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Early View

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Title

***MYCOPLASMA PNEUMONIAE* CARRIAGE EVADES INDUCTION OF PROTECTIVE MUCOSAL ANTIBODIES**

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Antibodies against *M. pneumoniae,* the most common bacterial cause of pneumonia in hospitalized children, are able to prevent adhesion of *M. pneumoniae* to epithelial cells, but are only induced during infection and not during asymptomatic carriage in children.

Abstract

Mycoplasma pneumoniae is the most common bacterial cause of pneumonia in children hospitalized for community-acquired pneumonia. Prevention of infection by vaccines may be an important strategy in the presence of emerging macrolide resistant *M. pneumoniae.* However, knowledge of immune responses to *M. pneumoniae* is limited, complicating vaccine design. We therefore studied the antibody response during *M. pneumoniae* infection and asymptomatic carriage.

In a nested case-control study (n=80) of *M. pneumoniae* carriers and matched controls we observed that carriage by *M. pneumoniae* does not lead to a rise in either mucosal or systemic *M. pneumoniae*-specific antibodies, even after months of persistent carriage. We replicated this finding in a second cohort (n=69) and also found that during *M. pneumoniae* community-acquired pneumonia, mucosal levels of *M. pneumoniae*-specific IgA and IgG did increase significantly. *In vitro* adhesion assays revealed that high levels of *M. pneumoniae*-specific antibodies in nasal secretions of paediatric patients prevented the adhesion of *M. pneumoniae* to respiratory epithelial cells.

In conclusion, our study demonstrates that *M. pneumoniae*-specific mucosal antibodies protect against bacterial adhesion to respiratory epithelial cells and are induced only during *M. pneumoniae* infection and not during asymptomatic carriage. This is strikingly different from carriage with bacteria such as *Streptococcus pneumoniae* where mucosal antibodies are induced by bacterial carriage.

Introduction

Upper respiratory tract carriage is essential for the life cycle of many bacterial respiratory pathogens, such as *Streptococcus pneumoniae, Staphylococcus aureus,* and *Mycoplasma pneumoniae*. *M. pneumoniae* highly depends on the host for essential nutrients, such as amino acids, cholesterol and folic acid, since it has limited metabolic capacity because of its small genome [1]. *M. pneumoniae* therefore strongly adheres to upper respiratory tract epithelial cells using a specialized multi-protein adhesion complex consisting of adhesins such as P1 [2]. *M. pneumoniae* carriage can last for several months and is in itself asymptomatic [3-5]. However, *M. pneumoniae* carriage can lead to disease since carriage can precede infection of the host resulting in symptomatic respiratory infection by *M. pneumoniae*. Furthermore, *M. pneumoniae* carriage also forms a reservoir for horizontal transmission to other hosts that are in close contact. Horizontal transmission often occurs within families, children in long-term care facilities and among military recruits [6-11].

Pneumonia is a major cause of morbidity and mortality among children worldwide and *M. pneumoniae* is the most common cause of bacterial pneumonia in children [12-14]. Although *M. pneumoniae* generally causes moderately-severe cases of pneumonia, *M. pneumoniae* can lead to severe pneumonia and can be accompanied by severe extrapulmonary manifestations [15]. Treatment of *M. pneumoniae* pneumonia can be challenging in countries with high prevalence of macrolide-resistant *M. pneumoniae*, since other classes of that target cell-wall synthesis are not effective, since Mycoplasmas lack a cell wall. Furthermore, quinolones and tetracyclines, which like macrolides work on other bacterial targets, are not routinely recommended in children because of concerns about toxicity. [16]. A *M. pneumoniae* vaccine is currently unavailable, but could potentially prevent morbidity and hospitalization in children, especially where the incidence of (macrolide-resistant) *M. pneumoniae* is high, i.e. East-Asia [14, 17]. The development of such a vaccine is hampered by a lack of knowledge of

mucosal immunity to *M. pneumoniae* carriage, since carriage is essential for subsequent infection and horizontal transmission.

The asymptomatic presence of a potential pathogen in the upper respiratory tract can be divided into two distinct phases: firstly, acquisition of carriage is the introduction of a micro-organism in a host and secondly, carriage is the persistent presence of a micro-organism that has been successfully introduced in the upper respiratory tract. However, data on what immune responses are required to clear *M. pneumoniae* from the nasal mucosa during each phase are limited. In contrast, this has been well described for *S. pneumoniae: S. pneumoniae*-specific antibodies have been shown to be important to prevent acquisition of *S. pneumoniae* carriage, whereas Th17-mediated recruitment of phagocytes is known to be important for clearance of persistent *S. pneumoniae* carriage [18-21]. Studies in patients with invasive *M. pneumoniae* infections and murine carriage studies suggest there could be a role for *M. pneumoniae*-specific antibodies in *M. pneumoniae* carriage [22-25].

In the present study we therefore determined the presence and the function of *M. pneumoniae*-specific antibodies in the upper respiratory tract of children. We hypothesized that mucosal antibodies are induced during upper respiratory tract carriage of *M. pneumoniae* in children and that these antibodies can interfere with *M. pneumoniae'*s most important virulence factor: attachment to the respiratory epithelium.

