Novel Serum and Urine Markers for Pediatric Appendicitis

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Abstract

Objectives: The objective was to describe the association between two novel biomarkers, calprotectin and leucine-rich alpha glycoprotein-1 (LRG), and appendicitis in children.

Methods: This was a prospective, cross-sectional study of children 3 to 18 years old presenting to a pediatric emergency department (ED) with possible appendicitis. Blood and urine samples were assayed for calprotectin and LRG via enzyme-linked immunosorbent assay (ELISA). Final diagnosis was determined by histopathology or telephone follow-up. Biomarker levels were compared for subjects with and without appendicitis. Recursive partitioning was used to identify thresholds that predicted appendicitis.

Results: Of 176 subjects, mean (±SD) age was 11.6 (±4.0) years and 52% were male. Fifty-eight patients (34%) were diagnosed with appendicitis. Median plasma calprotectin, serum LRG, and urine LRG levels were higher in appendicitis versus nonappendicitis (p < 0.008). When stratified by perforation status, median plasma calprotectin and serum LRG levels were higher in nonperforated appendicitis versus nonappendicitis (p < 0.01). Median serum LRG, urine LRG, and plasma calprotectin levels were higher in perforated appendicitis compared to nonperforated appendicitis (p < 0.05). Urine calprotectin did not differ among groups. A serum LRG < 40,150 ng/mL, a urine LRG < 42 ng/mL, and a plasma calprotectin < 159 ng/mL, each provided a sensitivity and negative predictive value of 100% to identify children at low risk for appendicitis, but with specificities ranging from 23% to 35%. The standard white blood cell (WBC) count achieved 100% sensitivity at a higher specificity than both novel biomarkers.

Conclusions: Plasma calprotectin and serum/urine LRG are elevated in pediatric appendicitis. No individual marker performed as well as the WBC count.
glycoprotein-1 (LRG).\textsuperscript{7,9} Calprotectin is a protein complex consisting of S-100:A8 and S-100:A9. The two S-100 proteins are found within the cytoplasm of neutrophils and are released by neutrophils that are degranulating.\textsuperscript{10} Calprotectin is thought to have antimicrobial activity, likely through zinc chelation.\textsuperscript{10} LRG is a protein secreted by liver cells and by neutrophils undergoing differentiation.\textsuperscript{11} Although its exact function is not known, it is up-regulated in patients with acute inflammatory and bacterial conditions.\textsuperscript{12,13} Investigators recently described favorable test performance characteristics for serum calprotectin for diagnosing appendicitis in adult patients.\textsuperscript{9} Other investigators identified LRG as being selectively enriched in the urine of pediatric patients with appendicitis.\textsuperscript{7} Although promising, more research is needed to understand the potential clinical utility of these biomarkers. Therefore, in this pilot study, we aimed to determine the association between serum and urine levels of calprotectin and LRG and appendicitis in children with suspected appendicitis and to identify calprotectin and LRG thresholds that could potentially be used to diagnose or exclude pediatric appendicitis.

**METHODS**

**Study Design**

We conducted a prospective, cross-sectional study in an urban, tertiary care pediatric emergency department (ED) with approximately 50,000 visits per year. We obtained written informed consent from all parents and assent from children more than 7 years of age. The study was approved by the local institutional review board.

**Study Setting and Population**

From July 2009 to April 2010, children 3 to 18 years of age who presented to the ED with acute abdominal pain of less than 96 hours’ duration, and who were being evaluated for possible appendicitis, were considered for enrollment. We defined “possible appendicitis” as the treating physician choosing to obtain blood tests, radiologic studies (CT and/or ultrasound [US]) or a surgical consultation for the purpose of diagnosing appendicitis. It is standard practice in our ED to obtain a white blood cell (WBC) count for all patients with suspected appendicitis. Radiologic studies or surgical consultations are obtained at the discretion of the treating physician. We excluded patients with any of the following conditions: pregnancy, prior abdominal surgery (e.g., gastrostomy tube, abdominal hernia repair), chronic illness that potentially affected the gastrointestinal system (e.g., cystic fibrosis, inflammatory bowel disease, sickle cell anemia, chronic pancreatitis, diabetes, immune suppression), or a medical condition that limited the conduct of an accurate history or physical examination (e.g., substantial language or developmental delay). We also excluded patients with radiologic studies (CT or US) of the abdomen performed prior to ED arrival and those who had a history of abdominal trauma within the preceding 7 days of ED evaluation. Once a patient was deemed eligible, the patient and family were approached for written informed consent and assent.

