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GLYCOPROTEIN A AS A BIOMARKER OF PULMONARY INFECTION AND INFLAMMATION IN CHILDREN WITH CYSTIC FIBROSIS

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Abstract

Background: Serum Glycoprotein A (GlycA) levels are increased in a variety of inflammatory disease states. However, GlycA has not been previously evaluated in children with cystic fibrosis (CF). We assessed the relationship between GlycA and pulmonary infection, inflammation, bronchial wall thickening (BWT) and bronchiectasis in young children with CF.

Methods: From 95 patients we obtained 311 paired serum and bronchoalveolar lavage (BAL) samples at multiple timepoints, with concurrent chest computed tomography on 168 occasions. Quantitative GlycA was determined using high-throughput nuclear magnetic resonance metabolomic testing. Participants were considered to be infected if ≥1 significant proinflammatory organism was isolated from their BAL. The presence of free neutrophil elastase above the limit of detection was considered evidence of inflammation. The relationships between GlycA levels and infection state, inflammation and bronchiectasis were examined using a generalised estimating equation approach.

Results: There was a positive relationship between GlycA (mean 1.01 mmol/L, range 0.68 - 1.92 mmol/L) and being infected with one or more proinflammatory organisms, even after adjusting for age and gender (OR 1.2 per 0.1 mmol/L, 95% CI 1.02, 1.4, p = 0.03). There was also a positive relationship between GlycA and neutrophil elastase (unadjusted OR 1.2 95% CI 1.01, 1.4, p = 0.04), not significant after adjustment. GlycA concentration was associated with BWT but not bronchiectasis.

Conclusions: Although GlycA levels were higher on average in those who had infection or neutrophilic inflammation, there was also considerable variability, limiting the clinical utility of this biomarker alone in determining early disease status in CF.

Introduction

Pulmonary disease remains the leading cause of morbidity and mortality in Cystic Fibrosis (CF)[1]. It has its origins early in life, often progressing without symptoms, and is characterised by pulmonary infection, inflammation and subsequent structural lung damage[2]. Detection of these processes in young children has to date required invasive

investigations such as chest computerised tomography (CT) scans and bronchoalveolar lavage (BAL) under anaesthesia, which are not without risk[3, 4]. Because of this, developing non-invasive breath or blood-based biomarkers of early lung pathology in CF is highly appealing as such tests may help identify those at greatest risk of progressive disease and those most likely to benefit from early interventions[5,6]without causing harm in the process.

Glycoprotein A (GlycA) levels reflect the abundance of mobile N-acetyl sugar groups found on glycoproteins in circulating blood which are involved in the human acute phase response[7]. In a variety of inflammatory disease states ranging from diabetes, cancer and atherosclerosis in adults[8] to acute Kawasaki disease in children[9], GlycA levels are elevated. Furthermore, in preschool children GlycA is associated with white cell count elevation and granulocyte proportion more closely than high sensitivity C-Reactive Protein is, suggesting that GlycA may be a superior marker of chronic cumulative inflammation in early life[10]. As the pathophysiology of early CF lung disease is characterized by inflammation and infection, we therefore considered GlycA to be a potentially salient biomarker not been previously evaluated with regard to its association with lung disease states in CF.

We aimed to assess the relationship between the inflammatory marker GlycA and CF-related pulmonary infection, inflammation, bronchial wall thickening (BWT) and bronchiectasis in young children.

Materials and Methods

A. GlycA samples

Between June 2010 and August 2016 we obtained serum to test GlycA levels from serial blood samples taken from 95 subjects at a single tertiary paediatric centre while undergoing flexible bronchoscopy and BAL under general anaesthesia as part of the previously-described Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST-CF) surveillance program[11]. Fasting peripheral blood was processed within four hours of collection at an on-site processing laboratory. Serum aliquots were frozen at negative 80˚C for batch analysis using a high-throughput proton nuclear magnetic resonance (NMR) metabolomics platform [Nightingale Ltd, Vantaa, Finland][12]. The NMR platform experimentation, quality control and applications are described elsewhere[13].

B. Bronchoalveolar lavage

BAL fluid was assessed using standard culture techniques by the clinical laboratory. Pulmonary infection was defined as colony count for a specific organism (excluding mixed oral flora) of at least 10^4 colony-forming units per mL (cfu/mL). For this study, patients were considered positive for infection if one or more of the significant pro-inflammatory organisms *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus species* and *Haemophilus influenzae* were isolated from their lavage [14,15]; analysis was also repeated with the latter organism excluded from the group.

