TITLE: BILE REFLUX IN PATIENTS WITH NERD IS ASSOCIATED WITH MORE SEVERE HEARTBURN AND LOWER VALUES OF MEAN NOCTURNAL BASELINE IMPEDANCE AND CHEMICAL CLEARANCE.

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NdB, ES: concepted designed the study; performed the research; collected and analysed the data; wrote the paper.

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Keypoints:

- Multichannel intraluminal impedance pH (MII-pH) monitoring and Bilitec are two techniques able to explore the underlying mechanisms associated to reflux symptoms. In particular, MII-pH can characterize the mucosal integrity, through the measurement of mean nocturnal baseline impedance (MNBI), and the chemical clearance, through the assessment of the post-reflux swallow-induced peristaltic wave (PSPW) index, providing further insights beyond the refractoriness to medical therapy in GERD
- In the present study, investigating patients with refractory GERD, we found that abnormal bile reflux is associated to greater impairment of mucosal integrity and chemical clearance, and when combined with acid reflux seems able to provoke more severe heartburn. Thus, abnormal bile reflux could be responsible of refractory GERD in patients assuming PPI therapy

ABSTRACT

Background. Mean nocturnal baseline impedance (MNBI) and post-reflux swallowed induced peristaltic wave (PSPW) index are novel impedance-based markers of reflux, but effect of bile reflux on these metrics is unknown. The aim of this study was to evaluate bile reflux, MNBI and PSPW index in patients with endoscopy-negative GERD partially responsive to PPI therapy.

Methods. All patients underwent off-PPI endoscopy, esophageal manometry, multichannel intraluminal impedance-pH (MII-pH) and bile reflux monitoring. Abnormal esophageal acid exposure time (AET) was required for inclusion. Symptom intensity (using 10-cm visual analog scales), conventional and novel MII-pH metrics were compared between patients with and without abnormal bile reflux.

Key results. We evaluated 42 NERD patients (29 males, mean age 53.4±13.8 yrs), mean AET 6.1±2%, of which 21 had abnormal bile reflux (group A, 10.2±4.9%), and 21 had normal bile reflux (group B, 0.4±0.1%, $p<0.05$ compared to group A). Heartburn reporting on PPI was higher in group A (7.2±2.1 vs 5.8±0.9; $p=0.002$), but AET, number of reflux events (acidic and weakly acidic) did not differ between the two groups. However, both PSPW index and MNBI were lower in group A ($p<0.001$). A strong inverse linear correlation was found between bile reflux and both MNBI (Pearson test; $R= -0.714$; $p<0.001$) and PSPW index ($R=-0.722$; $p<0.001$).

Conclusions and Inferences. Compared to acid reflux alone, the presence of bile in an acidic esophageal environment is associated with more severe heartburn, lesser relief from PPI therapy higher impairment of esophageal mucosal integrity and less effective chemical clearance.

KEY WORDS: GERD; biliary reflux; mean nocturnal baseline impedance, PSPW index; PPI.

BACKGROUND

Gastroesophageal reflux disease (GERD) is a chronic condition characterized by a heterogeneous spectrum of typical and atypical symptoms, of which heartburn is the most common symptom.[1,2]

Since proton pump inhibitors (PPIs) are effective in improving GERD related heartburn, response to empiric PPI therapy is used clinically to confirm GERD diagnosis in the absence of alarm symptoms, despite limited specificity due to overlap with functional esophageal disorders.[3-5] However, many patients respond only partially or do not respond to an 8-week course of full-dose PPI.[6] A normal upper endoscopy is found in the majority of such patients, which forms the basis for a diagnosis non-erosive reflux disease (NERD) when there is objective evidence that heartburn is reflux-related.[7,8]

