

Discovery and Validation of Urine Markers of Acute Pediatric Appendicitis Using High-Accuracy Mass Spectrometry

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Study objective: Molecular definition of disease has been changing all aspects of medical practice, from diagnosis and screening to understanding and treatment. Acute appendicitis is among many human conditions that are complicated by the heterogeneity of clinical presentation and shortage of diagnostic markers. Here, we sought to profile the urine of patients with appendicitis, with the goal of identifying new diagnostic markers.

Methods: Candidate markers were identified from the urine of children with histologically proven appendicitis by using high-accuracy mass spectrometry proteome profiling. These systemic and local markers were used to assess the probability of appendicitis in a blinded, prospective study of children being evaluated for acute abdominal pain in our emergency department. Tests of performance of the markers were evaluated against the pathologic diagnosis and histologic grade of appendicitis.

Results: Test performance of 57 identified candidate markers was studied in 67 patients, with median age of 11 years, 37% of whom had appendicitis. Several exhibited favorable diagnostic performance, including calgranulin A (S100-A8), α -1-acid glycoprotein 1 (orosomucoid), and leucine-rich α -2-glycoprotein (LRG), with the receiver operating characteristic area under the curve and values of 0.84 (95% confidence interval [CI] 0.72 to 0.95), 0.84 (95% CI 0.72 to 0.95), and 0.97 (95% CI 0.93 to 1.0), respectively. LRG was enriched in diseased appendices, and its abundance correlated with severity of appendicitis.

Conclusion: High-accuracy mass spectrometry urine proteome profiling allowed identification of diagnostic markers of acute appendicitis. Usage of LRG and other identified biomarkers may improve the diagnostic accuracy of clinical evaluations of appendicitis. [Ann Emerg Med. 2010;55:62-70.]

Please see page 63 for the Editor's Capsule Summary of this article.

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INTRODUCTION

Appendicitis is among many human diseases for which the diagnosis is complicated by the heterogeneity of its clinical presentation and shortage of diagnostic markers. As such, it remains the most common surgical emergency of children, with initial diagnostic accuracy additionally challenged because of nonspecific but similar symptoms of many other childhood conditions.¹ Delays in accurate diagnosis lead to increased

mortality, morbidity, and costs associated with the complications of appendicitis.²⁻⁴

The use of high-resolution computed tomography (CT) to identify appendiceal inflammation was hoped to improve both the diagnosis and treatment of acute appendicitis. Though variable, these improvements have been modest, with rates of unnecessary appendectomies and ruptures of 3% to 30% and 30% to 45%, respectively.⁵⁻¹⁰ Furthermore,

related medical care, including any operative care. All patients enrolled in the study received a final outcome.

For the discovery of candidate markers, thawed 10-mL urine aliquots were fractionated by using ultracentrifugation, cation exchange chromatography, protein precipitation, polyacrylamide gel electrophoresis, and reverse-phase liquid chromatography. Their protein composition was discovered by using liquid chromatography tandem mass spectrometry with a nanoflow HPLC system (Eksigent, Dublin, CA) coupled to a recently developed hybrid linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Scientific, Waltham, MA). The LTQ-Orbitrap enables an unprecedented combination of high detection sensitivity in the attomolar (10^{-18} M) range, and high mass accuracy of less than 2 parts per million (0.001 Da for a typical 500-Da peptide), as described in detail in Appendix E1 (available online at <http://www.annemergmed.com>). Validation of candidate markers was performed using 1 mL aliquots of coded specimens that were blinded to the final outcome, as described in Appendix E1 (available online at <http://www.annemergmed.com>). The entire experimental procedure is schematized in Figure 1.

Primary Data Analysis

During the discovery phase, candidate urine markers were ranked by calculating relative enrichment ratios of detection in appendicitis versus nonappendicitis groups by summing individual protein spectral counts normalized to the spectral counts of albumin to account for small differences in total protein abundance,²⁹ where relative enrichment ratios =

$$\frac{\sum_{\text{Appendicitis}} C_p / C_a}{\sum_{\text{Non-appendicitis}} C_p / C_a}$$

with C_p and C_a denoting spectral counts of candidate protein markers and albumin, respectively. Candidate markers were additionally ranked by assessing the prevalence of their detection among different specimens by using a uniformity parameter (U), calculated by dividing the number of appendicitis cases in which they were detected by the total number of appendicitis cases. Candidate markers were filtered to have U greater than 0.7 and relative enrichment ratios greater than 5 to remove those that were variably detected or insufficiently enriched, respectively. Support vector machine analysis and comparison of urine protein candidate markers with tissue gene expression profiles of diseased appendices were carried out as described in Appendix E1 (available online at <http://www.annemergmed.com>). The latter was based on a previous study.³⁰ For the validation phase, the test performance of the candidate marker was compared against the binary outcome of appendicitis or no appendicitis. Receiver operating characteristics were calculated with standard methods (SPSS, version 14.0; SPSS, Inc., Chicago, IL).

To prove the presence of the actual protein marker in diseased appendices, immunohistochemical staining of formalin-fixed, paraffin-embedded appendices was performed for the most promising marker by using the rabbit anti-leucine-rich α -2-glycoprotein (LRG) polyclonal antibody at 1:750 dilution (Atlas

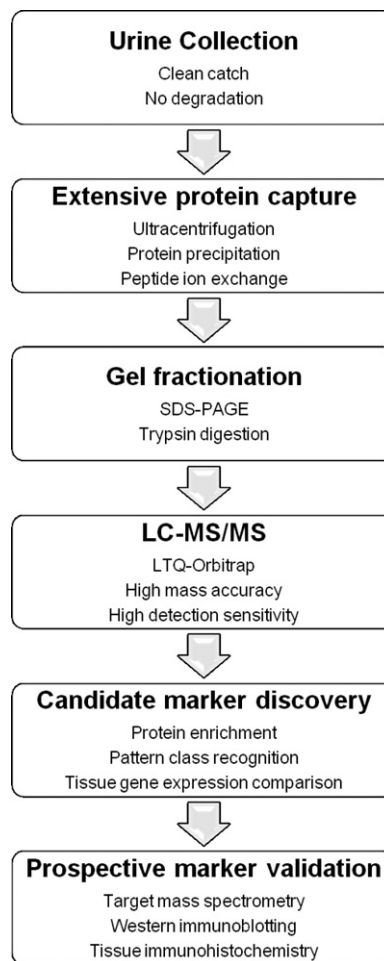


Figure 1. Experimental scheme, outlining methods used for protein capture and fractionation, identification for discovery urine proteomics, and validation of candidate diagnostic markers. The discovery phase of the study involved comparisons of 12 specimens obtained from 9 patients (6 patients without appendicitis and 3 patients with appendicitis) before and after they underwent appendectomies), whereas the validation phase of the study involved all 67 patients. LC-MS/MS, Liquid chromatography tandem mass spectrometry.

