

Original Article

Oxidized glutathione and uric acid as biomarkers of early cystic fibrosis lung disease



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Abstract

Background: In cystic fibrosis (CF) there is an urgent need for earlier diagnosis of pulmonary infections and inflammation using blood- and urine-based biomarkers.

Methods: Using mass spectrometry, oxidation products of glutathione and uric acid were measured in matched samples of bronchoalveolar lavage (BAL), serum and urine from 36 infants and children with CF, and related to markers of neutrophilic inflammation and infection in BAL.

Results: Oxidation products of glutathione (glutathione sulfonamide, GSA) and uric acid (allantoin), were elevated in BAL of children with pulmonary infections with *Pseudomonas aeruginosa* (PsA) compared to those without ($p < 0.05$) and correlated with other markers of neutrophilic inflammation. Serum GSA was significantly elevated in children with PsA infections ($p < 0.01$). Urinary GSA correlated with pulmonary GSA ($r = 0.42$, $p < 0.05$) and markers of neutrophilic inflammation.

Conclusions: This proof-of-concept study demonstrates that urinary GSA but not allantoin shows promise as a non-invasive marker of neutrophilic inflammation in early CF lung disease.

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1. Introduction

Lung disease in cystic fibrosis (CF) begins in early life, is progressive and characterized by neutrophil-dominated inflammation [1–3]. Free neutrophil elastase (NE) activity, detected in bronchoalveolar lavage (BAL) as early as 3 months of age indicates an increased risk of persistent, progressive bronchiectasis [1]. Despite best current therapy approximately 60–80% of

children with CF have radiological evidence of bronchiectasis before they reach school age [1,3]. Clearly a better approach aimed at preventing structural lung disease in early life is required.

Studies by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) have provided considerable insight into mechanisms underlying the onset and progress of CF lung disease using a BAL-based program [4]. Major risk factors for progressive lung disease include inflammation and infection, severe CF genotype and free NE activity in the BAL [1,5]. However, all of these can be present in the complete absence of clinically-apparent lung disease [1,2]. Acute pulmonary exacerbations, especially those requiring hospitalization, have been associated with loss of lung function and reduced lung function growth in children [6], but predicting these is problematic.

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The BAL-based program used by AREST CF is too invasive for frequent use and has limited ability to predict acute pulmonary exacerbations. Biomarkers of inflammation, infection or structural lung disease would aid management of young children, indicating who needs more intensive therapy. However, despite extensive efforts (reviewed in [7]) no blood or urine-based biomarker has yet entered clinical practice. In addition, biomarkers validated in older patients with established lung disease may not be valid in young children with early disease [8,9].

Neutrophils infiltrate the epithelium to combat infections [10] but release damaging proteases such as NE [1] and reactive oxygen species (ROS) [11]. Hypochlorous acid is produced through oxidation of chloride by myeloperoxidase (MPO) released by activated neutrophils [12]. Glutathione sulfonamide (GSA) is an oxidative metabolite of glutathione specific to hypochlorous acid (Supplementary Fig. 1A) [13]. Unlike oxidized glutathione (GSSG), GSA is not a substrate of glutathione reductase and provides a stable biomarker of neutrophil oxidant activity. Consequently, we postulated that GSA might be a systemic biomarker of pulmonary inflammation, infection and oxidative stress in CF.

Allantoin is the major oxidation product of uric acid when exposed to ROS including hypochlorous acid, hydroxyl radicals, and methaemoglobin/H₂O₂ (Supplementary Fig. 1B) [14,15] as well as when MPO directly oxidizes uric acid [16]. As allantoin is elevated in serum of patients with acute gout [17], we proposed that allantoin would be elevated during acute pulmonary inflammation in CF.

The aim of the present proof-of-concept study was to determine whether allantoin and GSA were present in BAL from the lungs of young children with CF, reflect current lung disease and whether they are elevated in serum and/or urine when children are infected.

2. Methods

Full details of the study population and methods used are provided in the online data supplement.