The recent recognition that abnormal findings on reflux monitoring may have a predictive value on symptom improvement has necessitated changes to the current NERD definition, preferably through a systematic approach.[9] With this aim, multichannel intraluminal impedance pH (MII-pH) monitoring has been proposed as the tool to distinguish NERD from heartburn not related to reflux, e.g. functional heartburn (FH).[10] This is primarily because two new impedance based metrics, mean nocturnal baseline impedance (MNBI) and post-reflux swallow-induced peristaltic wave (PSPW) index.[11,12] may categorize heartburn better than AET in terms of predicting PPI response and refractoriness, and in distinguishing GERD from FH.[11,13,14] An added advantage is that both of these metrics are independent of patients reporting symptoms and day-to-day variability of reflux monitoring.[15-17] Impaired mucosal integrity, reflected by lower esophageal baseline impedance values[18] can be easily and reliably evaluated by means of MNBI.[19] Adequacy of chemical clearance of residual mucosal acidification is measured by the PSPW index, which is abnormal in PPI refractory states.

While suboptimal esophageal chemical clearance has been well evaluated in the context of PPI refractoriness,[17,20,21] esophageal bile reflux has generally been under-evaluated. When both the

pyloric and esophago-gastric junction (EGJ) barrier function are impaired, duodeno-gastro esophageal reflux (DGER) can lead to presence of bile in the esophagus.[22] Utilizing a fiberoptic spectrophotometric probe that measures the absorbance spectrum of bilirubin (Bilitec®), studies have demonstrated increased bile acid concentration in esophageal aspirates in GERD patients with esophagitis.[23,24] Additionally, persistent acid reflux combined with Bilitec®-detected bile reflux may better explain persistent symptoms despite PPI therapy in patients with Barrett's esophagus.[25] However, the role of bile reflux in NERD, and effects on mucosal integrity and esophageal clearance are scarce to non-existent.

The aim of this observational study was to evaluate the role of esophageal bile reflux on the integrity of esophageal mucosa using MNBI, and esophageal chemical clearance using PSPW index, utilizing a combination of baseline symptomatic assessment, endoscopy, MII-pH monitoring, Bilitec monitoring, and patient follow-up in a cohort of well-characterized NERD patients with incomplete response to double PPI therapy.

MATERIALS AND METHODS

Patients

In this retrospective study, we analyzed prospectively collected data from consecutive adult endoscopy-negative patients, who were referred throughout 2018 to the outpatient's motility laboratories at the University of Pisa and Padua for troublesome heartburn with incomplete response to double dose PPI therapy (pantoprazole or esomeprazole 40mg bid) and an esophageal acid exposure higher than 4 recorded during reflux monitoring test. Exclusion criteria consisted of pregnancy (excluded by urine analysis) and/or breast feeding; eating disorders; history of thoracic, esophageal or gastric surgery; neoplasia; achalasia, esophago-gastric junction outflow obstruction, scleroderma, major disorders of peristalsis, underlying psychiatric illness or psychiatric therapies; use of non-steroidal anti-inflammatory drugs and aspirin. Heartburn unaffected or totally suppressed by PPI therapy constituted an additional exclusion criteria, as the study specifically targeted patients with partial PPI response. All patients signed an informed consent before undergoing clinical investigations. The study was designed and carried out in accordance with the Helsinki Declaration, and was approved by the local Institutional Review Boards.

Erosive esophagitis and other esophageal mucosal abnormalities were excluded by upper endoscopy performed after 4-week withdrawal of antisecretory therapy, within 6 months prior to the study inclusion. Double dose PPI treatment was recommended to each patient fulfilling study inclusion criteria after upper endoscopy in patients who previously has been treated with standard dose and did not recorded an adequate symptom improvement. Heartburn was scored using a 10-cm visual analogue scale (VAS) at baseline and after 4 weeks of PPI therapy.[26] A study investigator performed clinical evaluation of the patients using a validated questionnaire, including a careful medical history (with recording of height and weight), current medications, tobacco and alcohol consumption. Partial response to PPI therapy was defined as an on-PPI heartburn score between 5 and 8 on the 10 cm VAS after 8 weeks of double dose PPI therapy, meaning that the patients obtained some improvement in symptoms reporting during PPI treatment although not complete.