Antibodies, Stockholm, Sweden), OmniMap DAB anti-rabbit horse radish peroxidase detection kit and the Ventana Discovery XT automated slide processing platform, according to the manufacturer's instructions (Ventana Medical Systems, Tucson, AZ).

To confirm the detectability of a specific protein marker in urine, immunoblotting was performed on a sample of urine specimens. Specimens were precipitated and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described for targeted mass spectrometry (Appendix E1, available online at <http://www.annemergmed.com>). Western blotting was done blinded to final outcome, as described previously,³¹ using the rabbit anti-LRG polyclonal antibody at 1:2000 dilution, and the SuperSignal West Pico chemiluminescent reagent (Thermo).

Table 1. Presenting signs, symptoms and diagnostic studies of 67 patients with acute abdominal pain.

Characteristic	Final Diagnosis	
	Appendicitis	Nonappendicitis
Number	25	42
Sex, % male	56	40
Age, y	11±3.5	11±4.2
Duration of symptoms, days	2±1	2±1
Nausea or vomiting, %	72	52
Fever, %	52	48
Pain migration, %	36	14
RLQ pain or tenderness, %	100	95
Temperature at triage, °C	36.9±0.6	36.6±0.9
Peripheral WBC count, K cells/mm ³	15.7±5.2	11.0±6.4
Absolute neutrophil count, K cells/mm ³	12.8±5.4	8.5±6.6
Ultrasonographic imaging, %	88	74
Ultrasonographic diagnosis of appendicitis, %	64	0
CT imaging, %	60	64
CT diagnosis of appendicitis, %	93	7.4

RLQ, Right lower quadrant; K, thousand.
Values are reported as mean±SD, where appropriate, except for duration of symptoms which is reported as median±quartile.

RESULTS

During the 18-month course of this study, we enrolled 67 patients who presented to our ED and underwent evaluation for possible acute appendicitis. In agreement with earlier studies of the epidemiology and presentation of acute appendicitis in pediatric EDs, the mean age of our study population was 11 years, with presenting signs and symptoms described in Table 1. Twenty-five patients (37%) received a final diagnosis of appendicitis. All patients with appendicitis underwent appendectomies, 16% of whom were found to have a perforation. Two patients (7.4%) who received a preoperative diagnosis of appendicitis were found to have no gross or histologic evidence of appendicitis on undergoing appendectomy. Twenty-four percent of patients were found to have no specific cause of their abdominal pain, with the remaining patients found to have a variety of common and rare mimicking conditions (Table 2).

Candidate urine markers of appendicitis were identified from the analysis of 12 specimens collected at the onset of the study and distributed equally between patients with and without appendicitis (Appendix E1, available online at <http://www.annemergmed.com>). Table 3 lists the 32 candidate markers, identified by ranking their relative enrichment ratios (see Materials and Methods). These candidate proteins include known components of the acute phase response such as α -1-acid glycoprotein (orosomuroid), plasminogen, carbonic anhydrase, angiotensin-converting enzyme, and lipopolysaccharide binding protein, consistent with the systemic inflammatory response that accompanies acute appendicitis.

Table 2. Final diagnosis of the 67 study patients.

Final diagnosis	Number of Patients
Appendicitis	25
Nonspecific abdominal pain	16
Ovarian cyst or torsion	5
Constipation	5
Pyelonephritis or urinary tract infection	5
Renal calculus	2
Mesenteric adenitis	2
Gastroenteritis or gastritis	2
Influenza or scarlet fever	2
Intussusception	1
Inflammatory bowel disease	1
Diverticulitis	1

The candidate markers also include a number of cell adhesion proteins such as adipocyte specific adhesion molecule, a component of the epithelial and endothelial tight junctions; LRG, a marker of neutrophil differentiation involved in cell trafficking; vascular adhesion molecule 1, which mediates lymphocyte-endothelial adhesion; and lymphatic vessel endothelial hyaluronan acid receptor 1 involved in cell migration, consistent with earlier findings of leukocyte trafficking and infiltration into mucosal tissue that accompanies acute appendicitis.

Remaining top-ranking candidate markers do not appear to share any known functional or structural similarities, though some of them such as β -1,3-galactosyltransferase and VA0D1 have been shown to function specifically in the colonic epithelium and therefore may include components of the local and systemic appendicitis response. Additional candidate markers were identified by using support vector machine learning, as well as comparisons with tissue gene expression profiles of diseased appendices (Appendix E1, available online at <http://www.annemergmed.com>) (Tables E1 and E2, available online at <http://www.annemergmed.com>). In total, 57 candidate markers were identified.

In order to assess their diagnostic performance, we determined their relative concentrations in urine of all enrolled patients in a prospective fashion, with experimental measurements blinded to the patients' outcomes. Candidate proteins detected with sufficient uniformity among the 67 specimens examined are listed in Table 4. The remaining candidate proteins were detected in less than half of specimens, likely as a result of differences in processing of the discovery and validation specimens (Appendix E1, available online at <http://www.annemergmed.com>). Comparison of differences in urinary concentration between the appendicitis and nonappendicitis patient groups revealed LRG, S100-A8, and α -1-acid glycoprotein 1 (orosomuroid, ORM1) as exhibiting substantial apparent enrichment in the urine of patients with appendicitis (Figure 2).