2.1. Study population

Matched urine, serum and BAL samples were obtained from a previous study investigating the potential of YKL-40 as a serum and urinary biomarker for inflammation in CF [8]. Samples were collected from 36 infants and young children with CF who participated in the AREST CF early surveillance program. Details of the program, collection and measurements for cytokine concentrations, neutrophil elastase activity, cell counts and detection of infection and bronchiectasis have been described previously [1–3,5,8]. During 55 unique annual visits, a total of 55 BAL samples, 52 serum and 41 urine samples were obtained. Nine children visited twice, three children visited three times and one child visited five times. Measurements from the same child showed variability in all the analytes presented here. Variables related to each BAL sample were considered a separate event.

2.2. Measurement of GSA in BAL, serum and urine by LC–MS

GSA content was analysed by liquid chromatography with mass spectrometry (LC–MS) using multiple reaction monitoring on an Applied Biosystems 4000 QTrap as described before [18]. The relative standard deviations for intra- and inter-day precision were <10% and <15%, respectively [18]. Standard deviations were determined from a set of quality control BAL samples covering low, medium and high points on the respective calibration curves that were repeatedly frozen, thawed and analysed on five different days [18]. The lower limit of quantification for GSA (S/N > 10) in standard samples was 2 nM.

2.3. Measurement of allantoin in BAL, serum and urine by LC–MS

Allantoin was measured as described previously [19]. The relative standard deviations for intra-day and inter-day precision was <7% [19]. Standard deviations were determined from a set quality control plasma samples that were repeatedly frozen, thawed and analysed on four different days [19]. Artefactual production of allantoin from urate during sample preparation was also ruled out in this study [19]. The lower limit of quantification (S/N > 10) for allantoin in standard samples was 0.2 nM.

2.4. Measurement of 3-chlorotyrosine and methionine sulfoxide in BAL by LC–MS

The protein oxidation products 3-chlorotyrosine and methionine sulfoxide were measured as described in the online supplement. Chlorotyrosine concentrations were reported as chlorotyrosines per 1000 tyrosines. Methionine sulfoxide was reported as the % of total methionine species (methionine and methionine sulfoxide).

2.5. Measurement of MPO activity and protein by sandwich ELISA

MPO was determined by ELISA as described previously [20].

2.6. Measurement of urine creatinine

Urine creatinine was determined by Jaffe's reaction, where creatinine produces an orange coloured product with picric acid in alkaline medium [21]. The absorbance at 520 nm was measured and urine creatinine concentrations were determined using a standard curve.

2.7. Specific gravity analysis and normalization of urine concentrations of GSA and allantoin

Specific gravity of urine was measured on a refractometer (American Optical Corporation, Southbridge, MA), on which the specific gravity could be read directly and used to normalize urine analyte concentrations. To compare the effect of normalization by

specific gravity between groups we used the specific gravity ratio described in the online data supplement.

2.8. Statistical analysis

Statistical analyses were carried using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Pearson r correlation was used for regression analyses. Mann–Whitney rank-sum test was used for comparisons between groups.

3. Results

The AREST CF surveillance program provided samples from 55 unique annual visits by 36 children. These provided a total of 55 BAL samples, 50 serum and 41 urine samples. Variables related to each BAL sample was considered a separate event.

3.1. Relation of oxidative biomarkers in BAL to markers of neutrophilic inflammation and bronchiectasis

The oxidative biomarkers GSA, allantoin, methionine sulfoxide and 3-chlorotyrosine in BAL from children with CF were all related to airway MPO protein (Fig. 1). They were all correlated with each other (GSA vs. allantoin, $r = 0.8$, $p < 0.001$; GSA vs. 3-chlorotyrosine, $r = 0.5$, $p < 0.05$; GSA vs. methionine sulfoxide, $r = 0.4$, $p < 0.05$; allantoin vs. 3-chlorotyrosine, $r = 0.4$, $p < 0.05$; allantoin vs. methionine sulfoxide, $r = 0.3$, $p = 0.15$; 3-chlorotyrosine vs methionine sulfoxide, $r = 0.7$, $p < 0.001$; data not shown). Both airway GSA and allantoin were related to all other biomarkers of oxidative stress and levels of IL-1 β and IL-6 in BAL (Table 1). Airway allantoin was also related to

