Risks for cold frequency vary by sex: role of asthma, age, TLR7 and leukocyte subsets

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Asthma, age and blood markers of antiviral immunity associate with cold frequency in a sex-dependent manner. People with asthma have lower TLR7 gene expression than healthy people; only in men does this associate with cold frequency. https://bit.ly/3e3yWKy

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ABSTRACT Viral respiratory infections are usually benign but can trigger asthma exacerbations. The factors associated with upper respiratory tract infection (cold) frequency are not fully understood, nor is it clear whether such factors differ between women and men.

To determine which immunological and clinical variables associate with the frequency of self-reported respiratory infections (colds), 150 asthma cases and 151 controls were recruited. Associations between antiviral immune response variables: toll-like receptor (TLR)7/8 gene expression, plasmacytoid dendritic cell (pDC) numbers and interferon-α, tumour necrosis factor and interleukin-12 production, and asthma were then examined that might explain cold frequency.

People with asthma cases reported more colds per year (median 3 *versus* 2; p<0.001) and had lower baseline TLR7 gene expression (odds ratio 0.12; p=0.02) than controls. Associations between many variables and cold frequency differed between women and men. In women, high blood neutrophil counts (β=0.096, p=0.002), and younger age (β=−0.017, p<0.001), but not exposure to children, were independently associated with more frequent colds. In men, low TLR7 expression (β=−0.96, p=0.041) and high CLEC4C gene expression (a marker of pDC; β=0.88, p=0.008) were independently associated with more frequent colds. Poor asthma symptom control was independently associated with reduced TLR8 gene expression (β=−1.4, p=0.036) and high body mass index (β=0.041, p=0.004).

Asthma, age and markers of inflammation and antiviral immunity in peripheral blood are associated with frequent colds. Interestingly, the variables associated with cold frequency differed between women and men.

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Introduction

Respiratory viral infections are typically restricted to the upper respiratory tract and are caused by several single stranded RNA (ssRNA) viruses, most commonly the common cold virus: rhinovirus. Respiratory (cold) infections are common in all populations; however, some groups are more likely to develop complications, such as involvement of the lower respiratory tract and secondary bacterial infections.

Asthma increases the risk of being hospitalised for infections in general [1], and this susceptibility to infections may be the consequence of a deficient antiviral immune response. Respiratory infections place asthma patients at an increased risk of exacerbations [2] and they are also more likely to develop lower respiratory tract symptoms than those without asthma [3, 4]. While preschool-aged children with asthma have more frequent and prolonged respiratory infections than non-asthmatic children [5], it is less clear whether adults with asthma are also more susceptible to colds.

Toll-like receptor (TLR)7 and TLR8 genes are located on the X chromosome. The receptors they encode detect and respond to viral ssRNA, and are part of an efficient antiviral immune response [6]. Activation of these receptors induces the production of antiviral cytokines of which type I interferons (IFNs) are the most important. Plasmacytoid dendritic cells (pDCs) express high levels of TLR7 and are the main producer of type I IFN [7]. Deficiencies in these antiviral variables could therefore predispose to more frequent respiratory infections.

Several research groups have reported inconsistent findings in terms of deficient type I IFN production (reviewed in [8]) and rhinovirus-induced TLR7 expression and function in asthma [9]. Since several factors influence the type I IFN response, including variations in pDCs [7], sex and age [10], adjusting for these variables is critical. Most studies reporting deficiencies in the pDC-TLR7-IFN-axis function in asthma [5, 8, 11] have been based on relatively small sample sizes, and it remains uncertain if these variables are associated with cold susceptibility.

The primary aim of this study was to determine the clinical and antiviral immune response variables associated with cold frequency, and if those variables differ between men and women in a large study comprising 150 asthma cases and 151 controls. The secondary aim was to determine the extent to which antiviral immune variables are associated with asthma severity and control.

Materials and methods

Participants

An asthma case–control study was established after receiving ethical clearance from the University of Queensland and Metro South Human Research Ethics Committees. Participants were recruited from the community and from Princess Alexandra Hospital in 2014–2016. All participants provided written informed consent.

Inclusion criteria were: ⩾18 years of age, smoking history <20 pack-years of cigarettes and at least 2 weeks free from respiratory infection symptoms prior to enrolment. Cases had a current asthma diagnosis and reported asthma symptoms and medication use in the past year. Participants had not used oral corticosteroid treatment for ⩾four weeks nor inhaled corticosteroid medication for 24 h before sample collection. Control participants had never been diagnosed with asthma.

