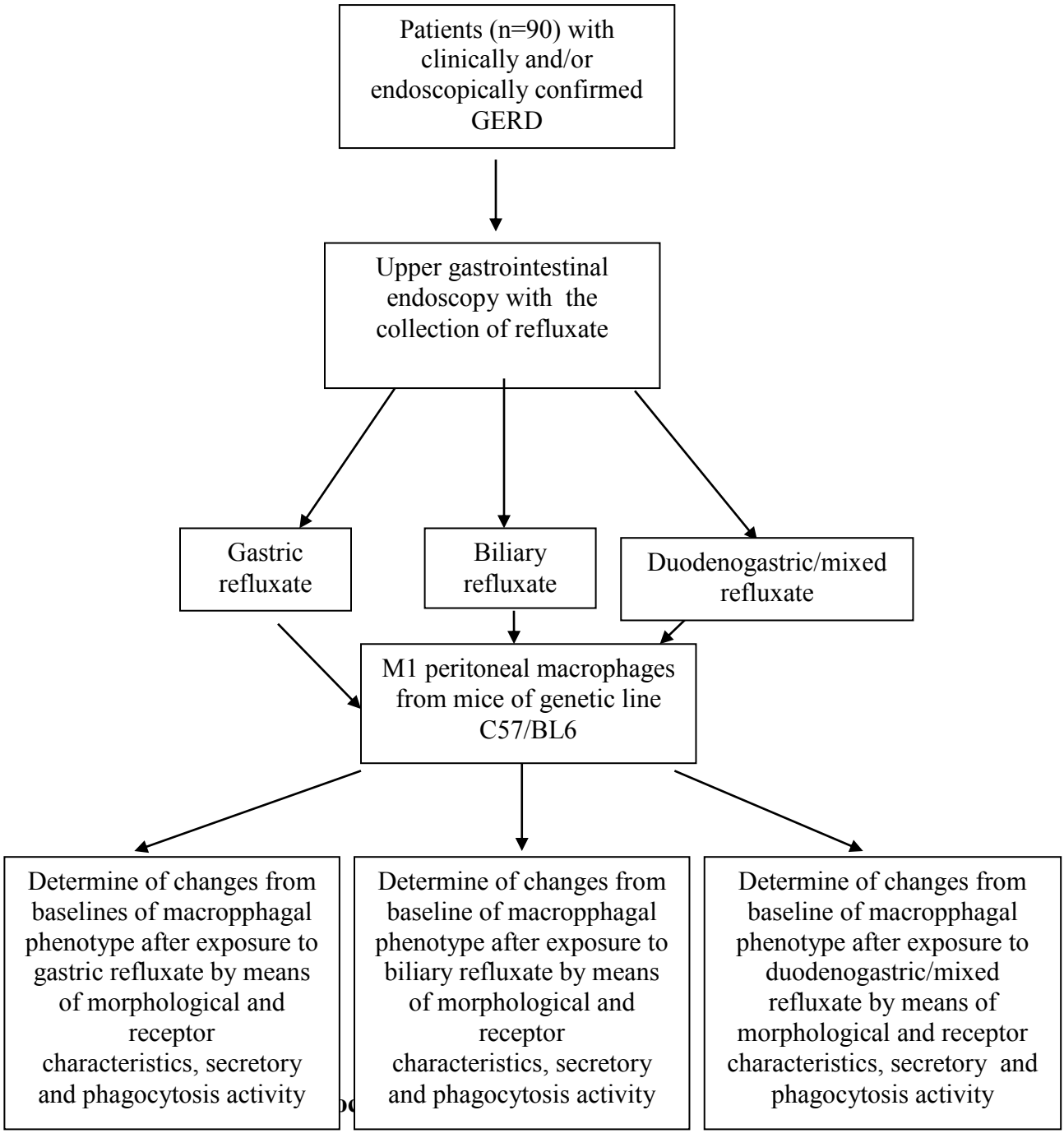
- macrophage phenotype assessment – 24 hours.

The total duration of the in vitro study to obtain one sample will be 36 hours.

