Stereotypical Metabolic Response to Endoscopic Retrograde Cholangiopancreatography Show Alterations in Pancreatic Function Regardless of Post-Procedure Pancreatitis

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OBJECTIVES: Metabolomics-based diagnosis or prediction of risk may improve patient outcomes and improve understanding of the pathogenesis of acute pancreatitis (AP). Endoscopic retrograde cholangiopancreatography (ERCP) is a risk factor for developing AP. This pilot study examined metabolomes of patients before and after ERCP, hypothesizing that metabolomics could differentiate between patients who did and did not develop post-ERCP pancreatitis, and that biomarkers associated with development of AP could be identified.

METHODS: Patients at high risk for developing post-ERCP pancreatitis were prospectively enrolled at the University of Minnesota from October 2012 to February 2014. Urine and serum samples were collected before ERCP, 2 h after ERCP, and daily thereafter if patients were admitted to the hospital with AP. Pancreatitis severity was calculated with Bedside Index for Severity in Acute Pancreatitis (BISAP) and Modified Glasgow scores. Patients who developed AP (n = 9) were matched to patients who did not develop AP (n = 18) by age and gender. Urine and serum metabolites were profiled with nuclear magnetic resonance spectroscopy. Partial least squares discriminant analysis (PLS-DA) was performed to detect changes in metabolic profiles associated with development of pancreatitis. Metabolic networks were constructed to probe functional relationships among metabolites.

RESULTS: Of the 113 enrolled patients, 9 developed mild AP according to BISAP and modified Glasgow scores. PLS-DA showed common differences between pre- and post-ERCP metabolic profiles in urine and serum regardless of AP status, characterized by increases in serum and urine ketones and serum glucose. Pre-ERCP lipase levels were somewhat elevated in those who went on to develop AP, though this did not reach statistical significance. Metabolic networks differed between patients with AP and those without after ERCP; however, metabolomics did not identify specific prognostic or diagnostic markers of ERCP-induced AP. Aspartate and asparagine were identified as well-connected hubs in post-ERCP serum networks of cases and were correlated with aspartate transaminase (AST) and white blood cell count levels. These features were not evident in controls. Serum aspartate was elevated in AP patients relative to those without AP after ERCP (P = 0.03).

CONCLUSIONS: In this pilot study, ERCP was found to induce global changes in urine and serum metabolomes indicative of alterations in pancreatic function and insulin resistance. This should be taken into consideration in future research on this topic. Post-ERCP serum metabolic networks indicate functional differences surrounding aspartate metabolism between patients with AP and those without. Further study must be done in larger patient populations to test elevated lipase as a prognostic biomarker associated with risk of developing AP and to examine active metabolic mechanisms at work.

Clinical and Translational Gastroenterology (2016) 7, e169; doi:10.1038/ctg.2016.26; published online 5 May 2016

Subject Category: Pancreas and Biliary Tract

INTRODUCTION

Disorders of the pancreas affect more than 1 million individuals in the United States and are estimated to result in nearly $3 billion in annual direct and indirect costs.1 Acute pancreatitis (AP) is the most common cause of hospitalization for pancreas-related disorders in the country, accounting for nearly 275,000 discharges.1 Because of significant advances in medical care, the vast majority of severe AP patients do not succumb to the initial injury, but many have complications marked by progression of early severe metabolic changes followed by a persistent systemic inflammatory response and immunologic events that in the most severe cases may lead to progressive organ failure and death.2–4 The mainstays of current treatment options for AP are limited to supportive care and intravenous fluid resuscitation.2,5

Endoscopic retrograde cholangiopancreatography (ERCP) is the most common cause of iatrogenic AP and is a continued
source of hospital admissions. Diagnostic ERCP has been replaced by less invasive procedures such as endoscopic ultrasound and magnetic resonance imaging that provide excellent visualization of the pancreaticobiliary system. However, therapeutic ERCP remains a common and necessary procedure. To date, research on medical therapies aimed at preventing or minimizing ERCP-induced pancreas injury have been limited. One exception is the recent use of prophylactic pancreas stents that have been shown to improve outcomes.