Asthma severity was measured indirectly using treatment intensity, according to the Global Initiative for Asthma (GINA) guidelines (https://ginasthma.org/). Participants also completed an Asthma Control Questionnaire (ACQ6), for assessing symptom control within the past week. Basic demographics, ancestry, self-reported cold frequency per year, work involving children and living with children aged under 15 years were documented for everyone (supplementary methods).

Sample processing

The following blood samples were collected: complete blood count, differential leukocyte count and total immunoglobulin (Ig)E (measured by PA Hospital Pathology Department); PAXgene (PreAnalytiX, Hombrechtikon, Switzerland) tubes for RNA extraction using Preanalytix blood RNA kit (Qiagen, Germantown, MD, USA); and heparinised blood samples for peripheral blood mononuclear cell (PBMC) isolation. PBMC were isolated as previously described [10] and stimulated *in vitro* for 24 h with either rhinovirus 16 (RV16) or the TLR8 specific agonist VTX-2337 (Sapphire Bioscience, Waterloo, Australia). Further details are in the supplementary material.

Cytokine measurement

ELISA of IFNα (pan-specific, Mabtech Ab, Stockholm, Sweden), IL12 (IL12 (p70), BD OptEIA, BD Biosciences, San Diego, CA, USA) and tumour necrosis factor (TNF) (BD Biosciences) were used to

measure cytokines in PBMC supernatants. Cytokine ELISAs were controlled for technical and biological variability with biological duplicates.

Gene expression quantification

RNA was reverse-transcribed to cDNA with sensiFAST cDNA synthesis kit (Bioline, London, UK). Relative quantitative RT-PCR was performed from the cDNA with PowerUp SYBR Green mastermix (Applied Biosystems, CA, USA) with Lightcycler 480 (Roche, Basel, Switzerland) machine. Custom primers were purchased from Geneworks (Adelaide, SA, Australia) (supplementary table E1). *TLR7*, *TLR8* and *CLEC4C* expression was obtained against the mean cycle threshold (Ct) of the two reference genes *B2M* (*beta-2-microglobulin*) and *UBC* (*polyubiquitin-C precursor*) using the comparative Ct method [12].

Statistical analysis

All statistical analyses were performed with R version 3.4.4 [13]. Variables were normalised using natural logarithm if required. Associations were tested with linear, logistic or ordinal logistic regression, as appropriate. Backward stepwise regression was used to fit the best multivariable model. The difference in sample distribution was tested with a nonparametric Mann–Whitney U-test, correlations between observations were tested with nonparametric Spearman's rank correlation test and differences in distribution between groups assessed with the Chi-squared test. A p-value of <0.05 was considered statistically significant.

Results

Participant demographics

Table 1 summarises the characteristics of the 150 asthma cases and 151 control participants. Self-identified ancestry varied between asthma and control groups, with more Asians in the control group and more Europeans in the asthma group. Both groups had a similar age distribution and minimal cigarette exposure. The asthma group reported frequent colds and greater exposure to children, had significantly higher body mass index (BMI), and significantly higher numbers of blood neutrophils, monocytes,

TABLE 1 Characteristics of the study groups

	Asthma	Control	p-value
Subjects n	150	151	
Female/male	97 (64.7)/53 (35.3)	90 (59.6)/61 (40.4)	0.431
Ancestry			
Unknown	1 (0.7)	1 (0.7)	
Africans	0 (0.0)	1 (0.7)	
Asians	6 (4.0)	26 (17.2)	
Europeans	133 (88.7)	105 (69.5)	
Mixed	10 (6.7)	18 (11.9)	
Cold frequency	3.00 (3.00–5.00)	2.00 (1.00–3.00)	**<0.001**
Has children[#]	42 (28.2)	24 (15.9)	**0.015**
Children at work[¶]	23 (15.4)	10 (6.6)	**0.024**
Age at donation years	35.00 (26.00–47.00)	32.00 (26.00–44.00)	0.309
Aged over 50 years	35 (23.3)	31 (20.5)	0.654
Smoked pack-years	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.871
BMI kg·m^{-2}	27.00 (23.00–30.00)	23.00 (22.00–26.00)	**<0.001**
Blood count ×10^{9}cells·L^{-1}			
White blood cells	6.50 (5.50–7.70)	5.90 (4.95–6.80)	**<0.001**
Platelets	269.00 (234.50–301.00)	240.00 (211.50–271.50)	**<0.001**
Neutrophils	3.68 (2.94–4.57)	3.21 (2.75–4.03)	**0.003**
Lymphocytes	1.92 (1.64–2.43)	1.87 (1.56–2.20)	0.072
Monocytes	0.53 (0.42–0.63)	0.46 (0.37–0.57)	**0.003**
Eosinophils	0.25 (0.15–0.39)	0.13 (0.08–0.19)	**<0.001**
Basophils	0.03 (0.02–0.04)	0.02 (0.01–0.03)	**<0.001**
IgE kU·L^{-1}	143.00 (51.75–346.75)	34.00 (13.00–100.00)	**<0.001**
***CLEC4C* mRNA**	0.11 (0.08–0.19)	0.11 (0.07–0.19)	0.349