Neutrophil elastase (NE) activity in BAL fluid was assessed as described previously[16]. The lower limit of detection of free NE activity was 100 $ng \cdot mL^{-1}$ and visits were defined as NE positive if the concentrations were above the lower limit of detection.

C. Radiology

Chest CT scans were performed immediately prior to BAL under the same general anaesthetic on 168 occasions as part of AREST-CF surveillance at annual review at one and three years of age. Limited-slice chest CT scans were performed prior to April 2011, and full volumetric inspiratory and expiratory scans were performed thereafter. Bronchiectasis was defined as any bronchial diameter to corresponding artery diameter ratio >1[17], determined by an expert clinical radiologist and reviewed by a panel of clinicians with experience in interpreting chest CT scans in young children with CF. These scans were also scored utilising PRAGMA-CF radiological scan scoring[18].

D. Analysis

We examined participant characteristics age, gender, CF genotype and mechanism of CF diagnosis as well as serum GlycA levels, CT scan findings and BAL characteristics indicative of infection and inflammation. Participant and BAL characteristics were summarised using counts and percentages for each category. The number of patients, their median age and interquartile range (IQR) are presented for consecutive paired GlycA-BAL samples. Box-and-whisker plots show the distribution of GlycA by age separately for the presence and absence of proinflammatory infection and inflammation. As free NE was below a detectable level for 71% of BAL samples, it was dichotomized into below a detectable level versus detected.

The relationship between GlycA and age was examined using a generalized estimating equation (GEE) method incorporating robust standard errors and an exchangeable correlation structure to account for repeated measures on patients. Associations between each of the binary outcomes absence/presence of

proinflammatory infection, inflammation, bronchiectasis, and both infection and inflammation, and the exposure GlycA, were examined as odds ratios (OR) estimated using the GEE method. Estimates were adjusted for age and gender. Both unadjusted and adjusted ORs and corresponding 95% CIs are presented for a 0.1 mmol/L change in GlycA. A Spearman rank correlation coefficient was calculated to assess the relationship between GlycA and the number of pro-inflammatory organisms isolated in each BAL. The rate of change in the number of pro-inflammatory organisms isolated in each BAL was calculated separately for each participant with at least two time-point measurements via a linear regression, and the rate of change in GlycA was similarly calculated. The relationship between the rates of change in GlycA and the number of pro-inflammatory organisms was examined via a linear regression, both unadjusted and adjusting for the number of pro-inflammatory organisms isolated in the first BAL of each participant. Receiver operating characteristic (ROC) analysis was performed to examine the ability of GlycA to diagnose the presence of proinflammatory infection. Analysis was performed using Stata version 15.1 (STATA Corp, College Station, TX).

Results

A. Population and samples

Subject characteristics gender, genotype and mode of presentation are described in table 1. Three subjects who did not have their genotype fully determined had sweat chloride results exceeding 60mmol/L, clinical presentations consistent with the diagnosis of CF, and bronchiectasis on imaging. Paired GlycA and BAL samples were collected on 311 occasions from 95 patients, aged 0.2 to 6.3 years at the time of collection. Two-thirds (n = 65) of patients had at least three paired samples while a

third (n = 33) had at least four samples (Table 2). The median (IQR) age at the first sample was 1.1 (0.34, 3.0) years. Of all paired GlycA-BAL samples collected, 96% (n = 300) were tested for free NE and 100% underwent culture to identify microorganisms; results are presented in table 3. A chest CT scan was performed at the same visit for 168 (54%) of the paired GlycA-BAL samples. Of the 168 CT scans performed, 51% (n = 86) were acquired using volumetric inspiratory and expiratory image acquisitions.

B. Relationship between GlycA and demographic / genetic factors

GlycA concentrations ranged from 0.68 to 1.92 (mean 1.01, SD 0.15) mmol/L. Higher GlycA concentrations were associated with increasing age (mean GlycA 0.03 mmol/L higher per year, 95% CI 0.02, 0.04, $p < 0.001$). We found no evidence of an association between GlycA concentration and either gender or genotype.