ERCP procedures offer the opportunity to study a human model of AP in a controlled setting (endoscopy suite) with a clearly defined etiology/noxious insult (ERCP) and onset of pancreatic injury (cannulation of papilla), thus minimizing numerous confounders of prior human AP research efforts. Quantitative metabolomics is a promising large-scale profiling technique that can be used to characterize the metabolome associated with AP. This study hypothesized that ERCP induces a different metabolic response in patients who develop ERCP-induced pancreatitis than those who do not. Furthermore, these differences could be used to probe mechanisms of injury and identify prognostic and diagnostic biomarkers of AP.

### Table 1 Demographics, procedures performed, and etiologies for enrolled patients

| Demographics          | No AP (n = 18) | AP (n = 9) |
|-----------------------|---------------|-----------|
| **Age**               | 47 ± 13       | 50 ± 16   |
| **Gender (number male)** | 4             | 2         |
| **Procedure performed** |              |           |
| Pancreatic manometry  | 4             | 1         |
| **Sphincterotomy**    |               |           |
| Biliary               | 2             | 1         |
| Pancreatic            | 3             | 3         |
| Dual                  | 1             | 1         |
| Knife                 | 0             | 0         |
| Dilatation            | 6             | 1         |
| Stent(s) placed       | 15            | 6         |
| Papillotomy           | 0             | 1         |
| Prior ERCP            | 4             | 2         |
| **Diagnosis**         |               |           |
| Prior acute pancreatitis | 8            | 6         |
| Recurrent acute pancreatitis | 5     | 3         |
| SOD                   | 11            | 6         |
| Pancreas divisum      | 3             | 1         |
| Side branch IPMN      | 0             | 1         |
| Pancreatic sphincter stenosis | 0   | 2         |
| Stenosis of PJ        | 0             | 1         |
| Papillary stenosis    | 2             | 0         |
| **Multiple diagnoses** | 2             | 5         |

AP, acute pancreatitis; ERCP, endoscopic retrograde cholangiopancreatography; IPMN, intraductal papillary mucinous neoplasm; PJ, pancreaticojejunostomy; SOD, sphincter of oddi dysfunction.

Non-AP cases were matched 2:1 with AP cases by age and gender. No statistically significant differences were observed between groups for procedures performed or diagnosis leading to ERCP. No statistically significant differences were observed between groups according to whether or not the patient had a prior ERCP. However, patients who went on to develop AP had a higher incidence of multiple diagnoses indicated than those who did not develop AP.

### METHODS

Patients at high risk for developing ERCP-induced AP were prospectively enrolled in this observational study at the University of Minnesota in accordance with the University’s institutional review board. Informed consent was obtained from patients who met the following enrollment criteria: choledocholithiasis, suspected sphincter of oddi dysfunction with planned manometry, precut sphincterotomy, known difficult cannulation, or history of post-ERCP pancreatitis. Patients were fasted overnight before the procedure. AP was defined as abdominal pain with elevation of amylase and lipase three times normal. Pancreatitis severity scores were calculated using Bedside Index for Severity in Acute Pancreatitis (BISAP) and modified Glasglow scores. All ERCP procedures were performed by the same physician (M.F.) on an outpatient basis.

Urine and serum (from lithium heparin tubes) samples were collected before the ERCP procedure, 2 h after the procedure, and daily thereafter if the patients were admitted to the hospital. Notably, three patients diagnosed with AP voluntarily chose not to be admitted to the hospital; one patient without AP was admitted to the hospital because of pain. All patients admitted to the hospital were kept for 2 days, except for one subject with AP who was kept in the hospital for 5 days. All samples were stored at −80 °C until analysis with nuclear magnetic resonance (NMR) spectroscopy.

