THE INVESTIGATOR’S PROTOCOL

1. **Title:** The Role of HIF-2α in the Pathogenesis of Reflux Esophagitis

2. **Principal investigator:** Stuart J. Spechler, MD  
   **Co-Investigators:** Rhonda Souza, MD; Shelby Melton, MD; Robert Genta, MD;  
   Kerry Dunbar, MD

3. **Sponsor of the study:** NIDDK

4. **Investigational New Drug (IND)/Investigational Device Exemption (IDE):** Not applicable

5. **Purpose of the study:** To elucidate the early histological events in the pathogenesis of reflux esophagitis in patients with GERD, and to correlate those events with esophageal expression of HIF-2α and pro-inflammatory cytokines, and with changes in esophageal proliferation.  
   **Study hypothesis:** In esophageal squamous epithelium, refluxed gastric juice activates HIF-2α, which triggers the production of inflammatory cytokines that mediate reflux esophagitis.

6. **Background and results of previous related research:** Traditional teaching holds that reflux esophagitis develops when refluxed gastric acid causes a caustic, chemical injury starting at the luminal surface of the esophagus. Using a rat model of reflux esophagitis in which an esophago-duodenostomy is created to produce reflux, we recently found that reflux esophagitis develops in a pattern that is more consistent with a cytokine-mediated injury than with a chemical burn. We observed that reflux esophagitis started with an inflammatory cell infiltration of the submucosa, not the luminal surface, and we showed that esophageal epithelial cells exposed to acid and bile salts secreted pro-inflammatory cytokines. Based on these findings, we proposed a new concept for the development of reflux esophagitis in which the reflux of gastric juice stimulates esophageal squamous cells to secrete cytokines that attract inflammatory cells that damage the esophageal mucosa. In esophageal cell lines, we have found that acid and bile salts activate hypoxia inducible factor (HIF)-2α, and we have good reason to suspect that this activated HIF-2α triggers the production of the inflammatory cytokines that mediate reflux esophagitis. If so, then therapies directed at HIF-2α or its downstream targets might have clinical utility for patients with GERD. Before pursuing such therapies, it is necessary to confirm that the HIF-2α-mediated effects observed in our cell lines also occur in esophageal epithelial cells in patients in vivo. Physicians virtually never see patients with “acute” GERD, and thus the early histological events in the pathogenesis of reflux esophagitis in humans are not known. We propose to induce acute GERD by temporarily stopping PPI therapy (for two weeks) in patients who have had endoscopically-documented, severe reflux esophagitis. Numerous studies have shown that reflux esophagitis recurs in nearly all such patients when PPI treatment is discontinued.
Definition of the population to which the study is directed, with justification:

Study patients will be veteran men and women of all ages who have had severe reflux esophagitis (Los Angeles grade C) documented by endoscopic examination.

Subject selection, inclusion and exclusion criteria:

Potential study subjects will be identified by reviewing our endoscopic database (ProVation® MD) at the Dallas VA Medical Center for patients who have had Los Angeles grade C reflux esophagitis documented during endoscopic examinations of the upper gastrointestinal tract. We will review the medical records (CPRS) of patients so identified for exclusion criteria, which include inability to provide informed consent, esophageal varices, treatment with warfarin, coagulopathy that precludes safe biopsy of the esophagus (including platelet count <100,000/mm³, INR>1.5), comorbidity that precludes safe participation in the study, allergy to fluorescein sodium, and pregnancy. Eligible patients will be sent a letter describing the study, explaining how they can call for more information, and informing them that they may be contacted by an investigator by telephone to follow up on their interest in study participation.

Number of subjects in the study:

We plan to enroll 30 veteran patients with a history of Los Angeles grade C reflux esophagitis.

Justification for the use of special subject populations:

The study is open to all veteran patients who had Los Angeles grade C reflux esophagitis documented during an endoscopy performed at the Dallas VA Medical Center. There are no exclusions based on gender or race.

Study design:

Patients who provide written, informed consent will have a medical history taken and a physical examination, and blood will be drawn for determination of platelet count and INR; women of child bearing potential will have a pregnancy test. Eligible patients will be treated with at least double-dose PPI therapy (omeprazole 20 mg or the corresponding conventional dose of any other PPI that they have been prescribed, given BID) for at least one month prior to the study. After at least one month of this PPI therapy, patients will report to the GI laboratory after taking their morning dose of PPI (Study Day 1). A validated symptom questionnaire (the GERD HQRL) will be administered to assess the severity of symptoms. An esophageal manometry will be performed to record esophageal motility and to determine the location of the lower esophageal sphincter. Next, an esophageal pH/impedance catheter will be passed through the nose for a 24-hour esophageal pH/impedance monitoring study to document their degree of acidic and non-acidic reflux during PPI therapy. Patients will take the evening dose of PPI while the catheter is in place, and then no more PPIs will be taken for the duration of the study. Patients may take antacid (Maalox®) as needed for heartburn for the 2-week duration of the study. The catheter will be removed on the following day (Study Day 2), and an endoscopic examination will be performed to document that there is no endoscopic evidence of reflux esophagitis. Patients with significant reflux esophagitis (Los Angeles grades B-D) will not be eligible for further study. During the endoscopic procedure, patients will also undergo confocal laser endomicroscopy. The confocal
endomicroscope is a standard gastrointestinal endoscope with a confocal microscope built into the tip that can provide images of the mucosa magnified up to 1000-fold. 5 ml of fluorescein sodium will be given intravenously, and the tip of the endoscope will be placed gently on the mucosa of the esophagus to acquire images of the esophageal mucosa. Images of the esophageal mucosa will be saved for later analysis of the size of intercellular spaces, numbers of interpapillary capillary loops, and other imaging features suggestive of GERD. Patients without reflux esophagitis will have 10 esophageal biopsy specimens taken at a level 1 to 3 cm proximal to the squamo-columnar junction (4 specimens will be fixed in formalin and embedded in paraffin, and 6 will be snap frozen). Patients will return one week later (Study Day 9) for a second endoscopic examination including confocal endomicroscopy, at which time the grade of esophagitis will be scored using the LA system, and 10 esophageal biopsy specimens will be taken as described above. If there are incompletely healed biopsy sites, care will be taken not to take biopsy specimens at those sites. On Study Day 15, the GERD HRQL questionnaire will be administered to document changes in GERD symptoms, esophageal manometry will be repeated to document any esophageal motility changes associated with the onset of esophagitis, and 24-hour esophageal pH/impedance monitoring will be repeated to document the degree of acidic and non-acidic reflux off PPI therapy. The pH/impedance catheter will be removed the next day (Study Day 16), and an endoscopic examination will be performed using the same procedure as described for study day 9 including confocal endomicroscopy. After this endoscopy, the study is terminated and patients will resume their PPI therapy as clinically indicated.

12. Description of procedures to be performed: Study patient procedures include three endoscopic examinations with confocal endomicroscopy and esophageal biopsy, two esophageal manometries and two 24-hour esophageal pH/impedance monitoring studies as described above. For all histological evaluation of esophageal biopsy specimens, the study pathologists will be blinded to the experimental time point at which the specimens were obtained. H&E-stained esophageal biopsy specimens will be evaluated using a scale ranging from 0 (absent) to 4 (severe) to score the degree of: a) inflammation in the epithelium b) basal zone hyperplasia and c) papillary hyperplasia. The scoring system will be used to determine the intensity of the neutrophilic, lymphocytic, and eosinophilic infiltrates individually; a composite inflammation score will be generated by the addition of each of these individual scores, using a system similar to that reported in our rat model of reflux esophagitis. The presence of erosions or ulcerations will also be recorded.

We will perform immunohistochemical (IHC) staining using anti-HIF-2α on paraffin-embedded sections of biopsy tissues. We will determine expression levels for pro-inflammatory cytokines using IHC staining and, in snap frozen biopsy specimens, ELISA assays, QRT-PCR and Western blotting. We will score the tissue specimens based on staining intensity and on the percentage of cells that stain for a particular protein. Staining intensity will be scored on a scale of 0 to 3 (0 = none; 1 = minimal; 2 = moderate; 3 = intense). We will determine each score individually and generate a combined score which will be the % positive cells x intensity of the positivity. The
combined scores from 5 different high power fields per section will be averaged to
derive a final score for that tissue specimen.
The proliferative compartment will be identified with primary antibodies to
proliferating cell nuclear antigen (PCNA), Ki-67, and cyclin D1; apoptosis rates will
be assessed by the TUNEL assay and by IHC staining for cleaved caspase 3.