C. Relationship between GlycA and infection

Proinflammatory organism(s) were detected in BAL fluid on 119 (38%) occasions (table 3). Only two participants had *Pseudomonas aeruginosa on >50% of BAL samples taken.* There was evidence of a relationship between GlycA level and being infected with one or more common proinflammatory organisms (*Aspergillus* species, *Pseudomonas aeruginosa, Staphylococcus aureus* or *Haemophilus influenzae*), even after adjusting for the effects of age and gender (OR 1.2 per 0.1 mmol/L, 95% CI 1.02, 1.4, p = 0.03; with *Haemophilus influenzae* excluded as a proinflammatory organism OR 1.2, 95% CI 1.02, 1.5, p = 0.03). However, patients with and without proinflammatory lung infection had GlycA levels that were wide-ranging with significant overlap, regardless of subject age (Figure 1). The area under a ROC curve

(AUC) constructed to examine the performance of GlycA levels in predicting the presence or absence of any proinflammatory organism was 0.64.

We found a weak correlation between GlycA and the number of pro-inflammatory organisms isolated in the paired BAL (Spearman's correlation coefficient 0.25, 95% CI 0.15, 0.36). Ninety-three of the 95 (98%) participants had at least two paired GlycA-BAL samples. We found no evidence of a relationship between the rate of change in GlycA and the rate of change in the number of pro-inflammatory organisms isolated from sequential BAL samples whether or not we adjusted for the number of pro-inflammatory organisms isolated in the first BAL of each participant (adjusted coefficient 0.30, 95% CI -0.44, 1.0, p =0.42).

D. Relationship between GlycA and BAL neutrophil elastase

Neutrophil elastase levels in BAL fluid were higher than the limit of detection on 87 (29%) occasions. There was evidence of a positive relationship between GlycA levels and Free NE above the limit of detection (unadjusted OR 1.2 95% CI 1.01, 1.4, p = 0.04) but not when adjusting for age and gender (OR 1.1 95% CI 0.97, 1.4, p = 0.12). As seen in Figure 2, GlycA levels in those with and without Free NE above the limit of detection were similar, regardless of age.

E. Relationship between GlycA and CT findings

Of the 81 patients who underwent CT chest imaging, 62 (77%) had bronchiectasis on at least one of their scans. After adjusting for age and gender, GlycA concentration was not associated with the presence or absence of bronchiectasis (OR 0.96 95% CI 0.75, 1.2, p = 0.76). BWT was present on 149 (89%) of CT scans. GlycA concentration was positively associated with the presence of BWT on concurrent

imaging (unadjusted OR 1.88 95% CI 1.16, 3.03, p = 0.01), even after adjusting for age and gender (OR 1.78 95% CI 1.2 – 2.64, p < 0.01).

F. Relationship between GlycA and combined lung disease markers

Although there was evidence of a relationship between GlycA levels and the combined presence of at least one pro-inflammatory organism and detectable free NE in BAL (unadjusted OR 1.2 95% CI 1.0, 1.5, p = 0.05; if *Haemophilus influenzae* excluded OR 1.2, 95% CI 1.0, 1.6, p = 0.05), there was no evidence of a relationship when adjusting for age and gender (OR 1.1 95% CI 0.91, 1.4, p = 0.29; if *Haemophilus influenzae* excluded OR 1.2, 95% CI 0.91, 1.5, p = 0.23).

Discussion

GlycA is a novel blood biomarker that has been shown to be elevated in various inflammatory conditions in both adults and children[7-9]. In our study cohort of participants with CF we demonstrated that GlycA levels were associated with the presence of proinflammatory organisms and/or detectable free NE in BAL fluid, as well as with BWT on CT imaging. However, after adjusting for age and gender the association with free NE no longer appeared significant. Furthermore, levels of GlycA varied greatly such that there was considerable overlap in those with different CF disease states and given our findings it seems unlikely that GlycA on its own could serve as a biomarker of early disease progression in clinical practice.

GlycA levels detected in each child from our CF population were at least double that reported in healthy children of a similar age, and were quite similar to those seen in acute Kawasaki Disease[9]. This may support the hypothesis that GlycA elevation in our cohort is a result of the inflammatory state associated with CF. As our subjects

were young, extra-pulmonary manifestations of CF such as diabetes and arthropathy that might themselves drive inflammation were not present. We therefore considered the most likely source of inflammation to be pulmonary disease, given that this is ubiquitous and has an early onset in CF. This is further supported by the fact that GlycA levels increased slightly with age in our study and we know that lung disease, infective burden and inflammation in CF are progressive[1].