All subjects fulfilling the above symptom-based criteria for partial PPI response underwent solid state esophageal high-resolution manometry (HRM) and simultaneously the two 24-h monitoring tests: MII-pH monitoring/Bilitec off PPI therapy (14-day wash-out). During the PPI withdrawal period, patients were only allowed to take alginates, on an as-needed basis to control heartburn.[27] HRM, MII-pH monitoring, and bile reflux monitoring were performed after an overnight fast.

High resolution manometry

All study patients underwent esophageal HRM using a 36-sensor solid state system (Sierra Scientific/Given Imaging, Los Angeles, CA, USA) capable of generating spatio-temporal Clouse plots of esophageal motility.[28-30] After naso-gastric catheter placement and a 5-min adaptation period, baseline measurements of EGJ pressure were acquired over a 30 second period of quiet rest without swallows. All patients were administered 10 test swallows of 5 mL water swallows administered by syringe in the supine position. Peristaltic performance after multiple rapid swallows was then assessed using five 2 mL water aliquots administered in rapid succession by syringe in the supine position.[31] EGJ morphology was assessed, and manometric findings were classified according to the current Chicago Classification (Version 3.0).[32,33]

Esophageal multichannel intraluminal impedance and pH monitoring

MII-pH monitoring was performed using a polyvinyl catheter (diameter: 2.3 mm), equipped with an antimony pH electrode and several 4 mm cylindrical electrodes at 2 cm intervals (Sandhill Scientific Inc. Highland Ranch, CO). Each pair of adjacent electrodes represented an impedance-measuring segment corresponding to one recording channel. The single-use MII-pH catheter was positioned with the pH electrode 5 cm and the six impedance recording channels at 3, 5, 7, 9, 15 and 17 cm above the proximal border of the lower esophageal sphincter (LES). All patients consumed foods and beverages exclusively during three standard meals (lunch at 1.00 p.m., dinner

at 8.00 p.m. and breakfast at 8.00 a.m. the next morning) on the basis of a Mediterranean diet,[34] without green vegetables, extra virgin olive oil, alcohol and coffee, to reduce the variability due to alimentary habits. The patients were invited to consume white rice or pasta with butter and parmesan (first dishes) and to choose among grilled fish or meat (chicken, beef, turkey), chees (emmenthal, parmesan, stracchino), ham (backed or dry cured) (second dishes) with potatoes or cauliflower. They were instructed to record the beginning and ending times of each meal. Patients were also instructed to remain in an upright position during the day and to indicate the night-time recumbent period (maximum 8 hours). Each patient was instructed to press the "event marker" button on the pH data-logger whenever they experienced reflux symptoms during the recording period. At the end of the recording period, data were edited using a dedicated software program (Bioview Analysis, Sandhill Scientific, Highlands Ranch, CO, USA).

MII-pH data analysis

MII-pH tracings were collected and reviewed manually by two expert investigators (NdB, ES). Meal periods were excluded from analysis. Impedance and pH data were used to determine the number and type of reflux episodes, and to calculate the acid exposure time (AET, percent time pH is <4.0 during the MII-pH study, normal <4.0%) in each patient.[34] Acidic, weakly acidic and weakly alkaline refluxes were identified using standard definitions[10] and the total number of reflux episodes was calculated (normal value <54).[34] Abnormal AET was required for inclusion in the study.

MNBI (Ohms) was assessed from the most distal impedance channel for 10 min periods during the overnight period at three time points (around 1.00 am, 2.00 am, and 3.00 am) avoiding swallows, reflux events and pH drops. The PSPW index, originally described by Frazzoni and co-workers,[35] was manually calculated and defined as the number of reflux events followed within 30s by a swallow-induced peristaltic wave as a proportion of the total number of reflux events.