6.5. Program Conduct

This is a prospective, experimental study consisting of the two parts: a clinical (patients) part and an animal (in vitro) part.
The clinical (patients) part of the study will be conducted at the Propaedeutic Clinic of Internal medicine, Gastroenterology, Hepatology of First MGMU n.a.. IM Sechenov.
The animal part of the study (in vitro) will be conducted at the Moscow State University of Medicine and Dentistry.
The in vitro part of the study will be conducted in M1 macrophages from mice of genetic line C57/BL6 (90±15 mice). To mirror the design of clinical part of the study and to ensure compliance with the enrolled patients' number macrophages from 30±5 mice will be exposed to acid refluctate, macrophages from another 30±5 mice – to biliary refluctate and from the other 30±5 mice – to mixed refluxate.

Figure 1. Study design



	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Timelines and Allowance Window	**Day 1 Baseline**	**Day 2 (±1Day)**	**Day 3 (±2 Days)**	**Day 4 (±2 Days)**	**Day 5**
Signed informed consent	X				
Assignment of patient identification number	X				
Inclusion and exclusion criteria assessment	X				
Allocation to the subgroups of GERD			X		
Demographics (date of birth, gender, race)	X				
Medical history	X				
Physical examination	X	X	X	X	X
FSSG assessment	X				
Blood sampling (macrophages)		X			
Hematology (RW, HIV, HbsAg, HCV)		X			
Upper gastrointestinal endoscopy with biopsy and collection of refluxate			X		
24-h esophageal pH-impedance test with esophageal high-resolution manometry				X	
Safety assessments*	X	X	X	X	X

* Any safety issues related to the invasive study procedures

6.6. Description of Activities

Visit 1 (Baseline, Day 0)

The procedures of visit:

- **Signed informed consent**

Patients considered eligible will be informed about the general aspects and procedures of the study and have to sign informed consent prior to any assessment or procedure is conducted.

- **Assignment of patient identification number**

Each patient will be assigned a two-digit patient identification number (ascending from 01 to 90).

- **Inclusion / exclusion criteria assessment**

The following criteria should be checked prior to study enrollment:

Inclusion criteria:

1. Signed informed consent
2. Gender: Male or female
3. Age: 18-65 years of age
3. Clinically and/or endoscopically confirmed diagnosis of GERD

Exclusion criteria

1. Current treatment or treatment with PPIs and/or H2RA 1 week prior inclusion
2. Female patients who are pregnant, planning to become pregnant or lactating
3. Any acute diseases or conditions, exacerbations of concomitant chronic diseases (including but not limited to inflammatory bowel disease (IBD), ulcer disease etc.) at study-start/ inclusion and/or which are not resolved 14 days prior to enrolment in the study.
4. Participation in a clinical trial in the past 3 months

5. Any condition which, in the opinion of investigator, makes the patient unsuitable for participation in the study

- **Demographics**

Includes information about date of birth, gender, race.

- **Medical history**

Medical history includes full medical information regarding GERD (duration of symptoms, results of previous examinations (upper gastrointestinal endoscopy, 24-h oesophageal pH-impedance or pH test, esophageal high-resolution manometry), previous and current treatment, comorbidities and concomitant medication, family history of the disease. Historic reports of undesirable events associated with past medications will be considered medical history.

- **Physical examination**

Includes the measurement of height, weight, blood pressure, pulse.

- **FSSG assessment**

FSSG (Frequency Scale for the Symptoms of GERD) – questionnaire with 12 questions related to acid reflux and dysmotility symptoms (See Appendix A). Questions 2,3,5, 8 and 11 are related to `dysmotility`, while other questions consider acid reflux symptoms. FSSG allows investigators to analyze different symptoms experienced by GERD patients [10]. Sum of both parts of the questionnaire will be analyzed for each patient. Permission to use the questionnaire for this study was obtained from its author/developer, professor Motoyasu Kusano.

• Safety Assessments

Any AEs related to the invasive study procedures, will be collected.
Only undesirable effects that are deemed at least possibly related to an Abbott medication by the investigator will be forwarded to Abbott affiliate PV.

Visit 2

- **Physical examination**

Includes the measurement of blood pressure, pulse

- **Hematology (RW, HIV, Hbs-Ag, HCV)**

Blood tests for RW, HIV, Hbs-Ag, HCV are mandatory and considered standard of medical care before any interventional procedures are performed (such as endoscopy and pH-impedancemetry).