Methods

Cohort 1: *M. pneumoniae* carriage in asymptomatic children

We performed a nested case-control study within a previously published cohort of asymptomatic children (n=405) who underwent elective surgery [3]. We considered 50% higher levels of mucosal *M. pneumoniae*-specific IgA in *M. pneumoniae* carriers compared to non-carrier controls to be a biologically relevant difference. Based on a sample size calculation with alpha set at 5%, desired power at 90%, and

expected loss of samples due to insufficient quantity or quality of available nasal lavage samples of 10%, we selected 40 *M. pneumoniae* carriers and 40 non-carrier controls. Cases of *M. pneumoniae* carriage were defined as those children without current or recent symptoms of respiratory tract disease who had a *M. pneumoniae* quantitative Polymerase Chain Reaction (qPCR) positive upper respiratory tract sample, i.e. pharyngeal swab and/or nasal lavage sample. All cases, i.e. *M. pneumoniae* carriers, were matched 1:1 based on age (less than 1 year age difference) and on date of inclusion to account for seasonality (less than 60 days apart) to non-carriers controls, defined as children without respiratory symptoms who were *M. pneumoniae* qPCR-negative. Upper respiratory tract samples were taken at inclusion and monthly follow-up samples were available for a subset of patients. Upper respiratory tract samples were analysed for the presence of *M. pneumoniae*-specific and total IgA, IgM and IgG antibody levels.

Cohort 2: *M. pneumoniae* carriage and respiratory tract infection

We analysed all available pharyngeal swab samples available from a prospective observational longitudinal cohort study [26] that included asymptomatic *M. pneumoniae* carriers, non-carrier controls, children with *M. pneumoniae* community-acquired pneumonia (*Mp*-CAP) and non-*M. pneumoniae*-CAP (Non-*Mp*-CAP). CAP was defined as a clinical diagnosis with fever >38.5°C and tachypnea according to BTS guidelines and children aged 3–18 years were included [26]. *M. pneumoniae* CAP patients were positive for pharyngeal swab *M. pneumoniae* qPCR and *M. pneumoniae*-specific Antibody Secreting Cells (ASCs) in peripheral blood, whereas non-*M. pneumoniae*-CAP patients were negative for both. Pharyngeal swab samples were taken at inclusion, visit 2 (2 weeks to 2 months post onset symptoms) and visit 3 (2–6 months post onset symptoms). Samples were measured for *M. pneumoniae*-specific and total mucosal and systemic antibody levels.

Detection and function of nasal lavage *M. pneumoniae*-specific and total Immunoglobulin

Levels of total and *M. pneumoniae*-specific IgA, IgM and IgG were measured using an in house ELISA assay (Supplemental information). Serum samples were considered seropositive for *M. pneumoniae* when measured specific antibody levels exceeded that of a reference control of pooled sera of patients without *M. pneumoniae* infection (Virion\Serion GmbH, Wurzburg, Germany). To assess a potential inhibitory effect of nasal lavage antibodies on *M. pneumoniae* epithelial adhesion, A549 respiratory epithelial cells were incubated with diluted nasal lavage samples and subsequently, 10^9 Colony Forming Units (CFUs) per well of *M. pneumoniae* strain M129 were added. After 4h incubation, unbound bacteria were washed off and adhering bacteria counted on PPLO agar plates (Supplemental information).

Statistical analysis

GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA), G*Power 3.1.9.7 (Universität Düsseldorf, Düsseldorf, Germany) and IBM SPSS Statistics 25 (IBM, Armonk, USA) were used for statistical analysis. Student's t-test and Mann-Whitney U test were used to compare groups. Pearson correlation coefficient and Spearman's rank correlation coefficient were used for statistical significance testing and quantifying the magnitude of correlations. Paired T-test and McNemar's test were used to compare multiple samples from the same patient and to compare matched patients. Tests are two-sided unless otherwise reported and alpha was set at 0.05.

Results

Asymptomatic carriage with *M. pneumoniae* does not increase mucosal *M. pneumoniae*-specific antibody levels.

To study the role of *M. pneumoniae*-specific antibodies in the upper respiratory tract we first investigated if mucosal *M. pneumoniae*-specific antibodies are induced in asymptomatic children during