Bilitec 2000

The fiber-optic spectrophotometer Bilitec 2000 was used to quantify bile reflux, concomitant with MII-pH recording. The system consists of a miniaturized 1.8 mm probe that beams light signals into the esophagus and back via a plastic fiberoptic bundle. Prior to analysis, the probe was calibrated in water. The catheter was introduced nasally and positioned 5 cm above the lower esophageal sphincter. Patients received a standardized diet during the test, as previousl mentioned.

Bile reflux was defined as an increase in bilirubin absorbance level ≥ 0.14 for at least 10 s. Pathological values were defined as the presence of abnormal bilirubin absorbance for more than 1.8% of the total 24-hour period.[36]

According to Bilitec 2000 findings, patients were divided into: i) Group A: patients with abnormal bile exposure (≥ 1.8%); ii) Group B: patients with normal bile exposure (< 1.8%).

Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normality of data distribution. Data normally distributed are expressed as median and interquartile range (IQR). Statistical tests to compare groups of subjects included the Student t test and analysis of variance for difference in mean values, the Mann–Whitney U test and the Kruskal–Wallis test for skewed variables, and the Pearson chi-squared test (with Yates continuity correction as appropriate) for differences in counts and frequency. Pearson analysis was performed to evaluate for correlation between MNBI and PSPW index values with bile exposure in patients with positive Bilitec studies. A p-value less than 0.05 was considered statistically significant. Analyses were performed using SPSS (version 21, IBM Corp, Armonk, NY)

RESULTS

Demographic and clinical characteristics

Sixty-nine endoscopy-negative heartburn patients who resulted partial responder to PPI double dose were screened for doing MII-pH and Bilitec 2000. Only 42 patients (60.8%) complete both tests. All details regarding enrollment were reported in the flow-chart of the consecutive screened patients (**Figure 1)**. Forty-two endoscopy-negative heartburn patients (29 M/13 F), with a mean age of 52 (IQR 45.5-63) years were enrolled for this study. Sixteen of 42 (38.1%) patients were smokers, 35/42 (83.3%) had at least one coffee per day and 22/42 (52.4%) reported consuming 1 alcohol unit (wine) per day. All patients were partial responders to PPI therapy according to the study definition (mean VAS for heartburn relief was 6.5±1.4).

Twenty-one out of 42 (50%) patients (Group A) had abnormal esophageal bile exposure. Indeed, they had significantly higher bile reflux as compared to Group B (9.6 IQR 8-13% vs 0.2 IQR 0.2-0.5; $p<0.0001$). There were no differences in baseline characteristics between the two groups, except for heartburn while on PPI therapy, which was higher in Group A ($p=0.002$). All data are reported in **Table 1**.

During HRM, an EGJ type III morphology was noted in 23/42 (54.7%) of patients (11 from group A and 12 from group B), 12/42 (28.6%) had EGJ type II morphology (7 from group A and 5 from group B) and 7/42 (16.7%) had EGJ type I morphology (3 from group A and 4 from group B) (p=ns). Only 5 patients (11.9%) had diagnosis of ineffective esophageal motility with 60% of ineffective swallows (3 from Group A and 2 from Group B) (p=ns). We observed 4 patients with fragmented peristalsis (2 from each group) with normal DCI but with large breaks (fragmented). All patients diagnosed as IEM and fragmented peristalsis failed MRS; additionally, 3 patients from group A with normal peristalsis recorded an ineffective MRS. In these patients the mean MRS/SS ratio was 0.6. There were no significant differences between MRS/SS ratio between the two groups (MRS/SS ratio 1 IQR 1-1.1 in group A and 1 IQR 1-1.2 in group B; p=NS).