### NMR spectroscopy

Centrifree Ultrafiltration devices (Millipore, Burlington, MA) were rinsed five times before filtering thawed serum. Equal parts of filtrate and 200 mM sodium phosphate buffer were mixed (250 μl) and 50 μl of 1 mM trimethylsilylpropionic acid (Sigma-Aldrich, St. Louis, MO) was added to the mixture to serve as internal standard. One-dimensional proton NMR spectra were acquired on a 700-MHz Bruker Avance NMR spectrometer with a 5-mm TXI proton-enhanced cryoprobe running TopSpin v. 2.16 (Bruker, Billerica, MA). A Carr–Purcell–Meiboom–Gill presaturation pulse sequence was used to acquire all serum spectra with 128 scans. Excitation sculpting with gradients was employed to reduce the water as a source of noise.

### Table 2 Mean clinical labs and scores obtained for enrolled subjects

| Outcome                  | No AP | AP |
|--------------------------|-------|----|
| Amylase (pre)            | 80.6  | 86.7 |
| Lipase (pre)             | 82.1a | 160.7a |
| BUN (post)               | 12.7  | 14 |
| TBR (post)               | 0.67  | 0.67 |
| Alkaline phosphatase (post) | 69.2 | 93.9 |
| AST (post)               | 28.6b | 47.6b |
| BISAP                    | 0.56  | — |
| MGS                      | 1.2   | — |

ALT, alanine aminotransferase; AP, acute pancreatitis; AST, aspartate aminotransferase; BISAP, Bedside Index for Severity in Acute Pancreatitis; BUN, blood urea nitrogen; MGS, modified Glasglow score; TBR, total bilirubin. Pre indicates values were obtained before endoscopic retrograde cholangiopancreatography (ERCP); post indicates values were obtained after ERCP.

aStatistical significance at P < 0.05.

bStatistical significance at P < 0.10 when comparing outcomes.
appended to the pulse sequence\textsuperscript{19} to provide superior water suppression. Thawed urine (1 ml) was mixed with 0.5 ml of 0.2 M sodium phosphate buffer. The solution was placed on ice for 10 min and then centrifuged at 7,000 \( \times \) g for 10 min. Then, 500 \( \mu \)l of the supernatant was withdrawn and combined with 50 \( \mu \)l of 1 M trimethylsilylpropionic acid.\textsuperscript{20} One-dimensional proton NMR spectra were acquired on a 700-MHz Bruker Avance NMR spectrometer with a 5-mm TXI proton-enhanced cryoprobe running TopSpin v. 2.16 (Bruker). A 1D NOESY (nuclear overhauser effect spectroscopy) pulse sequence was used to collect spectra of each sample.

Spectral profiling and quantification. Spectra from each biofluid type were fit using Chenomx NMR Suite version 7.7 (Edmonton, AB, Canada\textsuperscript{21}). Fine manual phasing and baseline corrections were applied to each spectrum before targeted profiling was performed. The identification and assignment of all metabolites was based on chemical shift relative to the designated internal standard and comparison with the published literature including the spectral library available in the Chenomx library and the Human Metabolome Database (www.hmdb.ca). For the urine, metabolite concentrations were divided by the osmolality of each urine sample (millimoles of solute per liter of urine) to correct for changes in the concentration of urine at all timepoints.\textsuperscript{22} Urea was removed from the urine data set because its signal is compromised by the NOESY pulse sequence.

Statistical analysis. Statistical analysis was conducted in the open-source R statistical program (v. 3.1.3)\textsuperscript{23} and Metaboanalyst v 3.0.\textsuperscript{24} Urine and serum metabolite concentrations were log-transformed and autoscaled before being analyzed by partial least squares discriminant analysis (PLS-DA), a common discrimination technique utilized in metabolomics\textsuperscript{25} that has been implemented previously in our lab.\textsuperscript{26,27} PLS-DA models were evaluated for accuracy and predictive power using cross-validation and permutation \( P \) values. Metabolites were subsequently ranked according to their respective variable importance of projection score. The top 10 metabolites represent the primary drivers of the calculated discrimination. Kruskal–Wallis rank-sum tests were used to calculate statistical significance.

