Enhanced Neutralizing Antibody Responses to Rhinovirus C and Age-Dependent Patterns of Infection

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Running title: Determinants of rhinovirus C illnesses in children

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10.11 Pediatrics: Respiratory Infections

At a Glance

What is the current scientific knowledge on this subject?

There are over 160 rhinovirus types in three species, and a greater understanding of at risk populations and the relative importance of rhinovirus types and species in causing illness would help to direct the development of specific treatments such as vaccines.

What does this study add to the field?

Young children with genetic predisposition are at high risk for rhinovirus-C illnesses, which are closely associated with wheezing. Rhinovirus-C is very immunogenic, and

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identification of types that are both common and virulent should help to facilitate development of a vaccine for this clinically important respiratory pathogen.

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ABSTRACT

Rationale: Rhinovirus C (RV-C) can cause asymptomatic infection and respiratory illnesses ranging from the common cold to severe wheezing.

Objectives: To identify how age and other individual-level factors are associated with susceptibility to RV-C illnesses.

Methods: Longitudinal data from the Childhood Origins of ASThma (COAST) birth cohort study were analyzed to determine relationships between age and RV-C infections. Neutralizing antibodies specific for rhinovirus A (RV-A) and RV-C (3 types each) were determined using a novel polymerase chain reaction-based assay. We pooled data from 14 study cohorts in the United States, Finland, and Australia and used mixed-effects logistic regression to identify factors related to the proportion of RV-C versus RV-A detection.

Measurements and Main Results: In COAST, RV-A and RV-C infections were similarly common in infancy, while RV-C was detected much less often than RV-A during both respiratory illnesses and scheduled surveillance visits ($p<0.001$, chi-square) in older children. The prevalence of neutralizing antibodies to RV-A or RV-C types was low (5%-27%) at age 2 years, but by age 16, RV-C seropositivity was more prevalent (78% vs. 18% for RV-A, $p<0.0001$). In the pooled analysis, the RV-C to RV-A detection ratio during illnesses was significantly related to age ($p<0.0001$), *CDHR3* genotype ($p<0.05$), and wheezing illnesses ($p<0.05$). Furthermore, certain RV types (e.g., C2, C11, A78, A12) were consistently more virulent and prevalent over time.

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Discussion: Knowledge of prevalent RV types, antibody responses, and populations at risk based on age and genetics may guide the development of vaccines or other novel therapies against this important respiratory pathogen.

Key Words: rhinovirus, genetics, wheezing, CDHR3, epidemiology

Abstract: 245 words

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INTRODUCTION

Rhinoviruses (RVs) are common causes of upper respiratory tract illnesses and exacerbations of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease [COPD] (1). In young children, RV infections also cause lower respiratory illnesses that can require hospitalization and even intensive care (2). There are three species of RVs (A, B, and C) based on genetic homology that are further subdivided into more than 160 types (3). Any RV can cause mild or asymptomatic respiratory illness; however, RV-B is less likely to cause severe illnesses or exacerbations of asthma (4, 5). Infections with RV-A and RV-C are common during childhood, but the relative contribution of these viruses to severe respiratory illnesses is controversial. Some studies have shown that RV-C infections are more likely than RV-A infections to cause wheezing illnesses and asthma exacerbations in children (2, 6-8), but other studies have found no species-specific associations (5, 9-11).

Several factors modify susceptibility to RV-C infections. A coding polymorphism (rs6967330) in the gene for CDHR3, which serves as the RV-C receptor, is associated with greater expression of this protein on the surface of cells and increased susceptibility to both RV-C illnesses and childhood asthma (12, 13). In addition, there is evidence that age might also affect the relative frequency of RV-A versus RV-C infections and illnesses. For example, RV-C was detected more often than RV-A in some studies of young children with respiratory illnesses (14-16), while some studies in adults have reported the opposite (17-19). The reasons for these observations are not fully understood but could be related to a greater naturally acquired immunity to RV-C over time. Infections with RV induce type-specific antibody responses that reduce the

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risk of reinfections. Natural infections with RV-A and RV-B induce neutralizing antibody (nAb) responses that protect against reinfection and persist for at least 2-4 years (20-24). Neutralizing antibody responses to RV-C have not previously been analyzed due to the lack of a suitable *in vitro* system to assay infectivity.

The primary goals of this study were to test the hypothesis that increasing age is associated with reduced frequency and severity of RV-C infections and illnesses, and to determine whether age-related reductions in RV-C illness frequency correspond with increased nAb responses to RV-C infections. We also sought to identify other personal factors that are associated with RV species susceptibility, and to describe relationships among RV species, type, and illness. To accomplish these objectives, we analyzed longitudinal sets of nasal and plasma samples from children from birth to age 18-19 years in the Childhood Origins of ASThma (COAST) birth cohort, which included high-risk children born to parents with allergy and/or asthma (25). In addition, we conducted a multicenter study by pooling data from 14 different studies across numerous cities located in the United States, Finland, and Australia. Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Study Subjects and Study Design

COAST birth cohort study. 289 children from the Madison, Wisconsin, area were enrolled in the COAST study at birth, 259 were followed prospectively to age 6 years and 210 to age 18-19 years, and additional children with asthma were enrolled more recently (25, 27). Families were asked to contact the study center each time the child

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had respiratory symptoms to enable collection of a nasal mucus sample for viral diagnostics. Nasal samples were also collected at scheduled study visits (2, 4, 6, 9, 12, 18, and 24 months and then annually), and respiratory illness symptoms were recorded if present. Plasma samples were collected during these annual visits.

Studies included in the pooled analysis. Investigators from 14 different cohort studies that had collected nasal mucus specimens from children during periods of illness and/or health (**Table E1**) contributed data to the pooled analysis. Five of these cohorts are participating in the Environmental Influences on Child Health Outcomes (ECHO) consortium. For each of these cohorts, real-time PCR and partial sequencing was used to identify RV species and type as previously described (28, 29). Each of these studies was approved by the local Human Research Ethics Committees, and participants provided informed consent. In addition to the viral diagnostics, 11 additional variables were included in the analysis. All cohorts had data on age, sex, race, and season of collection; nine cohorts provided data on aeroallergen sensitization (skin test or specific IgE measurement), asthma (parent report of asthma diagnosed by a health care provider), number of older siblings, history of breastfeeding, exposure to daycare, and illness type; and 6 of the 14 cohorts provided genotypes for the *CDHR3* variant rs6967330. These variables were harmonized for the pooled analysis (see online data supplement).

RV Neutralization Assay

RV-A16, -A36, -C2, -C15 and -C41 were clinical isolates cloned in plasmid vectors and produced by reverse genetics methods in WisL cells (human embryonic lung fibroblasts) (30-32); RV-A7 was isolated from nasal secretions and propagated in HeLa cells (31).

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HeLa-E8–adapted variants of RV-C isolates (33), possessing K41 mutation in 3A protein, were used for optimal replication in this cell line.

Neutralizing antibodies specific for RV-A and RV-C types were measured using a novel qPCR-based assay. Briefly, RV isolates were preincubated with serial two-fold dilutions of plasma samples, and this mixture was used to inoculate HeLa-E8 cells (34) that were engineered to express CDHR3 (RV-C receptor). Viral replication (progeny yields at 72 hours post-infection) was measured by qPCR. Neutralizing antibody titers (IC_{50}) were calculated by sigmoidal dose-response nonlinear fit analysis of virus replication curves. For the qualitative assay to identify the presence or absence of nAbs, the same infection procedure was used except that the number of plasma serial dilutions was limited to 3 (2-8-fold) (see online data supplement for additional details).

Statistical Analyses

Mixed-effects logistic regression was used to estimate the odds of infection with RV-C alone to infection with RV-A as a function of age (modeled using a natural cubic spline with 4 degrees of freedom). The model included subject as a random effect and age, race, and cohort as fixed effects.

Mixed-effects logistic regression models were used to assess the effects of other covariates (asthma history, allergy history, daycare exposure, number of older siblings, breastfeeding history, race, season of collection, CDHR3 rs6967330 asthma risk genotype, and illness type [**Table E2**]) on the RV-C to RV-A ratio. Additional details are provided in the online supplement.

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RESULTS

RV-A versus RV-C Infections and Illnesses in the COAST Study

In the COAST birth cohort study, we detected and typed RV clinical isolates in surveillance nasal samples collected during scheduled clinic visits (with or without respiratory symptoms) and in samples obtained during times of acute respiratory illnesses (symptom score of ≥ 5 (35)) during three age intervals: ages 0-5 years, 6-12 years, and 13-18 years. Partial sequence analysis confirmed detection of about 94% and 98% of known RV-A and RV-C types, respectively. While RV-A and RV-C infections and illnesses were similarly common in the first 3 years of life, in older children RV-C was detected significantly less often than RV-A during both respiratory illnesses and well visits ($p < 0.001$, chi-square, **Figure 1)**.

RV Neutralizing Antibody Responses

Both RV-A and RV-C isolates can infect transduced HeLa-E8 cells, which naturally express ICAM-1 and are engineered to express CDHR3 (33). Due to the absence of cytopathic effects (CPE) after infection with “wild-type” RV-C clinical isolates *in vitro*, we developed a novel, qPCR-based RV neutralization assay in HeLa-E8 cells. We then validated the assay by testing pre- and post-infection plasma samples from an RV-A16 experimental inoculation study (36). The nAb titers to RV-A16 determined by the assay correlated well (r_s=0.83, p=0.006) with those obtained by the traditional infectivity assay (endpoint dilution) that is based on CPE in WI-38 cells (**Figure E1**).

We next analyzed nAb responses to RV-A and RV-C types in plasma samples from three age groups (2, 10, and 16 years, same subjects) of COAST study participants

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(*n*=20) by qualitative nAb assay. We selected three clinical isolates representing RV-A and RV-C species to use in the neutralization assay. Two isolates from each species were phylogenetically related (A7 and A36, and C15 and C41), and the remaining ones (A16 and C2) were more distant from the first two isolates (**Figure E2**). The results demonstrated marked species-specific differences in the development of nAb responses (**Figure 2**). While nAbs to these RV-A and RV-C types were uncommon at age 2 years (5-27%), by age 10 and 16 years RV-C seropositivity was 70% and 78% compared to only 25% and 18% for RV-A, respectively ($p<0.0001$). RV-C–specific nAbs were significantly more prevalent compared to nAbs for RV-A in each of the tested age groups (ORs 3.9 [95% CI 1.6 to 9.5] at age 2, 4.0 [95% CI 2.0 to 7.7] at age 10, and 7.1 [95% CI 3.8 to 13.3] at age 16). There was no correlation between the detection frequency of the selected RV-A or RV-C types and the total number of positive nAb responses to them (**Figure E3).**

Neutralizing antibody detected at one timepoint was very likely to persist from one age to the next (**Figure 2**, $p<10^{-7}$, GEE logistic regression model). For RV-A types, positive antibodies persisted from age 2 to age 10 years in 67% (2/3) children and from age 10 to age 16 in 40% (6/15) children. For RV-C types, the corresponding numbers were 94% (15/16) and 90% (38/42) respectively. RV species was not significantly associated with persistence of antibody ($p=0.58$, Wald test for interaction), although this analysis was underpowered due to the low number of positive results for RV-A types.