bronchiectasis and neutrophil elastase ($r = 0.55$, $p < 0.05$ and $r = 0.37$, $p < 0.05$, respectively, Table 1). MPO protein in BAL was related to bronchiectasis and neutrophil elastase ($r = 0.78$, $p < 0.001$ and $r = 0.59$, $p < 0.001$, respectively, data not shown).

3.2. Effect of infection on biomarkers of neutrophilic inflammation in BAL

All measures of oxidative stress were elevated in the airways of CF children with PsA infections compared to uninfected children (Fig. 2). IL-8 and neutrophil elastase were also elevated with PsA infections (Supplementary Fig. 2). Children infected with any organisms other than PsA had higher levels of oxidative biomarkers, IL-8 and neutrophil elastase compared to uninfected children (Fig. 2, Supplementary Fig. 2), but this difference did not reach statistical significance, possibly due to the smaller sample size.

3.3. Comparisons between biomarkers in BAL, serum and urine

Levels of pulmonary, urinary or serum GSA and allantoin were not associated with sex, age, initial presentation with meconium ileus or regular antibiotic prophylaxis (data not shown).

GSA in serum was not correlated with MPO or GSA in BAL ($r = 0.26$, $p = 0.07$ and $r = 0.26$, $p = 0.07$, respectively, Table 1) or with GSA in urine ($r = 0.3$, $p = 0.08$, data not shown). Serum GSA was significantly associated with bronchiectasis ($r = 0.6$, $p < 0.01$, Table 1). GSA in urine was significantly correlated with GSA, allantoin, IL-1 β and IL-6 in BAL (Table 1). Serum or urine allantoin were not related to each other nor to allantoin in BAL (Table 1).

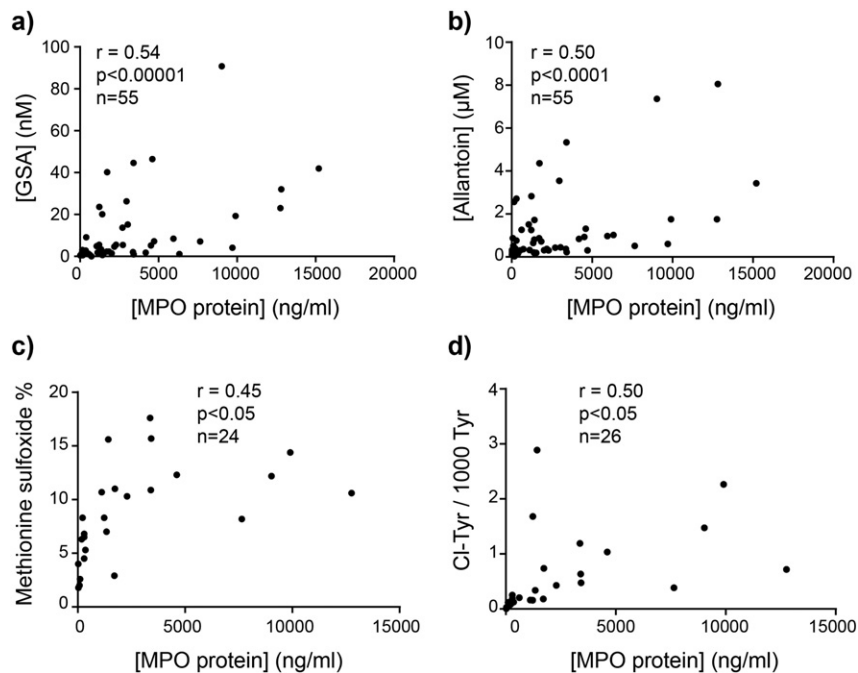


Fig. 1. Relationship between oxidative biomarkers and MPO in BAL. The relationship between a) GSA, b) allantoin, c) methionine sulfoxide and d) 3-chlorotyrosine and MPO protein in BAL from children with CF. Data were analysed using a Pearson product–moment correlation. Methionine sulfoxide is reported as the percent of total methionine species and 3-chlorotyrosine as 3-chlorotyrosines (Cl-Tyr) per 1000 tyrosines (Tyr). The smaller sample set for methionine sulfoxide and 3-chlorotyrosine is a result of limited sample availability.