M. pneumoniae carriage. We therefore performed a nested case-control study within cohort 1 and compared *M. pneumoniae* carriers to non-carrier controls (Table 1). Assessment of *M. pneumoniae*-specific IgA in nasal lavages revealed that *M*. *pneumoniae*-specific IgA levels were not significantly different between *M. pneumoniae* carriers and non-carrier controls (p=0.230, Figure 1A). Similar observations were made for *M. pneumoniae*-specific IgG (p=0.351) and IgM (p=0.385, Figure 1B and 1C). Importantly, nasal lavage *M. pneumoniae*-specific IgM levels were below the detection limit in 45.6% (n=36/79) of study subjects. Since *M. pneumoniae* carriers could have acquired *M. pneumoniae* too recently to have developed a mucosal antibody response [18, 27], we analysed monthly follow-up nasal lavage samples of *M. pneumoniae* carriers. No increase in levels of *M. pneumoniae*-specific IgA or IgG in nasal lavage was detected over the course of two months (Figure 1D and 1E). Furthermore, mean levels of total IgA, IgG and IgM in nasal lavage were not different between *M. pneumoniae* carriers and non-carrier controls (Suppl. Figure 1). On average nasal lavage levels of total IgA were over 70 times higher than total IgG levels, which in turn were 10-fold higher than total IgM levels (Suppl. Figure 1). In addition to mucosal antibody levels we also evaluated the presence of systemic *M. pneumoniae*-specific antibody levels in response to *M. pneumoniae* carriage, since systemic antibody responses to respiratory tract carriage have been shown for *S. pneumoniae* and *S. aureus* [18, 28]. Again, no significant difference in the prevalence of serum *M. pneumoniae*-specific IgM (19% vs. 11%), serum *M. pneumoniae*-specific IgA (0% vs. 2.8%) or serum *M. pneumoniae*-specific IgG (25% vs. 17%, Table 1) was detected between *M. pneumoniae* carriers and non-carrier controls at inclusion. Since our findings on *M. pneumoniae* are in contrast to data on *S. pneumoniae*, which does induce mucosal antibody production during carriage [18], we measured *M. pneumoniae*-specific immunoglobulins in nasal lavages of asymptomatic children from an independent cohort (cohort 2) [3, 26]. Within this second cohort we again compared *M. pneumoniae* carriers and non-carrier controls (n=29). Similar to our observations in cohort 1, both *M.*

pneumoniae-specific IgA (Suppl. Figure 2A) and *M. pneumoniae*-specific IgG (Suppl. Figure 2B) in nasal lavages were not significantly different between *M. pneumoniae* carriers and non-carrier controls. Taken together our data show that asymptomatic carriage of *M. pneumoniae* in the upper respiratory tract does neither induce a mucosal nor a systemic antibody response.

Table 1. *M. pneumoniae* carriers and age- and season-matched non-carrier controls from cohort 1

	Non-carrier controls (n=40)	*M. pneumoniae* carriers (n=40)	*p*-value
Sex Male Female	 25 15	 25 15	ns[a]
Age[b]	4,54 (0,4-16,0)	4,57 (0,4-16,8)	ns[c]
Year 2009 2010 2011	 11 16 13	 11 16 13	ns
Positive *M. pneumoniae*-specific IgM in serum[d]	11% (95%CI: 3-22%) (4/36)	19% (95%CI: 6-34%) (6/32)	ns[e]
Positive *M. pneumoniae*-specific IgA in serum[d]	2.8% (95%CI: 0-8%) (1/36)	0% (95%CI: 0-0%) (0/32)	ns[f]
Positive *M. pneumoniae*-specific IgG in serum[d]	17% (95%CI: 6-31%) (6/36)	25% (95%CI: 12-42%) (8/32)	ns[e]
URT *M. pneumoniae* load[g]	0 (0-0)	2653 (424-23075)	<0.001[h]

a) Related samples McNemar test, b) mean age (range) in years, c) paired t-test, d) percentage positive, e/f) Related Samples McNemar test, g) Median (Q1-Q3) upper respiratory tract *M. pneumoniae* load, copy numbers/mL, h) Related-samples Wilcoxon Signed Rank Test.

Mucosal specific antibody levels increase upon symptomatic *M. pneumoniae* infection

Next we assessed if during lower respiratory tract infection *M. pneumoniae* does induce the production of *M. pneumoniae*-specific antibodies in the upper respiratory tract, as this has been shown in experimental *M. pneumoniae* infection [25]. Hereto we examined nasal lavage samples from cohort 2, where *M. pneumoniae*-CAP patients were distinguished from non-*M. pneumoniae* CAP patients by the presence of *M. pneumoniae*-specific Antibody Secreting Cells in peripheral blood [26](Table 2). We compared *M. pneumoniae*-specific IgA and IgG in nasal lavage samples taken at least 10 days after the first clinical symptoms with antibody levels in nasal lavage samples from the same patients taken early during the infection (<10 days post onset of symptoms). We observed that *M. pneumoniae*-CAP patients developed mucosal *M. pneumoniae*-specific antibodies 10 days after onset of symptoms, consistent with the kinetics of antibody responses to other pathogens [29]. A significant increase in levels of both *M. pneumoniae*-specific IgA (geometric mean 3.66 vs. 32.8 AU/mL, p=0.033) as well as *M. pneumoniae*-specific IgG (geometric mean 2.35 vs. 26.6 AU/mL, p=0.038) was measured (Figure 2A and 2B). Mucosal antibody levels remained elevated in follow-up samples taken at 1-2 months post onset of symptoms. To confirm that these responses were specific to *M. pneumoniae* infection we assessed mucosal specific antibody levels of non-*M. pneumoniae* CAP patients. We found that both *M. pneumoniae*-specific IgA and specific IgG were significantly higher in nasal lavages of *M. pneumoniae* CAP patients (n=15) compared to non-*M. pneumoniae* CAP (n=18; Figure 2A and 2B). We made similar observations for systemic antibody responses, i.e. *M. pneumoniae*-specific IgA and IgG in serum (data not shown).