**Study Protocol**

We collected patient history and physical examination data via structured case report forms created specifically for this study. The treating pediatric emergency physicians completed the forms prior to knowledge of any radiological study results (CT or US), if obtained. Patients’ medical records were abstracted to obtain data from laboratory, radiology, pathology, and operative reports. A single research assistant entered data into SPSS (Version 18.0, SPSS, Inc., Chicago, IL); all data were double-checked for accuracy by one author. Enrollment occurred 24 hours a day, 7 days a week. We reviewed the daily ED admission log and electronic tracking system to identify potentially eligible patients who were not enrolled (i.e., missed). No formal sample size calculations were conducted, as the blood or urine levels of LRG or calprotectin by enzyme-linked immunosorbent assay (ELISA) have not been previously described for appendicitis.

**Serum Collection.** We obtained two additional blood samples (3 to 5 mL into a serum separator tube and 3 to 5 mL into a K\(^+\)-EDTA plasma tube) in the ED. These additional samples were centrifuged within 1 hour of collection at a speed of 1300 \(g\) for 10 minutes. During the hours of 09:00 to 16:00, trained laboratory technicians (located within a specialized laboratory of our hospital) then immediately divided the centrifuged serum sample into two aliquots and froze the samples at \(-80^\circ\text{C}\). After 16:00 on weekdays and at all times on weekends, the spun serum samples were stored at 4\(^\circ\text{C}\). The following business day, technicians divided each sample into two aliquots and froze the blood at \(-80^\circ\text{C}\). According to the manufacturer’s instructions, the reproducibility of the calprotectin assay was partially dependent on careful handling and processing of the K\(^+\)-EDTA plasma tube. Therefore, regardless of time of day or of week, after centrifugation, the plasma portion was removed from the K\(^+\)-EDTA tube and transferred to a cryopreservation tube, taking care not to disrupt the red blood cell pellet. This sample was subsequently frozen at \(-80^\circ\text{C}\). Samples were processed and assayed in accordance with published guidelines.\textsuperscript{14} The results for LRG or calprotectin were not made available to the treating clinician. The samples for the WBC count were obtained per standard procedure.

**Urine Collection.** Enrolled subjects provided a mid-stream clean-catch urine sample, collected in a sterile cup (at least 5 mL). During the hours of 09:00 and 16:00, the urine samples were transported to a specialized laboratory within our hospital where trained laboratory technicians spun the urine and froze the samples at \(-80^\circ\text{C}\). After 16:00 on weekdays and at all times on weekends, the urine samples were stored at 4\(^\circ\text{C}\) in a refrigerator located within our ED. The following business day, the urine samples were taken to the laboratory where they were processed as described for “serum collection.” Stability testing was conducted on urine samples kept at 4\(^\circ\text{C}\) for up to 48 hours, revealing little change in urine levels of LRG (data available upon request).
**Testing for Calprotectin and LRG.** Samples were thawed and assayed in batches. Quantification of calprotectin and LRG levels was performed via ELISA according to the manufacturer’s recommended procedures. For calprotectin analysis, samples were diluted (plasma 1:60, urine 1:10) in the manufacturer-supplied dilution buffer (Hycult Biotech, Uden, The Netherlands) so that results would be within the linear range of the assay. Similarly, for LRG analysis, serum samples were diluted 1:500 and urine samples were diluted 1:20 in the supplied dilution buffer (IBL America, Minneapolis, MN). Laboratory personnel were blinded to the diagnosis of enrolled patients.

**Measures**
The primary outcome was the presence or absence of appendicitis. The presence of appendicitis was determined by histopathology. Diagnosis of a perforated appendix was based upon review of the attending surgeon’s written postoperative diagnosis. For patients who did not have surgery, we determined the outcome by a follow-up telephone call 14 to 21 days following the index ED visit. If the family could not be reached, we conducted a review of the hospital electronic record system to assess for operations (i.e., appendectomy), hospitalizations, or ED visits during the follow-up period. Those assessing the outcome were masked to the biomarker levels.

**Data Analysis**
We conducted descriptive analyses for each biomarker, exploring ranges, means with standard deviations (SDs), and medians with interquartile ranges (IQRs). We assessed the association between each biomarker and the presence or absence of appendicitis (either nonperforated or perforated) with the Mann-Whitney U-test, as the biomarker levels were asymmetrically distributed. Next, we constructed receiver operator characteristic (ROC) curves to explore the performance of each marker to predict appendicitis (perforated and nonperforated). These statistical analyses were conducted using SPSS (Version 18.0). We used recursive partitioning to identify biomarker thresholds that potentially maximized the sensitivity and specificity to “rule-out” and “rule-in” appendicitis by varying the cost for misclassification (CART 6.0, Salford Systems, San Diego, CA). We calculated 95% confidence intervals (CIs) for the test characteristics of the individual biomarkers.