Functional relationships among metabolites were assessed with scale-free metabolic networks as a complement to PLS-DA analyses.\textsuperscript{28} A network analysis using the Weighted Gene Correlation Network Analysis (WGCNA) software

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**Figure 1** Partial least squares discriminant analysis (PLS-DA) scores plots (left) and loadings plots (right) for pre-and post-ERCP serum and urine obtained from all subjects, regardless of acute pancreatitis (AP) status. ERCP, endoscopic retrograde cholangiopancreatography. The scores plots show that samples obtained before ERCP (black circles) can reliably be separated from those obtained after ERCP (red circles). The loadings plots show which metabolites are most responsible for driving the separation observed. Model diagnostics are shown in Table 3 (serum: \( R^2 = 0.9, \quad P = 0.01 \); urine: \( R^2 = 0.7, \quad P = 0.02 \)).
package for R software was carried out on the normalized, log-transformed metabolite data for both urine and serum. The resulting network was displayed with VisANT software (http://visant.bu.edu/, Boston University).

RESULTS

Of the 113 patients enrolled into the study, 9 developed AP as a result of the ERCP procedure. Institutional incidence of ERCP-induced AP is 2%; this number rose to 8% in our enrolled patients. Those who developed AP were matched 1:2 by age and gender with controls who did not develop AP for comparison via metabolomics. Patient procedures and etiologies are reported in Table 1. No significant differences in these existed between patients who developed AP and those who did not. The notable exception is that AP patients had more instances of multiple diagnoses than non-AP patients. All cases of pancreatitis were mild, with a mean BISAP of 0.56 and a mean modified Glasgow score of 1.2 (Table 2).

Serum metabolic profiles contained 46 individual metabolites that were identified and quantified. Urine metabolic profiles contained 72 individual metabolites that were identified and quantified. These profiles were used to construct PLS-DA analyses to determine whether patients who develop AP from ERCP show differences in metabolism relative to those who do not. Metabolite means and s.d. values are reported in Supplementary Table S1 (serum) and Supplementary Table S2 (urine) online.

Response to the ERCP procedure is independent of AP status. PLS-DA analyses to discriminate samples obtained before ERCP from those obtained afterward were constructed to determine whether those who developed AP had a different metabolic response to the procedure than those who did not. According to the model statistics reported in the Supplementary Materials (Supplementary Table S3 online), the models could not reliably distinguish samples by timepoint in models where AP status was considered separately. Accordingly, a third PLS-DA analysis was performed in which samples were pooled regardless of AP status (Figure 1). These models were statistically significant ($P < 0.05$) for both urine and serum. Heatmaps of the top 10 variable importance of projection metabolites for serum and urine in the pooled models are shown in Figure 2. Notably, levels of the ketones acetoacetate and 3-hydroxybutyrate were significantly elevated after ERCP in both urine and serum (see Table 3). Serum glucose levels were also signifi-

![Figure 2](image-url)
Significantly increased after ERCP (0.50 vs. 0.61 mM, \( P = 0.00047 \), Table 3).

**Diagnostic markers of ERCP-induced pancreatitis.** Patients who developed AP had significantly higher levels of amylase (\( P = 0.006 \)) and lipase (\( P = 0.004 \)) after ERCP than those who did not (Table 2 and Figure 3). This is in accord with the use of amylase and lipase levels to diagnose pancreatitis as stated in the Methods. Urine and serum PLS-DA models of samples taken after ERCP could not reliably differentiate those who developed AP from those who did not at the post-ERCP timepoint, though these models had somewhat better statistical indicators than the pre-ERCP models (Supplementary Table S3 online). However, when daily samples collected from patients hospitalized with AP were included in the models, they were able to reliably distinguish patients who developed AP from those who did not develop AP in both urine and serum (Figure 4). Notably, the PLS loadings indicate that serum hypoxanthine and urine 1-methylnicotinamide, both markers of oxidative stress, are elevated in the samples from patients with AP (\( P = 9.4 \times 10^{-4} \) and \( 5.7 \times 10^{-4} \), respectively).