- **Blood sampling (monocytes/macrophages)**

The protocol of monocytes/macrophages isolation from venous blood

1. Blood will be collected in previously marked Vacuette system test tube (10-12 ml)
2. Blood in the test tube will be mixed with heparin (1 ml heparin – 10 ml blood)
3. Transportation of the marked test tubes in the laboratory of Cellular Biotechnology of Moscow State University of Medicine and Dentistry. The samples will be stored at temperature +4±2^{o}C until transportation and will be transported in a mobile refrigerator (+4±2^{o}C) to the laboratory of Cellular Biotechnology of Moscow State University of Medicine and Dentistry. Laboratory address: 105275, Moscow, Boris Zhigulenkova street, 23, building 1
4. Blood sample is stirred and placed into centrifuge.

5. Blood sample is centrifuged for 15 minutes at 3000 rpm.
6. After centrifugation the supernatant is separated from blood clot, in blood clot leukocytes are separated from erythrocytes, and leukocytes are placed in a pure plastic test tube.
7. Lysis buffer is prepared: 100 ml of distilled water per 0.825 g NH4Cl, 0.0037 g EDTA.
8. The leukocyte mass is mixed with 10 ml of lysis-buffer and mixed in vortex for 5 minutes (40 rpm).
9. The test tube is centrifuged for 4 min – 1000 rpm.
10. The supernatant is separated and drawn away.
11. We place 5 ml of growth medium RPMI1640 to the cell pellet and the tube is centrifuged for 4 min – 1000 rpm.
12. The supernatant is separated and the cell pellet is resuspended in 1 ml RPMI1640.
13. The cells are counted in a cell counter

• Safety Assessments

Any AEs related to the invasive study procedures, will be collected.
Only undesirable effects that are deemed at least possibly related to an Abbott medication by the investigator will be forwarded to Abbott affiliate PV.

Visit 3

- **Physical examination**

Includes the measurement of blood pressure, pulse

- **Upper gastrointestinal endoscopy with biopsy and collection of different types of refluxate**. All patients will undergo an upper gastrointestinal endoscopy with biopsy and

collection of different types of refluxate. The procedure should be performed on an empty stomach standard technique of anesthesia;10% lidocaine solution). The examination includes analysis of esophageal mucosa, expression of inflammatory changes, location, size, number of mucosal defects (erosions, ulcers, strictures, hemorrhages), as well as the appearance of the gastric and duodenal mucosa. . The modified Savary-Miller classification of esophagitis will be used to characterize changes in the esophageal mucosa.

Table 2. The modified Savary-Miller classification of esophagitis

I	single erosive or exudative lesion, oval or linear, taking only one longitudinal fold
II	noncircular multiple erosions or exudative lesions taking more than one longitudinal fold, with or without confluence
III	circular erosive or exudative lesion
IV	chronic lesions: ulcer(s), stricture(s), erosive or exudative lesion or short oesophagus, isolated or associated with lesions of grades I, II, or III
V	islands, finger-like forms or circumferential distribution of Barrett's epithelium isolated or associated with lesions of grades I to IV

For morphological research and monocyte/macrophage phenotype assessment biopsy sampling of distal esophagus will be performed according to the scheme: 4 fragments located at 12, 3, 6, 9 hours, 2 cm above the Z-line, as well as from individual foci of altered mucosa.

The refluxate samples required for the animal (in vitro) part of the study, will be placed into pure plastic test tubes and be stored at temperature +2-4 C until transportation to the Cellular Biotechnology of Moscow State University of Medicine and Dentistry. Transportation should be done at the temperature +2-4 C and be done within two hours after obtaining the refluxate.

- **Allocation to the subgroups of GERD**

Diagnosis of GERD (non-erosive and erosive) is made based on clinical symptoms (complaints of heartburn, regurgitation, belching, (non-cardiac) chest pain) and/or the results of an upper gastrointestinal endoscopy. Endoscopy should not have been done more than a month before inclusion. Diagnosis of Barrett's esophagus has to be endoscopically confirmed (incl. biopsy) and should not be older than 6 months prior to study enrolment.

Patients included into the study with a GERD diagnosis based on symptoms only and/or with an endoscopy performed more than one month ago prior to inclusion will be allocated to the subgroups on the result of endoscopy conducted at Visit 3.

• Safety Assessments

Information regarding complications of the procedure and patient complaints are to be collected and registered. After the procedure each patient will be placed under medical observation for at least for two hours. Any AEs related to the invasive study procedures, will be collected.
Only undesirable effects that are deemed at least possibly related to an Abbott medication by the investigator will be forwarded to Abbott affiliate PV

Visit 4

- **Physical examination**

Includes the measurement of blood pressure, pulse.