Higher mean GlycA levels correlated with the presence of multiple proinflammatory organisms, even after adjustment for the effects of age and gender. This suggests pulmonary infection plays a key role in increased inflammation detectable in blood samples using this biomarker. However, when examining patients using serial measurements, GlycA levels did not seem to rise and fall in concert with the number of different organisms isolated from paired lavages over time. There are many possible reasons for this. Our knowledge of the pulmonary microbiome in CF in young children continues to evolve[19] and ultimately, the number of different pro-inflammatory organisms isolated from BAL at a single point in time is unlikely to be a uniform marker of severity of infection. It is possible that GlycA levels do not have sufficient sensitivity to reflect subtle changes in early infection-associated inflammation. Additionally, inflammation is not always mediated through bacterial infection and even when it is the pro-inflammatory response is heterogeneous, affected by the particular strain of bacteria, interaction between organisms, and host factors.

Our study has a number of limitations. We chose our population as the group most likely to benefit from non-invasive biomarkers, but because participants were less than seven years of age, it is not possible to comment on how GlycA levels vary in

older children and adults with CF. Only a proportion of children were found to have bronchiectasis in our study, and this was mostly mild in severity. Although we found no association with current bronchiectasis, it is possible that inflammation as reflected by GlycA is predictive of future bronchiectasis as the latter likely reflects lung damage associated with prior insults. This may be supported by the fact that GlycA elevation was associated with BWT, commonly considered a precursor to bronchiectasis. It is also possible that in more established disease with chronic pseudomonal infection, there may be a stronger correlation with GlycA. However, very few participants in our cohort had evidence of chronic *Pseudomonas aeruginosa* infection so this hypothesis could not be tested.

Finally, GlycA levels exhibited a high degree of variability and spread, with the range of values obtained from participant groups overlapping greatly; the AUC of 0.64 for GlycA prediction of proinflammatory pulmonary infection is reflective of this. Our findings suggest that an isolated or age-adjusted GlycA threshold value could not be used to predict likelihood of significant pulmonary infection and/or inflammation with a degree of accuracy suitable for clinical use.

To summarize, GlycA is a novel blood biomarker of inflammation that appears to be elevated in young children with CF compared to published reports in healthy children. The GlycA level varies significantly regardless of bronchiectasis, pulmonary infection and inflammation which likely limits its individual discriminatory usefulness. The search for non-invasive markers continues[20] with the changes in GlycA we identified suggesting that there is indeed a systemic signal detectable in the blood, even if GlycA alone does not fulfil the role of a clinically useful early disease biomarker.

Author Contributions

Professor Sarath Ranganathan conceived the idea for the study. Dr Ajay Kevat wrote the first draft of the manuscript. Rosemary Carzino undertook data collection and revised the draft. Suzanna Vidmar completed statistical analyses. All authors contributed to planning, data analysis, review and writing of the final version of the manuscript.

Compliance with Ethical Standards

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Conflict of Interest Statement: The authors declare that they have no conflicts of interest.

Ethical approval: The study was approved by the Royal Children's Hospital Human Research Ethics Committee. All procedures performed were in accordance with the ethical standards of this committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Reference List

1. Cystic Fibrosis Foundation Patient Registry. 2017 Annual Data Report. Bethesda, Maryland, USA 2018. Available from: https://www.cff.org/Research/Researcher-Resources/Patient-Registry/.

2. Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, Robertson CF, Ranganathan SC, Sly PD, Stick SM. Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. Thorax. 2012; 67(6):509-16.

3. Niemann T, Colas L, Roser HW, Santangelo T, Faivre JB, Remy J, Remy-Jardin M, Bremerich J.. Estimated risk of radiation-induced cancer from paediatric chest CT: two-year cohort study. Pediatr Radiol. 2015; 45(3):329-36.

4. de Blic J, Marchac V, Scheinmann P. Complications of flexible bronchoscopy in children: prospective study of 1,328 procedures. Eur Respir J. 2002; 20(5):1271-6.

5. Gaisl T, Bregy L, Stebler N, Gaugg MT, Bruderer T, García-Gómez D, Moeller A, Singer F, Schwarz EI, Benden C, et al. Real-time exhaled breath analysis in patients with cystic fibrosis and controls. J Breath Res. 2018; 12(3):036013.

6. Shoki AH, Mayer-Hamblett N, Wilcox PG, Sin DD, Quon BS. Systematic review of blood biomarkers in cystic fibrosis pulmonary exacerbations. Chest. 2013; 144(5):1659-70.

7. Ritchie SC, Würtz P, Nath AP, Abraham G, Havulinna AS, Fearnley LG, Sarin AP, Kangas AJ, Soininen P, Aalto K, et al. The biomarker GlycA is associated with chronic inflammation and predicts long-term risk of severe infection. Cell Syst. 2015; 1(4):293-301.