Data are presented as n (%) or median (interquartile range), unless otherwise stated. n=study group size,[#:] Household with children under 15 years of age; [¶]:work involving contact with children. BMI:body mass index; Ig: immunoglobulin. p-value is shown for Mann-Whitney U-test between the two groups for continuous variables and Chi-squared test for the categorical variables. Bolded p-values are statistically significant.

eosinophils, basophils and platelets than the control group. As expected, total IgE levels were significantly higher in the asthma group than in the healthy group.

Table E2 describes the asthma group in further detail. Most asthma participants were in either GINA step 4 (37%) or GINA step 1 (35%) treatment groups. The relationships between GINA treatment step and asthma control (ACQ6 score) are shown in figure E1.

Asthma, age, blood neutrophil count and CLEC4C gene expression are independently associated with cold frequency

Different clinical and immunological factors were examined to establish associations with self-reported cold frequency. Baseline gene expression of *TLR7/8* and *CLEC4C*, reflecting the relative quantity of pDC [14], were measured in whole blood directly after sample collection, whereas TLR7/8 function was assessed by RV16- or TLR8-stimulated PBMC cytokine production. RV16-activated PBMCs produced substantial IFN-α (median 970 pg·mL^{-1}), whereas baseline (unstimulated) PBMC produced barely detectable amounts of IFN-α (table E6).

Univariate linear regression analysis showed that asthma, age, BMI, having children and blood eosinophil and neutrophil counts were associated with cold frequency (table 2). None of the measured antiviral response variables (baseline *TLR7/8* and *CLEC4C* mRNA, stimulated IFN-α, TNF and IL12 production) were associated with cold frequency by univariate analysis.

A multivariable linear regression model was used to mitigate the effects of potential confounders. Having asthma, younger age, blood neutrophil count and baseline *CLEC4C* expression were independently associated with cold frequency (table 2), whereas BMI was not independently associated. This analysis was also repeated in asthma cases and controls separately (table E3), and in those with BMI less than 25 (table E5).

Different variables associate with cold frequency in men and women

Selected antiviral immune variables were then compared between men and women with no sex related differences in cold frequency or baseline gene expression of *TLR7*, *TLR8* and *CLEC4C* observed (figure 1). RV16-stimulated IFN-α production was higher in women than in men (p=0.05), whereas TLR8-stimulated TNF and IL12 production was significantly lower in women (TNF p=0.003; IL12 p=0.001) (figure 2).

The observations that TLR7/8 function varies significantly with sex warranted the stratification of cold frequency analysis by sex (table 3). The multivariable model in women showed that having asthma, younger age and increased neutrophil count were independently associated with cold frequency. However, *CLEC4C* expression was not significantly associated with cold frequency in women. Working with children and high BMI were associated with frequent colds in the univariate models for women, but no longer

TABLE 2 Multivariable linear regression model for cold frequency

	Univariable models				Multivariable model			
	Estimated coefficient	**CI 2.5%**	**CI 97.5%**	**p-value**	**Estimated coefficient**	**CI 2.5%**	**CI 97.5%**	**p-value**
Dependent variable: cold frequency per year								
Asthma	0.47	0.34	0.60	**<0.001**	0.48	0.35	0.61	**<0.001**
Age at donation	−0.012	−0.018	−0.007	**<0.001**	−0.013	−0.018	−0.008	**<0.001**
BMI kg·m^{-2}	0.017	0.003	0.031	**0.021**				
Sex	−0.013	−0.16	0.13	0.86				
Has children	0.19	0.016	0.36	**0.032**				
Children at work	0.21	−0.022	0.45	0.076				
Neutrophil count ×10^{9} cells·L^{-1}	0.081	0.029	0.13	**0.002**	0.066	0.018	0.11	**0.007**
Eosinophil count ×10^{9} cells·L^{-1}	0.59	0.22	0.95	**0.002**				
CLEC4C mRNA	0.46	−0.058	0.98	0.081	0.55	0.094	1.0	**0.018**
TLR7 mRNA	−0.36	−0.85	0.13	0.14				
TLR8 mRNA	−0.22	−0.73	0.30	0.41				
IFNα ng·mL^{-1}	20	−37	77	0.49				
TNF ng·mL^{-1}	4.3	−15	24	0.67				
IL12 ng·mL^{-1}	150	−98	400	0.23				

Multivariable model included n=285 with 16 missing observations deleted. Adjusted R^2: 0.25, p<0.001. Cold frequency was natural log transformed, CLEC4C, Toll-Like receptor (TLR)7 and TLR8 gene expression at baseline were measured in resting whole blood, interferon (IFN) α production stimulated with RV16, tumour necrosis factor (TNF) and interleukin (IL)12 production stimulated with TLR8 agonist were measured in peripheral blood mononuclear cells.