Indeed, receiver operating characteristic curves for these markers exhibited excellent performance, with LRG having an area under the curve value of 0.97 (Figure 3; Table 4).

Table 3. Candidate urine marker proteins identified using relative enrichment ratio analysis.

Protein	Accession Number*	U [†]	Relative Enrichment Ratios [†]
Adipocyte specific adhesion molecule	IPI00024929	1.0	18
LRG	IPI00022417	1.0	9.5
Zinc- α -2-glycoprotein	IPI00166729	1.0	7.3
α -1-Acid glycoprotein 2	IPI00020091	1.0	5.8
MLKL	IPI00180781	1.0	5.5
α -1-Acid glycoprotein 1	IPI00022429	1.0	5.3
Plasminogen	IPI00019580	1.0	5.1
Carbonic anhydrase 1	IPI00215983	0.8	15
Angiotensin-converting enzyme 2	IPI00465187	0.8	12
Nicastroin	IPI00021983	0.8	12
Lipopolysaccharide binding protein	IPI00032311	0.8	11
Vascular adhesion molecule 1	IPI00018136	0.8	10
PDZK1 interacting protein 1	IPI00011858	0.8	7.5
SLC9A3	IPI00011184	0.8	7.5
Lymphatic vessel endothelial hyaluronan receptor 1	IPI00290856	0.8	6.9
FXR2	IPI00016250	0.7	N/A
SORBS1	IPI00002491	0.7	N/A
SLC4A1	IPI00022361	0.7	44
PRIC285	IPI00249305	0.7	14.9
TGFbeta2R	IPI00383479	0.7	11.3
SLC2A1	IPI00220194	0.7	10.7
Rcl	IPI00007926	0.7	9.7
VAOD1	IPI00034159	0.7	8.9
SLC13A3	IPI00103426	0.7	7.8
TTYH3	IPI00749429	0.7	7.3
SPRX2	IPI00004446	0.7	6.4
BAZ1B	IPI00216695	0.7	6.1
β -1,3-Galactosyltransferase	IPI00032034	0.7	6.1
Chromogranin A	IPI00383975	0.7	5.9
Novel protein	IPI00550644	0.7	5.5
SLC2A2	IPI00003905	0.7	5.2
FBLN7	IPI00167710	0.7	5.1

N/A, not detected in non-appendicitis specimens.

*International Protein Index (version 3.36; available at <http://www.ebi.ac.uk/IPI>).

[†]Values of $U=1$ indicate candidate markers detected in all appendicitis specimens, whereas values of relative enrichment ratios=1 indicate markers that exhibit no apparent enrichment in appendicitis compared with nonappendicitis groups.

Table 4. Urine marker proteins validated by targeted mass spectrometry.

Protein	ROC AUC	AUC 95% Confidence Interval
LRG	0.97	0.93–1.0
S100-A8	0.84	0.72–0.95
α -1-Acid glycoprotein 1	0.84	0.72–0.95
Plasminogen	0.79	0.67–0.91
Mannan-binding lectin serine protease 2	0.74	0.61–0.88
Zinc- α -2-glycoprotein	0.74	0.60–0.88
α -1-Antichymotrypsin	0.84	0.73–0.94
Apolipoprotein D	0.53	0.38–0.69

ROC, Receiver operating characteristic; AUC, area under the curve.

The listed confidence intervals were computed for single comparisons and do not include possible correction for multiple testing, which is expected to broaden them in proportion to the correlation and number of simultaneous tests.

Other prospectively validated markers with apparently good performance included S100-A8, orosomucoid 1, and α -1-antichymotrypsin (serpin A3); plasminogen, mannan-

binding lectin serine protease 2, and zinc- α -2-glycoprotein (AZGP) exhibited intermediate performance, and apolipoprotein D exhibited poor performance. These findings are consistent with most of these proteins being components of the general acute-phase response, during which they may be up-regulated by a variety of infectious and inflammatory conditions, including some that are represented in the nonappendicitis group (Table 2).

We assessed the relationship between apparent urine protein abundance of markers and the apparent severity of appendicitis by classifying appendectomy specimens with respect to the degree of neutrophil infiltration.¹⁸ As can be seen from Figure 4, LRG appears to be a marker of focal appendicitis, whereas S100-A8 appears to be a marker of progressive disease, reaching a peak level with moderate appendicitis. In addition to exhibiting excellent diagnostic performance, LRG was detected strongly in diseased as compared with normal appendices by tissue immunohistochemistry (Figure 4), consistent with its