13. Anticipated data and data analysis: We anticipate that reflux esophagitis in our
patients will follow the same histological sequence that we observed in our rat model
of reflux esophagitis, i.e. there will be an initial lymphocytic infiltration of the
submucosa that progresses to the mucosal surface, with basal cell and papillary
hyperplasia preceding the development of surface cell erosions. This is the opposite
sequence of events that would predicted from the prevailing chemical burn concept of
reflux esophagitis pathogenesis, in which one would expect initial damage to the
surface epithelium with neutrophilic infiltration and surface cell erosions preceding
basal cell and papillary hyperplasia. We also anticipate that HIF-2α expression will
increase as the inflammatory index increases, and that the increase in HIF-2α will
correlate with increased expression of cytokines and markers of proliferation in the
squamous epithelium.
Sample size determinations and selection of appropriate statistical analyses were
determined in consultation with Daisha J. Cipher, Ph.D., Associate Professor,
University of Texas at Arlington School of Nursing, and Biostatistical Consultant,
Dallas VA Research Corporation. Power analyses using SAS 9.2 indicate that a total
of 12 subjects are required to achieve our aims (based on one-sample repeated
measures analyses of inflammation over time using McNemar tests, with an
anticipated effect size of 80%, a study alpha of .05, and a beta of .10). We will
accommodate a 40% attrition rate (due to dropouts, inability to remain off PPI, and
the presence of significant esophagitis on the initial endoscopy) by enrolling 30 total
subjects. All study variables will be descriptively analyzed for measures of central
tendencies. The presence of esophageal inflammation will be compared from baseline
to 7 days, and baseline to 14 days with McNemar tests. Changes over time in raw
composite inflammation scores (with possible ranges from 0 to 4) will subsequently
be analyzed with a Friedman test. Increases in HIF-2α and inflammation from
baseline to 14 days will be re-coded as dichotomous variables, and those increases
will be tested for association with a phi coefficient. A significant phi coefficient will
be an indicator that as HIF-2α increases, inflammation increases. If this is the case,
subsequent phi coefficients will be computed to test for associations between
increases in HIF-2α and markers of proliferation (PCNA, Ki-67, cyclin D1), in HIF-
2α and markers of apoptosis (TUNEL, cleaved caspase-3), and in HIF-2α and pro-
inflammatory cytokine expression.

14. Provision for managing adverse reactions: Any emergency adverse event resulting
from participation in the study will be treated at the Dallas VA Medical Center. After
each endoscopy, patients will be observed in the holding area per standard of care for
post-endoscopy patient management. All patients will be instructed to notify the
study investigators immediately if they experience any delayed complications.
15. **Risk/Benefit Assessment:** The risks to study patients involve those associated with endoscopy with confocal laser endomicroscopy and esophageal biopsy, with esophageal manometry and pH/impedance monitoring, and with the discontinuation of PPI therapy. Serious complications of upper gastrointestinal endoscopy in patients who have no serious comorbidities (a requirement for study entry) are rare (fewer than 1 in 1,000 cases) and include aspiration, perforation and bleeding. The added risk associated with endomicroscopy is related to the administration of fluorescein sodium, which has been used for many years by ophthalmologists for retinal angiography. All patients who receive fluorescein develop transient yellow discoloration of the skin, which fades over several hours. Reported adverse events from fluorescein include nausea and vomiting, hypotension, injection site erythema, rash, and anaphylaxis. The largest study of fluorescein safety in gastroenterology involved 16 academic medical centers and 2,272 patients who had confocal laser endomicroscopy. Mild adverse events occurred in 1.4% of procedures, including transient hypotension (0.5%), nausea (0.39%), injection site erythema (0.35%), rash (0.04%), and mild epigastric pain (0.09%). There were no cases of anaphylaxis or serious adverse events. (Wallace MB, Meining A, Canto MI, et al; The safety of intravenous fluorescein for confocal laser endomicroscopy of the gastrointestinal tract; Aliment Pharmacol Ther 2010;31:548-52). In a review of 221,781 patients who received fluorescein for eye exams, serious adverse events included respiratory events (1.3800 or 0.03%), cardiac complications (1:5300 or .019%), seizures (1:13,900 or .007%) and one death). (Yannuzzi LA, rohrer MA, Tinde LJ et al, Fluorescein Angiography complication survey, Ophthalmology 1986:93(5):611 – 617). Esophageal manometry and pH/impedance monitoring involves the discomfort of having a thin-bore catheter passed through the nose into the esophagus, but there are virtually no serious adverse events associated with this clinical procedure. Discontinuation of PPI therapy in patients with Los Angeles grade C esophagitis is likely to result in an increase in GERD symptoms such as heartburn and regurgitation, and might result in the development of esophageal erosions. It is highly unlikely that discontinuation of PPI therapy will cause any serious or long-lasting damage to the esophagus, or any other serious complication. It is well known that GERD recurs in the large majority of patients with Los Angeles grade C esophagitis who discontinue PPI therapy. However, in the American Gastroenterological Association Institute’s technical review on the management of GERD (Kahrilas PJ, Shaheen NJ, Vaezi MF; American Gastroenterological Association Institute; Clinical Practice and Quality Management Committee. American Gastroenterological Association Institute technical review on the management of gastroesophageal reflux disease. Gastroenterology 2008; 135:1392-1413), the authors wrote: “There are no high quality data to suggest that continuous antisecretory therapy alters the natural history of reflux disease, other than to reduce the (already low) incidence of peptic stricture. There are also no data to the effect that intermittent esophageal erosions or some degree of residual symptomatology is harmful.” The authors also wrote “Certainly, the data do not support the contention that residual GERD symptoms predispose patients to the development of Barrett’s esophagus or esophageal adenocarcinoma…Beyond recurrence of symptoms and/or erosive disease, the risks
associated with cessation of therapy, including the possible development of Barrett’s esophagus, are minimal.”