Mucosal *M. pneumoniae*-specific antibodies correlate with *M. pneumoniae* load in the upper respiratory tract of children with *M. pneumoniae* CAP.

Although levels of mucosal *M. pneumoniae*-specific IgA were elevated in *M. pneumoniae* CAP patients following infection (>10 days post onset of symptoms), there was considerable variation in specific IgA levels between patients. We hypothesized that the height of the antibody response would be determined by the level of immune stimulation, which in turn would depend on the bacterial load. Indeed, *M. pneumoniae*-specific IgA levels in nasal lavages positively correlated (Spearman's rho=0.589, 95%CI: 0.133-0.850, p=0.021) with bacterial load in the upper respiratory tract as determined by *M. pneumoniae* copy numbers (Figure 2C). We observed that the IgG response followed a similar pattern as there was a strong correlation between mucosal *M. pneumoniae*-specific IgA and *M. pneumoniae*-specific IgG in *M. pneumoniae*-infected patients (Pearson's r=0.879, 95%CI: 0.756-0.946, p<0.001, Supplementary figure 2C).

In summary, in contrast to *M. pneumoniae* carriage, symptomatic lower respiratory tract infection by *M. pneumoniae* clearly induced the production of mucosal *M. pneumoniae*-specific IgA and IgG, levels of which showed a moderately strong correlation with *M. pneumoniae* load in the upper respiratory tract.

Table 2. Cohort 2 (Non-) *M. pneumoniae* community acquired pneumonia patients

	Non-*Mp* CAP controls[a] (n=22)	*M. pneumoniae* CAP[a] (n=18)	*p*-value
Sex			
Male	13	11	ns[b]
Female	9	7	
Age[c]	6,61 (5.4-8,0)	8,89 (7.9-9.6)	.033[d]
Year			
2016	20	15	ns[e]
2017	2	3	
URT *M. pneumoniae* load[f]	0 (0-0)	16799 (2215-82852)	.001[g]

M. pneumoniae-specific IgM ASC number[h]	n/a	185 (47-775)	n/a

a) *M. pneumoniae* pneumonia is defined as *M. pneumoniae* qPCR positive on URT sample and *M. pneumoniae*-specific IgM Antibody Secreting Cell positive in peripheral blood. Non-*M. pneumoniae* pneumonia patients were both *M. pneumoniae* qPCR and ASC negative, b) chi-squared test, c) Mean age (95%CI) in years, d) t-test with Welch's correction, e) chi-square test, f) median (Q1-Q3) upper respiratory tract *M. pneumoniae* load, copy numbers/mL, g) independent samples Mann-Whitney U-test, h) median (range) spots/10^6 PBMCs

Mucosal *M. pneumoniae*-specific antibodies are directed against components of the *M. pneumoniae* attachment organelle.

We then assessed against which *M. pneumoniae* epitopes the mucosal antibody response could be directed, since this information would be essential for the design of a *M. pneumoniae* vaccine. We hypothesized that the antibody response would for a substantial part be directed to *M. pneumoniae'*s attachment organelle and therefore focused on immunodominant *M. pneumoniae* proteins that are known to be involved in bacterial adhesion to the respiratory epithelium, i.e. P1, P30 and P116 [30, 31]. When comparing *M. pneumoniae*-CAP patient nasal lavages taken early versus those that were collected later during infection, we found that mucosal antibodies against P1, P30 as well as P116 were being induced in the upper respiratory tract during *M. pneumoniae* infection (Table 3). Antibody responses against these adhesins were absent in non-*M. pneumoniae* CAP patients, indicating that these antibodies were specific to infection with *M. pneumoniae* (supplementary table 2). IgA and IgG specific to the adhesion proteins were also present in *M. pneumoniae* carriers and non-carriers, albeit at lower levels than in *M. pneumoniae* CAP patients (Supplementary Table 2).

Table 3. Mucosal antibodies induced during *M. pneumoniae* CAP are directed against *M. pneumoniae* adhesin proteins

IgA	#1		#2		#3		#4		#5	
Patients:	<10d	>10d	<10d	>10d	<10d	>10d	<10d	>10d	<10d	>10d
P1$_{1287-1518}$	-	+++	-	+	++	+	-	+	-	++
P1$_{694-787}$	-	+++	-	-	++	-	-	-	-	+
P30$_{17-71}$	-	-	-	-	++	-	-	-	-	-
P30$_{98-274}$	+	++	+	+	+++	+	+	+	+	+
P116	+	+	-	+	+++	+	-	+	-	+

IgG	Non-carriers					*M. pneumoniae* carriers				
Patients:	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**
P1$_{1287-1518}$	-	-	+	+	-	-	+	+	-	+
P1$_{694-787}$	-	-	-	++	++	-	-	-	-	+++
P30$_{17-71}$	-	-	++	+	+	-	-	-	-	+
P30$_{98-274}$	+	+	+++	++	+	+	+	-	+	++
P116	-	+	+++	+	++	-	+	+	-	+++

Nasal lavage specific IgA and IgG antibody levels against *M. pneumoniae* protein fragments P1, P30 and P116 in individual *M. pneumoniae* CAP patients at both early (<10 days post OS) and later (>10 days post OS) time points. Below detection limit: (-), 1-1.5x detection limit: (+), 1.5-2x detection limit: (++), >2x detection limit: (+++)

Mucosal *M. pneumoniae*-specific antibodies prevent *M. pneumoniae* adhesion to respiratory epithelial cells.