Table 1

Associations between GSA and allantoin and airway markers of neutrophilic inflammation and oxidative stress and bronchiectasis.

Pearson product-moment correlation coefficient r , p -value and number of subjects (n) for each pair are shown.

	GSA in BAL			GSA in serum			GSA in urine		
	r	p	n	r	p	n	r	p	n
Myeloperoxidase	0.54	<0.001	55	0.26	0.07	50	0.23	0.18	36
GSA	–	–	–	0.26	0.07	50	0.42	<0.05	36
Allantoin	0.80	<0.001	55	0.17	0.24	50	0.50	<0.05	36
3-Chlorotyrosine	0.50	<0.001	26	0.10	0.64	26	0.16	0.57	26
Methionine sulfoxide	0.40	<0.05	25	0.37	0.08	25	–0.07	0.83	25
Bronchiectasis score	0.24	0.3	20	0.60	<0.01	20	0.26	0.29	20
Interleukin-8	0.06	0.6	54	0.06	0.07	50	0.33	0.05	36
Interleukin-1 β	0.46	<0.001	55	0.22	0.13	50	0.37	<0.05	36
Interleukin-6	0.63	<0.001	55	0.26	0.07	50	0.37	<0.05	36
Neutrophil elastase	0.17	0.2	55	–0.03	0.82	50	0.20	0.24	36

	Allantoin in BAL			Allantoin in serum			Allantoin in urine		
	r	p	n	r	P	n	r	p	n
Myeloperoxidase	0.50	<0.001	55	–0.29	<0.05	49	0.00	0.99	32
GSA	0.80	<0.001	55	–0.16	0.28	49	–0.12	0.51	32
Allantoin	–	–	–	–0.16	0.27	49	–0.14	0.46	32
3-Chlorotyrosine	0.40	<0.05	26	–0.19	0.37	26	–0.45	0.10	26
Methionine sulfoxide	0.30	0.15	25	–0.33	0.13	25	–0.41	0.12	25
Bronchiectasis score	0.55	<0.05	20	–0.24	0.33	20	0.05	0.86	20
Interleukin-8	0.18	0.20	54	–0.23	0.11	49	–0.07	0.70	32
Interleukin-1 β	0.58	<0.001	55	–0.22	0.14	49	0.25	0.15	32
Interleukin-6	0.46	<0.001	55	–0.16	0.29	49	–0.19	0.29	32
Neutrophil elastase	0.37	<0.05	55	–0.12	0.41	49	–0.12	0.28	32

3.4. Evaluation of GSA and allantoin in serum and urine as biomarkers of infection and bronchiectasis

Serum GSA was significantly elevated in children with PsA infections compared to uninfected children (Fig. 3a). With the exception of one sample, all serum samples with non-detectable GSA were from children without PsA infection (Fig. 3a). Children with bronchiectasis tended to have higher serum GSA compared to children without ($p = 0.06$, Fig. 3c). There was no effect of infection or bronchiectasis on serum allantoin (Fig. 3b and d).

In order to normalize urinary biomarker concentrations to urine dilution, we assessed specific gravity and creatinine as possible normalization factors. There was no difference in the specific gravity ratios between the infected and uninfected groups (Supplementary Fig. 3A). In contrast, urinary creatinine was elevated in children with PsA infections (Supplementary Fig. 3B), although this did not reach statistical significance. We used specific gravity ratios to normalize urinary GSA and allantoin concentrations. When compared to uninfected children, urinary GSA was elevated in children with infections (Fig. 4a). Children with bronchiectasis had slightly higher urinary GSA (Fig. 4b). Infection status and bronchiectasis had no effect on urinary allantoin (Fig. 4c and d).