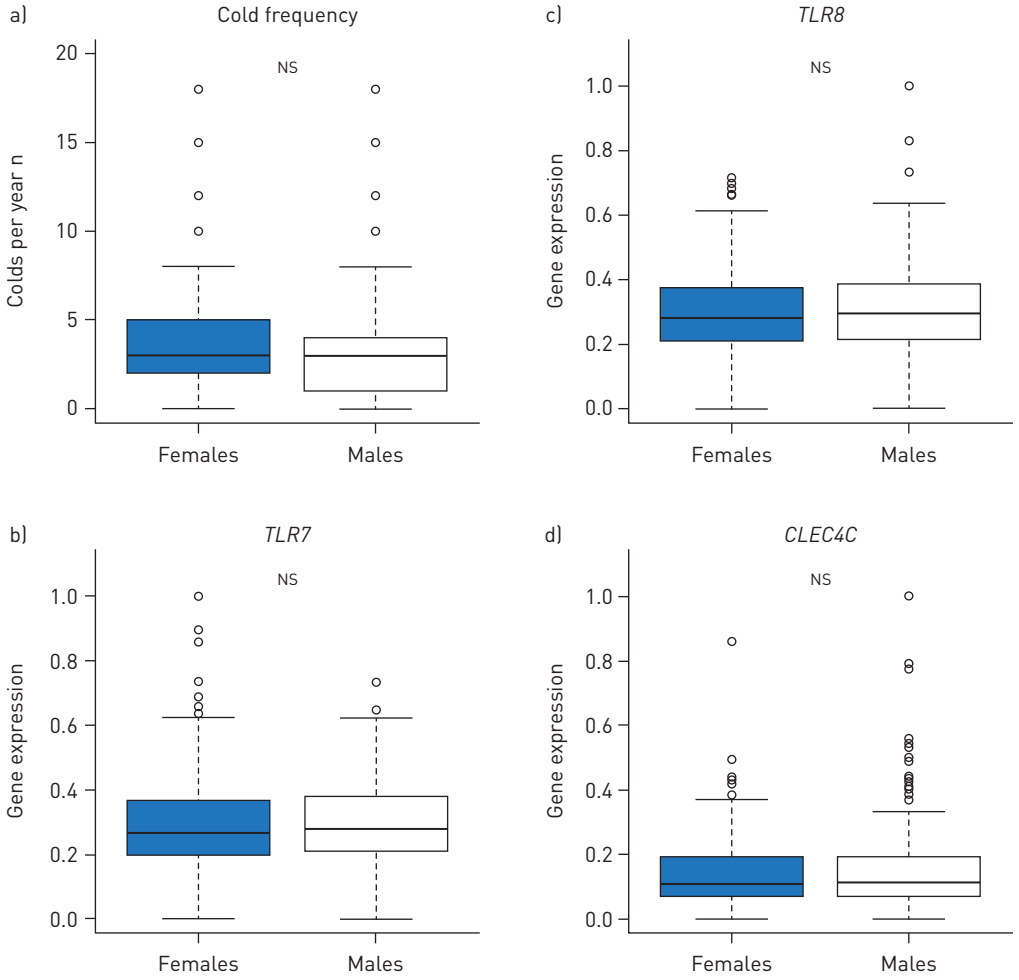

FIGURE 1 Cold frequency and gene expression of antiviral immune variables in men and women. Median and interquartile ranges presented as boxplots for both sexes for a) number of colds per year, b) toll-like receptor (TLR)7, c) TLR8 and d) CLEC4C gene expression in whole blood. NS: not significant Mann–Whitney U-test.

significant in the multivariable model. In men, having asthma, higher *CLEC4C* and lower *TLR7* expression were independently associated with frequent colds (table 3) but not age or neutrophil count.