• 24-h esophageal pH-impedance test with esophageal high-resolution manometry

To investigate number of reflux episodes and determine the type of refluxate (gastric, biliary, duodenogastric/mixed) in patients with different forms of acid reflux disease and complications thereof (Barrett`s esophagus) pH-impedance test with esophageal high-resolution manometry (HRM) will be done.

Calibration of device by means of two standard buffer solutions at temperature 37 C is necessary before the 24-hr esophageal pH-impedance test is performed.
Procedure of pH impedancemetry includes intranasal introduction of a probe into oesophagus and connection of the probe with the recording device and 24-hour wearing of the probe. Installation of the probe is carried out on an empty stomach.

High-resolution manometry (HRM) will be done with a 22-channel water-perfused catheter and Solar GI system (Medical Measurements Systems, Enschede, the Netherlands).

In GERD patients will be analyzed following HRM parameters: the distal contractile integral (DCI) and the lower esophageal sphincter resting pressure (LES RP).

24-h pH-impedance monitoring will be done in both distal oesophagus and in the stomach according to standard of practice. Esophageal pH sensor will be positioned 5 cm above the manometrically located border of the LES. Measurement with a second pH sensor in the stomach, 10cm below the LES in order to monitor intragastric acidity will be done as a standard routine procedure.

The probe is removed after 24 hours and the results from the recording unit are transferred to the computer database for further analysis.

Standard measuring parameters will be collected: percent of time when pH is less than 4, total number of reflux episodes, number of acid and alkaline reflux episodes, duration of longest reflux episode.

• Safety Assessments

Information regarding complications of the procedure and patient complaints are collected and registered. After the procedure each patient is placed under medical observation for at least two hours. Any AEs related to the invasive study procedures, will be collected. Only undesirable effects that are deemed at least possibly related to an Abbott medication by the investigator will be forwarded to Abbott affiliate PV

Visit 5

- Removing of the device for 24-h esophageal pH-impedance test.

The results from the recording unit are transferred to the computer database for further analysis**.**

- Physical examination

Includes the measurement of blood pressure, pulse.

- Safety Assessments

Information regarding complications of the procedure and patient complaints are collected and registered. Any AEs related to the invasive study procedures, will be collected. Only undesirable effects that are deemed at least possibly related to an Abbott medication by the investigator will be forwarded to Abbott affiliate PV.

Conduct of the Animal (in-vitro) part of the study.

The animal part of the study will be conducted in accordance with all applicable regulatory requirements: International Guiding Principles for Biomedical Research Involving Animal, APS's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, Good Laboratory Practice.

C57/BL6 mice selected for the study would be of an appropriate genetic strain and quality, and the minimum number of animals required to obtain scientifically valid results will be defined according to the number of patients. Mice will be housed in the accredited vivarium of Moscow State University of Medicine and Dentistry to ensure that the general health of the animals is safeguarded and that undue stress is avoided. Special attention will be given to the space

allocation for each animal, and adequate standards of hygiene should be maintained as well as protection against predators, vermin, and other pests. Facilities for quarantine and isolation will be provided. Entry should normally be restricted to authorized persons. Environmental needs such as temperature, humidity, ventilation, lighting, and social interaction will be consistent with the needs of the species concerned. Noise and odor levels will be minimal. Proper facilities will be provided for the disposal of animals and animal waste. Animals will receive a supply of foodstuffs appropriate to their requirements and of a quality and quantity adequate to preserve their health, and they will have free access to potable water, unless the object of the experiment is to study the effects of variations of these nutritional requirements. Veterinary care, including a programme of health surveillance and disease prevention, will be available. Sick or injured animals should, according to circumstances, either receive appropriate veterinary care or be painlessly killed.

All mice will be infused 2 ml of 4% thioglycolates 4 days before the experiment starts. All mice will receive etherization before the experiment. Peritoneal macrophages will be isolated from peritoneal fluid of C57/BL6 mice. Peritoneal lavage fluid will be centrifuged for 4 minutes - 1000 rpm, then supernatant and cell pellet will be divided. Peritoneal macrophages will be cultured in RPMI1640 culture medium with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg /ml streptomycin on 48-well plate by $0.5x10^6$ macrophages/well.

In vitro experiments on animal biological material (according to GLP and International Guiding Principles for Biomedical Research Involving Animal).

