Rhinovirus Species-Specific Antibodies Differentially Reflect Clinical Outcomes in Health and Asthma

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Spyridon Megremis*, Katarzyna Niespodziana*, Clarissa Cabauatan, Paraskevi Xepapadaki, Marek L. Kowalski, Tuomas Jartti, Claus Bachert, Susetta Finotto, Peter West, Sophia Stamataki, Anna Lewandowska-Polak, Heikki Lukkarinen, Nan Zhang, Theodor Zimmermann, Frank Stolz, Angela Neubauer, Mübeccel Akdis, Evangelos Andreakos, Rudolf Valenta*, Nikolaos G. Papadopoulos*

Spyridon Megremis, PhD; University of Manchester, Division of Infection, Immunity and Respiratory Medicine

Katarzyna Niespodziana, PhD; Medical University of Vienna, Division of Immunopathology, Department of Pathophysiology and Allergy Research

Clarissa Cabauatan; Medical University of Vienna, Division of Immunopathology, Department of Pathophysiology and Allergy Research

Paraskevi Xepapadaki, PhD; National and Kapodistrian University of Athens, Allergy Department, 2nd Pediatric Clinic

Marek L. Kowalski, PhD; Medical University of Lodz, Department of Immunology, Rheumatology and Allergy

Tuomas Jartti, PhD; Department of Paediatrics Turku University Hospital and University of Turku, Turku, Finland

Claus Bachert, PhD; Ghent University, Head Upper Airways Research Laboratory

Susetta Finotto, PhD; Department of Molecular Pneumology, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen

Peter West, PhD; University of Manchester, Division of Infection, Immunity and Respiratory Medicine

Sophia Stamataki, MD; Athens General Children's Hospital "Pan & Aglaia Kyriakou", Greece

Anna Lewandowska-Polak, MD; Medical University of Lodz, Department of Rheumatology, Poland

Heikki Lukkarinen, PhD; Department of Paediatrics Turku University Hospital and University of Turku, Turku, Finland

Nan Zhang, PhD; Ghent University, Head Upper Airways Research Laboratory

Theodor Zimmermann, MD; Children's Hospital, Department of Allergy and Pneumology, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen

Frank Stolz; Biomay AG, Vienna, Austria

Angela Neubauer, PhD; Biomay AG, Vienna, Austria

Mubeccel Akdis, PhD; University of Zurich, Swiss Institute of Allergy and Asthma Research

Evangelos Andreakos, PhD; Biomedical Research Foundation, Academy of Athens, Greece

Rudolf Valenta, PhD; Medical University of Vienna, Division of Immunopathology, Department of Pathophysiology and Allergy Research, and NRC Institute of Immunology FMBA of Russia, Moscow, Russia

Nikolaos Papadopoulos; MD, PhD, University of Manchester, Division of Infection, Immunity and Respiratory Medicine; National and Kapodistrian University of Athens, Allergy Department, 2nd Pediatric Clinic

* Equally contributed

Corresponding author: Nikolaos Papadopoulos; MD, PhD, University of Manchester, Division of Infection, Immunity and Respiratory Medicine; and National and Kapodistrian University of Athens, Allergy Department, 2nd Pediatric Clinic. ngpallergy@gmail.com, 41, Fidippidou Athens 115 27 Greece, T: +30 (210) 7776964, F: +30 (210) 7777693.

NP, RV, SM and KN contributed to the conception and design of the work.

KN, CC, PX, MK, TJ, CB, SF, SS, ALP, HL, NZ, TZ, FS, AN, MA, EA , NP and RV contributed to the acquisition of the data. SM analysed the data. SM and NP, interpreted the data SM and NP drafted the work.SM, KN, RV and NP revised the final draft., All authors approved the final version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Running head: Rhinovirus antibody response in asthmatic children

1.21 Infectious Mechanisms

Total word count: 3910

At a glance commentary:

Patients with asthma are more susceptible to symptomatic rhinovirus infection and a suboptimal antiviral response is often associated with increased viral replication. However, the antibody response against RVs is misdirected towards an inaccessible virus region. We screened the largest repertoire of RV proteins and peptides up to now and identified species-specific antibodies in pre-school age children with or without asthma. The humoral response to different RV species varied in both groups and suggests differential infectivity pattern between RV species. In healthy pre-schoolers, RV antibodies accumulate with colds in a linear trajectory. RV antibody levels in asthma patients are higher than in healthy children. However, they are not related to previous infections and they are 'maxed-out' even in patients with no reported infections suggesting a saturated system. Importantly, in asthma, RV-A and RV-C antibodies increased with disease severity and wheeze episodes. Higher antibody levels in asthma may be due to a compromised innate immune response, leading to increased exposure of the adaptive immunity to the virus but with no apparent protection.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org"

Key words: Rhinovirus, Asthma, Antibody, Predicta, Chip

Abstract:

Rationale: Rhinoviruses are major triggers of common cold and acute asthma exacerbations; Rhinovirus species A, B and C may have distinct clinical impact; however, little is known regarding RV species-specific antibody responses in health and asthma.