Baseline gene expression of TLR7 and BMI are independently associated with asthma

Having shown that asthma is independently associated with cold frequency in both women and men (tables 2 and 3), the TLR7/8-related immunological variables were therefore assessed. Of these, only lower baseline *TLR7* expression and higher BMI were associated with asthma (table 4). Of note, RV16-induced

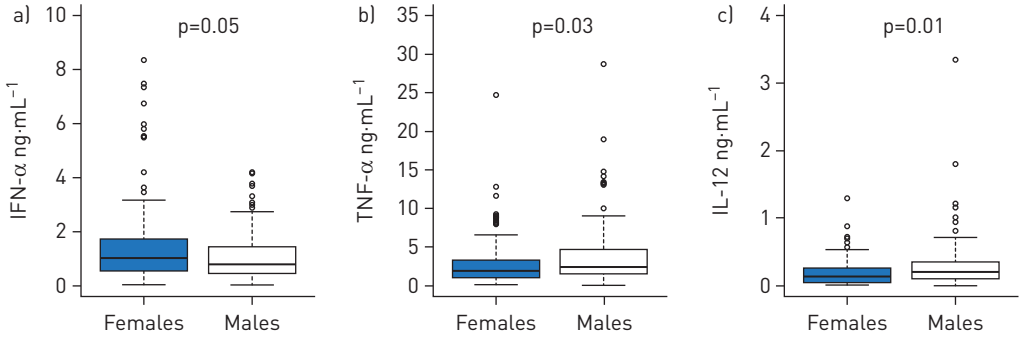

FIGURE 2 Cytokine production in men and women. Median and interquartile ranges presented as boxplots for both sexes for a) RV-induced IFNα and TLR8 agonist-induced b) TNF and c) IL12 cytokine production in PBMC after 24 h stimulation. p-value is shown for significant Mann-Whitney U-test.

TABLE 3 Multivariable linear regression models for cold frequency by sex

	Univariable models				Multivariable model			
	Estimated coefficient	**CI 2.5%**	**CI 97.5%**	**p-value**	**Estimated coefficient**	**CI 2.5%**	**CI 97.5%**	**p-value**
Females only								
Dependent variable: cold frequency per year								
Asthma	0.47	0.30	0.65	**<0.001**	0.47	0.31	0.62	**<0.001**
Age at donation	−0.016	−0.023	−0.009	**<0.001**	−0.017	−0.023	−0.011	**<0.001**
BMI kg·m^{-2}	0.02	0.004	0.037	**0.018**				
Has children	0.18	−0.042	0.39	0.11				
Children at work	0.29	0.022	0.56	**0.034**				
Neutrophil count ×10^9 cells·L^{-1}	0.14	0.068	0.20	**<0.001**	0.096	0.036	0.16	**0.002**
Eosinophil count ×10^9 cells·L^{-1}	0.91	0.38	1.5	**<0.001**				
CLEC4C mRNA	0.40	−0.44	1.2	0.35				
TLR7 mRNA	−0.22	−0.81	0.36	0.45				
TLR8 mRNA	−0.31	−1.0	0.39	0.39				
IFNα ng·mL^{-1}	25	−41	91	0.46				
TNF ng·mL^{-1}	−3.6	−32	25	0.81				
IL12 ng·mL^{-1}	−61	−590	460	0.82				
Males only								
Dependent variable: cold frequency per year								
Asthma	0.46	0.25	0.67	**<0.001**	0.46	0.25	0.67	**<0.001**
Age at donation	−0.007	−0.016	0.001	0.086				
BMI kg·m^{-2}	0.007	−0.021	0.034	0.64				
Has children	−0.077	−0.58	0.43	0.76				
Children at work	0.21	−0.076	0.49	0.15				
Neutrophil count ×10^9 cells·L^{-1}	0.0016	−0.085	0.088	0.97				
Eosinophil count ×10^9 cells·L^{-1}	0.30	−0.20	0.81	0.24				
CLEC4C mRNA	0.51	−0.15	1.2	0.13	0.88	0.23	1.5	**0.008**
TLR7 mRNA	−0.75	−1.7	0.18	0.11	−0.96	−1.88	−0.038	**0.041**
TLR8 mRNA	−0.096	−0.86	0.67	0.80				
IFNα ng·mL^{-1}	−0.032	−120	120	1.0				
TNF ng·mL^{-1}	13	−15	40	0.36				
IL12 ng·mL^{-1}	230	−52	510	0.11				

Multivariable model included n=174 with 9 missing observations deleted. Adjusted R^2: 0.29, p<0.001. Multivariable model included n=106 with 6 missing observations deleted. Adjusted R^2: 0.19, p<0.001. Cold frequency was natural log transformed, CLEC4C, Toll-like receptor (TLR)7 and TLR8 gene expression at baseline were measured in resting whole blood, interferon (IFN)α production stimulated with RV16, tumour necrosis factor (TNF) and interleukin (IL)12 production stimulated with TLR8 agonist were measured in peripheral blood mononuclear cells.