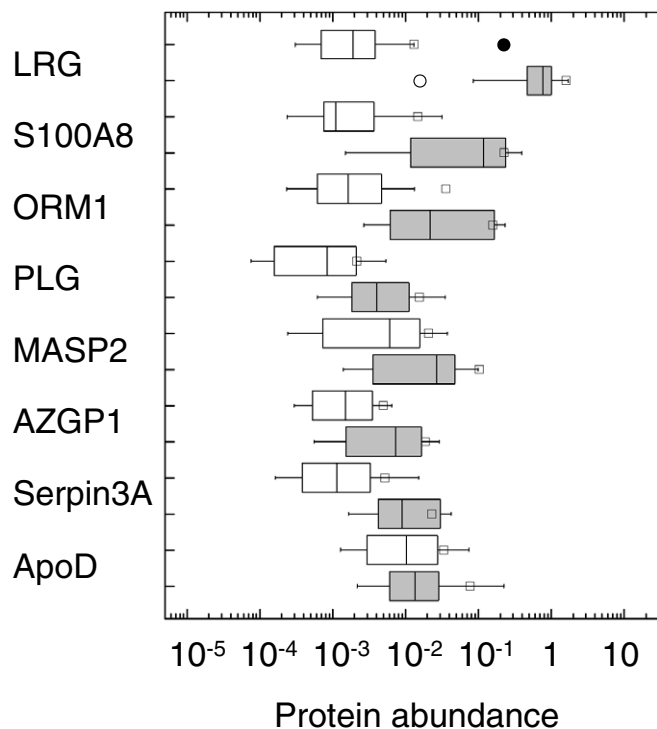


Figure 2. Box plots of the relative urine protein abundance (logarithm normalized ion current units) of the validated candidate diagnostic markers for the non-appendicitis (open) and appendicitis (gray) patient groups for LRG, calgranulin A (S100-A8), α -1-acid glycoprotein 1 (ORM1), plasminogen (PLG), mannan-binding lectin serine protease 2, zinc- α -2-glycoprotein (AZGP1), α -1-antichymotrypsin (serpin3A), and apolipoprotein D (ApoD). Normalized value of 1 corresponds to the apparent abundance of internal reference standard (Appendix E1, available online at <http://www.annemergmed.com>). Boxes contain the 25% to 75% interquartile range, with the dividing bars representing medians and whiskers representing the 10% to 90% range. Square symbols represent means. Abundance of LRG in patients with pyelonephritis (solid dot, ●) and those who underwent appendectomies with findings of histologically normal appendices (open dot, ○).

biological function and proposed role in appendicitis (see below). Its enrichment in urine of patients with appendicitis relative to those with other conditions was confirmed by using Western immunoblotting (Figure 3B), suggesting that clinical diagnostic immunoassays may be devised.

LIMITATIONS

We have not tested urine protein markers of acute appendicitis in patients evaluated in settings other than the ED or in older adult patients, who may experience other causes of abdominal pain from those observed in our cohort. Though our cohort included patients with short duration of symptoms of less than 1 day, the median duration of symptoms was 2 days, and the diagnostic performance of identified markers may be

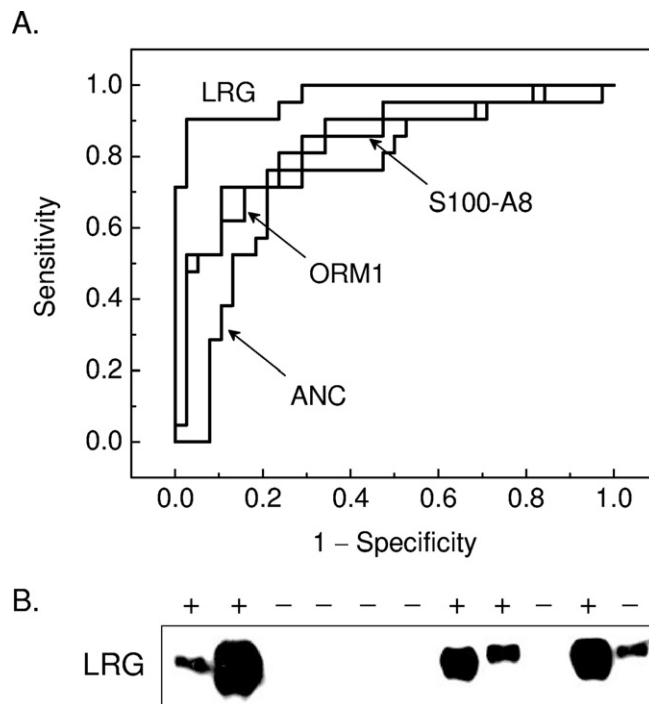


Figure 3. A, Receiver operating characteristics of urine protein markers validated by targeted mass spectrometry, demonstrating the relative diagnostic performance of LRG, calgranulin A (S100-A8), α -1-acid glycoprotein 1 (ORM1), and peripheral blood absolute neutrophil count (ANC). B, Enrichment of LRG in a random sample of urine of patients with histologically proven appendicitis (+) compared with those without (-) by using Western immunoblotting. LRG signal was observed in 5 of 5 patients with appendicitis and no signal was observed in 5 of 6 patients without appendicitis. Development of quantitative LRG urine immunoblotting and assessment of its diagnostic performance in interventional studies are important directions of future work.

different earlier in the disease course. Likewise, urine protein markers identified by our study will require further study in individuals with underlying renal or urologic disease, as well as in patients with extreme dehydration. Though our mass spectrometry measurements included internal correction for variable urine concentration by incorporating albumin normalization, clinical LRG testing using immunoassays such as analytical or dipstick enzyme-linked immunosorbent assays may require assessments or corrections for variable or age-dependent urine concentration.

DISCUSSION

The use of high-resolution CT and ultrasonography has led to improvements in the diagnosis of acute appendicitis, with respect to both the rates of complications and unnecessary appendectomies.⁵⁻¹⁰ However, significant diagnostic challenges remain, largely because of the nonspecific nature of signs and symptoms of many conditions that can mimic acute