Objectives: To describe and compare total and rhinovirus species-specific antibody levels in healthy and asthmatic children, away from an acute event.

Methods: Serum samples from 163 preschool children with mild to moderate asthma and 72 healthy controls from the multinational Predicta cohort were analysed using the recently developed PreDicta rhinovirus antibody chip.

Main results: Rhinovirus antibody levels varied, with rhinovirus C and rhinovirus A being higher than rhinovirus B in both groups. Compared to controls, asthma was characterised by significantly higher levels of antibodies to rhinovirus A and rhinovirus C, but not rhinovirus B. Rhinovirus antibody levels positively correlated with the number of common colds over the previous year in healthy children, and wheeze episodes in asthmatics. Antibody levels also positively correlated with asthma severity but not with current asthma control.

Conclusions: The variable humoral response to rhinovirus species in both groups, suggests a differential infectivity pattern between rhinovirus species. In healthy pre-schoolers, rhinovirus antibodies accumulate with colds. In asthma, rhinovirus A and rhinovirus C antibodies are much higher and further increase with disease severity and wheeze episodes. Higher antibody levels in asthma may be due to a compromised innate immune response, leading to increased exposure of the adaptive immunity to the virus. Importantly, there is no apparent protection with increasing levels of antibodies.

Abstract word count: 250

Introduction:

Asthma is a major contemporary epidemic[1]. A considerable proportion of the asthma burden, is attributed to acute exacerbations, which almost invariably[2] follow an upper respiratory tract infection (URIs), most often due to Rhinoviruses (RVs)[3,4]. In addition to exacerbations, RVs promote asthma in multiple ways[5,6,7]. Asthmatics are more susceptible to symptomatic RV infection and a suboptimal antiviral response is associated with increased viral replication and cytotoxicity[8].

There are 81 RV-A, 33 RV-B and 33 RV-C full genome sequences available in addition to 358 yet unclassified partial sequences (NCBI Taxonomy Browser). RV-C genotypes are more diverse than RV-A or RV-B[9] and recombination is frequent[10], especially for RV-A and RV-C species[11]. RV species are widespread and continuously co-circulating throughout the world[12]. RV-A and RV-C species are associated with severe infections and hospitalisation in young children, especially those with asthma[13,14].

Following an RV infection, serum-neutralising antibody titres increase for about a year and high pre-existing neutralising antibody titres have been associated with resistance to reinfection[15]. RV species-specific and cross-reactive signal can be defined[16], although, antibody responses against RV-A and RV-C species are highly cross-reactive. There is low correlation between the RV genotype detected during a symptomatic or recovering period and RV antibody titres[17-19], possibly due to the high sequence homology observed between RV species. Thus, understanding the full extent of RV epitope diversity is required to develop a vaccine with wide species coverage[20].

During an exacerbation, asthmatic children have higher total anti-RV antibody titres than non-asthmatic[17] and rhinovirus VP1-specific IgG_1 levels tend to be higher in adults with asthma

than healthy controls, prior to an experimental infection[21]. Even though, it has been suggested that the immune response to RV-C species is less efficacious than RV-A and RV-B[17,21], T-cell responses to RV-A and RV-C are of similar magnitude[22].

With advancing technology and an increasing number of sequences available, we are now able to identify species-specific antibodies with increased resolution. In this study, we take advantage of our recently developed PreDicta RV chip to describe RV species-specific antibody responses in pre-school age children with or without asthma.

Methods

Study population: The PreDicta pediatric cohort is a 2-year prospective multicentre study, part of the EU FP7 program PreDicta and has been described elsewhere[23]. We have analysed available serum samples from 163 children, 4-6 years of age, with a diagnosis of mild to moderate severity asthma confirmed by a doctor of the participating study centre using pre-specified criteria. Seventy-two healthy children of matched age, with no history of asthma/wheeze, served as cross-sectional controls (Table E1, online data supplement). The study was approved by all participants' institutional ethics committees, and written informed consent was obtained from all parents.

Chip-based measurement of RV-specific antibody levels in human serum samples: Serum RV-specific antibody levels were measured using the newly developed PreDicta RV chip[19] . Briefly, the PreDicta microarray contains 130 synthesized linear RV peptides and proteins including, 20 recombinant RV capsid proteins, VP1-VP4, and 15 recombinant VP1 fragments from five representative RV strains, RV89, RV14, RV16, RV2, and RVC. In addition, it also contains 5 recombinant non-structural proteins from RV89. Details on the chip-based

measurement of RV-specific antibody levels in human serum samples are provided in the online data supplement.