Multicenter Analysis of RV-A and RV-C Infections

Results of viral diagnostics. The 14 study cohorts collected 17,664 samples of nasal mucus from study participants and detected at least one RV in 10,185 samples (**Table**

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1). Of the 10,185 pooled samples, 6,643 were collected during illnesses and 3,542 were collected during periods of health (e.g., at well-child or routine study visits). Similar numbers of specimens tested positive for RV-A and RV-C. RV-B was detected more often in samples from asymptomatic children compared to samples obtained during illness (857/3,542 [24%] vs. 589/6,643 [9%], two proportion z-test $p < 0.001$).

We detected 178 types, including several provisionally assigned types (designated "pat"), in the pooled analysis. The median detection rate for any given type was 0.45% (range 0.0% to 2.7%). The most frequently detected RV types in illness samples were A78, C02, C11, A12 and A101 in descending order, while 33 of the RV types were detected in <0.1% (6 or fewer) of illness samples (**Table E3**). The rate of detection for the frequently detected types was consistently high over the 12-year sampling period (**Figure E4**). In samples from asymptomatic children, B17, B6, B103, A21, and B91 were detected most frequently in descending order (**Table E3**). We also compared the frequency of types detected during illnesses to that of types detected during healthy periods (**Figure 3**). Twenty-two types (4 A, 18 C) were significantly more likely to be detected during illnesses, while 23 types (4 A, 18 B, 1 C) were more often detected in healthy children.

Analysis of factors related to RV-A and RV-C infections. Consistent with findings in COAST, increasing age was strongly correlated with the ratio of RV-C to RV-A infections in the pooled data (**Figure 4 and Figure E5)**. RV-C infections were more prevalent in young children up until about age 5 years, followed by a steady decline in the ratio such that RV-A infections predominated in adolescents. In order to evaluate other potential predictors of the RV-C to RV-A ratio, we performed logistic regression on

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each of the covariates separately while adjusting for age and participant (some studies included multiple samples per participant). Because there was significant variety among the cohort studies in age and covariates measured, the analyses were performed in discrete age intervals (**Figure 5**). Two covariates were significant predictors of increased RV-C infections compared to RV-A infections. The *CDHR3* rs6967330 asthma risk allele (A) significantly increased the risk for RV-C illness (**Figure 6A**). In addition, the RV-C to RV-A ratio was related to the presence of lower respiratory illness (LRI) with wheezing but not LRI without wheezing (**Figures 6B and 6C**).

Season of collection was also related to the RV-C to RV-A ratio; children were more likely to be infected with RV-C versus RV-A in fall and winter compared to spring for 5-6 of the 12 age windows (**Figure E6**). Other personal factors, including race, aeroallergen sensitization, asthma history, history of breastfeeding, exposure to daycare, and number of older siblings, were not consistently related to the RV-C to RV-A ratio (**Figure E7** and data not shown).

DISCUSSION

RV infections are important causes of LRI and wheezing during infancy and in children and adults with asthma and other chronic respiratory diseases, and understanding the contributions of different viruses is needed to guide efforts to develop specific treatments. While there is consensus that RV-B is less likely to cause severe respiratory illnesses, the relative roles of RV-A and RV-C have been uncertain. In the COAST birth cohort study, RV-A and RV-C infections and illnesses were both common in the first 3 years of life, but RV-C was less often detected than RV-A in older children during both respiratory illnesses and scheduled surveillance visits. Analysis of nAb responses to

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selected RV-A and RV-C types in the COAST cohort demonstrated progressively greater frequency of protective responses to RV-C over time, suggesting that species-specific differences in the development of nAb responses may contribute to the frequency of illnesses with RV-C versus RV-A. Pooling data from 14 cohorts confirmed that RV-C illnesses are more prevalent in preschoolers. These data also provide evidence that RV-C (compared to RV-A) is specifically associated with wheezing LRI, and confirm that a single nucleotide polymorphism (rs6967330) in the *CDHR3* gene promotes RV-C infections and illnesses throughout childhood.

Previous studies have demonstrated that RV infection rates peak during infancy and gradually decline with age (37). In parallel, nAb responses to RV-A and RV-B types generally increase during childhood and early adult years and plateau with nAb to approximately 40% of RV types after 35-40 years. RV infections continue at a lower rate throughout adult life, suggesting that protective immunity is either not fully developed to each virus type (low nAb titers) or is not maintained (38). Our new data provide definitive evidence that, in comparison to RV-A, young children have similar rates of RV-C infections but proportionally higher rates of RV-C illnesses, and that increasing age is associated with steady reductions in RV-C detection rates during sickness or health. While some previous studies have detected this trend, this pooled data analysis estimated the odds of detecting RV-C compared to RV-A over a broad age range and in a large and diverse study population with over 10,000 nasal samples typed.

The significant age-related decline in the RV-C to RV-A ratio during RV infections was associated with marked increases in titers of nAb specific for RV-C types. Notably, this was true for RV-C types that were commonly detected (RV-C2 and RV-C15), and also

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for RV-C types that were less commonly detected (RV-C41). While this finding needs to be confirmed by testing for nAb responses to other RV-C types, this raises the possibility that RV-C infections induce antibody responses that can cross-neutralize other RV-C types.

Prior studies of antibody responses to RV-C measured IgG antibody binding to synthetic viral peptides by ELISA. Interestingly, antibody responses specific for RV-A and RV-C peptides were found to be highly cross-reactive (39-41). In addition, serum IgG1 antibody titers to RV-C were significantly lower than those to RV-A and RV-B suggesting that humoral immune response to RV-C may be muted (40, 41). In contrast, a recent study utilizing an RV peptide array that included representative capsid protein sequences from all three species reported higher antibody levels to RV-A and RV-C compared to RV-B in both healthy and asthmatic children (42). The authors also reported that asthma was associated with higher antibody levels that did not seem to confer enhanced protection. Antibody responses to viral capsid proteins (VP1) of RV-A and RV-C were also associated with severity of symptoms of previous respiratory illnesses (39, 43).

Previous studies of antibody responses to RV-C did not include measurement of nAbs (40-42). Infectivity assays in HeLa-H1 cells or lung fibroblasts, which were used for RV-A and RV-B neutralization (44), are not suitable for RV-C because these cells lack CDHR3 expression required for viral binding and replication (34, 45, 46). We developed a novel qPCR based neutralization assay for both RV-A and RV-C to analyze nAbs in the absence of visible cytopathic effects; the results of the new assay correlated closely

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with those of a standard CPE-based assay (36). Our findings demonstrate that natural humoral immunity to RV-C develops at an accelerated rate compared to RV-A.

These findings suggest that RV-C types are more immunogenic compared to RV-A, or are more likely to induce cross-neutralizing antibody. Neutralizing antigenic sites in RV-A and RV-B are located at the highest points of the capsid surface in hypervariable regions of VP1, VP2 and VP3 (47-50). Little is known about neutralizing sites in RV-C, which has unique structural features and receptor interactions (51, 52). The first extracellular domain (EC1) of CDHR3 binds to residues located in a shallow groove formed primarily by VP2 and VP3, with some contribution from VP1 (52). The accessibility of the binding site suggests that certain epitopes in this region could elicit nAbs that block receptor binding. In addition, our data confirm that many RV-C types are more virulent compared to other RV types, and previous studies have demonstrated that viremia (detection of viral RNA in serum specimens) is much more common with RV-C infections compared to infections with other RV species (53, 54). Virulence of RV-C types could also be enhanced by a muted T helper-1 response to RV-C versus RV-A, which could impair antiviral responses (55). Because symptom severity and peak viral titer during acute infections are positively related to induction of RV-specific nAb responses (26, 56), the increased virulence of RV-C could contribute to the greater prevalence and perhaps duration of nAb responses.

The pooled analysis provided descriptive information on over 10,000 samples that tested positive for RV. Several RV types (RV-C2, RV-A78, RV-C11, and RV-A12) were frequently detected and found at high rates throughout most of the years during the two decade period of analysis. Similarly, a recent meta-analysis found that RV-A12, RV-

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A78, RV-C15, and RV-C2 were reported in more published studies than any other RV-A or RV-C types (57). The consistency of these findings suggests that there are certain RV types such as RV-C2 and RV-A78 that are either more virulent or more readily transmitted. Notably, the affinity of CDHR3 binding for four RV-C types was recently estimated by measuring how multiple single mutations in a soluble EC1 domain affected its ability to inhibit viral replication (52). The results indicate that CDHR3 binding is tightest for C02, and progressively less for C15, C41, and C45. This estimate of binding affinity corresponded with the frequency with which these viruses were detected in our pooled analysis of illness samples, suggesting that the strength of viral binding to CDHR3 could contribute to virulence and/or transmission. Direct measurements of binding of RV-C types to CDHR3 are needed to test this hypothesis.

This study has several strengths and some limitations to consider when interpreting the results. Longitudinal sampling in COAST from birth to adolescence allowed assessments of relative contributions of RV types across childhood within the same children. The pooled sample analysis provided a large sample and included studies that were diverse in terms of geography, climates, years of sampling, ethnicity, and home and community environments. Several studies collected samples from children with and without illness, which enabled analysis across illness and health. Viral typing was performed by partial sequencing using the same methods. Limitations included the need to harmonize variables such as illness type that were defined differently by each study cohort, which could have led to reduced precision. Several of the studies collected samples and information over a relatively short age range, which necessitated analyzing risk factors for RV-C and RV-A infections in age windows so that age would not be

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confounded with study site. Finally, we measured neutralizing antibody responses to 6 RV types, and data for additional RV types would be helpful to confirm our findings and to identify viral characteristics besides species that can influence the development of neutralizing antibody responses.

In conclusion, this study and others identify young children and those who are genetically predisposed as populations at high-risk for RV-C wheezing illnesses, who could potentially benefit from preventive approaches such as vaccination. Obstacles to the development of RV-specific vaccines have included the large number of RV types and a paucity of information about RV-C epidemiology and protective serologic responses. To overcome these limitations, this study has identified key subsets of RV-C types that are more prevalent and more virulent, and have demonstrated that RV-C infections elicit potent and durable nAb responses. Therefore, building on these data it may now be more feasible to develop an RV-C vaccine for use early in life to prevent rhinovirus-provoked wheezing illnesses and reduce a major health burden in high-risk children.

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FIGURE LEGENDS

Figure 1. Relationship of age to frequency of rhinovirus (RV)-C and RV-A detection during illnesses in the COAST birth cohort. RV types and species (A, B, and C) were determined in nasal samples by partial sequencing. Detection rates (%) of RV-A and RV-C at different ages are shown as bar plots. Odds ratios (ORs) refer to odds of RV-C relative to RV-A at older ages compared to younger ages. Groups were compared using Fisher's exact test. P values of < 0.05 were considered to indicate statistical significance. Illnesses: OR = 0.31 (95% CI 0.21-0.45), $p < 0.0001$; scheduled visits: OR = 0.31 (0.14-0.64), $p = 0.0006$. *N* represents the number of samples analyzed for each age category.

Figure 2. Neutralizing antibody (nAb) responses to selected RV-A and RV-C types in the COAST study children. Heatmaps show the presence of nAbs to indicated RV-A (blue) and RV-C (red) types in plasma (ages 2, 10, and 16 years, each row represents serial sampling from the same subjects) from COAST study participants (n = 20). Odds ratios (ORs) refer to odds of finding nAbs to RV-C relative to RV-A at different ages. Age 2: OR = 3.9 (95% CI 1.6 to 9.5); age 10: OR = 4.0 (95% CI 2.0 to 7.7); age 16: OR = 7.1 (95% CI 3.8 to 13.3). The differences in nAb responses to RV-A versus RV-C were highly significant at all ages tested. ($p < 0.0001$, GEE logistic regression).