There were no differences in total, upright or recumbent AET, or the number of reflux episodes (acid, weakly acid or weakly alkaline) between the two groups. (p=ns). The total number of heartburn events reported during the analysis was higher in Group A patients (p=0.04). The SI and SAP were both positive respectively in 19 patients in Group A and in 16 patients in Group B (p=ns). Interestingly, MNBI was lower in patients from group A as compared to those in group B (1014; IQR 926-1296 versus 1748; IQR 1563-2001; $p<0.0001$). Similarly, PSPW index was also lower in Group A compared with group B (35; IQR 33-38 versus 45; IQR 42-51; $p<0.001$). Details regarding findings from MII-pH monitoring are reported in Table 2.

A strong inverse linear correlation was found between esophageal bile exposure and both MNBI ($R=-0.714$; $p<0.001$) and PSPW index ($R=-0.722$; $p<0.001$). These data are depicted in Figures 1 and 2.

DISCUSSION

While bile reflux has been implicated in GERD-related complications such as severe erosive esophagitis and Barrett' esophagus,[25] no studies to date have explored the role of bile reflux in the pathogenesis of NERD or response to PPIs in NERD. In this study evaluating the relationship between bile reflux and partial heartburn response to PPIs, we demonstrate that: a) patients with abnormal bile reflux have more severe heartburn despite similar AET values as those with normal bile reflux; b) patients with abnormal bile reflux showed lower MNBI and PSPW index, with strong negative inverse correlations between bile reflux and both these metrics. Based on our results, we can argue that these findings have an important clinical value in NERD patients because the presence of bile reflux cannot be successfully treated with PPI therapy even with double dosage. In line with this assumption, bile reflux may be considered a pathophysiological marker of disease activity, thus emphasizing its role in PPI refractoriness and explaining the lower value of both chemical clearance and MNBI.

Bile reflux was first documented using spectrophotometry (Bilitec®) in 1990,[37] and subsequent studies showed a role of DGER in the genesis of GERD-related symptoms.[38,39] Gasiorowska et al.[40] demonstrated in a large group of patients with GERD-related symptoms that 67% of poor responders to PPI treatment had documented DGER. Further, Kunsch et al.[41] observed that bile reflux was reduced by pantoprazole only in patients with PPI-responsive reflux symptoms but was unaffected in PPI-refractory cases. Our results also show that persistent troublesome residual heartburn during PPI treatment occurs in patients with abnormal biliary esophageal exposure, thus highlighting the role of bile reflux in triggering reflux symptoms in NERD patients with incomplete PPI response.

The role of the DGER has always been suspected to be directly involved in the pathophysiology of GERD. Farré et al.[42] showed that a mix of acid and biliary salts induced more intercellular dilated spaces (DIS) on esophageal mucosa compared with acid alone. The same authors observed a progressive reduction of trans epithelial resistance (TEER) associated with

increase in concentration of biliary salts; the same results were not recorded with acid alone.

Moreover, Padron MA and colleagues[43] demonstrated in their *in vitro* study higher levels of apoptosis among esophageal cell cultures during infusion of acid and biliary salts (deoxycholic acid) compared to that observed after acid infusion alone. Our data corroborates these findings, since we found a strong linear inverse correlation between bile reflux and MNBI, suggesting an important role of bile in impairment of mucosal integrity in patients with proven NERD. Moreover, the strong inverse linear correlation between bile exposure and PSPW index suggests that the vagal esophago-salivary reflex and timely delivery of saliva in the distal esophagus following a reflux episode can be impaired in DGER. These findings could also potentially indicate bile may not be an adequate stimulus for initiation of the esophago-salivary reflex in contrast to acid alone.

Previously, Vaezi M and Richter J.[44] showed that abnormal esophageal bile reflux was more frequently detected in patients with Barrett's esophagus and reflux esophagitis as compared to NERD patients. This study strongly supported the hypothesis that acid and bile were able to affect esophageal mucosa more than acid alone, but the NERD group consisted of 16 cases only. Our series consisted of 42 patients with well documented NERD, and our results showed for the first time that bile reflux determines impairment of mucosal damage that goes undetected at endoscopy. With these results, it can be assumed that patients with abnormal acid and biliary reflux seems affects by the reflux events more than those with abnormal acid reflux alone.