**RESULTS**

**Study Population**
Over the 10-month study period, 248 patients 3 to 18 years of age presented to the ED and were considered for enrollment (i.e., possible appendicitis), of whom 192 were eligible for study participation and 176 were enrolled (92% capture rate). The number of blood and urine samples for analysis varied slightly. Of the 176 patients enrolled, plasma samples were obtained from 153 subjects, serum samples from 148, and urine from 137 (Figure 1). Most of the serum and plasma sample processing errors were due to insufficient quantities of blood. For the first 4 weeks of the study, urine samples were not collected after 18:00 or on the weekend. This resulted in the majority of missed urine samples (n = 39, coded as sample processing errors). Eligible patients who were not enrolled were slightly younger (mean ± SD age = 9.6 ± 4.3 years), had lower rates of abdominal imaging (68%), and had lower rates of appendicitis (25%). However, these values did not differ significantly from the enrolled population.

**Clinical Characteristics and Outcomes**
The clinical characteristics of the 176 enrolled patients are shown in Table 1. The mean ±SD age of enrolled patients was 11.6 (±4.0) years and 92 (52%) were male.
Fifty-eight patients were diagnosed with appendicitis (34%), of whom 15 (25%) had a perforated appendix. Although those with appendicitis were more likely to be male, there was substantial overlap in the signs and symptoms of those with and without appendicitis. Of enrolled patients without appendicitis, the most common final diagnosis included nonspecific abdominal pain, constipation, and gastroenteritis. We completed telephone follow-up on 107 (99%) of the 108 patients who did not undergo an operation; none had an appendectomy during the follow-up period. The medical records of the one patient lost to telephone follow-up revealed no further ED visits, operations, or hospitalizations within 2 months of enrollment.

Biomarker Levels
Median levels of LRG (serum and urine) and calprotectin (plasma only) were statistically higher in patients with appendicitis compared to those without appendicitis (Table 2). When biomarker levels were stratified by appendicitis status (nonappendicitis, non-perforated appendicitis, or perforated appendicitis), median blood levels for plasma calprotectin and serum LRG were higher in patients with perforated appendicitis compared to those patients with non-perforated appendicitis (p < 0.05) and higher in patients with nonperforated appendicitis compared to those without appendicitis (p < 0.01; Table 3). Urine LRG levels were also elevated in perforated appendicitis compared to nonperforated appendicitis (p < 0.001). However, median urine LRG levels were not significantly elevated when comparing patients with nonperforated appendicitis to those without appendicitis. Last, urine calprotectin showed no statistical differences among groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No Appendicitis, n = 118</th>
<th>Appendicitis (Perforated and Nonperforated), n = 58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>57 (48.3)</td>
<td>35 (60.3)</td>
</tr>
<tr>
<td>Age in years, mean (SD)</td>
<td>11.6 (± 4.1)</td>
<td>11.8 (± 3.7)</td>
</tr>
<tr>
<td>Duration of abdominal pain &lt; 24 hours, n (%)</td>
<td>68 (55.9)</td>
<td>37 (63.8)</td>
</tr>
<tr>
<td>Temperature (°F), mean (SD)</td>
<td>99.1 (± 1.5)</td>
<td>99.0 (± 1.0)</td>
</tr>
<tr>
<td>Abdominal tenderness in right lower quadrant, n (%)</td>
<td>113 (95.8)</td>
<td>58 (100)</td>
</tr>
<tr>
<td>Abdominal CT imaging, n (%)</td>
<td>94 (79.7)</td>
<td>44 (79.9)</td>
</tr>
</tbody>
</table>

Table 2
Biomarker Levels: Appendicitis Compared to No Appendicitis

<table>
<thead>
<tr>
<th>Biomarker*, ng/mL</th>
<th>No Appendicitis, n = 118</th>
<th>Appendicitis (Perforated and Nonperforated), n = 58</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum LRG</td>
<td>53,593 (29,898–117,492)</td>
<td>95,396 (67,198–144,734)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine LRG</td>
<td>225.2 (46.5–1,442.8)</td>
<td>683.5 (122.3–3,832.3)</td>
<td>0.008</td>
</tr>
<tr>
<td>Plasma calprotectin</td>
<td>221.9 (147.3–329.8)</td>
<td>330.1 (246.5–466.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine calprotectin</td>
<td>10.3 (5.0–46.7)</td>
<td>11.7 (5.5–31.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Median biomarker levels are presented with IQR (25%–75%)
LRG = leucine-rich alpha glycoprotein-1.
*All biomarker levels reported as median with IQR (25% to 75%).