The finding that mucosal specific IgA and IgG antibodies were not elevated in *M. pneumoniae* carriers, raised the question if these antibodies have any protective role [32]. Especially since we found that mucosal antibodies are directed against *M. pneumoniae* proteins P1, P30 and P116, which are involved in attachment to epithelium. We thus assessed the ability of *M. pneumoniae*-specific antibodies from nasal lavages of children to prevent adhesion of *M. pneumoniae* to respiratory epithelium in an *in vitro* adhesion assay. We found that nasal lavages containing low levels of *M. pneumoniae*-specific IgA and IgG did not block the adhesion of *M. pneumoniae* to A549 cells, whereas nasal lavages containing high levels did prevent *M. pneumoniae* adhesion (Figure 3A and 3B). This interference with bacterial

adhesion was more strongly associated with *M. pneumoniae*-specific IgA levels (Spearman's rho=-0.544 (95%CI: -0.806 – -0.197), *p*=0.001) than with specific IgG levels (Spearman's rho=-0.358 (95%CI: -0.690 – 0.050), *p*=0.041). Importantly, interference with bacterial adhesion was not correlated with levels of total IgA, total IgG or levels of total protein in nasal lavages (data not shown), which excluded that these factors confounded our observation that *M. pneumoniae*-specific antibodies prevented *M. pneumoniae* adhesion to respiratory epithelial cells.

Together these results show that *M. pneumoniae*-specific antibodies present in the upper respiratory tract of children can prevent *M. pneumoniae* adhesion to respiratory epithelial cells.

Discussion

Our study provides novel insights into humoral immunity to *M. pneumoniae* carriage in children. These data are the basis for the design of a *M. pneumoniae* vaccine that interferes with carriage and subsequently *M. pneumoniae* infection and transmission, ultimately lowering the burden of pneumonia on child health [33]. Furthermore, our study increases our understanding of the dynamics of upper respiratory carriage by respiratory pathogens by showing the differences in mucosal antibody response between *M. pneumoniae* and other pathogens such as *S. pneumoniae* and *S. aureus.*

Surprisingly, mean levels of mucosal and systemic *M. pneumoniae*-specific and total antibodies were not elevated in children carrying *M. pneumoniae* when compared to non-carrier controls, even after two months of persistent carriage, which should be sufficient for the induction of an antibody response. Several studies have shown that systemic *M. pneumoniae*-specific immunoglobulins can be detected in *M. pneumoniae* carriers and non-carrier controls suggesting that these are the result of recent infection in those patients [3, 26]. The absence of an antibody response to *M. pneumoniae* carriage is in contrast with findings on *S. pneumoniae* and *S. aureus* carriage, where carriage with these pathogens has been shown to elevate specific, mucosal or systemic IgA and IgG [18, 28]. It seems

unlikely that our results can explained by an inability to mount a mature antibody response, since the majority of the children in our cohorts were over four years old and none had underlying diseases. The absence of antibody induction in response to *M. pneumoniae* carriage is therefore probably related to bacteria-intrinsic factors and/or differences in bacterial behaviour compared to *S. pneumoniae* and *S. aureus.* Indeed, during carriage *M. pneumoniae* is thought to rest on the upper respiratory tract epithelium [25], whereas *S. pneumoniae* can cross the epithelium into the lamina propria even during carriage [34]. These so-called micro-invasions of *S. pneumoniae* and subsequent activation of the immune system could lead to induction of an antibody response during *S. pneumoniae* carriage. Another explanation for the absence of an antibody response to *M. pneumoniae* carriage could be that *M. pneumoniae* cell membranes contain less immunogenic lipoproteins or glycolipids compared to *S. pneumoniae* and *S. aureus,* resulting in less immune activation. Furthermore, *M. pneumoniae* could possibly even actively downregulate the host immune response.

We show that mucosal *M. pneumoniae*-specific antibodies are directed against key proteins of *M. pneumoniae*'s attachment organelle, i.e. P1, P30 and P116, which is in line with previous studies that associated the presence of antibodies in the upper respiratory tract with protection against *M. pneumoniae.* Furthermore, our *in vitro* data indicate that when present at high levels, *M. pneumoniae*-specific antibodies in nasal lavages of children inhibit *M. pneumoniae* adherence to respiratory epithelial cells. Sufficiently high antibody levels are likely to be present in the upper respiratory tract of children, since in our *in vitro* adhesion assay we used 10-fold diluted samples, that still efficiently blocked bacterial adhesion. In our adhesion assay we did not evaluate if specific antibodies could clear existing carriage of *M. pneumoniae,* since in our in vitro assay *M. pneumoniae*-specific antibodies were added before bacterial suspensions. However, in a murine model of *M. pneumoniae* post-infectious carriage, we previously observed that bacterial loads were higher in the upper respiratory tract of B cell deficient mice compared to wild-type mice, suggesting specific antibodies can clear existing carriage.