The most important limitation is that we did not include a control group with complete lack of response to PPI treatment. We opted for this approach since in our experience the number of patients completely refractory to PPIs usually represent a functional esophageal disorder rather than pathologic reflux disease. A different potential limitation was the double probe analysis (MII-pH and Bilitec) that might be troubling for patients even if, in our opinion, it might have little influence on the results of our study. Finally, we have to acknowledge that there are few data in literature reporting that a white diet could reduce acid exposure and influence bilitec results[45]. However, we believe that at least in part this limitation has been obviated due to the fact that the white diet we

chosen has been based on animal protein and fats, that has been previously associated to greater reflux burden.[46]

In conclusion, our results show that patients with DGER complain of more severe heartburn and lower symptom relief during PPI therapy. Lower MNBI values in patients with abnormal bile reflux reflect more severe impairment of mucosal integrity, possibly explaining more severe heartburn and lower responsiveness to PPI therapy.

TABLE PAGE

Table 1. Baseline characteristics of 42 NERD patients partially responsive to PPI therapy. All data are reported as median and IQR)

	Group A (21)	**Group B (21)**	***p***
Male/Female ratio	15/6	14/7	0.972
Mean Age (IQR)	53 (46-63)	52 (42-63)	0.225
Mean BMI (IQR)	26 (25-28)	25 (24-26)	0.585
Smoking (%)	9 (42.9%)	7 (33.3%)	0.525
Coffee (%)	19 (90.6%)	16 (76.2%)	0.403
Alcohol (%)	11 (52.4%)	11 (52.4%)	1

LEGEND: Group A= positive for biliary reflux; Group B=negative for biliary reflux

NERD = non-erosive reflux disease; IQR= interquartile range

Table 2: On-PPI heartburn grading and off-PPI MII-pH 24-h findings in 42 NERD patients partially responsive to PPI therapy. (All values are reported as median; IQR)

	Group A (21)	**Group B (21)**	***p***
Heartburn (baseline-OFF PPI)	9; 9-10	9; 8-9.3	0.734
Heartburn (ON PPI)	**8; 7-8**	**5.5; 5-6.3**	**0.002**
AET (%)	8.7; 6.9-9.8	9.3; 6.9-10	0.818
AET upright (%)	23.8; 18.6-27	25.6; 19.8-28.4	0.489
AET recumbent (%)	6.1; 6-6.4	6.1; 6-6.5	0.522
Total reflux Events (n)	66.7; 59.8-73.9	69.4; 60.1-78.4	0.489
Acid Reflux Events (n)	34.1; 29.5-40.3	39; 31.6-45.3	0.095
Weakly Acidic Reflux (n)	22.8; 19.6-26.4	25.3; 21.9-30	0.121
Weakly Alkaline Reflux (n)	3; 2-3.7	3.5; 3-4.3	0.167
SI and SAP positive	19/21	16/21	0.41
Recorded heartburn events (n)	**4; 3-5**	**3; 3-4.7**	**0.04**
MNBI (Ohms)	**1014; 926-1296**	**1748; 1563-2001**	**0.0001**
PSPW Index (%)	**35; 33-38**	**45; 42-51**	**0.0001**

LEGEND: Group A= positive for biliary reflux; Group B=negative for biliary reflux

NERD = non-erosive reflux disease; AET = acid exposure time; SI = symptom index; SAP = symptom association probability; MNBI = mean nocturnal baseline impedance; PSPW = post-reflux swallow-induced peristaltic wave; IQR= interquartile range.

FIGURE PAGE

Figure 1. Flow-chart of all screened patients

Figure 2. Linear correlation between the MNBI and esophageal bile exposure

Figure 3. Linear correlation between PSPW index and esophageal bile exposure

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