DISCUSSION
Given the complexity of identifying appendicitis in children, clinicians have sought imaging and laboratory options to aid in the diagnosis. Investigators have studied an array of biologic markers, including the WBC count, absolute neutrophil count, C-reactive protein, procalcitonin, and cytokines such as IL-6 and IL-8.15–18 None of these markers have displayed substantial discriminatory accuracy due to the overlap in inflammatory response between appendicitis and the numerous...
infections that stimulate an immune response. In this study, we assessed two novel proteins whose increased expression is thought to represent increased neutrophil activity as a direct result of a focal inflammatory process. Although we found that calprotectin and LRG levels are increased in children with appendicitis, neither individual marker was as accurate as the standard WBC count.

Leucine-rich alpha glycoprotein-1 has been previously demonstrated to be elevated in patients with bacterial conditions. Investigators recently described elevated levels of LRG in the urine of patients with appendicitis. The protein is expressed by neutrophils undergoing differentiation, by the liver, and in high endothelial venules of the mesentery (such as the mesoappendix). Although LRG’s exact function is not known, it is thought to play a role in the activation and/or chemotaxis of neutrophils as they enter areas of inflammation. Our data suggest that LRG can be a sensitive but not specific marker for appendicitis, likely due to the array of inflammatory (especially bacterial) conditions that lead to LRG up-regulation. Conversely, low LRG levels in the blood or urine could potentially have clinical utility to identify a subset of patients at low risk for appendicitis.

Calprotectin has also been described as a marker of inflammation. In inflammatory conditions, the S-100A8 and S100-A9 proteins are released by neutrophils, macrophages, and monocytes. Calprotectin is released at the site of a localized inflammatory process and elevated blood levels serve as a marker of increased neutrophil activity. Previous reports have described using calprotectin levels to monitor the degree of inflammation in juvenile rheumatoid arthritis and inflammatory bowel disease (IBD). For example, investigators recently demonstrated that fecal calprotectin levels could be used to assess the severity of mucosal inflammation in IBD and the response to treatment. In a recent study mainly of adults with possible appendicitis, calprotectin had an AUC of 0.71, sensitivity of 92.7% (95% CI = 80.6% to 97.5%), and specificity of 33.6% (95% CI = 43.3% to 61.3%) at a threshold of 20 units (units not defined in text). Our results revealed a similar overall AUC (0.68). However, we found that although low calprotectin levels could predict the absence of appendicitis, the specificity of the biomarker was low at this threshold (100% sensitivity and 27% specificity). Future studies will need to further explore the utility of calprotectin in combination with other biomarkers.

In addition to blood measurements, we assayed calprotectin and LRG in the urine. Urine assays have potential advantages compared to serum/plasma measurements in that urine is usually less painful to obtain, certain proteins may be selectively concentrated in the urine, and urine assays have the potential for more widespread use and acceptance across settings (e.g., office and ED). Similar to a previously published article, we showed a relationship between the presence and absence of appendicitis and urine LRG levels. The renal threshold for LRG is not known, nor is it known whether LRG is selectively filtered into the urine. In addition, it is unclear why we did not detect a relationship
between urine calprotectin and appendicitis; perhaps calprotectin is modified during passage through the renal collecting system, preventing its detection in the urine by our ELISA test kits. It should be noted that we detected LRG and calprotectin in the urine (and serum) via a commercially available ELISA kit and not a Western blot. The ELISA testing was performed in a clinical laboratory environment and provides quantitative results, whereas Western blot is primarily used in a research setting and is semiquantitative. Our ability to detect differential levels of the protein using ELISA is encouraging for future attempts to develop a rapid urine or serum assay.

LIMITATIONS

This was a single-center pilot study conducted to determine whether calprotectin or LRG warrant further investigation. Additional larger studies are needed to further understand whether these novel biomarkers provide marginal benefit when combined with clinical findings (e.g., as part of a prediction rule) and standard laboratory tests such as the WBC count and differential. Our preliminary exploratory analyses suggested that combining LRG or calprotectin with the WBC count may be useful. Research should also assess the effect of dehydration on serum, plasma, and urine levels of these biomarkers and determine the range of biomarker values in other disease conditions. If found to be clinically useful, more rapid measurement techniques must be developed rather than the greater than 4-hour processing time needed to complete testing in our research laboratory. We cannot exclude the possibility that our sample processing methods were not adequate to prevent degradation of assayed proteins in the blood or urine. However, we did adhere to published standards for biomarker processing and used a special laboratory with particular expertise in biomarker discovery. Last, we based our final diagnosis on histopathology and operative reports for those who underwent an appendectomy rather than having each pathology specimen evaluated by a blinded pathologist.

CONCLUSIONS

Plasma calprotectin and both serum and urine leucine-rich alpha glycoprotein-1 are elevated in children with appendicitis and are low in those without appendicitis. The white blood cell count performed better than either new biomarker for the purpose of diagnosing appendicitis.

References


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