Interestingly, passive immunization with serum from infected wild-type did not rescue clearance of *M. pneumoniae* from the upper respiratory tract in B cell deficient mice, indicating that mucosal instead of systemic antibodies are essential to clear *M. pneumoniae* carriage [25].

M. pneumoniae-specific mucosal IgA showed a stronger correlation with ability to block bacterial adhesion compared to *M*. *pneumoniae*-specific mucosal IgG. This does not preclude a role for IgG in blocking bacterial adhesion, but rather suggests that specific IgA is more important. Co-linearity between *M. pneumoniae*-specific IgA and IgG levels prevented us from dissecting the relative importance of both isotypes. Importantly, *M. pneumoniae*-specific IgG could additionally contribute to immunity to *M. pneumoniae* carriage by activating complement and opsonizing bacteria to enhance phagocytosis [35].

In our cohorts of *M. pneumoniae* carriers only one patient had strongly elevated *M. pneumoniae*-specific antibodies. Given that *M. pneumoniae* infection results in robust induction of mucosal antibodies that remain in the upper respiratory tract for months, this observation suggests that primary *M. pneumoniae* carriage is much more prevalent than post-infectious carriage. Furthermore, our finding also suggests that immunity after *M. pneumoniae* infection might offer protection against post-infectious *M. pneumoniae* carriage. This is supported by a study in human volunteers where pre-existing levels of respiratory tract *M. pneumoniae*-specific IgA correlated with protection against experimental infection [32].

Our study has several strengths and limitations. An important strength of our study is that both cohorts of patients contain data for multiple time-points on *M. pneumoniae* carriers and *M. pneumoniae* CAP patients. Furthermore, both cohorts contain relevant matched control groups of non-carriers and non-*M. pneumoniae* CAP patients. A limitation is that due to the design of the study we were unable to evaluate if mucosal *M. pneumoniae*-specific antibodies are able to protect children against a subsequent episode of pneumonia.

In summary, in this study we show that only during symptomatic infection and not during asymptomatic carriage with *M. pneumoniae*, there is an induction of mucosal and systemic *M. pneumoniae*-specific antibodies. This is a striking disparity with immunity to other respiratory pathogens such as *S. pneumoniae* and *S. aureus,* suggesting that the immune response to microbial carriage in the respiratory tract differs between pathogens. Mucosal *M. pneumoniae*-specific antibodies effectively prevented adhesion to respiratory epithelial cells. Considering that *M. pneumoniae* largely depends on host nutrients, maintaining adherence to the upper respiratory tract epithelium by preventing mucosal antibody induction could be essential for *M. pneumoniae* survival and transmission to other hosts.

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Author contributions

RdG, AvR and WU conceived the project, interpreted data and drafted the manuscript. RdG, SE, PMS, AP, TH conducted and analysed experiments. PMS and ES collected patient samples. PMS and LV interpreted data. All authors critically reviewed the manuscript and made intellectual contributions to the study.