Functional differences in post-ERCP serum metabolic networks were observed immediately after ERCP in patients who developed AP compared with those who did not. The serum networks associated with the post-ERCP timepoint are shown in Figure 5. Node colors indicate groups of metabolites with functional relationships. The serum metabolic network in those who develop AP (top) shows fewer connections between metabolites than the network for those who did not develop AP (bottom). Aspartate and asparagine are well-connected hubs in those who develop AP. These metabolites are positively correlated with aspartate transaminase (AST; \( P = 0.0006 \)) and white blood cell counts (\( P = 0.005 \)) in patients who develop AP. Aspartate and asparagine are unconnected in the serum metabolic network of those who did not develop AP. Post-ERCP aspartate levels were significantly higher in subjects with AP (0.0051 mM AP vs. 0.0035 mM no AP, \( P = 0.03 \)). Urine networks and pre-ERCP serum networks did not demonstrate any functional differences according to AP status.

**Prognostic markers of ERCP-induced pancreatitis.** Patients who went on to develop AP showed a trend toward higher pre-ERCP lipase levels relative to those who did not.
develop AP ($P=0.09$, Table 2 and Figure 3). PLS-DA models could not reliably distinguish pre-ERCP urine or serum samples of patients who went on to develop AP from those who did not.

**DISCUSSION**

The goal of this work was to identify a unique metabolic response linked to ERCP-induced AP. Prognostic biomarkers were sought to aid in identification of patients at risk for developing AP from the procedure, and diagnostic markers were sought to identify potential mechanisms of injury leading to ERCP-induced pancreatitis. This study was not able to identify a metabolic response to ERCP that was independent of AP status. Rather, this analysis showed that the ERCP procedure evoked a common response regardless of whether the patient developed AP or not. This study also did not identify strong prognostic markers of AP, though pre-ERCP lipase levels were somewhat elevated in those who went on to develop AP. No novel diagnostic markers were identified; however, this study did identify differences in functional relationships between serum metabolites aspartate and asparagine at the post-ERCP timepoint.

This study identified a common metabolic response to the ERCP procedure that was independent of AP status. Notably, the response contained an increase in ketones acetoacetate and 3-hydroxybutyrate in both urine and serum. It is possible that the increase in ketone levels resulting from ERCP is related to preprocedure fasting. However, only 2 h elapsed between collection of pre-ERCP samples and post-ERCP samples. Because of this, the global increase in ketones is unlikely to be due solely to fasting status. Furthermore, another group investigating AP with NMR-based metabolo-mics also found elevated ketones in serum and urine associated with AP. This group attributed their findings to insulin resistance and altered pancreatic function resulting in hyperketonemia.
Figure 5  Post-ERCP serum metabolic networks for patients who developed acute pancreatitis (AP; top) and patients who did not (bottom). ERCP, endoscopic retrograde cholangiopancreatography. The serum metabolic network associated with those who developed AP has fewer connections (green lines) than the serum metabolic network associated with patients who did not develop AP (gray lines). Line thickness is proportional to the interaction strength between metabolites. Connections between metabolites (indicated by lines) and functional groupings of metabolites (indicated by node colors) differ between the two groups, indicating different functional metabolic states between those with AP and those without.
Serum lipase levels may indicate subclinical pancreatic disease and deserve additional study. This interpretation of our results is supported by other investigators who note that elevated serum lipase levels are more accurate than serum amylase levels in the diagnosis of acute pancreatitis (AP). These traits were not identified in the post-ERCP serum metabolic networks of patients who did not develop AP.