TABLE 4 Multivariable logistic regression model for asthma

	Univariable models				Multivariable model			
	Odds ratio	**CI 2.5%**	**CI 97.5%**	**p-value**	**Odds ratio**	**CI 2.5%**	**CI 97.5%**	**p-value**
Dependent variable: asthma								
Sex	0.81	0.50	1.3	0.37				
Age at donation	1.0	0.99	1.0	0.34				
BMI kg·m^{-2}	1.1	1.1	1.2	**<0.001**	1.12	1.06	1.18	**<0.001**
CLEC4C mRNA	0.44	0.076	2.33	0.34				
TLR7 mRNA	0.12	0.023	0.62	**0.013**	0.12	0.020	0.69	**0.020**
TLR8 mRNA	0.26	0.047	1.3	0.11				
IFNα ng·mL^{-1}	0.94	0.77	1.1	0.48				
TNF ng·mL^{-1}	1.0	0.97	1.1	0.31	1.1	1.0	1.2	0.050
IL12 ng·mL^{-1}	1.9	0.81	5.2	0.18				

Multivariable model included n=297 with 4 missing observations deleted. Pseudo R^2: 0.13, p<0.001. CLEC4C, Toll-like receptor (TLR)7 and TLR8 gene expression at baseline were measured in resting whole blood, interferon (IFN)α production stimulated with RV16, tumour necrosis factor (TNF) and interleukin (IL) 12 production stimulated with TLR8 agonist were measured in peripheral blood mononuclear cells.

TABLE 5 Multivariable linear regression model for asthma control and severity

	Univariable models				Multivariable model			
	Estimated coefficient	CI 2.5%	CI 97.5%	p-value	Estimated coefficient	CI 2.5%	CI 97.5%	p-value
Dependent variable: asthma control (ACQ6 score)								
Sex	−0.036	−0.35	0.28	0.82	0.033	−0.28	0.35	0.84
Age at donation	0.016	0.005	0.027	**0.005**				
BMI kg·m^{-2}	0.040	0.015	0.066	**0.002**	0.041	0.015	0.067	**0.004**
CLEC4C mRNA	−0.071	−1.3	1.1	0.91				
TLR7 mRNA	−0.12	−1.3	1.0	0.83	0.88	−0.42	2.2	0.18
TLR8 mRNA	−1.0	−2.1	0.12	0.079	−1.4	−2.7	−0.093	**0.036**
IFNα ng·mL^{-1}	−0.024	−0.17	0.12	0.74				
TNF ng·mL^{-1}	−0.021	−0.060	0.017	0.28				
IL12 ng·mL^{-1}	−0.063	−0.51	0.39	0.78				
Dependent variable: asthma severity (GINA step)								
Sex	1.0	0.53	1.9	0.98	1.4	0.70	2.7	0.36
Age at donation	1.04	1.02	1.07	**<0.001**	1.03	1.01	1.06	**0.005**
BMI kg·m^{-2}	1.1	1.1	1.2	**<0.001**	1.1	1.0	1.2	**0.004**
CLEC4C mRNA	0.13	0.010	1.2	0.093				
TLR7 mRNA	0.13	0.015	1.0	0.057	0.23	0.024	2.0	0.19
TLR8 mRNA	0.19	0.021	1.5	0.12				
IFNα ng·mL^{-1}	0.85	0.62	1.1	0.39				
TNF ng·mL^{-1}	0.96	0.88	1.0	0.73				
IL12 ng·mL^{-1}	1.5	0.70	4.1	0.36				

ACQ: Asthma Control Questionnaire; GINA: Global Initiative for Asthma. Multivariable model included n=150. Multivariable model included n=147 with 3 missing observations deleted. Adjusted R^2: 0.06, p=0.010. CLEC4C, Toll-like receptor (TLR)7 and TLR8 gene expression at baseline were measured in resting whole blood, interferon (IFN)α production stimulated with RV16, tumour necrosis factor (TNF) and interleukin (IL)12 production stimulated with TLR8 agonist were measured in peripheral blood mononuclear cells.

IFNα production was not associated with the presence or absence of asthma. A multivariable logistic regression model was fitted to account for confounders. The negative association between *TLR7* mRNA and asthma remained significant when adjusted for BMI and TLR8-induced TNF production (table 4). The association between baseline *TLR7* expression and asthma was no longer significant when men and women were analysed separately (table E4).

The association between baseline *TLR7* gene expression and asthma was no longer significant when men and women were analysed separately (table E4).