Figure 3. Frequency of detection for each RV type and ratio of detection proportions for sick versus well visits in the pooled dataset. The species is color coded, and individual types are numbered within each species. The size of the plotting characters is proportional to the negative logarithm of the p value, and plotting characters in boxes

AJRCCM Articles in Press. Published December 24, 2020 as 10.1164/rccm.202010-3753OC

are significant at the 5% level (unadjusted for multiple comparisons). Error bars represent 95% confidence intervals for the ratio of detection proportions.

Figure 4. Ratio of RV-C to RV-A in illness samples from the pooled dataset with respect to age. Individual points represent the RV-C to RV-A detection ratio for each year of age. The line and shaded area represent the logistic regression model fitted to the sample-level data and the associated 95% confidence intervals, respectively.

Figure 5. Age distribution of 14 study cohorts included in pooled sample analysis. Symbols represent samples included in this analysis by cohort and the age of sample acquisition. Age windows were used in the multivariable analyses, and start and end points were assigned based on ages at which a study or studies started contributing samples or stopped contributing samples to the overall pool.

Figure 6. Analysis of covariates related to the RV-C to RV-A ratio. Odds ratios (logistic regression) were calculated to compare the RV-C/RV-A ratio to the *CDHR3* asthma risk allele (panel A, rs6967330$_{G\rightarrow A}$, 1 or 2 risk alleles compared to 0) and to illness type (B, LRI vs. URI; C, wheezing LRI vs. URI). Odds ratios for each age interval were calculated, along with an overall odds ratio including all ages. Whisker bars represent 95% confidence intervals. Abbreviation: wLRI, LRI with wheezing.

AJRCCM Articles in Press. Published December 24, 2020 as 10.1164/rccm.202010-3753OC

Table 1. Viruses detected in rhinovirus (RV)-positive samples.

Virus	Sample Type		
	All	**Sick**	**Well**
Solo RV-A	4379	2890	1489
Solo RV-B	1197	453	744
Solo RV-C	4235	3067	1168
Mixed RV	374	233	141
Total	**10185**	**6643**	**3542**

AJRCCM Articles in Press. Published December 24, 2020 as 10.1164/rccm.202010-3753OC