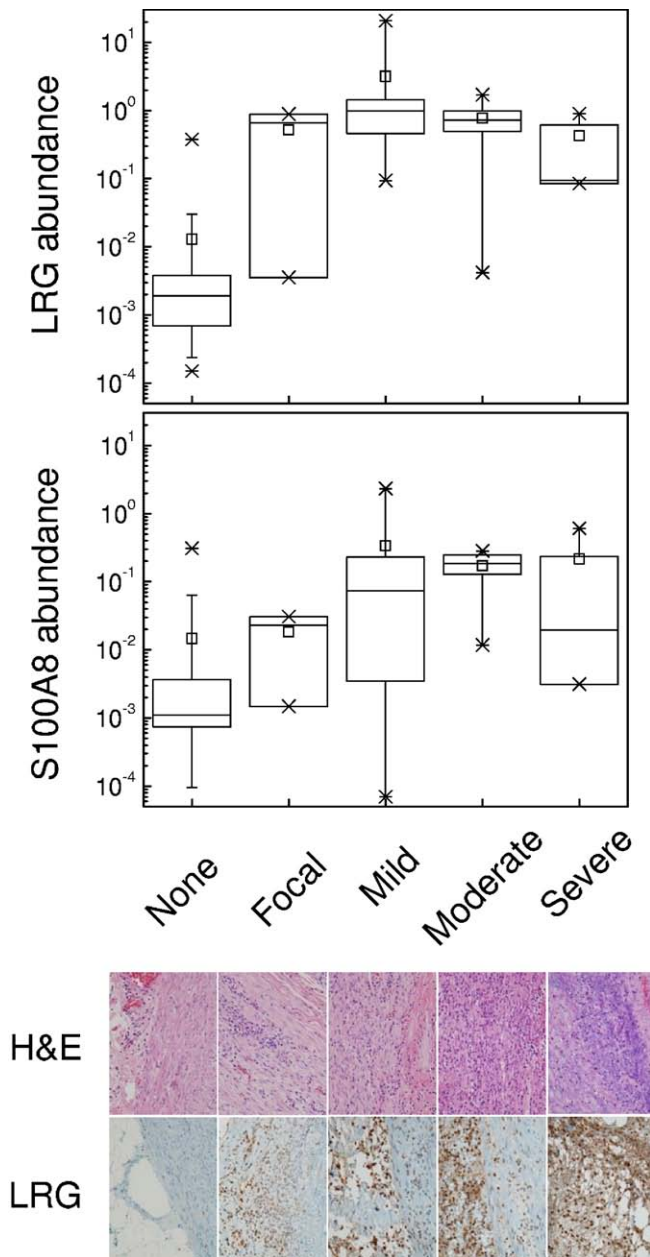


Figure 4. Top panel: Box plots of the relative urine protein abundance (normalized ion current units) of LRG and calgranulin A (S100-A8) as a function of appendicitis severity, as assessed with histologic classification. Crosses represent 1% to 99% range. Note that the group with histologically normal appendices includes patients who underwent appendectomies and patients without clinical diagnosis of appendicitis. Bottom panel: Representative micrographs of appendectomy specimens and immunohistochemistry staining against LRG.

appendicitis. Similarly, CT and ultrasonographic findings can often be indeterminate or equivocal.³²

Numerous studies have sought to identify biomarkers to aid the diagnosis of appendicitis, with the total peripheral WBC

count, absolute neutrophil count, and serum C-reactive protein levels being most useful, but still limited with respect to their sensitivity and specificity.³³⁻³⁶ Recent attempts to identify new and improved diagnostic markers, such as CD44, interleukin-6, interleukin-8, and 5-hydroxyindole acetate, produced limited improvements compared with the existing ones,¹²⁻¹⁷ likely as a result of being closely correlated with the existing markers of the general acute-phase response or not specific for the distinct immune mechanisms that characterize acute appendicitis.

By taking advantage of the latest generation of mass spectrometers that combine high accuracy with high sensitivity and carrying out exhaustive protein capture and fractionation of routinely collected urine specimens, we developed a method that enables discovery and validation of multiple diagnostic markers, thereby overcoming the limitations of conventional approaches based on single-hypothesis testing. Because of the depth of discovery achieved, identifying more than 2,000 unique proteins in total, urine proteomic profiling, like gene expression profiling, may be susceptible to noise and selection bias. To minimize these potential problems, 12 discovery urine proteomes were compared not only between patients with histologically proven appendicitis and those without but also with the same patients after they recovered from appendectomies (Appendix E1, available online at <http://www.annemergmed.com>), thereby minimizing individual differences caused by age, sex, physiologic state, or genetic variation. High-stringency identification criteria were used, essentially eliminating false protein identifications. The discriminatory power of candidate diagnostic markers was assessed by examining the level and uniformity of their enrichment in patients with appendicitis (Table 3), by using pattern-recognition class-prediction learning algorithms (Table E1, available online at <http://www.annemergmed.com>), and by comparing discovered urine protein markers with tissue gene expression profiles of diseased appendices (Table E2, available online at <http://www.annemergmed.com>).³⁰

As a result, the 57 discovered candidate urinary markers constitute an extensive characterization of the molecular response that accompanies acute appendicitis, including both systemically and locally produced molecules that participate in the systemic inflammatory response or its localization to the appendiceal tissue. Seven of these candidate markers were validated successfully, including LRG in particular (Figure 3; Table 4). LRG is expressed by differentiating neutrophils, liver, and high endothelial venules of the mesentery, including the mesoappendix, functioning in leukocyte activation, and chemotaxis, respectively.^{37,38} Its enrichment in the urine of patients with acute appendicitis suggests that it may be shed by locally activated neutrophils or local inflammatory sites such as the mesoappendix, through which neutrophils likely traffic (Figure 4) (Appendix E1, available online at <http://www.annemergmed.com>). As such, it is likely a specific marker of local inflammatory processes such as those that specifically characterize acute appendicitis, as opposed to general markers of

systemic response such as the acute-phase reactants, and macroscopic markers of local inflammation such as those observed using ultrasonographic and CT imaging.

LRG appears to be enriched in the urine of patients with appendicitis in the absence of macroscopic inflammatory changes, as evidenced by its accurate diagnosis of appendicitis of 2 patients who exhibited normal imaging findings but had evidence of acute appendicitis on histologic examination, as well as its accurate diagnosis of the absence of appendicitis in a patient without histologic evidence of appendicitis, but who underwent appendectomy as a result of findings of appendiceal enlargement on CT. Last, LRG appears to be enriched in the urine of patients with pyelonephritis, consistent with its proposed role in local inflammatory processes. Consequently, its diagnostic performance of acute appendicitis will likely depend on accurate ability to rule out other local tissue infections, such as pyelonephritis, abscesses, and pelvic inflammatory disease, consistent with early studies.³⁹ LRG appears to be strongly expressed in diseased appendices, suggesting that it may underlie a principal pathway of appendiceal inflammation by localizing or sustaining the local neutrophilic infiltration that specifically characterizes acute appendicitis.^{18,19,30} The mechanisms by which LRG and other local cytokines accumulate in urine, as well as their relationship to the pathophysiology of acute appendicitis, are important directions of future work.