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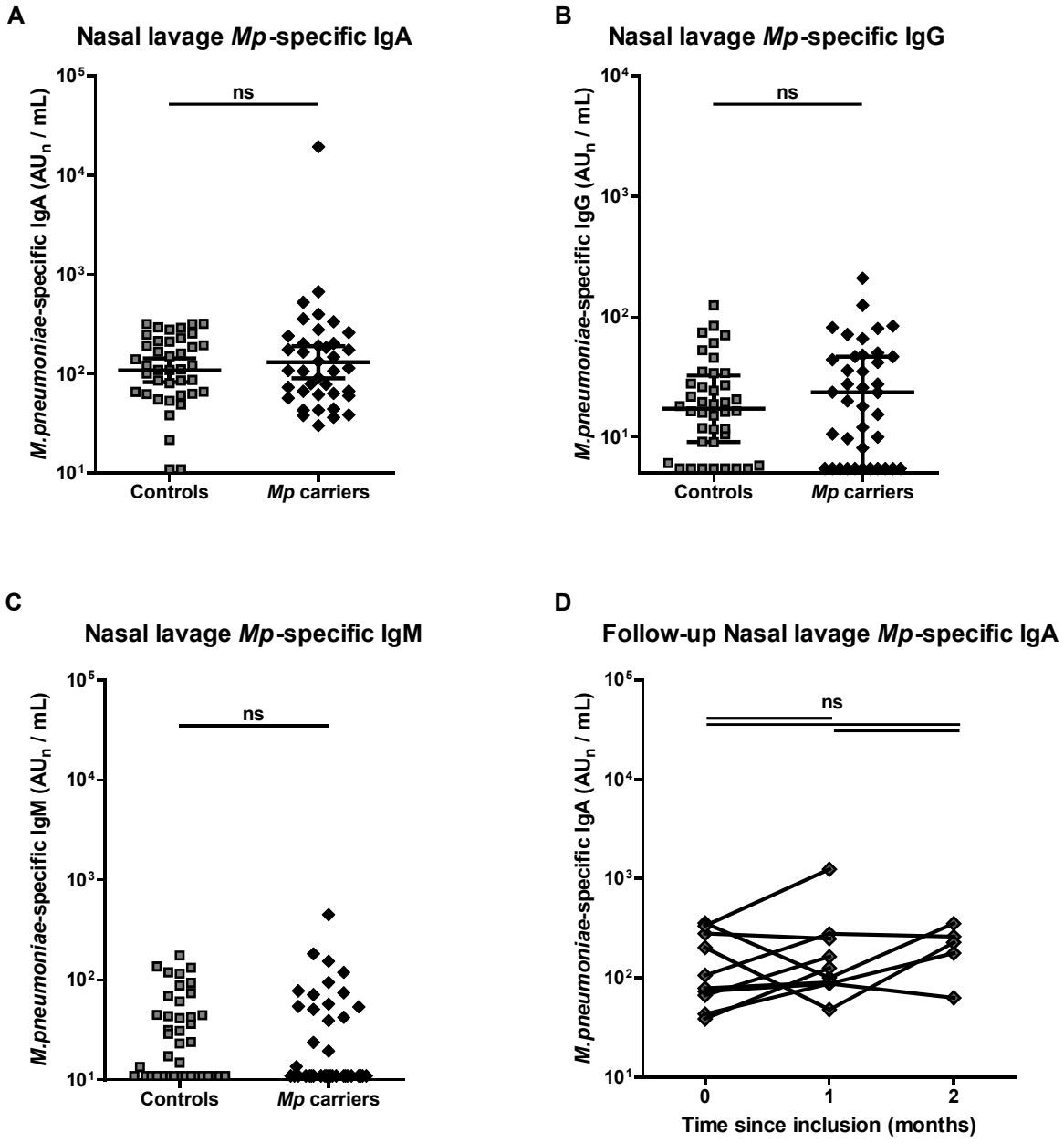
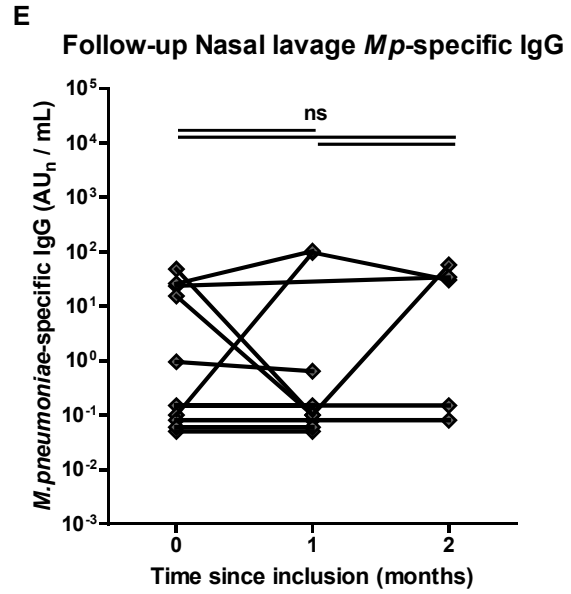
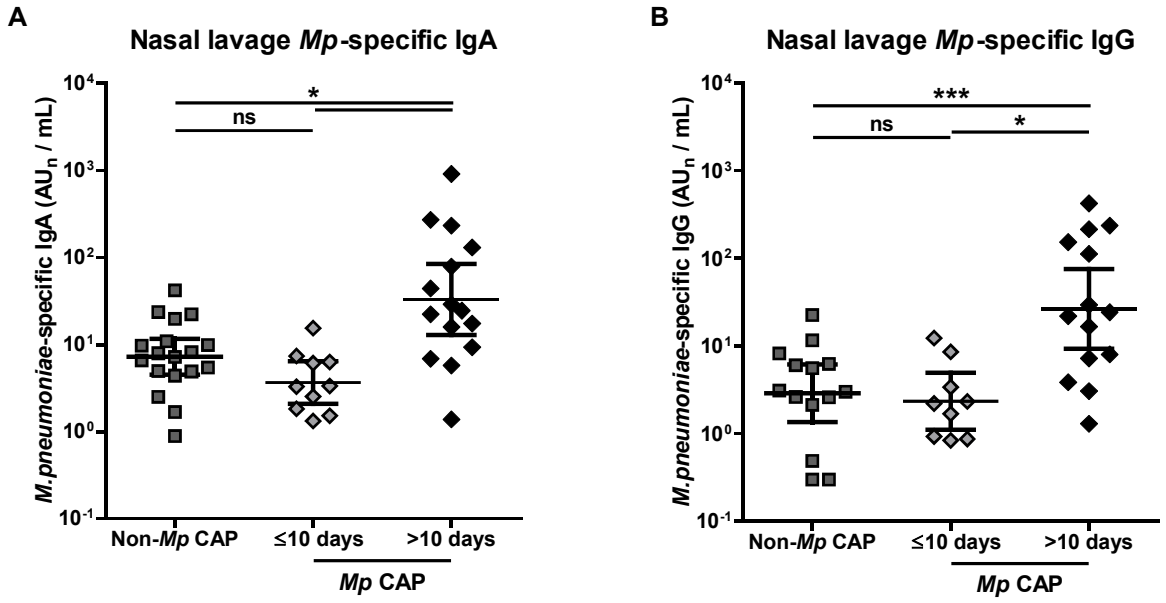
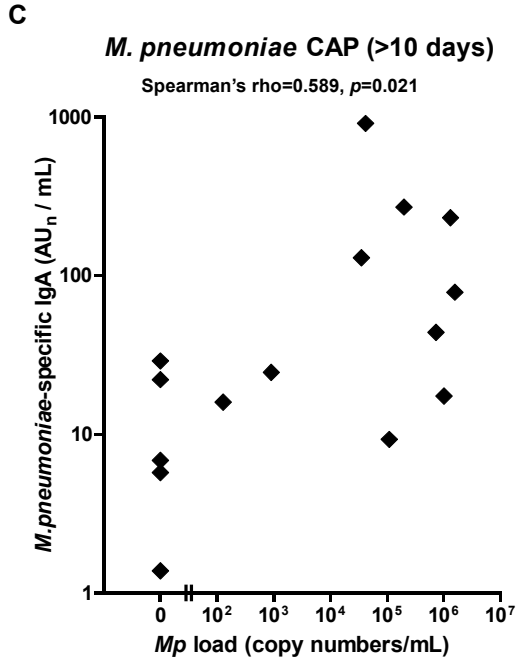