Noise reduction and determination of peptide specificity: Prior to comparing measurements between groups, the data were processed in order to: (i) exclude non-informative signal and (ii) determine the specificity of peptide signal towards RV A, B and C (Figure E1-E4, online data supplement). The final dataset included antibody signals from RV-A (n=14), RV-B (n=9), and RV-C (n=2) species-specific peptides (Figure E4 and Table E2, online data supplement). A detailed description of the analysis can be found in the online data supplement.

Significance tests:

A detailed description of the analysis can be found in the online data supplement.

Results

Differential antibody levels against RV-A, B & C

In the overall population, RV-A (95% CI: 1.646-1.728) and RV-C (95% CI: 1.748-1.845) specific signal levels were significantly higher than RV-B (95% CI: 1.123-1.248) (Figure 1A). This was the case in both healthy control children (RV-A 95% CI: 1.518-1.669, RV-C 95% CI: 1.577-1.753 and RV-B 95% CI: 1.054-1.298) (Figure 1B) and asthma patients (RV-A 95% CI: 1.679-1.776, RV-C 95% CI: 1.797-1.911 and RV-B 95% CI: 1.116-1.262) (Figure 1C). Moreover, in the asthma group, as well as in the overall population, RV-C specific antibody signal was higher than that to RV-A (Figures 1C, 1A).

Children with asthma have higher RV-A and RV-C, but not RV-B, antibody levels than healthy controls. The average RV antibody signal was significantly higher in asthma patients (95% CI: 1.457-1.543) than healthy participants (95% CI: 1.342-1.468) (Figure 2A). When analysed

individually, the average RV-A (Figure 2B) and RV-C (Figure 2D) species-specific signals were significantly higher in asthma (RV-A 95% CI: 1.679-1.776, RV-C 95% CI: 1.797-1.911) than controls (RV-A 95% CI: 1.518-1.669, RV-C 95% CI: 1.577-1.753), while no differences were observed for RV-B (Figure 2C). Significant differences between the asthma and control groups were also found for the peptides identifying both RV-A and RV-C (RV-A/C, p: 0.0089) and those identifying all RV species (RV-A/B/C, p: 0.0014) but not those identifying RV-A and RV-B (RV-A/B, p: 0.4137).

Participants were grouped (K=2, unsupervised K means clustering) based on the measured RV species-specific and mixed antibody signal into high and low responders. Significantly more asthma patients were classified as high responders when compared with healthy donors, but only for the RV-A specific (Figure 2E), RV-C specific (Figure 2F) and RV-A/C peptides (Figure 2G). Regression analysis suggested that asthma patients were significantly more likely to be high responders to RV-A (95% CI: 0.233-0.775) and RV-C (95% CI: 0.199-0.661) species-specific and RV-A/C (95% CI: 0.169-0.716) mixed-signal peptide measurements. No differences were observed in groups of high and low responders to RV-B specific, RV-A/B, and RV-A/B/C mixed peptides.

RV antibody levels reflect the number of upper respiratory infections (URIs) in the last 12 months in healthy children but not in asthma patients. Linear regression was used to investigate the relationship between RV antibody signal and number of reported upper respiratory infections (URIs) for a time window of 12 months prior to inclusion in the study (Figure 3). URI data were available for 64/72 healthy donors and 160/163 asthma patients. RV antibody signal increased linearly with the number of reported URIs in healthy donors (Figure 3A), but not in asthma patients (Figure 3E). This was observed in all RV species (Figure 3B-D

and 3F-3H). Asthma patients which reported no or few URIs during the last 12 months had RV antibody signals at the same level as healthy patients with multiple URIs (Supplementary Figure E5). Moreover, asthmatics reporting no URIs (Mean: 1.650, S.D.: 0.18) during the past year exhibited significantly higher (p: 0.0001) RV antibody signal than healthy donors with no history of URIs (Mean: 1.036, S.D.: 0.04).

Association between RV antibody levels and lower respiratory infections

Donors were grouped based on the number of lower respiratory infections (LRI=0 and LRI>1). In healthy participants, only RV-B specific antibody levels were significantly lower (Unpaired t test with Welch's correction, p: 0.0269) in children with more than one LRI in the previous year (Mean: 0.7449, SEM ± 0.155, n=6) compared to children with no reported LRIs (Mean: 1.216, SEM ± 0.07052, n=58). RV-A specific antibody levels were elevated in children with no LRIs (Mean: 1.616, SEM ± 0.03916, n=58) compared to children with more than one LRI (1.433 ± 0.1418, n=6) but did not reach statistical significance. No differences were observed in asthma patients.

Association between RV antibody levels and susceptibility to the spread of upper respiratory infections to the lower respiratory tract.