Though the availability of clinical mass spectrometry is expanding rapidly, it is currently limited to large academic centers. However, detection of LRG in urine of patients with appendicitis by using Western immunoblotting suggests that widely available clinical diagnostic immunoassays may be devised (Figure 3B). Indeed, measurement of serum concentrations of LRG was recently demonstrated by using enzyme-linked immunosorbent assay.⁴⁰ This can be developed into analytical clinical laboratory urine tests or a dipstick format for rapid point-of-care testing. We were able to detect LRG using small, 1-mL volumes of urine (Figure 3B), which would be readily obtainable from patients of all ages.

Testing of these markers in multi-institutional, interventional studies is an important direction of future work. In all, this work promises to establish a paradigm for the identification of clinically useful markers of human disease.

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APPENDIX E1.

Discovery and validation of urine markers of acute pediatric appendicitis by using high accuracy mass spectrometry.

Discovery of Diagnostic Markers by Using Urine Proteomic Profiling

To identify candidate urinary markers of acute appendicitis, we assembled a discovery urine proteome data set, derived from the analysis of 12 specimens, without any clinical urinalysis abnormalities, collected at the onset of the study and distributed equally between patients with and without appendicitis. Six of these specimens were collected from patients who were found to have histologic evidence of appendicitis (2 mild, 3 moderate, 1 severe). Three specimens were collected from patients without appendicitis (1 with nonspecific abdominal pain, 1 with constipation, 1 with mesenteric adenitis). From 3 patients with appendicitis, we collected additional control specimens at their routine postsurgical evaluation 6 to 8 weeks after undergoing appendectomies, at which time they were asymptomatic and in their usual state of health. These specimens were included in the analysis to minimize the potential effect of individual variability in urinary composition that may arise because of age, sex, physiologic state, or possible genetic variation.

The urine proteome compositions of these 12 (9 original urine samples from index encounter and 3 from follow-up) were discovered by using protein capture and fractionation coupled with high-accuracy mass spectrometry, as described in detail below and schematized in Figure 1. Because urine is a complex mixture with abundant proteins such as albumin obscuring the detection of less-concentrated, potentially diagnostic proteins such as secreted cytokines and mediators of the inflammatory response, we devised a fractionation method that reduced mixture complexity while minimizing loss of material (Figure 1).

Aliquots were thawed and centrifuged at 17,000 *g* for 15 minutes at 10°C to sediment cellular fragments. Absence of intact cells in the sediment was confirmed by light microscopy (data not shown). Subsequently, supernatant was centrifuged at 210,000 *g* for 60 minutes at 4°C to sediment vesicles and high-molecular-weight complexes. Resultant pellets were resuspended in 0.5 mL of 0.1× Laemmli buffer, concentrated 10-fold to 0.05 mL by vacuum centrifugation and stored at –80°C.

Supernatant remaining after ultracentrifugation was diluted 5-fold with 0.1 M acetic acid, 10% (volume/volume) methanol, pH 2.7 (Buffer A) and incubated with 1 mL 50% (volume/volume) slurry of SP Sephadex (40–120 μm beads; Amersham) for 30 minutes at 4°C to adsorb peptides that are less than 30 kDa molecular weight. On washing of the beads twice with Buffer A, peptides were eluted by incubating the beads in 5 mL of 0.5-M ammonium acetate, 10% (volume/volume) methanol, pH 7 for 30 minutes at 4°C. Eluted peptides were purified by reverse phase chromatography by using PepClean C-18 spin columns, according to manufacturer's instructions (Pierce). Residual purification solvents were removed by vacuum centrifugation, and small pro-

teins and peptides were resuspended in aqueous 50-mM ammonium bicarbonate buffer (pH 8.5).

Proteins remaining in solution after cation exchange were precipitated by adding trichloroacetic acid to 20% (w/v), with deoxycholate to 0.02% (w/v) and Triton X-100 to 2.5% (volume/volume) as carriers, and incubating the samples for 16 hours at 4°C. Precipitates were sedimented at 10,000 *g* for 15 minutes at 4°C and pellets were washed twice with neat acetone at 4°C, with residual acetone removed by air drying. Dried pellets were resuspended in 0.1 mL of 1× Laemmli buffer.

Laemmli buffer suspended fractions (from 17,000 *g* and 210,000 *g* centrifugation, and from protein precipitation) were incubated at 70°C for 15 min and separated by using NuPage 10% polyacrylamide Bis-Tris gels according to manufacturer's instructions (Invitrogen). Gels were washed three times with distilled water, fixed with 5% (volume/volume) acetic acid in 50% (volume/volume) aqueous methanol for 15 minutes at room temperature, and stained with Coomassie. Each gel lane was cut into 6 fragments, and each fragment was cut into roughly 1-mm³ particles, which were subsequently washed 3 times with water and once with acetonitrile.

Protein-containing gel particles and cation exchange-purified proteins were reduced with 10 mM dithiothreitol in 50-mM ammonium bicarbonate (pH 8.5) at 56°C for 45 minutes. They were subsequently alkylated with 55-mM iodoacetamide in 50-mM ammonium bicarbonate (pH 8.5) at room temperature in darkness for 30 minutes. Gel particles were washed 3 times with 50-mM ammonium bicarbonate (pH 8.5) before digestion. Alkylated peptides were purified by using PepClean C-18 spin columns as described above to remove residual iodoacetamide from the cation exchange fraction. They were then digested with 12.5 ng/ μL sequencing-grade bovine trypsin in 50-mM ammonium bicarbonate (pH 8.5) at 37°C for 16 hours. Tryptic products were purified by using PepClean C-18 spin columns as described above, vacuum centrifuged, and stored at –80°C.