Figure 1

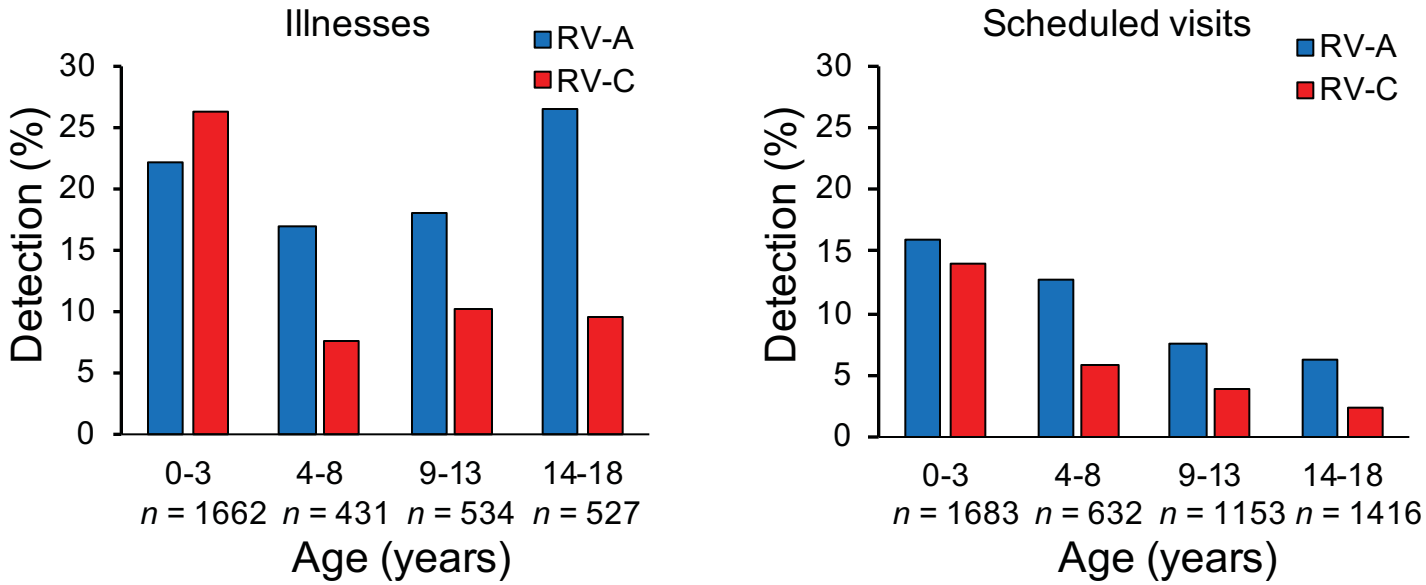

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Figure 2

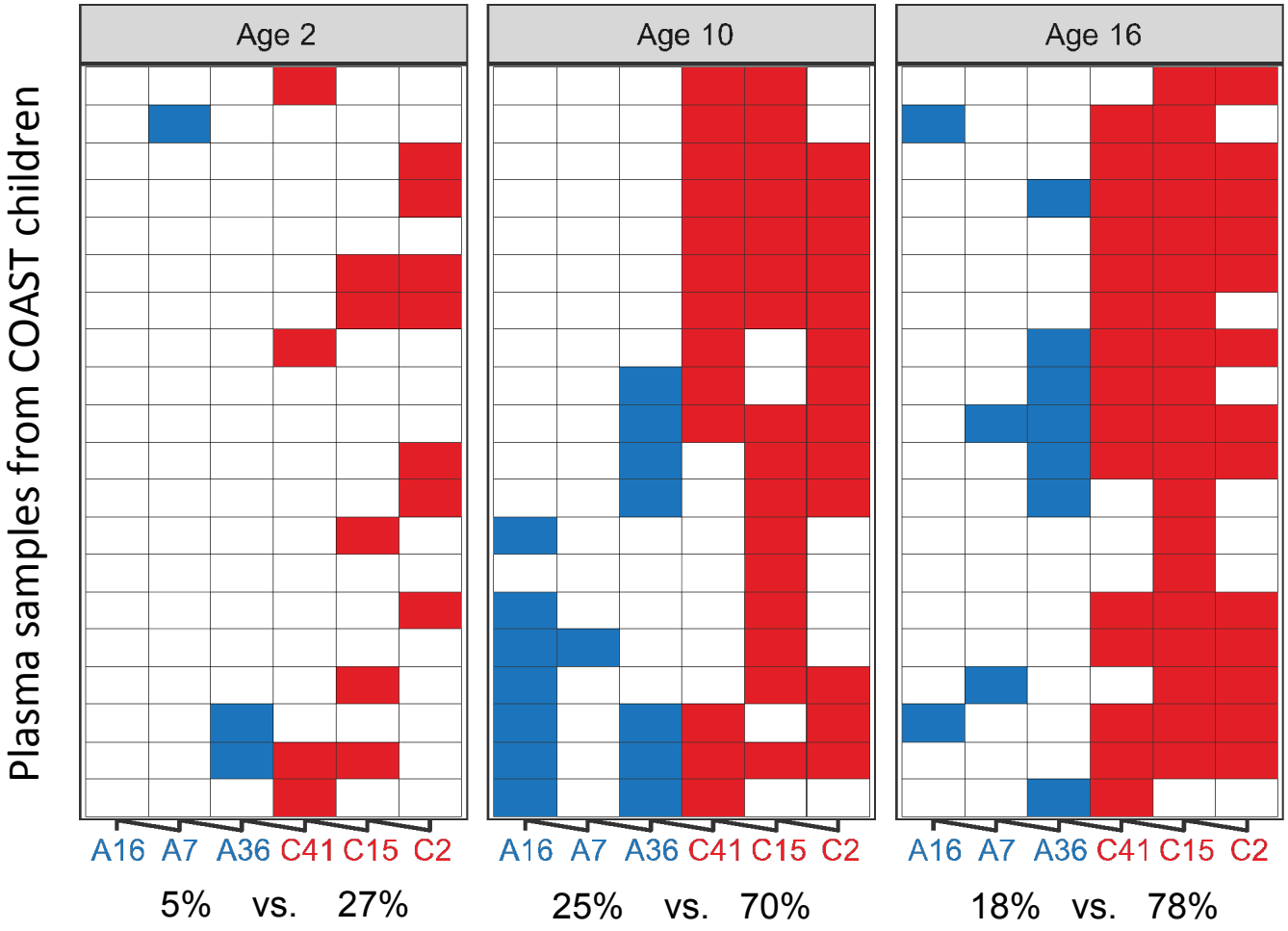

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Figure 3

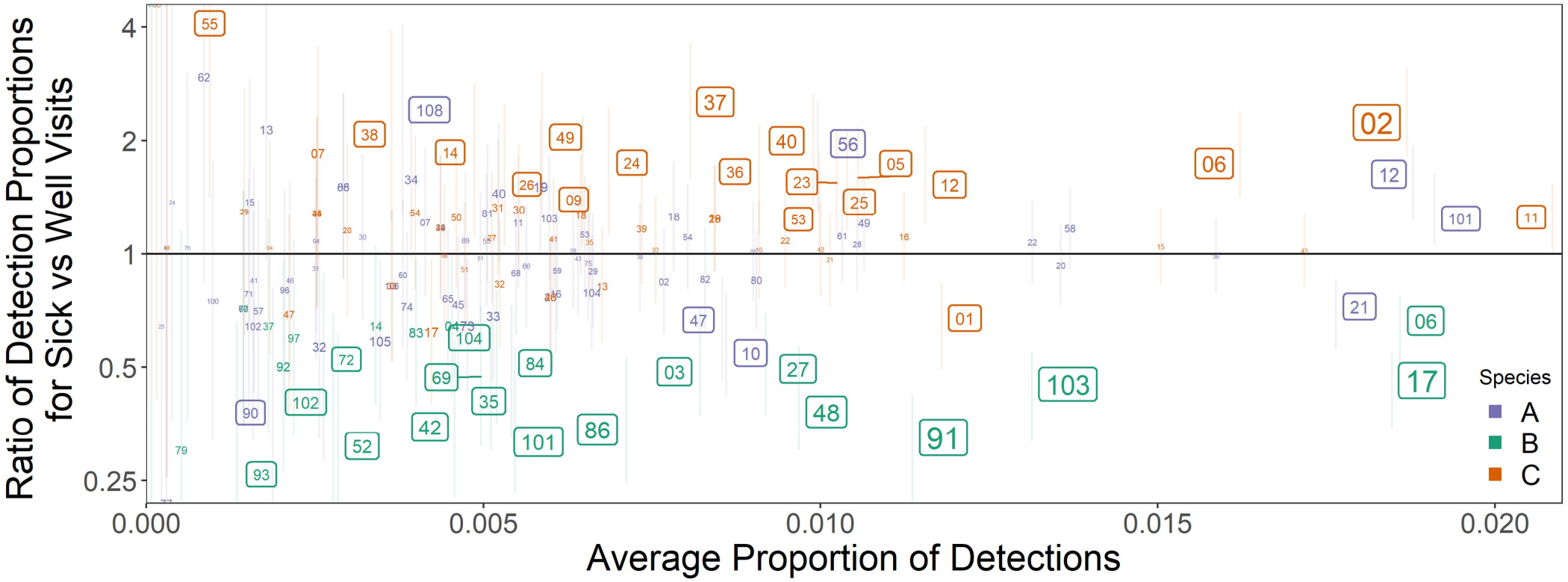

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Figure 4

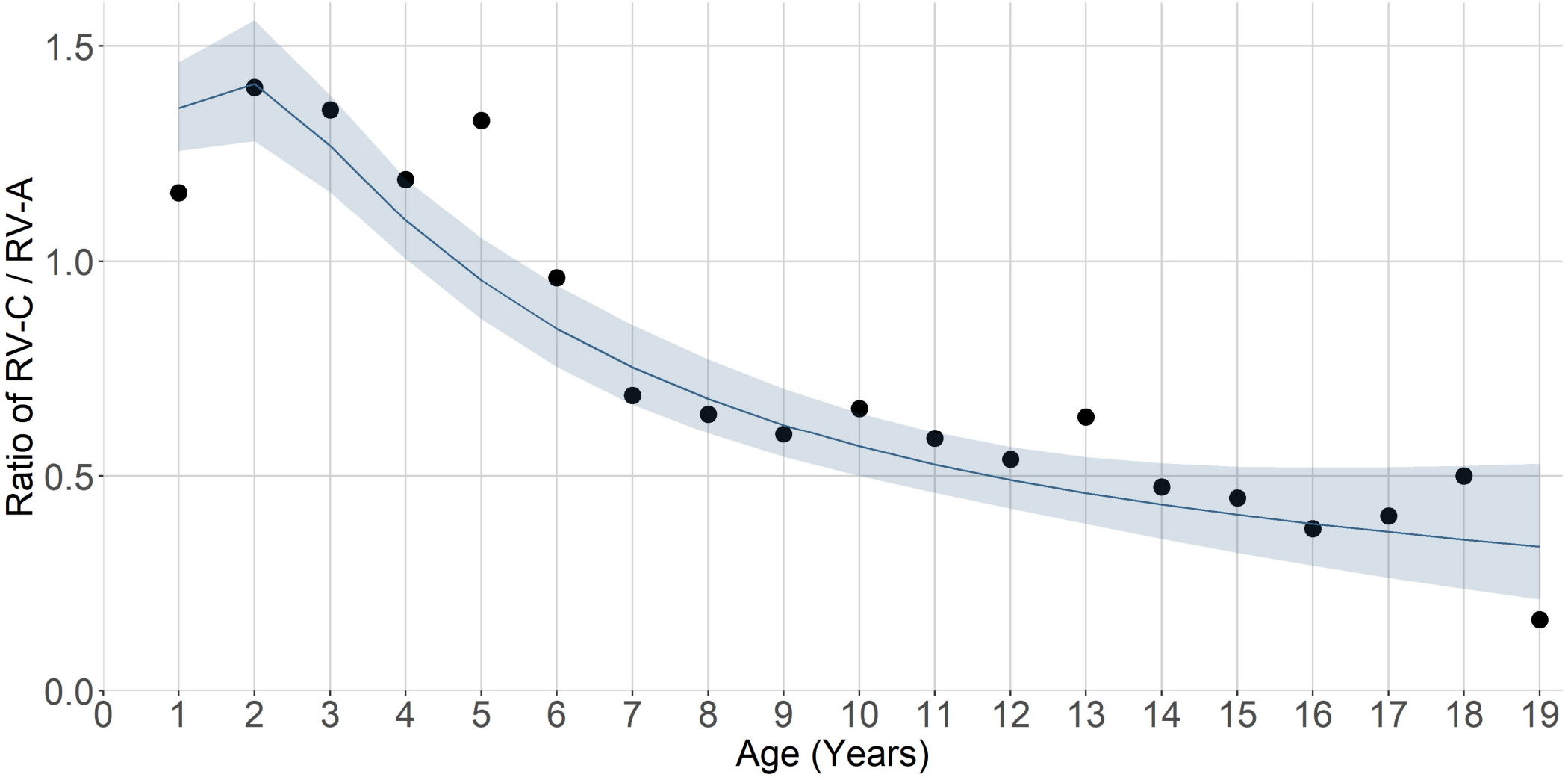

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Figure 5

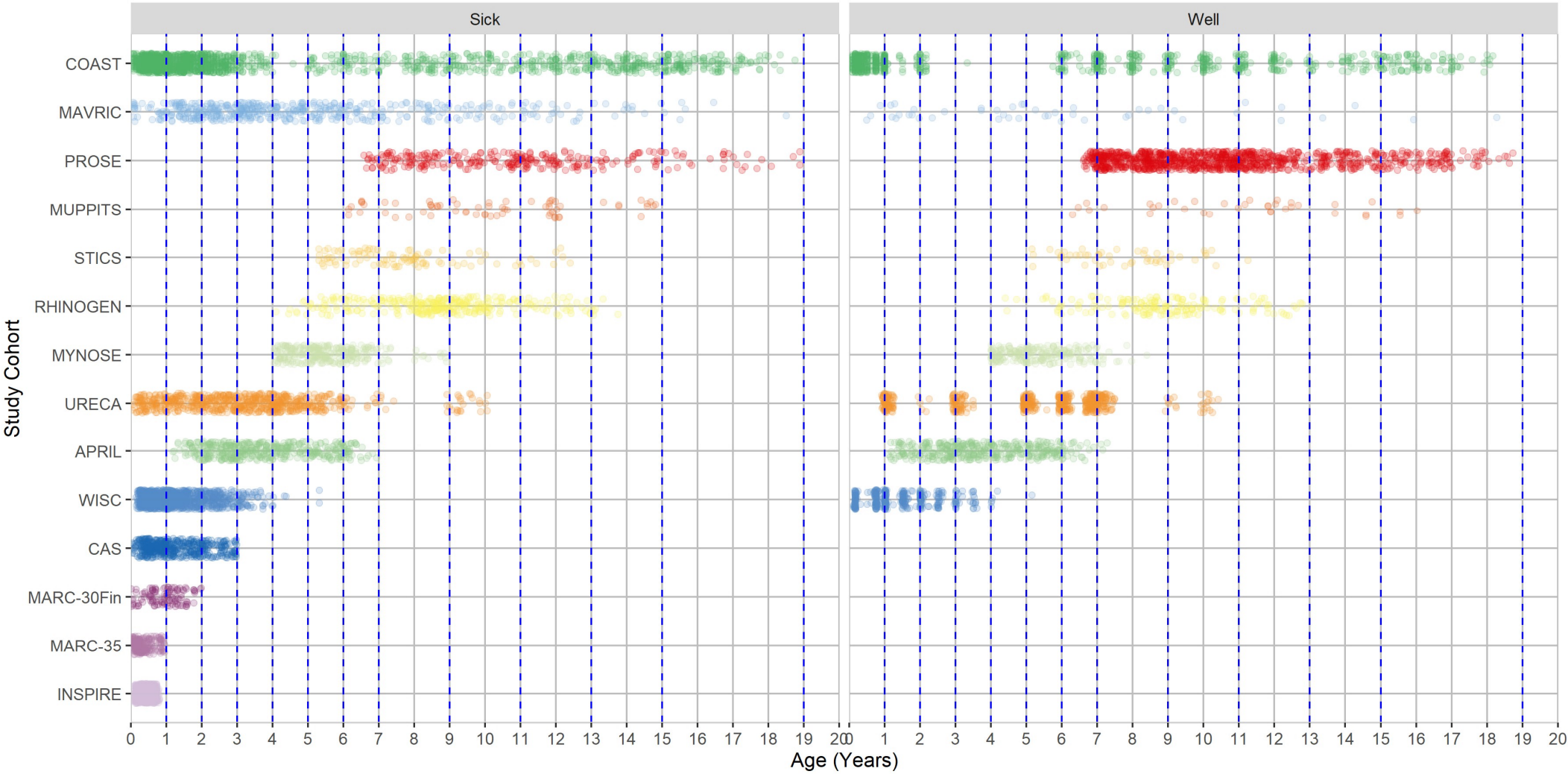

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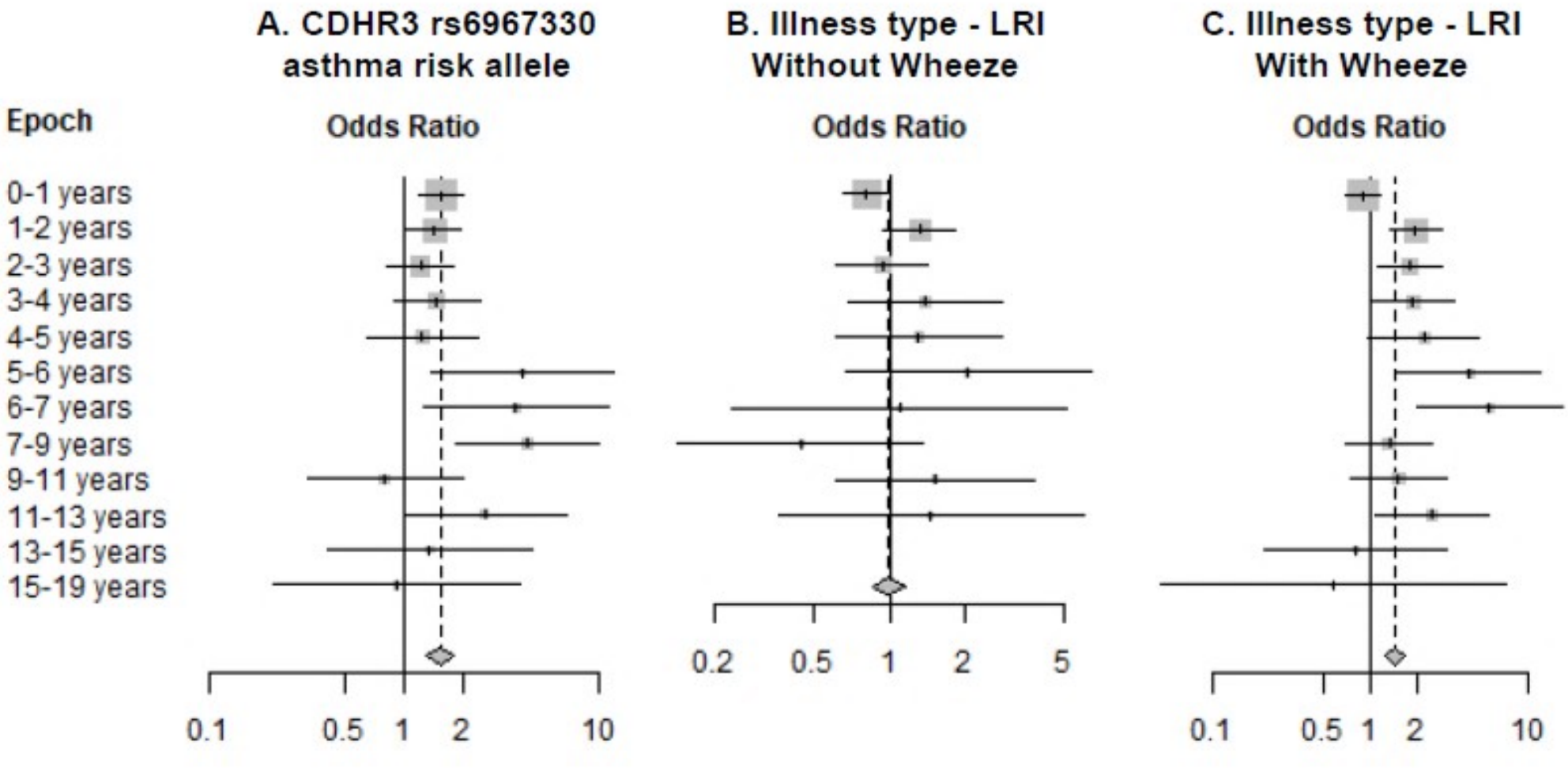
Figure 6



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Online Data Supplement

Enhanced Neutralizing Antibody Responses to Rhinovirus C and Age-Dependent Patterns of Infection

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*JEG and YAB contributed equally to the project.