Isolation and cultivation of macrophages. Macrophages will be isolated from biological material during centrifugation at 1000 rpm within 4 minutes. The supernatant will be rejected, and the packed cells will be resuspended in RPMI-1640 nutritional medium. Then the concentration cells in RPMI 1640 will be make up to 1x106/ml. [11]. After the procedure of macrophages isolation the degree of macrophages survival should be no less than 95% (a standard technique with trypan blue). Cell suspension will be distributed in the 48-well plate expected 0.5x106 cells per well containing 0.5 ml of RPMI 1640 medium. Macrophage cultivation will be carried out at 37ºC and 5% CO2.

Determination of macrophage phenotype. At least there are three types of markers for definition of macrophages phenotype: 1) morphological marker, such as form of the cells, 2) receptor markers, such as CD25, CD80, CD163 and CD206 expression [12], and 3) functional markers, such as cytokine production or phagocytosis ability of cells [13]. Therefore we can define at least three types of macrophage phenotypes - morphological, receptor and functional. We will execute an assessment of all three types of indicators.

The morphological macrophage phenotype was defined with the use of an ordinary light microscope with magnification power 15x40. Quantity of rounded-form macrophages, typical for M1 phenotype, and fibroblast-like cells, typical for M2 phenotype [13-18] was calculated among 100 cells in 5 fields of vision in a well [19]. Rounded cells had an equal ratio of length to width or less than 2:1. If the ratio of length to width was more than 2:1, the form of a cell was regarded as fibroblast-like [20]. The morphological phenotype of macrophage population will be estimated as a ratio of absolute count of rounded-form macrophages to fibroblast-like ones.

The receptor macrophage phenotype was determined by percentage of expression of superficial and cellular receptors - CD80 for M1 phenotype and CD206 for M2 phenotype [12, 13], in the isolated macrophages during flow cytometry by means of CD80 and CD206 monoclonal antibodies [Beckman Coulter, USA; BD Pharmingen, USA]. Analysis was performed according to the producers' instructions. The receptor phenotype of macrophage population will be estimated as a ratio of CD markers expression typical for M1 phenotype and CD markers of M2 phenotype [18, 21].

The functional macrophage phenotype was estimated by cytokine (pro-inflammatory, such as IL-12 (M1 markers) and anti-inflammatory, such as IL-10 (M2 markers)) production [22]. Cytokine concentrations will be defined by flow cytometry [Beckman Coulter FC500, USA] with multiplex definition kits. The functional phenotype of macrophage population will be estimated as a ratio of functional markers of M1 and M2 phenotypes.

Determination of macrophage phenotype after exposure to human acid refluctate, human biliary refluctate and mixed human refluctate will be performed by the above mentioned methods (the part of "Determination of macrophage phenotype") and the changes vs initial results will be registered.

***In vitro* experiments on human biological material** (according to GLP, GCP):

Isolation and cultivation of macrophages. Macrophages will be isolated from biological material (blood) or derived from blood monocytes. Macrophages will be resuspended in RPMI-1640 nutritional medium. Then the concentration of cells in RPMI 1640 will be adjusted to reflect a concentration of $1x10^6$/ml. [11]. After macrophage isolation the degree of macrophage survival should not be less than 95% (standard technique with trypan blue). The cell suspension will be distributed in a 48-well plate, with each well containing an expected 0.5x106 cells per well containing 0.5 ml of RPMI 1640 medium. Macrophage cultivation will be carried out at 37ºC and 5% CO2.

Determination of macrophage phenotype will be performed using the same methods as for animal biological materials.

Macrophage plasticity will be defined by standard criteria similar to macrophage phenotype detection**.** We will use the definition of morphological phenotype (ordinary light microscope with magnification power 15x40), receptor phenotype (percentage of expression of superficial and cellular receptors - CD80 for M1 phenotype and CD206 for M2 phenotype [12, 13], in the isolated macrophages during flow cytometry by means of CD80 and CD206 monoclonal antibodies [Beckman Coulter, USA; BD Pharmingen, USA].) and functional phenotype (NO and cytokine (pro-inflammatory, such as IL-12 (M1 markers) and anti-inflammatory, such as IL-10 (M2 markers)) production [22]. After determining the macrophage phenotype (see «Determination of macrophage phenotype»), the phenotypic plasticity (PhP) will be calculated. PhP of M1 will be calculated as percent change in the M1/M2 ratio under the action of M1 reprogramming factors and PhP of M2 will be calculated as percent change in the M2/M1 ratio under the action of M2 reprogramming factors. The phenotype marker ratio in standard culture conditions (10% FBS) will be taken as 100%.