We have performed nonparametric correlation of the number of URIs (Mean: 5.762, SEM ± 0.304) and LRIs (Mean: 1.306, SEM ± 0.193) in the asthma group (n=160) using Spearman's test. The variables were negatively correlated (r: -0.280, p: 0.000). We explored the effect of RV antibody levels on the URIs versus LRIs correlation. RV-A antibody levels as a co-factor did not affect the negative correlation (r: -0.321, p: 0.000). The same applied for RV-B antibody levels (r: -0.325, p: 0.000) and RV-C (r: -0.326, p: 0.000). Age did not affect the negative correlation (r: - 0.315, p: 0.000). In the healthy group (n=64) the correlation between URIS

(Mean 4.109, SEM ± 0.335) and LRIs (Mean 0.109, SEM ± 0.045) was not significant (r: -0.158, p: 0.212).

In children with asthma, RV-A and RV-C, but not RV-B, antibody levels positively correlate with the number of asthma-related episodes. The relation of RV antibody levels and the reported events of lower respiratory symptomatology for the past 12 months were investigated (Figure 4). Data regarding the number of respiratory episodes were available for 160 out of 163 asthma patients. The RV-A (Figure 4B) and RV-C (Figure 4D) specific signal were positively correlated with the number of wheeze episodes. The correlations were not affected by the number of reported URTIs.

Correlation of RV antibody levels with asthma severity.

Children with asthma were characterised by the clinical investigators as intermittent (n=47) or persistent (n=115) (GINA). Higher RV antibody signal levels were present in persistent (95% CI: 1.486-1.591) than in intermittent asthmatics (95% CI: 1.340-1.486) (Figure 5A). This was also the case for RV-A specific antibody signal (persistent 95% CI: 1.713-1.829, intermittent 95% CI: 1.545-1.716) (Figure 5B). RV-C specific antibody signal was higher in persistent asthma in comparison to healthy controls (95% CI: 1.820-1.955 vs 95% CI: 1.577-1.753), but not in comparison to intermittent asthma (Figure 5D). No significant differences were observed between the groups in relation to RV-B (Figure 5C). Classification of the participants into the three groups based on their antibody profiles was also investigated through discriminant function analysis (Supplementary Figure E6).

RV antibody levels are not related to current asthma control.

Asthma patients were grouped based on disease control into controlled (n=80) and partially controlled/uncontrolled (n=81). No differences in antibody levels were observed between

controlled and uncontrolled asthma in any RV group (Figure 6). Patients with not well controlled asthma at the time of inclusion (95% CI: 1.478-1.604) had significantly higher RV antibody signal than healthy donors (95% CI: 1.342-1.468) (Figure 6A). The RV-A specific antibody signal was significantly higher in patients with partially controlled and uncontrolled asthma (95% CI: 1.686-1.835) than in healthy donors (95% CI: 1.518-1.669) (Figure 6B), and slightly increased in children with well-controlled asthma (95% CI: 1.637-1.764). RV-C specific signal was significantly higher in partially controlled-uncontrolled (95% CI: 1.770-1.949) and well controlled (95% CI: 1.777-1.931) asthmatic children than in healthy donors (95% CI: 1.577-1.753) (Figure 6D). No significant differences were observed in RV-B specific antibody signal (Figure 6C).

Seasonal variation in RV antibody levels is observed only in healthy children. The RV antibody signal was analysed based on the season of inclusion to the study (Figure 7). In healthy donors, significant differences were observed amongst children of whom samples were obtained during summer (Figure 7A). This variation was evident in the RV-A (Figure 7B) and RV-C (Figure 7D) species-specific antibody signal, but not in RV-B (Figure 7C). No seasonal variation was observed in antibody levels of asthma patients (Figure 7E-7H).

Atopic status does not affect RV antibody levels.

Asthma patients were stratified in atopic (n=81) and non-atopic (n=79) and the average antibody signal per donor was compared using Welch's t test (Supplementary Figure E7). No differences were observed between the two groups in RV-A (A.), RV-B (D.) and RV-C (G.) Patients were further stratified based on the number of previously reported LRIs (LRI=0, and LRI>1,). No differences were observed in RV-A antibody levels in atopic and non-atopic patients with LRI=0 (B.) and LRI>1 (C.). No differences were observed in RV-B antibody levels

in atopic and non-atopic patients with LRI=0 (E.) and LRI>1 (F.). No differences were observed in RV-C antibody levels in atopic and non-atopic patients with LRI=0 (E.). Atopic patients with LRI>1 had increased RV-C antibody levels compared to non-atopic patients with LRI>1 (I.). Atopy was not related to the number of upper (ExpB: 1.046, p: 0.282) or lower (ExpB: 1.025, p: 0.874) respiratory infections tested with binary regression.