†See Acknowledgments for full listing of collaborators.

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METHODS

Data harmonization

In addition to age, 9 variables were collected from some or all of the 14 participating cohorts: aeroallergen sensitization (skin test or allergen-specific IgE), asthma history (parental report of asthma diagnosed by a health care provider), season of collection, race, number of older siblings, history of breastfeeding, CDHR3 asthma risk genotype, exposure to daycare, and illness type.

For allergy, studies collected information differently on the number of positive tests to aeroallergens. Some studies collected this as an all-or-nothing binary categorical data, whereas others recorded a numerical value for the number of aeroallergens. To harmonize these data, those studies that collected more specific information on the number of sensitization to aeroallergens were converted to a binary categorical variable (no sensitization to aeroallergens vs sensitization to at least one aeroallergen). In a similar manner, the number of older siblings was harmonized to a binary categorical variable (no older siblings vs. one or more older siblings). The CDHR3 asthma risk genotype contains three categories: AA–homozygous for mutant (risk) allele, AG–heterozygous, and GG–homozygous for wild-type allele. Because samples belonging to the homozygous mutant allele were scarce, this variable was also converted to a binary classification (homozygous wild-type vs. one or more mutant allele). Season of collection was coded as a four-category variable for each of the four seasons. The sample was deemed to be collected in “Spring” if the collection date was in the months of March, April, or May. The rest of the seasons were also assigned as 3-month blocks. However, for those samples that were part of studies in Australia, the months were

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offset by 6 months such that “Spring” was assigned months of September, October, and November. Race was defined differently by almost every study cohort. As such, we decided on a simple classification for consistency across all 14 studies: a four-category variable with White, Black, Other, and Multirace. Finally, illness type consisted of three categories: upper respiratory illness (URI), lower respiratory illness (LRI), and wheezing LRI. This classification was determined based on symptom scores and the presence of wheezing prior to sample collection for those studies that had this information available.

Measurement of RV-C nAb

We selected RV clinical isolates for use in the nAb assays from those that were available (cloned or cultured from clinical isolates) at the beginning of this study. The RV-A and RV-C viruses selected included two more frequently detected types and one less frequently detected type within each species, as determined from our analysis of COAST samples for each RV species. In addition, we selected two RV types that were closely related based on capsid amino acid sequence (Figure E2) and one type was more distant. We also selected RV-C types possessing an adaptive mutation in the 3A protein for optimal replication in Hela-E8 cells.

Neutralizing antibodies (nAbs) specific for the six RV types listed above were detected using a novel quantitative PCR-based assay. HeLa-E8 cells (35) were grown in suspension in 125 mL Erlenmeyer flasks (37°C, 190 rpm) and passaged twice a week. The cells were seeded in 24-well plates at a density of 1.6×10^5 cells per well and infected on the next day with RV isolates preincubated with plasma samples. Before infection, serial two-fold dilutions (2-fold to 1024-fold) of heat-inactivated plasma in 0.5% gelatin (25 µL) or negative control (0.5% gelatin alone) were made and then mixed with

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1×10^5 plaque-forming unit equivalents (PFUe) of each RV isolate (25 µL). The serum-antibody mixture (50 µL) was then allowed to incubate at room temperature for 30 minutes before being moved to 34°C for additional 30 minutes. Prior to infection, cells were washed with phosphate buffered saline (PBS), and 150 µL 0.5% gelatin was added to each well. The virus was allowed to bind and enter into cells for 2 hours before adding 0.5 mL of growth medium to each well. After 72 hours of incubation at 34°C, growth medium was removed, and cells were lysed in 350 µL of the RLT buffer. Total RNA was extracted with RNeasy mini kit (Qiagen) and stored at -80^0C before RT-qPCR. TaqMan Reverse Transcription Reagents and Power SYBR Green PCR master mix (Life Technologies) were used for reverse transcription and quantification of cell-associated viral RNA with pan-RV primers and a standard curve as described (31). nAb titers (EC_{50}) were calculated by sigmoidal dose-response nonlinear fit analysis of virus replication curves. For the qualitative assay to identify just the presence or absence of nAbs, the same infection procedure was used as described above, but the total number of plasma sample serial dilutions was limited to three (2-fold to 8-fold) and a one-log inhibition of viral titer by plasma compared to negative control was used as a threshold for positive nAb response.

Quantitation of RV replication

TaqMan Reverse Transcription Reagents (Life Technologies) were used for reverse transcription of viral RNA (3.85 µL) with virus-specific reverse primer (5'-AAACACGGACACCCAAAGTAGT-3') per manufacturer's recommendation. qPCR was performed using Power SYBR green PCR master mix (Applied Biosystems) with pan-RV forward (5'-CCTCCGGCCCTGAAT-3') and reverse (5'-GGTCCCATCCCGCAATT-

AJRCCM Articles in Press. Published December 24, 2020 as 10.1164/rccm.202010-3753OC

3') primers and run on 7300 Real-time PCR system (Applied Biosystems) as described (31). RV concentrations were determined by RT-qPCR with a standard curve obtained from serial 10-fold dilutions of RV-A16 with known infectivity titer and expressed as plaque-forming unit equivalents (PFUe).

Statistical analysis

Subsets of data based on the presence or absence of respiratory symptoms were created from the pooled dataset. In the analyses comparing infection rates of RV-C and RV-A types, samples with more than one RV species detected were excluded. Samples in which an RV species was detected along with a non-RV virus were excluded from the pooled dataset.

Mixed-effects logistic regression models were used to assess the effects of other covariates (asthma history, allergy history, daycare exposure, number of older siblings, breastfeeding history, race, season of collection, CDHR3 rs6967330 asthma risk genotype, and illness type [**Table E2**]) on the RV-C to RV-A ratio. Each model included subject as a random effect and age (modeled using a natural cubic spline with 2 degrees of freedom) as a fixed effect. For each covariate, a separate model was fitted for each of the following age windows: 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-9, 9-11, 11-13, 13-15, 15-19. Within each age window, data are available for a common set of cohorts. Age breaks correspond to ages at which cohorts started or stopped contributing data.

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Table E1. Participating studies

Protocol	PI	Institution	Study Design and Sample	Subjects* (n)	Samples* (n)
APRIL (Treatment of Preschool Children With Upper Respiratory Tract Illnesses Using Azithromycin)	Daniel Jackson	UW-Madison	Interventional study of preschool children with a history of recurrent severe lower respiratory tract illnesses to determine whether early treatment with azithromycin (vs. placebo) treatment can prevent wheezing illnesses.	451	829
CAS (Childhood Asthma Study)	Patrick Holt and Peter Sly	University of Western Australia and University of Queensland	This observational birth cohort study consists of children at high risk for allergy and asthma (at least 1 parent with a doctor-diagnosed history of asthma, hay fever or eczema) who were recruited prenatally 1996-1998 and followed for development of respiratory illnesses, allergic diseases and asthma.	170	455
COAST (Childhood Origins of Asthma)	Daniel Jackson and Robert F. Lemanske, Jr.	UW-Madison	Birth cohort study of children born to families with at least one parent with allergic diseases or asthma	277	2300
INSPIRE (Infant Susceptibility to Pulmonary Infections and Asthma Following RSV Exposure)	Tina Hartert	Vanderbilt University	Prospective observational birth cohort study to determine the impact of early-life environmental factors on children's development and respiratory health.	536	693
MARC-30 Finland (the 30th Multicenter Airway Research Collaboration in Finland cohort)	Tuomas Jartti	University of Turku, Finland	Observational cohort study to identify early immunological events in children susceptible to RV-induced early wheezing and the efficacy of intervention with systemic corticosteroid to modify these events.	110	110
MARC-35 (the 35th Multicenter Airway	Carlos Camargo	Massachusetts General Hospital,	This 17-center prospective cohort study of infants with severe bronchiolitis addresses the role of different viruses (e.g., RV, RSV [and their species and genotypes]), clinical characteristics (e.g.,	204	204

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Protocol	PI	Institution	Study Design and Sample	Subjects* (n)	Samples* (n)
Research Collaboration cohort)		Harvard Medical School	severity of illness), immunity-related factors (e.g., IgE, vitamin D), and multiple omics (e.g., genome, epigenome, transcriptome, metabolome, microbiome) in the development of recurrent wheeze and childhood asthma (and its phenotypes).		
MAVRIC (Mechanisms of Acute Viral Respiratory Infection in Children)	Ingrid Laing and Peter Le Souëf	Telethon Kids Institute, The University of Western Australia	Prospective observational cohort study of children who presented to the children's hospital emergency department with lower respiratory tract illnesses.	526	526
MUPPITS-1 (Mechanisms Underlying Asthma Exacerbations Prevented and Persistent With Immune-Based Therapy)	Daniel Jackson	UW-Madison	Prospective, longitudinal, nested case-control study designed to identify changes in gene transcription predictive of and associated with asthma exacerbations in children ages 6 to 17 years with difficult-to-control, exacerbation-prone asthma.	70	89
My NOSE (Sinusitis in children and the nasopharyngeal microbiome)	Ellen Wald	UW-Madison	Observational cohort study to investigate the relationship between viral infections of the upper respiratory tract, perturbations of the nasopharyngeal microbiome in children, and the risk of acute bacterial sinusitis over a one year period.	509	509
PROSE (Preventative Omalizumab or Step-up therapy for Severe Fall Exacerbations)	William Busse	UW-Madison	Randomized clinical trial comparing three treatments — omalizumab, corticosteroid therapy boost, and placebo — in reducing fall exacerbations in inner-city children and adolescents with allergic persistent asthma	444	1295
RhinoGen (Genetic Influences on Rhinovirus (RV)-Influenced Asthma)	James Gern	UW-Madison	Prospective cohort study including children ages 4-12 with or without asthma	283	477
STICS (Step-up Yellow Zone Inhaled	Daniel Jackson	UW-Madison	Double-blind, parallel-group interventional study to determine whether, in children 5-11 years receiving low-dose inhaled corticosteroids (ICS), quintupling	108	146

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Protocol	PI	Institution	Study Design and Sample	Subjects* (n)	Samples* (n)
Corticosteroids to Prevent Exacerbations)			the dose of inhaled corticosteroids at the onset of symptoms previously associated with upper respiratory illnesses and subsequent asthma exacerbations reduces the rate of severe asthma exacerbations treated with oral corticosteroids.		
URECA (Urban Environmental factors and Childhood Asthma)	James Gern	UW-Madison, Boston University, Columbia University, Johns Hopkins University, Washington University	Birth cohort study of urban children born to families in neighborhoods with high rates of poverty and with at least one parent with allergic diseases or asthma	442	1365
WISC (Wisconsin Infant Study Cohort)	James Gern and Christine Seroogy	UW-Madison and Marshfield Clinic Research Foundation	Prospective birth cohort study of two groups of children: those born to farming families, and those from small towns or rural areas in Wisconsin without farm exposures.	214	1331
Total samples and subjects				4344	10329

*Numbers of subjects and samples represent those with at least one detectable rhinovirus and included in the pooled analysis.