7.0 SAFETY

There will be no investigational product in the study.

It is planned to assign the standard treatment for GERD and complications thereof (Barrett's oesophagus) on the measurement results of 24-h esophageal pH-impedance test - on the next day after visit #5.

However some medication may be prescribed before the study completion in case of need and in the interest of patient. In case any Abbott product will be part of the patient's treatment regimeany safety issue or safety signal, which would negatively impact the benefit-risk ratio of the concerned Abbott product and that is detected during conduct of the study is to be reported promptly to the Affiliate safety representative.

8.0 ETHICS AND QUALITY

The study will be conducted in accordance with protocol and all applicable regulatory requirements: ICH Guideline for Good Clinical Practice (GCP), guiding principles of the Declaration of Helsinki, guiding principles of Good Laboratory Practice (GLP), International Guiding Principles for Biomedical Research Involving Animal and all applicable local regulations.

Patient's written authorization for use and/or disclose personal and/or health data must be obtained prior to enrolling each patient in the program and patients not willing to provide such written authorization will not be included in the program. However, all reasonable efforts will be made in order to avoid subject identifying information (such as name, address, etc.).

The protocol and relevant program documents will be submitted for review and approval of Regulatory Authorities (if applicable) and Local Ethics Committee(s) (LECs) and the study will start only after obtaining written approval from the Regulatory Authorities (if applicable) and LECs of both Institutions.

All data will be captured and handled in such a way so as to not reveal identity of individual patients and hence patient confidentiality will be maintained at all times.

The physician/investigator will be responsible for ensuring that a quality control and quality assurance system is in place to ensure that the program is conducted and data generated, documented, and reported in compliance with the protocol, accepted standards of Good Clinical Practice, and any applicable local laws and regulations. The investigators shall be solely responsible for regulatory and legal compliance.

The animal part of the study will be conducted in accordance with all applicable regulatory requirements: International Guiding Principles for Biomedical Research Involving Animal, APS's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, GLP (as described above).

9.0 STATISTICAL METHODS

This is an experimental, interventional, non-randomized study. The primary purpose of the this study is exploratory in its general sense and no hypothesis is being tested, therefore no sample size calculation was performed.

Planned enrollment in this study constitutes 90 patients in clinical part and 90 subjects (mice) in animal (in vitro) part.

Statistical analysis will be performed using the STATISTICA 8.0 software for Windows (StatSoft Inc, the USA).

Normality of data distribution will be tested by using either the Shapiro-Wilk or Kolmogorov-Smirnov test.

In case of normal (Gaussian) distribution of data significance of differences will be determined by Student's t-test. In cases with non-normal distribution we will use non - parametrical U-test according to the method of Mann-Whitney for independent samples.

Tests resulting in p-values less than or equal to 0.05 will be reported as "statistically significant».

Continuous data will be summarized using descriptive statistics, and the following parameters will be reported: number of observations (n), mean and standard deviation (SD); minimum (Min), and maximum (Max); median. 95 % CI will be calculated.

Categorical data will be presented using frequency (n) and percentage (%).