Discussion

This study provides several novel insights into the RV-specific antibody repertoire of preschool children, in both health and asthma: (i) A clear differential of RV species antibody levels was demonstrated in this multinational cohort (Supplementary Figure E8), in both health and asthma. (ii) Asthma is characterised by higher levels of antibodies to RV-A and RV-C, but not RV-B, suggesting differential susceptibility to these species. (iii) RV antibody levels reflect the number of common colds in healthy children, and wheeze episodes in asthmatics. (iv) In the asthma group, the number of URIs was negative correlated with the number of LRIs. This observation was not affected by RV species-specific antibody levels (v) RV antibody levels correlate with asthma severity but not with current asthma control, suggesting accumulation over longer periods of time. (vi) The presence of atopy does not affect RV antibody levels and susceptibility to LRIs.

A newly developed technology was used which allows the measurement of 130 different RVproteins and peptides providing unprecedented power of analysis. This allowed a data-driven identification of species-specific and mixed (cross-reactive) antibodies using a combination of phylogenetic and unsupervised clustering to discriminate between expected and observed specificity. Subsequently, RV-A, RV-B and RV-C species-specific peptides were used to identify differences between asthma patients and healthy controls.

306 The antibody signal follows closely the degree of sequence homology (i.e. RV-A> RV-C> RV-B).
307 Robust signal was generated against peptides derived from the VP1 region of all three RV
308 species further confirming our earlier finding that the N-terminus of VP1 represents a major
309 epitope of rhinovirus-specific antibodies[19,24]. It has long been thought that RV antibodies
310 cause a large change to the structure of the viral coat which neutralises the virus and stops
311 infectivity[25]. However, we have shown that the viral capsid is dynamic and undergoes
312 uncoating when RV is bound to ICAM-1, thus exposing the normally inaccessible N terminus
313 of VP1 and misdirecting the antibody response[19,24].
314 Antibody levels against RV species were significantly different, with RV-C > RV-A > RV-B, in
315 asthma patients. RV-A and RV-C antibodies were higher than RV-B in healthy donors. A
316 previous report has suggested that total and specific RV-A IgG_1 titres are higher than both RV-
317 B and RV-C[17] and the species-specific titres to RV-C are extremely low in both asthmatic and
318 non-asthmatic children, although they both have high RV-C titres to cross-reactive RV-A & RV-
319 C antigens. Differential detection of antibody levels against the three RV species may be
320 attributed to differential exposure, differential immune response and the ability to analyse a
321 diverse repertoire of strains, and epitopes; First, RV-C species exhibit higher within-group
322 diversity than A and B species[9], suggesting that exposure to a certain RV-C strain might not
323 influence the immune response against other RV-C strains. This is further supported by the
324 lack of difference between RV-A and RV-C in our healthy controls. Second, our recent
325 observations associating RV-A and RV-C antibodylevels with more severe asthma outcomes
326 and respiratory symptomatology, suggests a correlation of antibody response and immune
327 status. Finally, we have analysed a diverse collection of RV proteins and peptides, with only a

few overlaps with the Iwasaki study[17] investigating antibody levels against RV in asthma; 14 RV-A (1 common: A01B), 9 RV-B (2 common: B14, B69) and 2 RV-C (none common).

Children with asthma have higher RV-total and RV-A and RV-C species-specific antibodies than non-asthmatic healthy children. Iwasaki et al[17] also reported similar differences (higher RV-total, RV-A, and to a lesser extent RV-B) when comparing antibody levels in asthmatic children during an exacerbation, with healthy controls. Furthermore, we have also previously observed increased VP1-specific IgG_1 levels in adult asthmatic patients (age 19-54 years)[21]. It thus appears that starting from at least the preschool years; patients with asthma develop high levels of antibodies against specific RV species A and C. In a responder analysis, many more asthma patients than healthy participants are high responders to RV-A, RV-C and RV-A/C mixed peptides. The differential antibody response to RV subtypes, paralleling their reported clinical impact in asthma, and the apparent lack of overall antibody-mediated protection from respiratory morbidity in asthma, points towards the innate immunity as the key limiting factor of RV virulence[26,27]: a defective innate response to RV-A and RV-C in asthma may explain both the higher levels of antibodies and increased morbidity from these viruses[28]. Indeed, it was recently demonstrated that children, independent of their asthma status, have a competent CD4+ T-cell recall response to RV-A and RV-C[29].