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Table E2. Illness type definitions used by contributing studies

Study Name	Illness Type Definition
APRIL	N/A
CAS	Samples taken at time of acute respiratory illness. Parent asked the question: Does your child have a wheezy/rattly chest? - Yes → LRI, No → URI If Yes, then was it wheeze or rattle? - Wheeze → wheezing LRI, rattle → LRI
COAST	Sick visit score card available that asked yes/no questions for fever, cough (mild, moderate, or severe), runny nose (mild or severe), hoarse, wheeze, cyanosis, retractions, tachypnea. If wheezing and/or cyanosis, retractions, or tachypnea present → wheezing LRI If severe cough without wheezing LRI indicators → LRI If mild cough or runny nose without any LRI or wheezing LRI indicators → URI
INSPIRE	Categorization of LRI and URI was based on respiratory severity score which captures wheezing, physician diagnosis and/or chart review
MARC-30 Finland	wLRI was defined according to NAEPP criteria of wheezing “high-pitched whistling sounds when breathing out”
MARC-35	All participants (infants aged <12 months) were hospitalized for bronchiolitis and so all were classified as having a LRI. Information on the presence or absence of wheezing was collected during the acute illnesses.
MAVRIC	This was based primarily on the ED physicians discharge diagnosis. The categories available were acute asthma; wheeze, no specific trigger; virus-induced wheeze; allergen-induced wheeze; exercise-induced wheeze; bronchiolitis; or lower respiratory tract infection. This variable was combined with data collected (when available) on the severity of wheezing that is used as part of the exacerbation severity scores. In children over 2 years of age, the wheeze categories are: normal breathing or end-expiratory wheezing; expiratory wheezing; inspiratory and expiratory wheezing, diminished breath sounds or both. In children under 2 years of age the categories are: none and well; end-expiratory only; entire expiration & inspiration with stethoscope only; loud wheezing audible without stethoscope (or silent chest in the presence of tachypnoea).

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Study Name	Illness Type Definition
	In addition, data from the following was also used. A positive response by the parent completing the respiratory history questionnaire to the question "This illness – What symptoms have there been and how long have they been/were they present?" Answered: Wheeze. If data on the two wheeze variables were missing, and the ED physician's diagnosis was "acute asthma", this was coded as a LRI with wheeze as it is very unlikely that children presenting to the Perth Children's hospital would be given this diagnosis if wheeze was not present.
MUPPITS	Not applicable.
MYNOSE	URI defined as at least 48 hours of acute respiratory symptoms including nasal congestion, nasal discharge or cough.
PROSE	4 level wheeze and cold symptom scores available for each sample (none, mild, moderate, severe). - If wheeze score was moderate to severe → wheezing LRI - If wheeze was none or mild and cough was severe → LRI - If cough was mild or moderate and wheeze was none or mild → URI
RHINOGEN	4 level asthma and cold symptom scores available for each sample during collection period (none, mild, moderate, severe) - At least mild asthma symptoms (occasional cough or wheeze, does not affect daily activities) → wheezing LRI - No asthma symptoms with severe cold symptoms (cannot breathe through nose and not able to sleep well) → LRI - All other respiratory illnesses → URI
STICS	N/A
URECA	Symptom score of 0 to 20 - Score of 5, 6, 7, 8, 9 → URI - Score of 10 or greater → LRI - Score of 10 or greater with wheeze → wheezing LRI
WISC	4 level daily cold, cough, wheeze symptom scores available (none, mild, moderate, severe). - At least mild wheezing symptoms → wheezing LRI - Severe cough without wheezing LRI indicators → LRI - All other illnesses with at least mild cough or cold symptoms → URI

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Table E3. Detection of RV types in samples obtained from ill and healthy children.*

RV Type	Number of Samples	
	Illness Samples N = 6643	Well Samples N = 3542
A78	194	58
C02	191	30
C11	163	65
A12	158	45
A101	152	56
C06	151	34
C43	121	58
C15	110	53
A36	109	54
A58	101	47
C12	101	29
C40	98	22
A56	94	22
C25	94	24
B06	91	86
C05	92	24
C23	91	28
A21	90	76
A22	89	44
A20	86	52
C16	84	38
C53	84	27
C37	83	12
C36	81	21
A49	80	33
C42	75	35
Cpat19	74	40
A61	72	32
A28	71	34
C21	71	39
A01	69	61
C28	69	27
C22	68	32
C10	66	31
C19	65	24
B17	63	103
C01	63	59
C24	62	19
A88	59	31
A18	58	23
C09	57	15
C49	57	13
A54	56	26

RV Type	Number of Samples	
	Illness Samples N = 6643	Well Samples N = 3542
A80	55	35
C39	55	29
C03	53	28
A82	52	35
C35	51	23
A38	49	26
C18	49	20
A19	48	14
C26	47	13
A40	46	15
A53	46	20
A02	45	31
A103	45	18
C14	45	11
C31	45	15
A75	43	27
C30	42	16
C41	42	20
A09	41	21
A59	41	22
A11	40	16
A16	40	24
A29	40	25
A43	40	25
A47	40	45
A81	40	14
B103	39	78
B27	39	49
A104	38	29
C13	38	27
A10	37	41
A108	37	6
A66	37	21
C27	37	17
C38	37	6
C50	37	14
C44	36	17
A55	35	16
A23	34	25
A51	34	17
C46	34	26
A34	33	9
A68	33	22

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Table E3 (cont.)

RV Type	Number of Samples	
	Illness Samples N = 6643	Well Samples N = 3542
A89	33	15
C54	33	13
A07	32	12
A39	32	13
Cpat22	32	9
B03	31	45
B91	31	75
B48	30	56
C32	30	22
C08	29	16
C51	29	20
B69	28	26
A33	26	24
Cpat16	26	9
A08	25	7
A44	24	13
A65	24	18
A85	24	8
C20	24	9
A30	23	10
A45	23	20
A60	23	14
A73	23	23
B04	23	20
C07	23	5
C33	23	15
C45	23	7
C17	22	19
B35	21	30
B84	21	30
A106	20	15
B104	20	27
B83	20	21
B86	20	41
C34	20	8
A74	19	16
A94	19	9
A13	17	3
Cpat21	17	19
A31	16	10
B14	16	15
B42	15	27
A105	14	16

RV Type	Number of Samples	
	Illness Samples N = 6643	Well Samples N = 3542
B101	14	32
Cpat28	14	3
A15	13	4
A46	12	8
B72	12	13
C04	12	6
C55	12	1
A71	11	7
A96	11	8
B97	11	11
C29	11	4
Cpat24	11	10
A32	10	12
A41	10	7
A57	10	8
A62	10	1
B70	10	7
C47	10	11
A98	9	6
AW48*	9	7
A67	8	6
B102	8	17
B37	8	8
B92	8	10
A102	7	7
B52	7	16
A76	6	3
A90	6	10
A100	5	4
AW22*	4	1
A24	3	2
AW49*	3	2
A25	2	2
A63	2	1
A87	2	0
B93	2	8
C48	2	1
C56	2	0
A03	1	0
A06	1	0
B26	1	0
B79	1	3
C59	1	0

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Table E3 (cont.)

RV Type	Number of Samples	
	Illness Samples N = 6643	Well Samples N = 3542
Cpat14	1	0
Cpat20	1	0
A50	0	0
A64	0	0
A77	0	0
A107	0	0
A109	0	0
B05	0	0
B99	0	0
B100	0	0
B105	0	0
B106	0	0
C52	0	0
C57	0	0

*The table includes all known RV types, sorted by the number of times each RV type was detected in illness samples from the pooled data set. Abbreviations: pat, provisionally assigned type.

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Supplemental Figure Legends

Figure E1. Anti-RV-A16 nAb titers determined by traditional $TCID_{50}$ assay vs. novel qPCR-based assay. (A) nAb titers in plasma samples (n = 9) from RV-A16 inoculation study subjects (37) were first determined by $TCID_{50}$ infectivity assay in embryonic lung fibroblasts (WI-38) and then retested by novel qPCR-based assay in HeLa-E8 cells. Spearman's rank correlation: r_s = 0.82707, p (2-tailed) = 0.00595 (B) Correlation of nAb titers determined by two infectivity assays. R^2, Pearson Product-Moment Correlation Coefficient.

Figure E2. RV-A and RV-C types selected for the virus neutralization assay. Neighbor-joining phylogenetic tree based on capsid protein amino-acid sequences of currently recognized RV-A and RV-C types. Tree branch representing RV-B types was condensed. The evolutionary distances were computed using the p-distance method in MEGA 7 software and are in the units of the number of base differences per site. All major nodes are labelled with bootstrap values (500 replicates, with its value more than 75%). Branch lengths are proportional to amino acid similarity (p-distance). Enteroviruses (EV-A71 and EV-D68) were included as an outgroup. Selected RV-A and RV-C types are indicated by filled red and green circles, respectively.

Figure E3. Detection frequency of and seropositivity to RV types used in the virus neutralization assay. (A) Horizontal bars show detection frequencies for the RV types in the COAST samples set. (B) Seropositivity to selected RV types in COAST children (n = 20).

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Figure E4. Descriptive analysis of RV types detected in the pooled analysis. The histogram represents the frequency distribution for the how often each type was detected in illness samples. Individual graphs depict the proportion of detection for the most prevalent types (RV-A78, RV-C2, RV-C11, RV-C02, RV-A12 and RV-A101) during each year in the pooled analysis.

Figure E5. Association between age and RV-C to RV-A ratio by individual study cohorts. Lines represent regressions for each individual study.

Figure E6. Relationships between race and season of collection on the RV-C to RV-A ratio in illness samples.

Figure E7. Association between other personal factors and exposures on the RV-C to RV-A ratio in illness samples.

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Figure E1

A

Serum	$TCID_{50}$ titer	qPCR titer (IC_{50})
0174A	5.7	1.9
0175A	2.8	4.6
0069 V6	4.0	4.7
0076 V6	8.0	7.9
0084 V6	5.7	5.9
0162 V6	4.0	7.6
0078 V6	512.0	500.0
0081 V6	11.0	27.0
0169 V6	8.0	18.5