4. Discussion

The data from the present study show that levels of GSA and allantoin in the BAL correlate with neutrophil-derived oxidation

activity in the lungs of infants and young children with early CF lung disease. Indicators of oxidative stress were greater in children with infections, especially with PsA, indicating their potential utility as biomarkers in CF. GSA in urine correlated with GSA in BAL ($p < 0.05$), but the correlation between serum and pulmonary GSA failed to reach statistical significance ($p = 0.07$). Urinary GSA was also associated with other markers of neutrophilic inflammation in BAL. There were no correlations between pulmonary allantoin and levels in serum ($p = 0.27$) or urine ($p = 0.46$). These data suggest that measuring GSA in urine may provide a useful and non-invasive biomarker of pulmonary neutrophilic inflammation.

With the understanding that CF lung disease begins early in life, often unaccompanied by respiratory symptoms, a greater emphasis has developed on being able to identify which children are at greatest risk and require more intensive treatment [4]. The acquisition of PsA is thought to be a critical event that is associated with a worse prognosis [22] and cannot be predicted by lifestyle factors [23]. Early detection is important as aggressive early treatment early is usually successful in eradicating the organism [24]. However, early detection is difficult as the median age of acquisition in the AREST CF surveillance program is around 2 years of age and pulmonary infection with PsA can occur in the absence of clinically-apparent lung disease [1,2,24]. Young children cannot easily expectorate sputum, even in the presence of a moist cough and cough swabs or oropharyngeal swabs do not reliably detect lower airway infection with PsA [22,24]. Previous attempts at validating biomarkers of PsA infection developed in adults in young children have been disappointing [8,9]. Cyanide in