In asthma, increased number of URIs was correlated with decreased number of LRIs but was not mediated by RV antibody levels. Moreover, RV antibody levels did not correlate with the number of URIs or LRIs as in the healthy group. Importantly, asthmatic children reported significantly higher number of upper and lower respiratory infections compared to healthy children[23]. In healthy children, RV antibody levels were robustly correlated with the number of reported respiratory infections in the last 12 months, in a linear manner. Children with

LRIs>1 had significantly lower RV-B and slightly decreased RV-A antibody levels. Unfortunately, the small number of LRIs did not allow robust characterisation of upper and lower respiratory tract infections and RV antibodies. We believe that in healthy children RV antibody accumulation is akin to the number of RV infections and protective from re-infection and spread to the lower tract. Therefore, in this asthma age group, RV antibodies accumulate without conferring (at least clinically relevant) cross-protection. . In contrast, RV antibodies in asthmatics correlated with previous wheeze episodes. This may be due to (i) different kinetics of antibody accumulation in this population i.e. children with asthma may have already accumulated high levels of RV antibodies at earlier times and are now expanding their repertoire only after more severe infections, associated with wheeze, and/or (ii) symptom interpretation in children with asthma: it is possible that several of the events identified as URI may be in fact triggered by other factors. Whether asthma patients have more URIs than normal individuals is still disputed, as it is possibly confounded by different symptom thresholds in asthmatic versus normal populations[4,6]. It is clear however that people with asthma suffer from more frequent lower respiratory infections and have more severe and longer-lasting LRT symptoms[31]. In a cohort of younger children sampled during an acute episode of wheeze[18] Stenberg-Hammar et al demonstrated that more respiratory symptoms were significantly associated with increases in RV-A and RV-C specific IgG_1. Moreover, RV-B specific antibody levels showed a tendency to be negatively correlated with the number of reported lower respiratory infections in healthy participants but not in asthma patients, and in the small number of healthy children that had a LRI, RV antibody levels were significantly lower than those who did not.

In consequence with the above, RV antibodies were also associated with asthma severity in our cohort, with children with persistent asthma having higher levels of RV-A and RV-C antibodies than those with intermittent disease. It is reasonable that patients with more severe disease had accumulated larger amount of RV antibodies due to a higher number of previous infections. It should be noted that children with severe persistent asthma were excluded from the study. In contrast, asthma control, reflecting disease activity one month before the antibody sampling, was not significantly correlated to RV antibody levels, even though there was a tendency of higher RV-A and -C antibodies in asthmatics with not well controlled disease.

Seasonality was not pronounced, however, total RV, RV-A and RV-C antibody levels of healthy children were at their lowest during the summer, consistent with the epidemiology of RVs and our understanding of RV antibody production kinetics[15,32,33]. However, this was not the case in asthma patients suggesting an absence of correlation between RV antibody levels and RV epidemiology in this age group.

Currently no models exist that can explain how pre-existing antibodies may affect the generation of protective responses to RV as a faction of the number of respiratory infections and/or infecting RV strain and if this may potentially be altered in asthma. Our findings can be summarised in a hypothetical graph based on the epitope masking model[34] (Supplementary Figure E9). Longitudinal studies, such PreDicta, are in great need to understand the built up and extent of the RV antibody repertoire in health and asthma.

The major strengths of the study are the high number of proteins and peptides used, the unsupervised data-driven approach to discriminate between RV species-specific and mixed signal, the multicentre/multinational setting, the narrow range of age and the exclusion of

extreme asthma severity cases. A limitation in this study is the retrospective reporting of events, either infections or wheeze episodes which may suffer from recall bias. However, the robustness of the correlation between reported URI and antibody levels in healthy children suggests that this is not the case, at least in this group. Nevertheless, it is possible that the interpretation of respiratory symptoms in asthmatics, particularly in persistent cases, is not easy and can underpin the lack of association in this group. In contrast, wheezing episodes are conceivably more memorable in asthma cases.

In conclusion, we have used the novel PreDicta RV antibody chip to characterise the species-specific antibody repertoire of healthy and asthmatic pre-school age children. In health, RV antibodies reflect previous URIs, while in asthma they reflect previous episodes of wheeze and disease severity. There are clear differences in RV antibody levels between normal and asthmatic children, as well as within the asthmatic population, suggesting that these measurements could be further explored as potential biomarkers. The humoral immune response to RV subgroups is variable with higher levels of RV-C and RV-A antibodies; however there is no apparent protection with increasing levels of antibodies. Longitudinal trajectories of RV antibody levels over time, in association with disease activity, will provide further insights on their role in disease progression.