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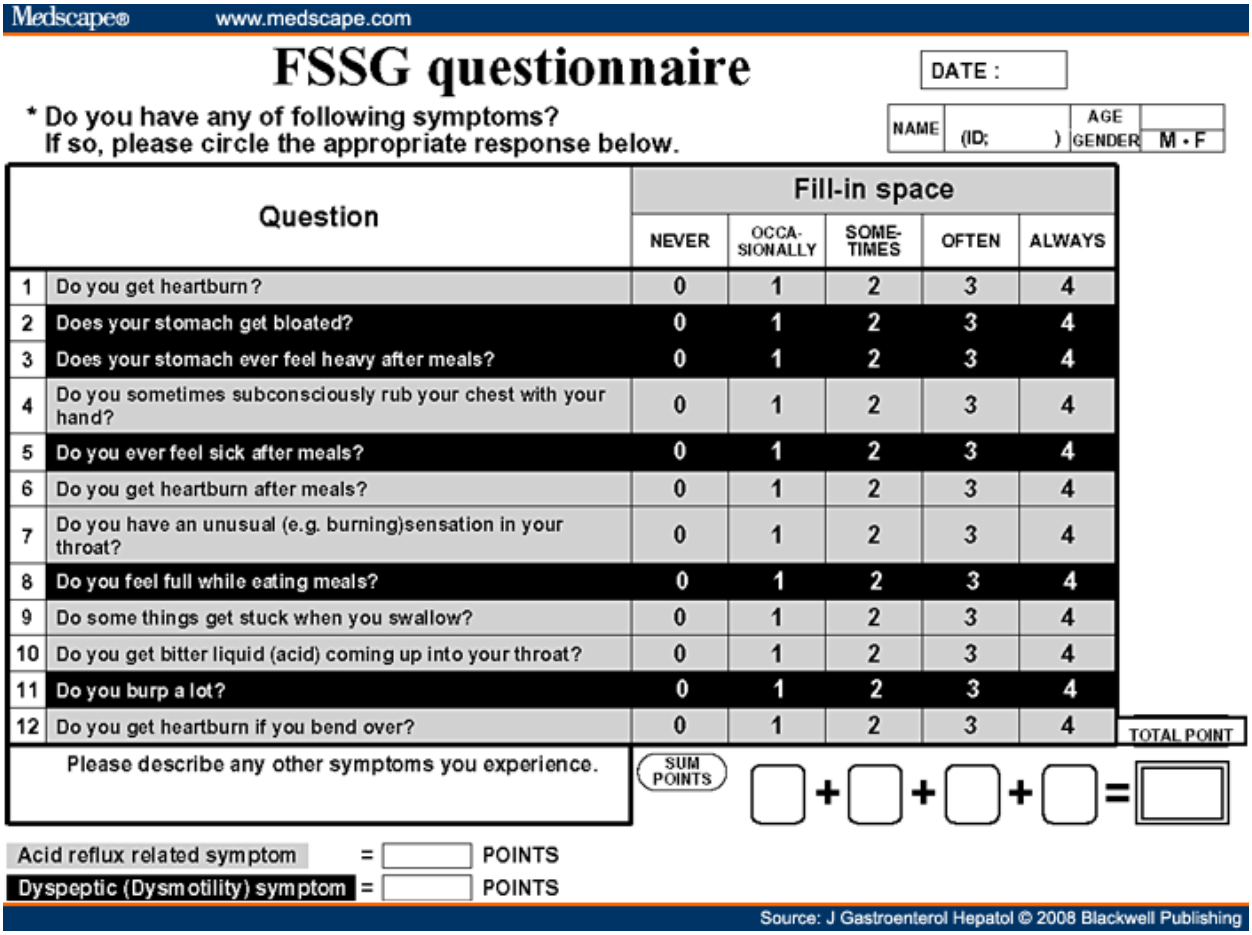
13.0 APPENDIX A: FSSG



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FSSG questionnaire

DATE :

NAME (ID;) AGE GENDER M • F

* Do you have any of following symptoms?
If so, please circle the appropriate response below.

	Question	Fill-in space				
		NEVER	OCCA-SIONALLY	SOME-TIMES	OFTEN	ALWAYS
1	Do you get heartburn?	0	1	2	3	4
2	Does your stomach get bloated?	0	1	2	3	4
3	Does your stomach ever feel heavy after meals?	0	1	2	3	4
4	Do you sometimes subconsciously rub your chest with your hand?	0	1	2	3	4
5	Do you ever feel sick after meals?	0	1	2	3	4
6	Do you get heartburn after meals?	0	1	2	3	4
7	Do you have an unusual (e.g. burning)sensation in your throat?	0	1	2	3	4
8	Do you feel full while eating meals?	0	1	2	3	4
9	Do some things get stuck when you swallow?	0	1	2	3	4
10	Do you get bitter liquid (acid) coming up into your throat?	0	1	2	3	4
11	Do you burp a lot?	0	1	2	3	4
12	Do you get heartburn if you bend over?	0	1	2	3	4

TOTAL POINT

Please describe any other symptoms you experience.

SUM POINTS ☐ + ☐ + ☐ + ☐ = ☐

Acid reflux related symptom = ☐ POINTS

Dyspeptic (Dysmotility) symptom = ☐ POINTS

Source: J Gastroenterol Hepatol © 2008 Blackwell Publishing