TLR8 gene expression and BMI associate with asthma control whereas age and BMI associate with asthma severity

Asthma control and severity were worse with advancing age and elevated BMI; however, the immune response variables did not associate with these measures. Clinical and immune response variables were tested for an association with ACQ6 score and treatment intensity because asthma is a very heterogeneous condition. Baseline *TLR8* expression was inversely proportional to ACQ6 score when adjusted for sex, BMI and *TLR7* expression (table 5).

However, none of the immune response variables were associated with treatment intensity, after accounting for confounders. BMI was independently associated with both ACQ6 score and treatment intensity, whereas advancing age was significantly associated with treatment intensity only.

Discussion

This study of 301 participants identified four key variables independently associated with self-reported cold frequency, namely having asthma, young age, high blood neutrophil count, and higher *CLEC4C* expression in a sex-dependent fashion. In women, having asthma, high blood neutrophils and younger age were independently associated with frequent colds; whereas, in men, having asthma, low *TLR7* and high *CLEC4C* baseline expression were independently associated with frequent colds. Baseline *TLR7* expression was lower in asthma patients than controls, while baseline *TLR8* expression was inversely proportional to asthma control when adjusted for other variables.

The finding that participants with asthma reported more frequent colds than controls aligns with the previous studies showing more severe rhinovirus infections [3]. Corne *et al.* [3] reported slightly more

rhinovirus infections in those with asthma compared to healthy participants (odds ratio 1.15), but the confidence intervals were wide, and the differences were not statistically significant. Others have reported more frequent colds in pre-school children [5], and increased hospitalisations for infections in asthma [1]. Importantly, vulnerability to virus infections in adult asthma also includes mild cold infections and is not restricted to severe infections requiring hospitalisation. This finding further highlights the burden of respiratory infections experienced by people with asthma.

Our study suggests an important link between systemic inflammation and colds, though interestingly the specific leukocyte subsets associated with cold frequency differ between women (high neutrophil counts) and men (high pDC counts). High blood neutrophils can be seen in neutrophilic asthma [15] and obesity-related inflammation [16]. High BMI was associated with cold frequency by univariable analysis, but not when adjusted for other variables (table 2). Hence, the link between neutrophil counts and cold frequency appears independent of obesity. Similarly, blood eosinophils were associated with cold frequency by univariable analysis, but not when adjusted for other variables (table 2). A very large Danish study recently reported that high neutrophil counts in asthma patients are associated with exacerbation risk [17]. As virus infections trigger many exacerbations, blood neutrophil counts could therefore be a marker of both frequent colds and exacerbation risk. Although this study does not provide information on likely mechanisms, persistent subclinical infection might contribute to elevated neutrophil counts, considering that study participants were free of cold symptoms for at least 4 weeks prior to blood sampling. We hypothesise that those with asthma and high immune cell counts may have a reduced ability to clear infections and/or resolve inflammation, which is then a risk factor for subsequent exacerbations. The other possibility, that high neutrophil counts predisposes to frequent colds, seems less likely.

Younger participants (especially women) were more likely to have frequent colds, and while having children was associated with frequent colds in the univariable analysis, it was not significant in the multivariable regression model. It is possible that these associations would have been stronger if the analysis was restricted to children less than 6 years, as this is the age group who experience the most frequent colds and who shed higher loads of virus. Though immune memory in older adults might confer some protection from colds, the fact that age was not associated with cold frequency in men supports the alternative hypothesis that sex hormones influence antiviral immunity, an issue we will address in the future. The fact that neutrophil count was not associated with cold frequency in men, further emphasises the differential determinants for cold frequency in each sex.

Our finding that several antiviral variables differed between men and women, supports previous reports [10]. TLR7 is important for detecting RNA viruses; however, the association between low *TLR7* expression and increased colds was only observed in men in conjunction with high *CLEC4C* expression. This finding was unexpected, as pDC are thought to be the primary cell that expresses TLR7. However, others have reported a similar phenomenon whereby lower TLR7 function in men only is independent of pDC numbers [18]. Elevated pDC numbers have previously been associated with atopy (reviewed in [19]), and also may be a consequence of subclinical virus infection as discussed above.

Type I IFN, IL12 and TNF production were measured as markers of TLR7/8 function, but contrary to expectations, no significant associations with cold frequency were observed. Perhaps TLR7/8 receptor availability and other downstream pathways play more important roles in host defence against cold viruses than the cytokines we measured. Detailed transcriptomic studies are needed to address this issue more fully.