References

1. Eder W, Ege MJ, von Mutius E. The asthma epidemic. The New England journal of medicine 2006;355:2226-35.
2. Guibas GV, Megremis S, West P, Papadopoulos NG. Contributing factors to the development of childhood asthma: working toward risk minimization. Expert review of clinical immunology 2015;11:721-35.
3. Papadopoulos NG, Christodoulou I, Rohde G, et al. Viruses and bacteria in acute asthma exacerbations--a GA(2) LEN-DARE systematic review. Allergy 2011;66:458-68.
4. Busse WW, Lemanske RF, Jr., Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. Lancet 2010;376:826-34.
5. Jackson DJ, Gangnon RE, Evans MD, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. American journal of respiratory and critical care medicine 2008;178:667-72.
6. Xepapadaki P, Papadopoulos NG, Bossios A, Manoussakis E, Manousakas T, Saxoni-Papageorgiou P. Duration of postviral airway hyperresponsiveness in children with asthma: effect of atopy. The Journal of allergy and clinical immunology 2005;116:299-304.
7. Papadopoulos NG, Xepapadaki P, Mallia P, et al. Mechanisms of virus-induced asthma exacerbations: state-of-the-art. A GA2LEN and InterAirways document. Allergy 2007;62:457-70.
8. Ritchie AI, Farne HA, Singanayagam A, Jackson DJ, Mallia P, Johnston SL. Pathogenesis of Viral Infection in Exacerbations of Airway Disease. Annals of the American Thoracic Society 2015;12 Suppl 2:S115-32.
9. Palmenberg AC, Spiro D, Kuzmickas R, et al. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. Science 2009;324:55-9.
10. Palmenberg AC, Gern JE. Classification and evolution of human rhinoviruses. Methods in molecular biology 2015;1221:1-10.
11. McIntyre CL, McWilliam Leitch EC, Savolainen-Kopra C, Hovi T, Simmonds P. Analysis of genetic diversity and sites of recombination in human rhinovirus species C. Journal of virology 2010;84:10297-310.
12. Briese T, Renwick N, Venter M, et al. Global distribution of novel rhinovirus genotype. Emerging infectious diseases 2008;14:944-7.
13. Bochkov YA, Gern JE. Clinical and molecular features of human rhinovirus C. Microbes and infection 2012;14:485-94.
14. Bonnelykke K, Coleman AT, Evans MD, et al. CDHR3 Genetics and Rhinovirus C Respiratory Illnesses. American journal of respiratory and critical care medicine 2017.
15. Barclay WS, al-Nakib W, Higgins PG, Tyrrell DA. The time course of the humoral immune response to rhinovirus infection. Epidemiology and infection 1989;103:659-69.
16. Iwasaki J, Smith WA, Stone SR, Thomas WR, Hales BJ. Species-specific and cross-reactive IgG1 antibody binding to viral capsid protein 1 (VP1) antigens of human rhinovirus species A, B and C. PloS one 2013;8:e70552.
17. Iwasaki J, Smith WA, Khoo SK, et al. Comparison of rhinovirus antibody titers in children with asthma exacerbations and species-specific rhinovirus infection. The Journal of allergy and clinical immunology 2014;134:25-32.
18. Stenberg-Hammar K, Niespodziana K, Soderhall C, et al. Rhinovirus-specific antibody responses in preschool children with acute wheeze reflect severity of respiratory symptoms. Allergy 2016;71:1728-35.

19. Niespodziana K, Stenberg-Hammar K, Megremis S, et al. PreDicta chip-based high resolution diagnosis of rhinovirus-induced wheeze. Nat Commun 2018;9:2382.
20. Papadopoulos NG, Megremis S, Kitsioulis NA, Vangelatou O, West P, Xepapadaki P. Promising approaches for the treatment and prevention of viral respiratory illnesses. The Journal of allergy and clinical immunology 2017;140:921-32.
21. Niespodziana K, Cabauatan CR, Jackson DJ, et al. Rhinovirus-induced VP1-specific Antibodies are Group-specific and Associated With Severity of Respiratory Symptoms. EBioMedicine 2015;2:64-70.
22. Gaido CM, Stone S, Chopra A, Thomas WR, Le Souef PN, Hales BJ. Immunodominant T-Cell Epitopes in the VP1 Capsid Protein of Rhinovirus Species A and C. Journal of virology 2016;90:10459-71.
23. Xepapadaki P, Bachert C, Finotto S, et al. Contribution of repeated infections in asthma persistence from preschool to school age: design and characteristics of the PreDicta cohort. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology 2018.
24. Niespodziana K, Napora K, Cabauatan C, et al. Misdirected antibody responses against an N-terminal epitope on human rhinovirus VP1 as explanation for recurrent RV infections. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2012;26:1001-8.
25. Katpally U, Fu TM, Freed DC, Casimiro DR, Smith TJ. Antibodies to the buried N terminus of rhinovirus VP4 exhibit cross-serotypic neutralization. Journal of virology 2009;83:7040-8.
26. Chen Y, Hamati E, Lee PK, et al. Rhinovirus induces airway epithelial gene expression through double-stranded RNA and IFN-dependent pathways. American journal of respiratory cell and molecular biology 2006;34:192-203.
27. Custovic A, Belgrave D, Lin L, et al. Cytokine Responses to Rhinovirus and Development of Asthma, Allergic Sensitization, and Respiratory Infections during Childhood. American journal of respiratory and critical care medicine 2018;197:1265-74.
28. Ritchie AI, Jackson DJ, Edwards MR, Johnston SL. Airway Epithelial Orchestration of Innate Immune Function in Response to Virus Infection. A Focus on Asthma. Annals of the American Thoracic Society 2016;13 Suppl 1:S55-63.
29. Gaido CM, Granland C, Laing IA, et al. T-cell responses against rhinovirus species A and C in asthmatic and healthy children. Immunity, inflammation and disease 2017.
30. Paraskevi Xepapadaki CB, Susetta Finotto, Tuomas Jartti, George N. Konstantinou, Alexander Kiefer, Marek Kowalski, Anna Lewandowska-Polak, Heikki Lukkarinen, Eirini Roumpedaki, Anna Sobanska, Ina Sintobin, Tytti Vuorinen, Nan Zhang, Theodor Zimmermann, Nikolaos G. Papadopoulos. Contribution of repeated infections in asthma persistence from preschool to school age: design and characteristics of the PreDicta cohort Pediatric Allergy and Immunology 2018.
31. Corne JM, Marshall C, Smith S, et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. Lancet 2002;359:831-4.
32. Lee WM, Lemanske RF, Jr., Evans MD, et al. Human rhinovirus species and season of infection determine illness severity. American journal of respiratory and critical care medicine 2012;186:886-91.
33. Monto AS. The seasonality of rhinovirus infections and its implications for clinical recognition. Clinical therapeutics 2002;24:1987-97.
34. Zarnitsyna VI, Lavine J, Ellebedy A, Ahmed R, Antia R. Multi-epitope Models Explain How Pre-existing Antibodies Affect the Generation of Broadly Protective Responses to Influenza. PLoS pathogens 2016;12:e1005692.