B

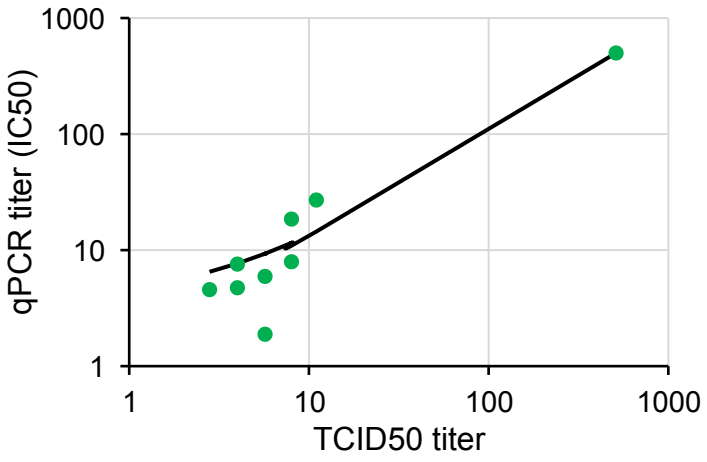

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Figure E2

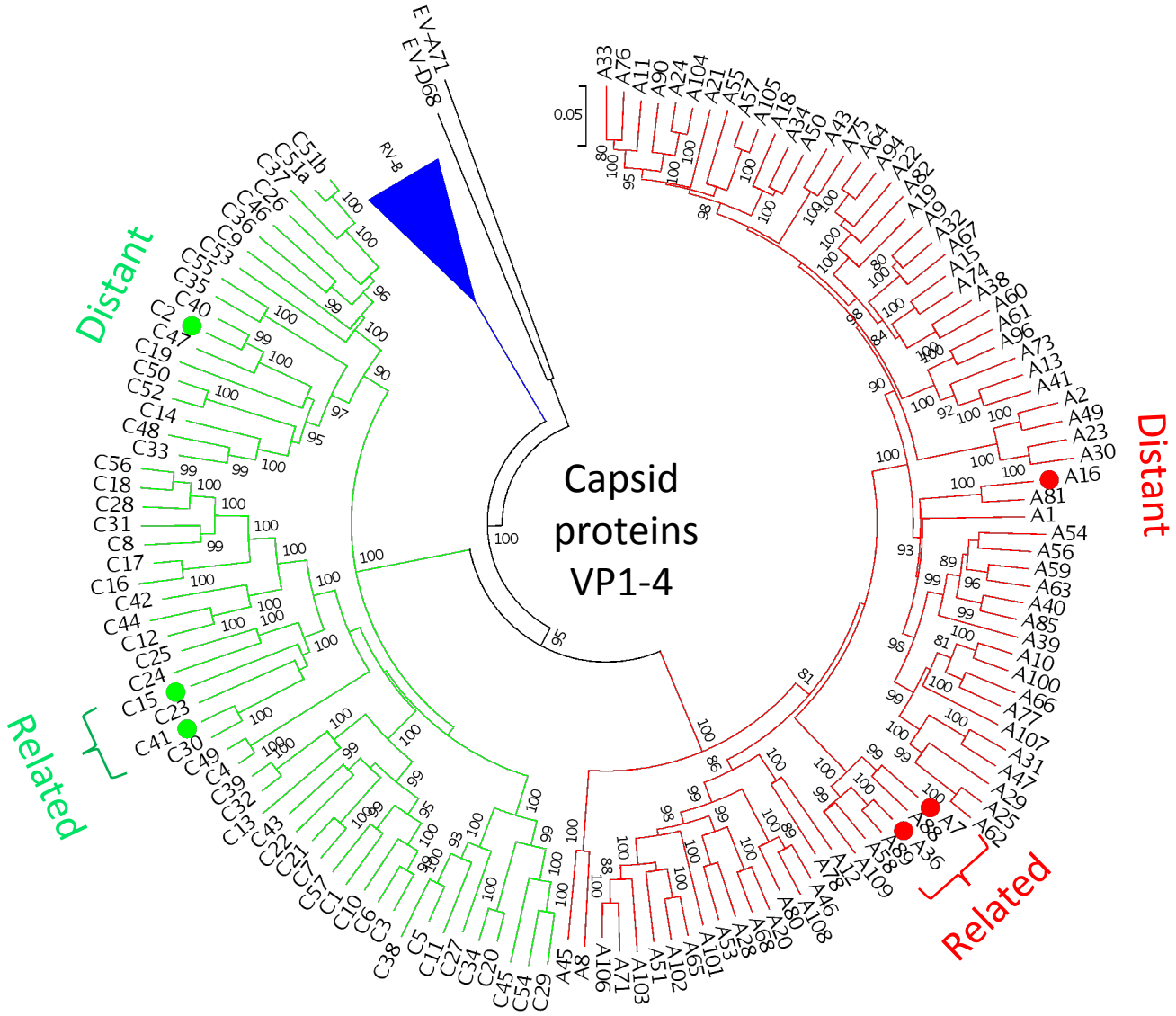

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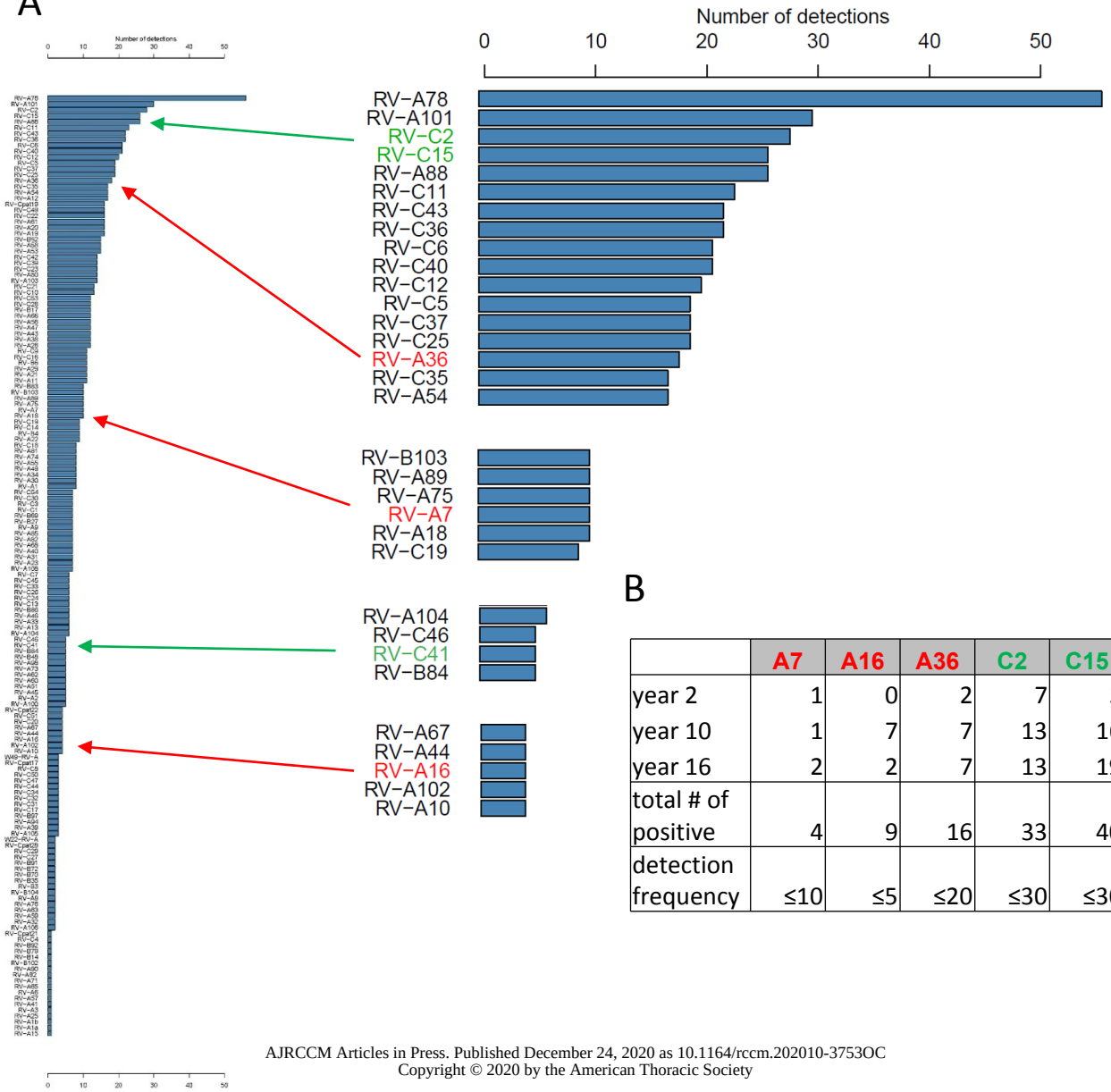
Figure E3

A



B

	A7	A16	A36	C2	C15	C41
year 2	1	0	2	7	5	4
year 10	1	7	7	13	16	13
year 16	2	2	7	13	19	15
total # of positive	4	9	16	33	40	32
detection frequency	≤10	≤5	≤20	≤30	≤30	≤5

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Figure S4.