8. Dungan K, Binkley P, Osei K. GlycA is a novel marker of inflammation among non-critically ill hospitalized patients with type 2 diabetes. Inflammation. 2015; 38(3):1357-63.

9. Connelly MA, Shimizu C, Winegar DA, Shalaurova I, Pourfarzib R, Otvos JD, Kanegaye JT, Tremoulet AH, Burns JC. Differences in GlycA and lipoprotein particle parameters may help distinguish acute Kawasaki disease from other febrile illnesses in children. BMC Pediatr. 2016; 16(1):151.

10. Collier F, Ellul S, Juonala M, Ponsonby A, Vuillermin P, Saffery R, Burgner D. Glycoprotein acetyls (GlycA) at 12 months are associated with high-sensitivity C-reactive protein and early life inflammatory immune measures. Pediatr Res. 2019; 85:584-585.

11. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, Murray CP, Stick SM. Risk factors for bronchiectasis in children with cystic fibrosis. N Engl J Med. 2013; 368(21):1963-70.

12. Soininen P, Kangas AJ, Würtz P, Suna T, Ala-Korpela M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. Circ Cardiovasc Genet. 2015; 8(1):192-206.

13. Würtz P, Kangas AJ, Soininen P, Lawlor DA, Davey Smith G, Ala-Korpela M. Quantitative serum nuclear magnetic resonance metabolomics in large-scale epidemiology: A primer on -omic technologies. Am J Epidemiol. 2017; 186(9):1084-96.

14. Gangell C, Gard S, Douglas T, Park J, de Klerk N, Keil T, Brennan S, Ranganathan S, Robins-Browne R, Sly PD. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. Clin Infect Dis. 2011; 53(5):425-32.

15. King P. Haemophilus influenzae and the lung (Haemophilus and the lung). Clin Transl Med. 2012; 1(1):10.

16. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, Stick SM, Robinson PJ, Robertson CF, Ranganathan SC. Lung disease at diagnosis in infants

with cystic fibrosis detected by newborn screening. Am J Respir Crit Care Med. 2009; 180(2):146-52.

17. Kuhn JP, Brody AS. High-resolution CT of pediatric lung disease. Radiol Clin North Am. 2002; 40(1):89-110.

18. Rosenow T, Oudraad MC, Murray CP, Turkovic L, Kuo W, de Bruijne M, Ranganathan SC, Tiddens HA, Stick SM. PRAGMA-CF. A quantitative structural lung disease computed tomography outcome in young children with cystic fibrosis. Am J Respir Crit Care Med. 2015; 191(10):1158-65.

19. Frayman KB, Armstrong DS, Grimwood K, Ranganathan SC. The airway microbiota in early cystic fibrosis lung disease. Pediatr Pulmonol. 2017; 52(11):1384-404.

20. Laguna TA, Williams CB, Nunez MG, Welchlin-Bradford C, Moen CE, Reilly CS, Wendt CH. Biomarkers of inflammation in infants with cystic fibrosis. Respir Res. 2018; 19:6.

Table 1: Participant Characteristics

Demographics	***N (%)***
	(n=95)
Female sex	43 (45)
CF Genotype	
Homozygous ΔF508	42 (44)
Heterozygous ΔF508	46 (48)
Other	7 (7.4)
CF Diagnosis	
Newborn screening	66 (69)
Meconium ileus	13 (14)
Respiratory symptoms	4 (4.2)
Family history prompting sweat test	3 (3.2)
Failure to thrive	2 (2.1)
Other	7 (7.4)

Table 2: Participant age at the time of each paired GlycA-BAL sample

Sample No.	No. of Participants	Median (IQR) age (year)
1	95	1.1 (0.34, 3.0)
2	93	2.1 (1.2, 4.0)
3	65	3.0 (2.1, 4.0)
4	33	4.0 (3.1, 5.0)
5	16	4.7 (4.1, 6.0)
6	7	6.0 (5.7, 6.1)
7	2	6.2 (6.0, 6.3)

Table 3: Bronchoalveolar Lavage Characteristics

Characteristic	N (%)
Inflammation	*300 (100)*
Free NE above the limit of detection	87 (29)
Infection	*311 (100)*
Any proinflammatory infection	100 (32)

(h. influenza excluded)	
Any proinflammatory infection	119 (38)
(h. influenza included)	
>1 proinflammatory organism	28 (9.0)
(h. influenza included)	