Figure legends

Figure 1: RV species-specific antibody signal levels in healthy and asthmatic preschool children. In all participants of the study, the highest RV signal is observed against RV-C peptides followed by RV-A and RV-B antibody levels (A). In healthy donors, RV-A and RV-C signals were higher than RV-B (B). In asthma patients, the highest RV antibody signal is observed against RV-C peptides followed by RV-A and RV-B (C). Differences are significant at the 0.05 level. ANOVA & Post Hoc (Tukey's test).

Figure 2: Differential accumulation of RV species antibody signal levels between asthma patients and healthy donors. Children with asthma have higher RV antibody signal compared to healthy donors (A). Asthma patients have higher species-specific antibody signal against RV-A (B) and RV-C (D), but not against RV-B (C). The majority of asthma patients could be assigned as 'high responders' against RV-A, 55.2% vs 34.3%) (E), RV-C, 60.7% vs 35.9% (F) and RV-A/C, 56.4% vs 32.8% peptides (G), compared to healthy donors. Low responders: white portion of bar plot. High responders: Black portion of bar plot. Differences were significant at the 0.05 level, unpaired T test with Welch's correction.

Figure 3: Correlation of RV antibody signal levels and reported URIs. RV antibody levels were linearly and positively correlated with the number of URIs in healthy donors (A) but not in asthma patients (E). In healthy donors, RV-A (B), RV-B (C) and RV-C (D) specific signal increased linearly with increasing number of URIS. In asthma patients, none of the RV-A (F), RV-B (G) or RV-C (H) specific signal was correlated with the number of URIs. Differences were significant at the 0.05 level (Linear regression).

Figure 4: Evaluation of RV antibody signal levels and reported asthma-related episodes in asthma patients. RV-A (B) and RV-C (D) specific antibody levels were linearly and positively correlated with the number of previous wheeze episodes. No correlation was observed for the total RV (A) and RV-B specific antibody levels (C). Correlations were significant at the 0.05 level (Linear regression).

Figure 5: Differential levels of RV antibodies in patients with intermittent and persistent asthma. Children with more severe asthma have higher total RV (A) and RV-A (B) specific antibody levels than asthmatic children with intermittent asthma. RV-C specific antibody levels were higher only in severe asthmatics when compared to healthy controls (D). No differences were observed in RV-B specific antibody levels (C). Differences were significant at the 0.05 level using ANOVA with Post Hoc (Tukey's test).

Figure 6: RV antibody levels in asthma patients with different disease control. Data are presented for RV (A) and RV-A (B), RV-B (C) and RV-C (D) specific peptides. Differences were significant at the 0.05 level using ANOVA with Post Hoc (Tukey's test).

Figure 7: Seasonal variation in RV antibody signal levels. RV (A), RV-A (B) and RV-C (D) antibody levels in healthy children differ between seasons. In asthma patients no fluctuation of RV antibodies is observed (E-H). Differences were significant at the 0.05 level, ANOVA with Post Hoc (Tukey's test).