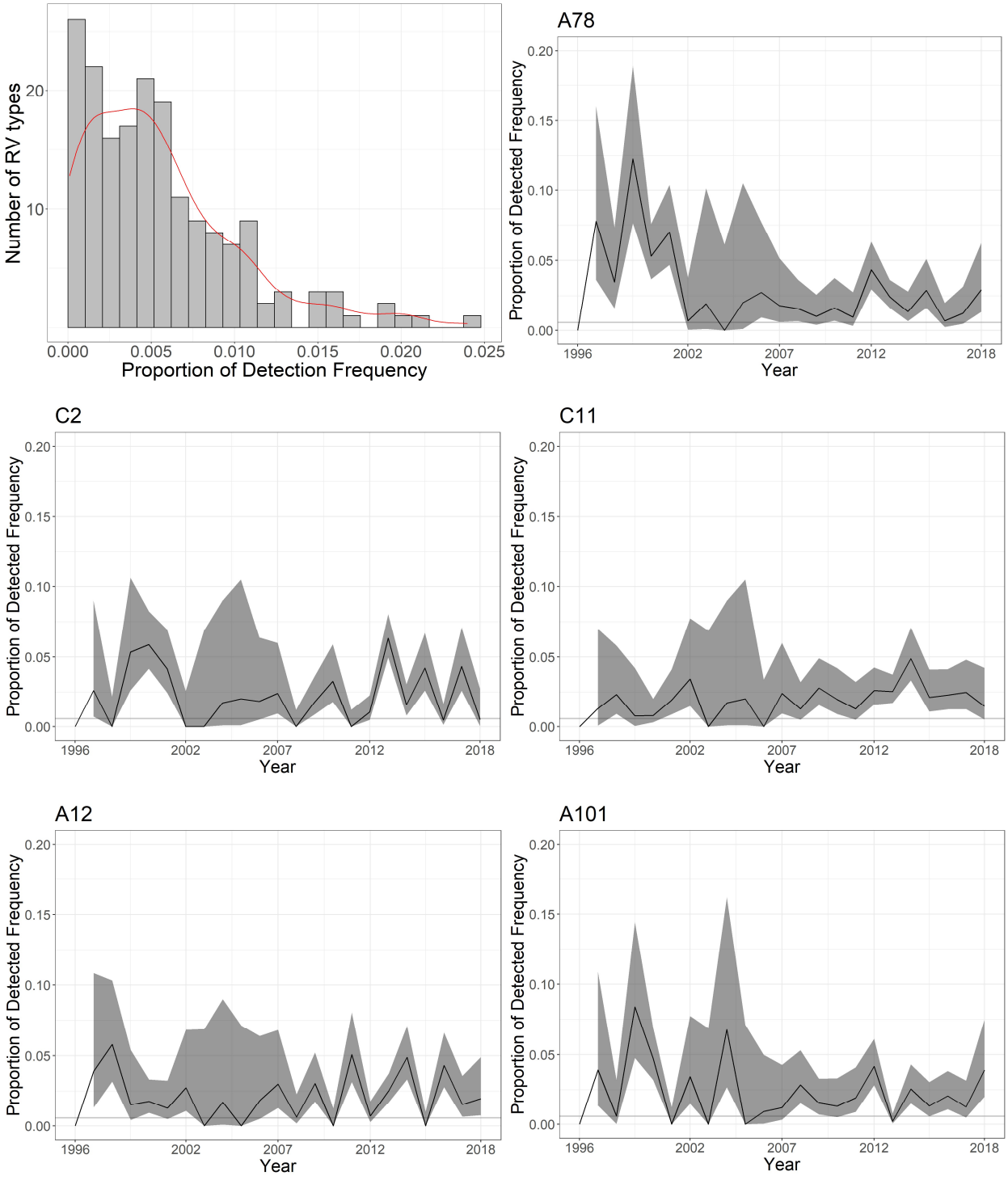

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Figure E5

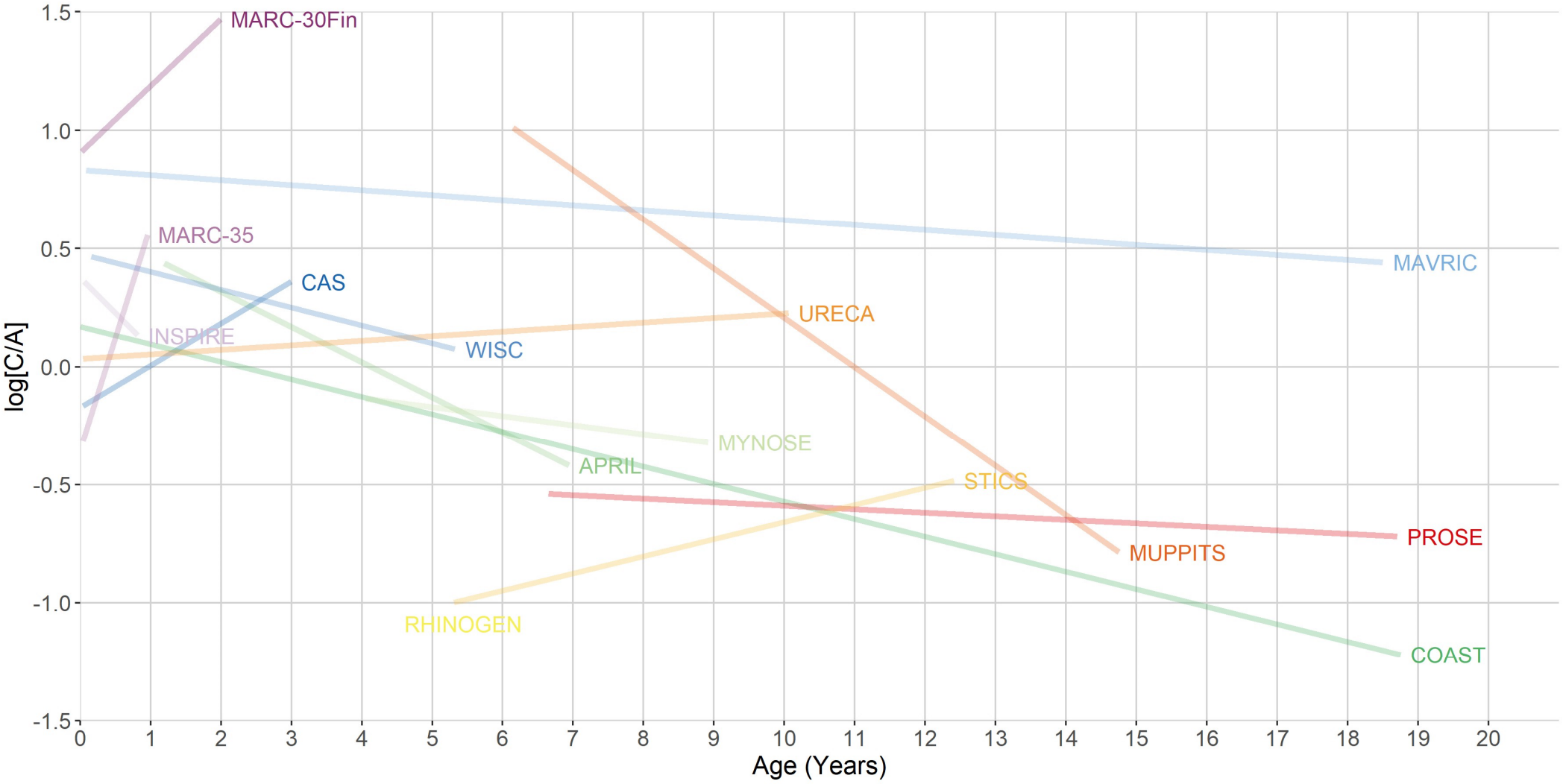

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Figure E6

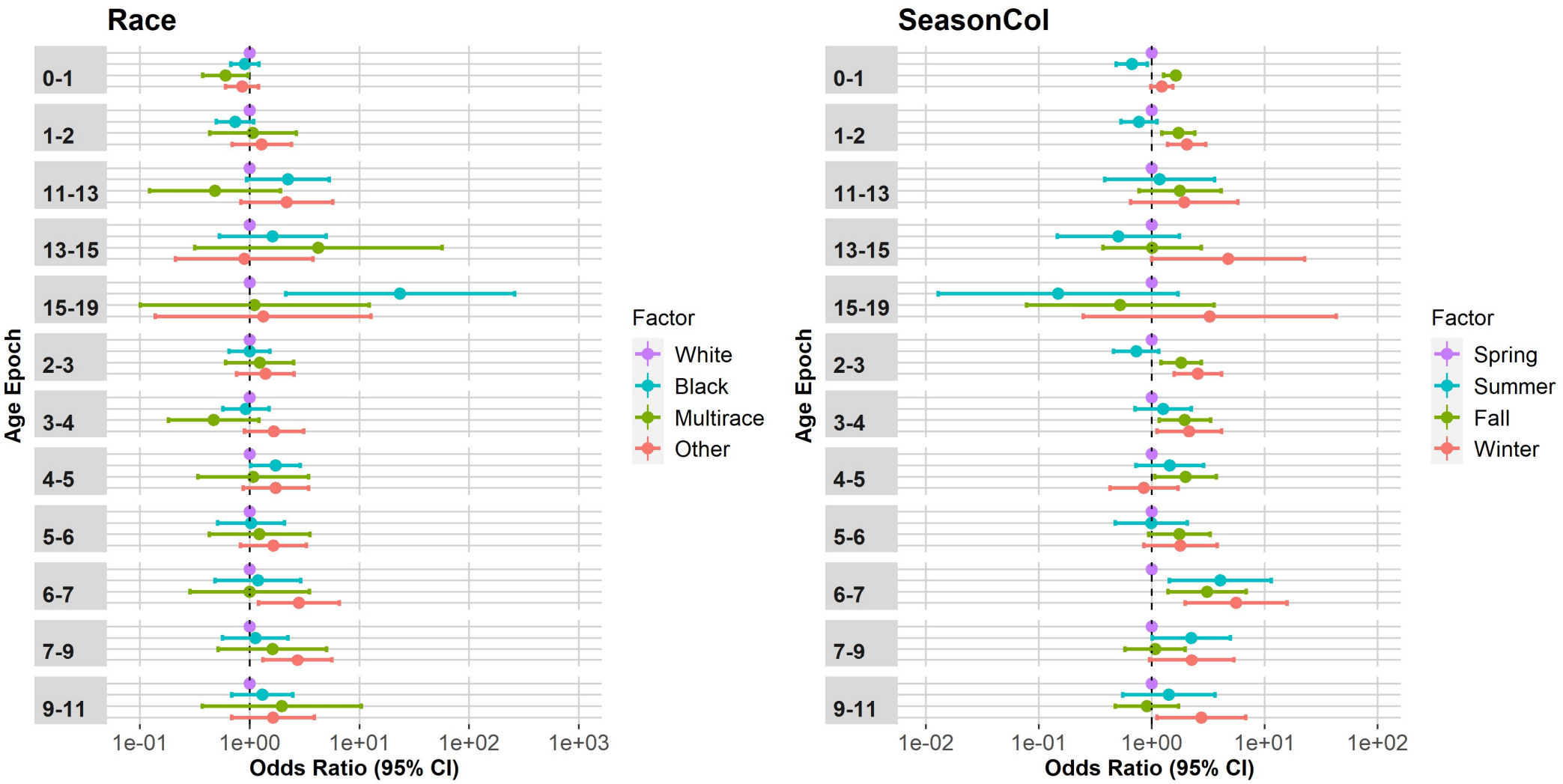

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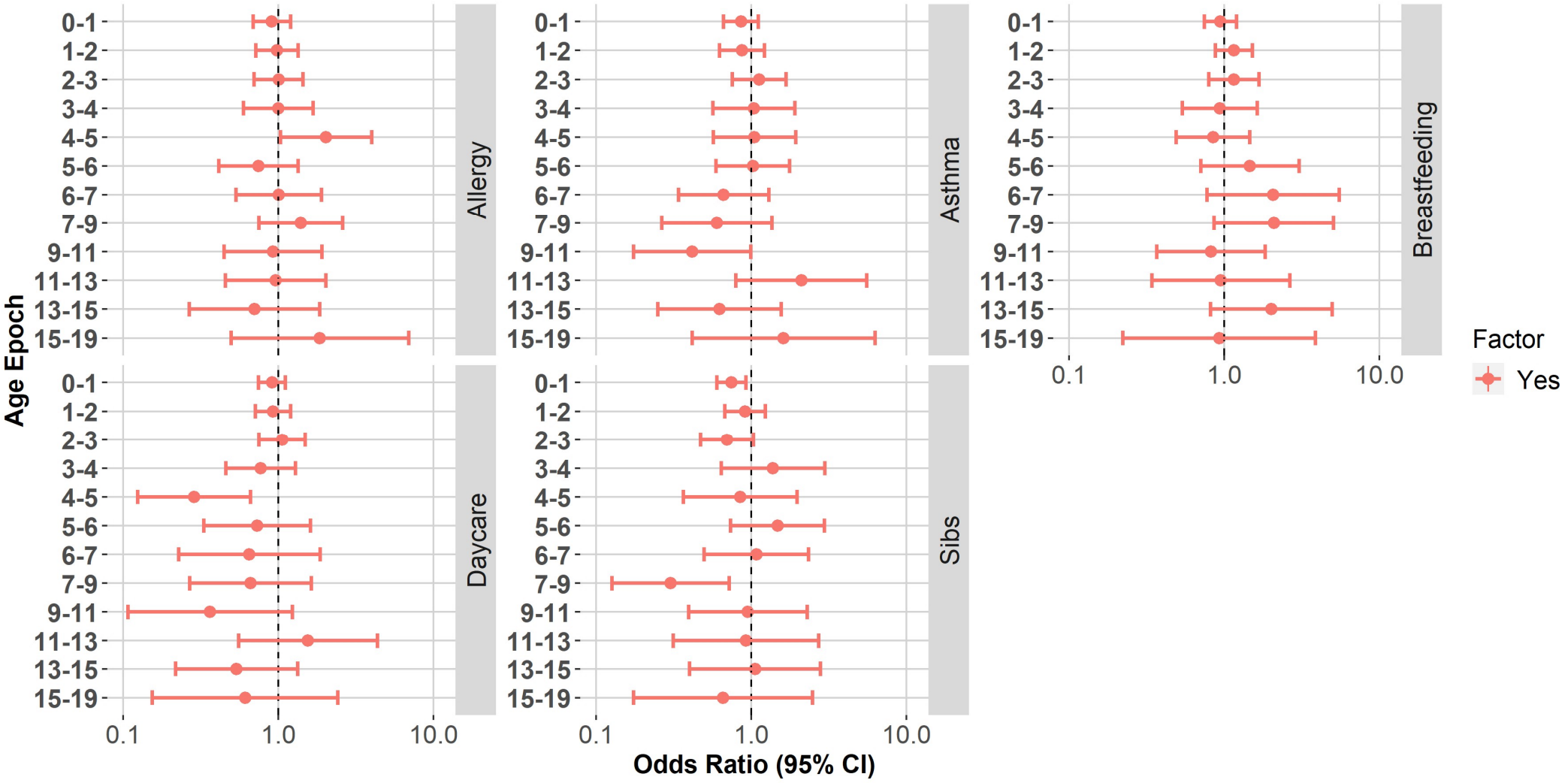
Figure E7



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