Numerous studies that have documented the efficacy of PPI therapy for reflux esophagitis have included groups treated with placebo for 8 weeks to 6 months without apparent serious consequences other than the persistence or return of GERD symptoms and reflux esophagitis. Furthermore, our animal model of reflux esophagitis suggests that esophageal ulcerations do not develop for several weeks after the onset of severe GERD. We propose to discontinue PPI treatment for only two weeks in our study patients. The patients will be allowed to take antacids, which can relieve heartburn even though they have no documented efficacy for healing reflux esophagitis. All patients will be informed that, if they experience intolerable GERD symptoms despite antacid therapy, they should withdraw from the study.

Study participation may be of no immediate benefit to the research subject. However, it is important to understand the early pathophysiology of GERD at the cellular and molecular levels if new medical treatments are to be developed to improve symptom control and to prevent the development of GERD complications like Barrett’s esophagus and esophageal adenocarcinoma. Although the PPIs have been extremely effective for healing reflux esophagitis, up to 40% of patients treated with PPIs continue to experience bothersome GERD symptoms. Furthermore, the frequency of GERD symptoms and complications that do not respond to PPI treatment might be due to the reflux-induced release of pro-inflammatory and pro-proliferative cytokines mediated by HIF-2α. If so, then therapies directed at HIF-2α or its downstream targets might be developed, and such therapies might result in better symptom control and prevention of GERD complications.

16. Data Safety Monitoring Plan
Does this study have a Data Safety Monitoring Board (DSMB)?    YES    NO

This is a single-center study that is not a Phase III trial. Therefore, a Data Safety Monitoring Boards (DSMB) is not required. To ensure that the safety of the patients and the integrity of our data are preserved, Dr. Spechler will review the data on a regular basis. The goal of this periodic review of the collected data is to search for trends of adverse events or ill-effects so that the health of current and perspective patients is not jeopardized.

17. Planned procedures for obtaining informed consent:  As described above, potential study patients identified through review of our endoscopic database (ProVation® MD) and medical records (CPRS) will be sent a letter describing the study, explaining how they can call for more information, and informing them that they may be contacted by an investigator by telephone to follow up on their interest in study participation. The principal investigator or a co-investigator will explain the study and proposed procedures to potential subjects. For patients who express interest in participating, the physician investigator will go over the consent form, answer any questions, and address any concerns. The study consent will be entered into CPRS.
18. Documentation of informed consent: A research enrollment note documenting informed consent will be entered into CPRS for all patients enrolled in the study. Only the PI, Dr. Spechler, will have responsibilities for entering the appropriate research enrollment accept or research enrollment decline note in the electronic medical record.

19. Payment to subject for their participation: Participation in this study entails substantial inconvenience (three endoscopic examinations, two esophageal manometries, two 24-hour esophageal pH/impedance monitoring studies), and the potential discomfort involved in the recurrence of GERD when PPIs are stopped for two weeks. We propose to compensate patients as follows:

- $125 for each endoscopy (3 total=$375)
- $200 for each esophageal manometry with 24-hour pH/impedance monitoring (2 total=$400)
- $225 for completing the entire study

Therefore, the total compensation for a patient who completes the entire study is $1,000.

20. Provision for assuring confidentiality and protecting the subject’s privacy: The investigators maintain confidentiality of all research records, and no one outside of the IRB, VA staff involved with the study, and appropriate officials of the VA will be permitted access to those records. The study records will be kept in a locked, confidential area in the Gastroenterology Division. Patient names will not be included in any publication prepared as a result of this study.

In accordance with HIPAA, the consent form describes to the subject what protected health information (PHI) will be obtained and/or stored and for what purpose, as well as a list of who may have access to this data, including outside agencies. All records will be maintained in a locked cabinet in the research team’s locked office. Computerized data will be de-identified and stored on a password protected computer in a locked office of the research team.

In accordance with VA guidelines, all records of this research study will continue to be securely maintained for a minimum of five years from the date of completion of the study. The records will be kept in a locked file cabinet or locked room with limited access. If the PI leaves the VA facility, the original research records will be retained by the institution.

21. Dissemination of research results: Study results will be submitted for publication in peer-reviewed journals. In addition, preliminary results may be submitted in abstract form for presentation at scientific meetings including the annual meetings of the American Gastroenterological Association (DDW) or the American College of Gastroenterology. A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law.