Fractions containing tryptic peptides dissolved in aqueous 5% (volume/volume) acetonitrile and 0.1% (volume/volume) formic acid were resolved and ionized by using nanoflow high-performance liquid chromatography (nanoLC; Eksigent) coupled to the LTQ-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a 15-cm fused silica capillary with 100- μm inner diameter, in-house packed with Magic C18 resin (200 Å, 5 μm ; Michrom Bioresources). Peptide mixtures were injected onto the column at a flow rate of 1000 nL/min and resolved at 400 nL/min with 45-minute linear acetonitrile gradients from 5% to 40% (volume/volume) aqueous acetonitrile in 0.1% (volume/volume) formic acid. Mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using the lock mass for internal mass calibration, with the resolution of 60,000 and *m/z* range of 350 to 2000. The 6 most intense multiply charged ions were sequentially fragmented by using collision-induced dissociation, and spectra of their fragments were recorded in the linear ion trap,

with the dynamic exclusion of precursor ions already selected for tandem mass spectrometry of 60 seconds.

Custom-written software was used to extract the 200 most intense peaks from each tandem mass spectrometry spectrum and to generate mascot generic format files. Peak lists were searched against the human International Protein Index database (version 3.36; available at <http://www.ebi.ac.uk/IPI>) by using Mascot (version 2.1.04; Matrix Science), allowing for variable formation of *N*-pyroglutamate, asparagine and glutamine deamidation, *N*-acetylation, and methionine oxidation, requiring full trypsin cleavage of identified peptides with 2 possible miscleavages, and mass tolerances of 5 parts per million and 0.8 Da for the precursor and fragment ions, respectively. Searches allowing semitryptic peptides did not affect overall search yields (data not shown). Spectral counts were calculated by summing the number of fragment ion spectra assigned to each unique precursor peptide.

Assessment of identification accuracy was carried out by searching a decoy database composed of reversed protein sequences of the target IPI database. Frequency of apparent false-positive identifications was calculated by merging individual target and decoy searches for each sample. An initial estimate of the apparent false-positive rate was obtained by dividing the number of peptide identifications with a Mascot score greater than the identity score obtained from the target search by the number of peptide identifications with a score higher than the identity score threshold extracted from the decoy search.¹ Only proteins identified on the basis of more than 2 peptides were included in the comparison.

As a result, we were able to identify 2,362 proteins in routinely collected urine specimens with the apparent rate of false identifications of less than 1%, as ascertained from decoy database searching.¹ More than 1,200 identified proteins have not been detected in previous proteomic studies of urine, and more than 300 proteins appear to be filtered from serum and expressed in distal tissues, including the intestine. For the discovery of candidate appendicitis markers, we further increased the stringency of peptide identifications to less than 0.1% false identifications, yielding essentially no false protein identifications for proteins identified on the basis of multiple peptides. For example, proteins identified on the basis of 10 unique peptides (median for the entire data set), have an approximate identification error frequency of 10^{-19} .

To identify candidate markers of appendicitis, we took advantage of the quantitative information provided by tandem mass spectrometry by recording the number of fragment ion spectra assigned to each unique precursor peptide, which are proportional to peptide abundance² and have been used for relative quantification of components of complex protein mixtures.³ Though the composition and concentration of urine vary with physiologic state, there was less than $10 \pm 10\%$ (mean \pm SD) difference in total protein abundance among individual specimens, similar to that of earlier studies of urine of children.⁴⁻⁶ Individual protein spectral counts, calculated by summing spectral counts of unique peptides assigned to distinct proteins, were normalized relative to the spectral counts of albumin to account for these small differences in total protein abundance.³

To maximize the depth of candidate marker discovery, we subjected the discovery urine proteome to support vector machine learning to identify candidate urine markers that may be enriched as a group but not necessarily individually, as required by the relative enrichment ratios analysis above. This approach is implemented in a biomarker discovery program BDVAL that uses cross-validation to identify predictive biomarkers (Fabien Campagne, unpublished data; BDVAL available at <http://icb.med.cornell.edu/wiki/index.php/BDVAL>), similar to established methods for microarray class discovery.⁷ Because of the low number of samples, we performed cross-validation with 4 folds, repeated 5 times with random fold assignments (12 samples total, 6 cases, 6 controls). In this setting, 20 individual evaluation models (5×4) were trained. Each model was trained with a set of 50 features (normalized protein abundance levels). In each split, consisting of 9 training samples and 3 test samples, a Student *t* test prefiltering step prioritized up to 400 features whose average value differed the most between cases and controls in the training set. The 400 intermediate features were ranked by decreasing support vector machine weights, and the top 50 features were used to train the evaluation model (models were implemented as a support vector machine, implemented in libSVM with linear kernel, and margin parameter $C=1$). At the end of the evaluation, the lists of features were inspected to determine how many times a given feature has been used in any one of the 20 evaluation models. We considered features for validation only if they were found in at least 50% of the evaluation models generated (10 models in this case).

Table E1 lists 17 proteins identified by support vector machine analysis, which include several proteins that were identified by relative enrichment ratios analysis, as well as many that were not, including additional components of the acute-phase response, such as serum amyloid A, α -1-antichymotrypsin, and bikunin (AMBP). Notably, exclusion of control specimens collected from asymptomatic patients after they underwent appendectomies increased the number of candidate markers to 273 by additionally including a variety of proteins unlikely to be related to the appendicitis response, such as the universal tyrosine kinase Src, for example, suggesting that individually variant factors such as those that influence protein filtration and urine production may significantly affect biomarker discovery studies.

Candidate Validation-Targeted Mass Spectrometry

Thawed 1-mL urine aliquots were precipitated by adding trichloroacetic acid to 20% (w/v) and incubating the samples for 1 hour at 4°C. Precipitates were sedimented at 10,000 *g* for 15 minutes at 4°C, and pellets were washed twice with neat acetone at 4°C, with residual acetone removed by air drying. Dried pellets were resuspended in Laemmli buffer, resolved by SDS-PAGE alkylated, and digested with trypsin as described above. To each sample, 0.4 μ g of single-stranded binding protein purified from *Escherichia coli* (USB) was added to serve as a reference standard.