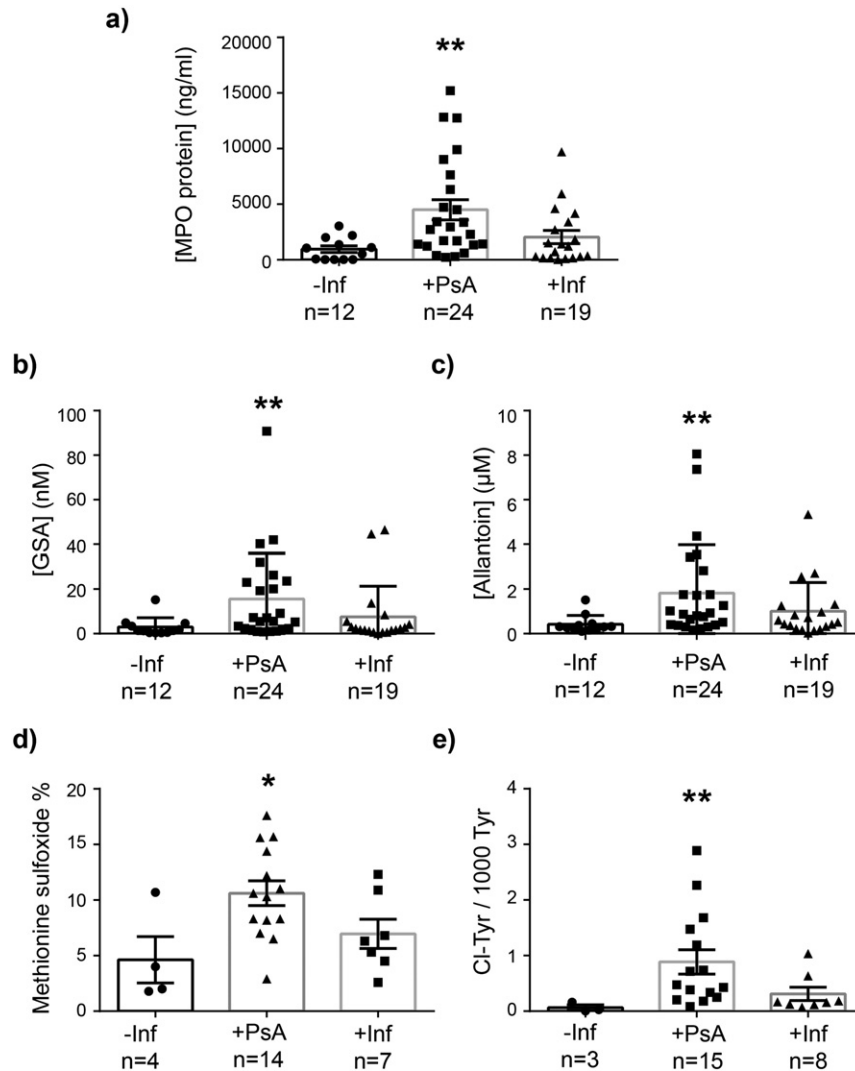


Fig. 2. Effect of infection on biomarkers of neutrophilic inflammation in BAL. Children with CF were split into those that were uninfected (–Inf), infected with *Pseudomonas aeruginosa* (+PsA) and infected with any organism other than PsA (+Inf) and the concentration in BAL of a) MPO protein, b) GSA, c) allantoin, d) methionine sulfoxide and e) 3-chlorotyrosine was determined. Methionine sulfoxide is reported as the percent of total methionine species and 3-chlorotyrosine as 3-chlorotyrosines (Cl-Tyr) per 1000 tyrosines (Tyr). The smaller sample set for methionine sulfoxide and 3-chlorotyrosine is a result of limited sample availability. Individual values are shown by symbols and the mean is represented by the bar \pm SEM. Mann–Whitney rank test: * $P < 0.05$, ** $P < 0.01$ compared with the uninfected group.

sputum was postulated as a specific marker of infection with PsA but cyanide levels in BAL obtained from young children with CF were more correlated with neutrophil number and activation [9]. While not specific for infection with PsA, an increased level of GSA in urine could indicate which children require more intensive investigation, including BAL.

Biomarkers of neutrophil-induced oxidative stress have previously been shown to be elevated in BAL obtained from children with CF and to relate to clinical and radiological indicators of lung disease [11,25–27]. However, BAL is too invasive for frequent use. In addition, a randomized trial of BAL-directed therapy in CF, in which 50% of participants underwent BAL at the onset of an exacerbation requiring hospitalization to determine appropriate antibiotic therapy questioned the use of BAL during acute illness. At the final assessment at the age of 5 years, there were no differences in

the presence or severity of bronchiectasis between the BAL-directed and conventional treatment arms [28]. Thus, additional, preferably non-invasive methods for detecting the onset or following the progress of CF lung disease is required. As needle-related distress is common in children with CF [29], a urine-based biomarker would be preferable to one detected in serum.

GSA is a specific and stable by-product of glutathione oxidation by hypochlorous acid, the production of which is catalysed by MPO [13]. Thus, GSA is an indicator of neutrophil-dominated inflammation, as seen in CF and is not specific to any particular infecting organism. GSA has previously been reported to correlate with functional consequences of neutrophil oxidant activity in the lung in children with CF and to be increased in the presence of pulmonary infection [25]. GSA has also shown to be increased in the lungs