Network analysis showed that metabolites aspartate and asparagine were identified as hubs in the post-ERCP serum metabolic network of patients with AP. These metabolites are highly connected in the network and also have a high interaction strength with each other. In contrast, aspartate and asparagine are unconnected in the post-ERCP serum metabolic network of patients without AP. These differences may suggest that the conversion of aspartate to asparagine (Figure 6) is occurring in AP patients and not in those without AP. These metabolites are also correlated with AST levels in AP patients only. Because AST levels are effective markers of pancreatitis due to biliary obstruction, this may point to ERCP-induced trauma or obstruction in the etiology of pancreatitis in these subjects. The correlation of these metabolites with AST and white blood cell counts is an interesting relationship. It may simply be a component of an inflammatory response to the ERCP procedure; however, we were unable to identify a clear connection between these findings in the literature. This finding, if confirmed in other studies, is an area for future investigation.

The source of suggested prognostic value of serum lipase levels is somewhat unclear. It has been shown that serum lipase levels are more accurate than serum amylase levels in the diagnosis of AP. Although there was no difference in the incidence of AP in their medical history (6/9 AP vs. 8/18 no AP, P = 0.49), patients who went on to develop AP did have higher instances of multiple diagnoses coming in to the ERCP procedure. Thus, these patients may have entered the procedure with subclinical AP. This interpretation of our results is supported by other investigators who note that elevated serum lipase levels may indicate subclinical pancreatic disease, even when subjects are asymptomatic. The link between subclinical pancreatic disease and elevated serum lipase could be exploited to determine risk of ERCP-induced AP, and deserves further study.

Ultimately, this study was not able to identify a metabolic response to ERCP that is uniquely dependent upon whether or not the subject developed AP from the procedure. Two major limitations prevented us from having stronger results. First, of the 113 patients enrolled in the study, only 9 developed AP. Second, all cases of AP in this study were classified as mild. It is likely that a larger study with more subjects and more severe disease would result in statistically significant PLS-DA models and ameliorate overfitting, thereby identifying differences between pre- and post-ERCP that demonstrate unique responses to the procedure according to AP status.

In conclusion, this pilot study was unable to confirm the hypothesis that ERCP induces a different metabolic response in patients who develop AP as a result of the procedure. Instead, a common metabolic response to ERCP was identified, regardless of whether the patients developed AP or not. This may be an important finding to consider in future post-ERCP and acute pancreatitis research. Increases in urine and serum ketones and serum glucose after ERCP suggest alterations in pancreatic function and insulin resistance as a result of ERCP in all subjects. Functional differences in serum metabolic networks related to aspartate metabolism were identified in patients with AP. Finally, this study also suggests that elevated levels of lipase before ERCP may be a prognostic marker for ERCP-induced pancreatitis. A larger study with more cases of AP and more severe disease is warranted.

**CONFLICT OF INTEREST**

Guarantor of the article: Elizabeth R. Lusczek, PhD.
Specific author contributions: Elizabeth R. Lusczek: principal investigator; planning study, interpreting data, and drafting manuscript. Sydne Muratore: collecting data and drafting manuscript. Kristen Colling and Martin Freeman: planning study, interpreting data, and drafting manuscript. All authors have approved the final manuscript.

Financial support: Funding was provided by the American College of Gastroenterology, grant ACG-CR-031-2012.
Potential competing interests: None.

Acknowledgments. This pilot study was funded by the American College of Gastroenterology (award number ACG-CR-031-2012). Funding for NMR instrumentation was provided by the University of Minnesota’s Office of the Vice President for Research, the Medical School, the College of Biological Science, NIH, NSF, and the Minnesota Medical Foundation.

**Study Highlights**

**WHAT IS CURRENT KNOWLEDGE**

- Endoscopic retrograde cholangiopancreatography (ERCP) is a risk factor for developing acute pancreatitis.

**WHAT IS NEW HERE**

- Elevated serum lipase before ERCP may be a prognostic marker for ERCP-induced pancreatitis.
- The ERCP procedure affects metabolism regardless of whether or not patients develop pancreatitis.
- Elevated serum lipase may be indicative of preclinical pancreatic disease and deserves additional study.
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