Target nanoliquid chromatography–tandem mass spectrometry was accomplished by using the LTQ-Orbitrap mass spectrometer, using the parameters described above but operated in an inclusion list-dependent acquisition mode, searching detected

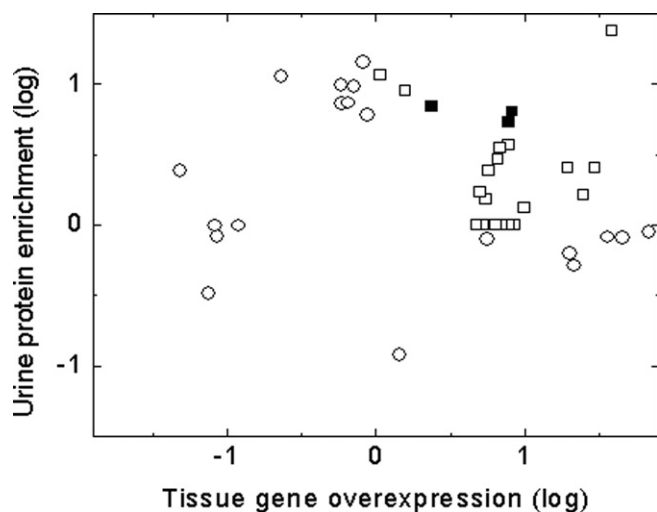


Figure E1. Relative enrichment of candidate urine protein markers as a function of appendicitis tissue overexpression of the corresponding genes, demonstrating that more than 50% of candidate markers with tissue overexpression exhibit urine enrichment (□), but that only 3 of these (■) were identified as candidate markers by urine proteome profiling.

precursor ions against m/z values of candidate marker peptides with a tolerance of 0.05 Da, using an inclusion list of masses and charges of candidate marker peptides, derived from the analysis of the discovery proteomes. The 6 most intense matched ions were sequentially fragmented by using collision-induced dissociation, and spectra of their fragments were recorded in the linear ion trap, with the dynamic exclusion of precursor ions already selected for tandem mass spectrometry of 60 seconds. Such an approach is superior to conventional data-dependent acquisition methods by minimizing the detection of nontarget peptides.⁸ Differences in apparent protein abundance were normalized relative to exogenously added single stranded binding reference standard to account for instrumental variability. Absence of single stranded binding from urine specimens without its addition was confirmed by searching the data against the database of *Escherichia coli* proteins (data not shown).

Urine Markers of Appendiceal Inflammatory Response

Because acute appendicitis is characterized by the increased expression of distinct chemoattractants in the gut mucosa¹⁰ and specific infiltration of neutrophils,¹¹ we wondered whether markers of acute appendicitis identified from studies of appendiceal tissue may be detected in the urine of patients with appendicitis. To this end, we compared candidate urine protein markers as identified by using urine proteome profiling (Table 3), with tissue markers identified in a different study by using microarray gene expression of diseased appendices.¹² Figure E1 plots relative enrichment ratio values of the 40 most uniformly detected ($U > 0.7$) candidate urine markers as a function of the tissue overexpression of their respective microarray profiled genes. Of these, more than

Table E1. Candidate urine marker proteins identified using support vector machine analysis.

Protein	Accession Number
Serum amyloid A protein	IPI00552578
α -1-Antichymotrypsin	IPI00550991
Supervillin	IPI00412650
Mannan-binding lectin serine protease 2	IPI00306378
Inter- α -trypsin inhibitor	IPI00218192
VIP36	IPI00009950
Prostaglandin-H2 D-isomerase	IPI00013179
α -1-Acid glycoprotein 2	IPI00020091
AMBP	IPI00022426
α -1-Acid glycoprotein 1	IPI00022429
CD14	IPI00029260
Hemoglobin α	IPI00410714
Apolipoprotein D	IPI00006662
Hemoglobin β	IPI00654755
LRG	IPI00022417
Zinc- α -2-glycoprotein	IPI00166729

Table E2. Candidate urine marker proteins identified by comparisons with corresponding tissue gene overexpression.

Protein	Accession Number	Affymetrix Gene ID*	Fold Gene Overexpression*
S100-A8	IPI00007047	214370_at	67
S100-A9	IPI00027462	203535_at	45
Amyloid-like protein 2	IPI00031030	214456_x_at	38
Versican	IPI00009802	211571_s_at	11
SPRX2	IPI00004446	205499_at	8.1
α -1-Acid glycoprotein 1	IPI00022429	205041_s_at	7.8
Interleukin-1 receptor antagonist protein	IPI00000045	212657_s_at	4.3
Lymphatic vessel endothelial hyaluronan acid receptor 1	IPI00290856	220037_s_at	2.0

*From Murphy et al.¹²

50% exhibit a positive correlation between tissue overexpression and urine enrichment (Figure E1), consistent with the notion that tissue gene expression profiles may be used to suggest candidate disease markers. However, only 3 of the genes that are overexpressed in diseased as opposed to normal appendices were also identified as candidate markers by urine proteome profiling: SPRX2, lymphatic vessel endothelial hyaluronan acid receptor 1 (LYVE1), and α -1-acid glycoprotein 1 (orosomucoïd 1), suggesting that detection of markers of local disease in the urine is not solely dependent on tissue overexpression but likely also requires other factors, such as shedding, circulation in blood, and accumulation in urine. Table E2 lists urine protein markers that were enriched in the urines of patients with appendicitis with corresponding genes that were overexpressed in diseased appendices.

In contrast to LRG, which is expressed exclusively by the neutrophils, liver, and mesentery, S100-A8 is a cytokine expressed by diverse tissues, including a variety of endothelial and epithelial cells.^{13,14} It is up-regulated specifically in inflammatory states, including the processes of neutrophil activation and migration.

Findings of its overexpression in appendiceal tissue during acute appendicitis¹² and enrichment in the urine of appendicitis patients suggest that, like LRG, it is also a marker of local inflammation, though its expression in a wide variety of tissues may affect its diagnostic specificity, consistent with its slightly reduced dynamic range and performance compared with those of LRG (Table 4) (Figure 3). Accordingly, it has been found to be up-regulated in a wide variety of conditions, including inflammatory bowel disease,¹⁵ arthritis,¹⁶ Kawasaki vasculitis,¹⁷ cancer,¹⁸ and sepsis.¹⁹

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