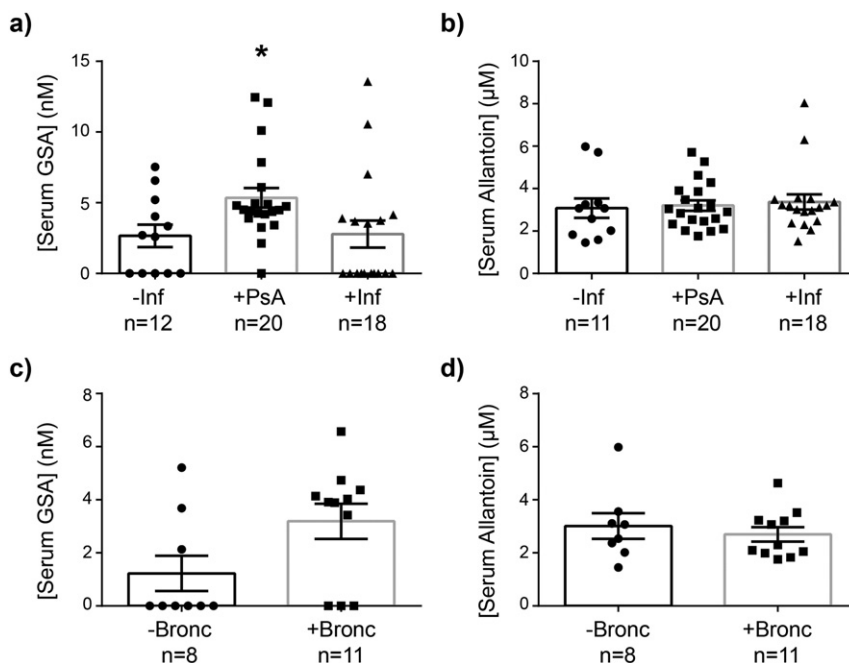


Fig. 3. Effect of infection and bronchiectasis on serum GSA and allantoin in children with CF. Children with CF were split into those that were uninfected (–Inf), infected with *Pseudomonas aeruginosa* (+PsA) and infected with any organism other than PsA (+Inf) and the serum concentration of (a) GSA and (b) allantoin was determined. Children were split into those with (+) and without (–) bronchiectasis (bronc) and the serum concentration of (c) GSA and (d) allantoin was determined. The smaller sample set for allantoin is a result of limited sample availability. Bronchiectasis data was not always available accounting for the smaller sample set in c) and d) compared to a) and b). Individual values are shown by symbols and the mean is represented by the bar ± SEM. Mann–Whitney rank test: *P < 0.05, compared with the uninfected group.