In contrast to men, only the "clinical" variables were associated with cold frequency in women. Younger age was associated with more frequent colds in women, so we speculate that declining oestrogen production with age may impact on cold frequency. Because oestrogen modifies pDC responses through TLR7 [20] and both TLR7/8 expression [21], the relationship between TLR7 function and oestrogen might obscure the associations between TLR7/pDC and cold frequency seen in men. Additional aspects of antiviral immunity (other than those studied here) may contribute to cold frequency in women. The *TLR7* gene on the X-chromosome has been reported to escape X-chromosome inactivation (XCI) [22], however the similar *TLR7* expression observed in men and women in this study (data not shown) suggests otherwise. Rather, it seems more likely that oestrogens or other female hormones may be modifying key molecular pathways downstream of TLR7.

Changes in antiviral immune responses in asthma patients were identified in this study and reported by others [8, 9, 19], which may explain higher cold frequency in asthma. Our group previously reported lower rhinovirus-stimulated expression of *TLR7* and other related genes in asthma than in controls [9]. Type 2 inflammation can reduce TLR7 activity in a mouse model [23], and sputum eosinophilia correlates with lower airway TLR7 expression in human asthma [23]. Impaired TLR7 function may thus facilitate frequent or severe respiratory infections. TLR7 also acts to suppress type 2 immunity in mouse studies: depletion of

TLR7 in mice contributes to a Th2 biased response to influenza [24], while TLR7-activation in B-cells and pDC suppresses type 2 responses [25, 26]. The ability of TLR7 to further suppress Th17 cells provides another link between asthma inflammation and TLR7 activity [27, 28].

As asthma is recognised as a heterogeneous disease [29], pinpointing the asthma subgroups most afflicted by impaired antiviral immunity is vital for better targeted asthma treatment. We found that poor asthma control was associated with impaired *TLR8* expression. Because TLR8-induced type 1 immunity antagonises type 2 immunity [30], it is possible that reduced *TLR8* expression facilitates poor asthma symptom control by enhancing type 2 inflammation. A cross-regulatory relationship exists between TLR7 and TLR8, possibly *via* competition for a common chaperone protein Unc93B1 [31, 32]. However, very few cells express both receptors, and the regression model (table 5) implies that *TLR8* expression associates with asthma symptom control independent of *TLR7* expression. Further studies are needed to dissect the complex relationships between TLR7/8 function and asthma.

In contrast, asthma severity was not associated with any of the antiviral immune response variables tested. Contrary to expectations, the asthma group had similar rhinovirus-stimulated IFN-α production to the control group. The sample size in this study was much larger than many previous studies, so the study was unlikely to be underpowered. Increased IFN responses in asthma have been reported in relation to asthma exacerbations and type 2-polarised environment [33, 34], while other groups have described distinct asthma subtypes where IFN production during an exacerbation can be either high or low [35, 36]. Both the lowest and highest type I IFN response to rhinovirus were associated with having asthma in a study of 11-year-old children [11]. Type I IFN response thus appears to be quite heterogeneous within the asthma population.

Asthma is known to increase infection risk [1] as is obesity: obese people are more susceptible to influenza infections [37], and overweight children are susceptible to respiratory infections [38]. While high BMI was associated with cold frequency by univariable analysis, it was not significantly associated with cold frequency when adjusted for other variables in the multivariable model (table 2). A separate analysis restricted to those with a BMI<25 $kg{\cdot}m^{-2}$ (Table E5), confirmed these findings. Obesity is a risk factor for asthma development and is associated with more severe disease [39]. Given that high BMI was associated with both asthma symptom control and treatment intensity (table 5), we recommend considering BMI at the design phase in future asthma and antiviral immunity studies.

Since the frequency of colds was self-reported, a note of caution is due, given that the study may have selectively attracted participants with frequent colds. Recalling the numbers of past infections may be inaccurate. However, the median frequency of two colds per year in the healthy controls is in line with earlier reports [40] and the large sample size of 301 in this study should partly mitigate these factors. Importantly, several biological variables were identified that were independently associated with cold frequency, indicating that self-reported colds has some validity as a relevant outcome. We also considered the possibility that people with asthma might over-report colds or attribute symptoms (*e.g.* cough) to a cold rather than to asthma. However, this seems less likely as we observed no association between cold frequency and ACQ6 score or between cold frequency and asthma severity. Similarly, adjusting for hay fever in our analyses did not alter our findings.

Conclusion

The four key variables of asthma, young age, *CLEC4C* expression and blood neutrophil count are associated with frequent colds and differ between men and women. This implies that men are more susceptible to variations in antiviral immunity, whereas women are susceptible to the influence of inflammation, age and possibly hormones. Asthma patients report a greater burden of respiratory infections, which may be partly mediated by altered *TLR7/8* function. Further studies are needed to better understand interactions between sex, hormones, obesity, antiviral immunity and susceptibility to respiratory infections in asthma.

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