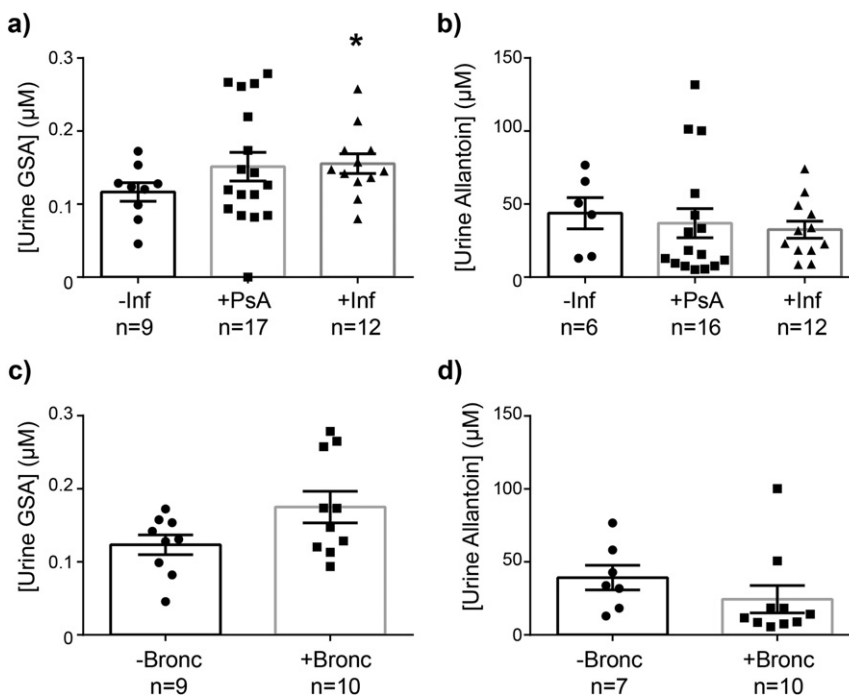


Fig. 4. Effect of infection and bronchiectasis on urinary GSA and allantoin in children with CF. Children with CF were split into those that were uninfected (–Inf), infected with *Pseudomonas aeruginosa* (+PsA) and infected with any organism other than PsA (+Inf) and a) GSA and b) allantoin was determined. Children were split into those with (+) and without (–) bronchiectasis (bronc) and the urinary concentration of c) GSA and d) allantoin was determined. The smaller sample set for allantoin is a result of limited sample availability. Bronchiectasis data was not always available accounting for a smaller sample set in c) and d) compared to a) and b). Concentrations were normalized using specific gravity ratios. Individual values are shown by symbols and the mean is represented by the bar ± SEM. Mann–Whitney rank test: *P < 0.05 compared with the uninfected group.

of β ENaC mice [30], in tracheal aspirates from ventilated preterm infants [31] and in murine blood and urine as an indicator of oxidative stress in a lupus nephritis model [32]. GSA in BAL of young children with CF has been shown to correlate with levels of MPO and to indicate protein damage in the lungs, evidence by increased levels of chlorinated proteins [25]. The significant correlation between GSA in BAL and in urine (and the trend for a correlation in serum) provides hope that high urinary GSA ($>0.2 \mu\text{M}$) may be useful in indicating the presence of pulmonary infections.

Allantoin, an oxidation product of uric acid, has been measured in plasma as a biomarker of oxidative stress in acute gout, rheumatoid arthritis and diabetes [17,33,34], but has not yet been studied in cystic fibrosis. Here, we report a mean serum allantoin concentration of $3.2 \pm 1.3 \mu\text{M}$, which was similar to that of the control group of healthy adults in the gout study ($2.6 \mu\text{M}$) [17]. The children in the present study were clinically stable and were not suffering from acute pulmonary exacerbations. Indeed, they had low CRP levels indicating a lack of systemic inflammation [8]. Allantoin in the BAL did correlate with measures of neutrophilic inflammation and of oxidative stress in the lungs. However, the lack of correlation between pulmonary allantoin and levels in the serum or urine suggest that this may not be a useful urinary marker of CF lung disease.

Biomarker studies using spot urine are greatly influenced by the normalization factor used to account for urine dilution [35]. We used specific gravity as the normalization factor instead of the commonly used urinary creatinine [36,37]. Creatinine is known to be affected by a number of factors including age, sex and antibiotic use [38,39]. We did not observe a relationship between creatinine and age, gender or the use of antibiotics in this population, possibly due to the small sample size. However, we did find that creatinine concentrations were higher in children with infections than in those without. An increased tubular secretion of creatinine has previously been observed for CF individuals [40], but the effect of infection was not studied. Elevated urine creatinine may be an indicator of lung infection in cystic fibrosis and it may be of interest to explore the mechanism contributing to this association further. However, these results do indicate that urinary creatinine is not suitable for normalizing concentrations of urinary analytes in children with CF. We did not find differences in urine specific gravity related to infection status and suggest that using specific gravity for normalization is more appropriate.

We do need to acknowledge limitations with the present study. The sample size is small and the study is not powered to investigate associations between biomarkers of neutrophilic inflammation and clinical disease. In addition, the study population were a convenience sample in whom samples of BAL, serum and urine collected at the same time were available in the AREST CF biobank. Samples were requested from uninfected children, those infected with PsA and those infected with organisms other than PsA. As such these children may not be representative of the total AREST CF population. Thus, the results we present here should be interpreted as proof-of-concept data. Larger prospective studies will be required to determine the true potential of measuring

urinary GSA as biomarker of neutrophil-induced oxidative lung damage in young children with CF.

In summary, we show that measuring urinary GSA has the potential to indicate the presence of pulmonary infection and neutrophil-induced oxidative stress and warrants further investigation as a non-invasive biomarker in CF.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jcf.2016.10.012>.

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