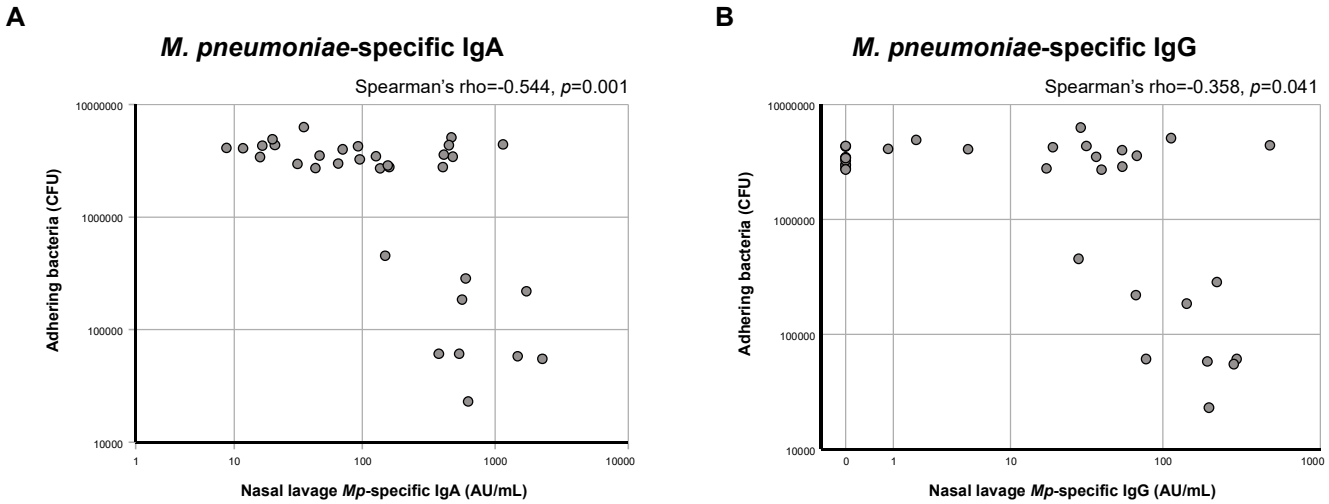


***M. pneumoniae* upper respiratory tract colonization does not induce a mucosal *M. pneumoniae*-specific antibody response.**
(A-C) Cohort 1 nasal lavage samples of age- and season-matched *M. pneumoniae* carriers (n=39/40) and healthy controls (n=40/40) were analyzed by ELISA for levels of (A) *M. pneumoniae-specific* IgA (B) *M. pneumoniae-specific* IgG and (C) *M. pneumoniae*-specific IgM. (D-E) *M. pneumoniae*-specific IgA and IgG levels in nasal lavage of *M. pneumoniae* carriers (n=10) over the course of 2-months. Lines and error bars represent geometric means with 95% CI. ns for non-significant (Paired T-test).





M. pneumoniae infection induces an increase in mucosal _M. pneumoniae_-specific antibody levels
(A-B) Cohort 2 nasal lavage samples of *M. pneumoniae* CAP (n=10-15/18) and non-*M. pneumoniae* CAP patients (n=18/22) were analyzed by ELISA for levels of (A) *M. pneumoniae*-specific IgA and (B) *M. pneumoniae*-specific IgG. Lines and error bars represent geometric means with 95% CI. (C) Genomic copy numbers of *M. pneumoniae* in the URT as determined by qPCR in relation to URT levels of *M. pneumoniae*-specific IgA in *M. pneumoniae* CAP patients at >10 days post OS (15/18). *p<0.05, ***p<0.001, ns for non-significant (Paired T-test for ≤10 days vs. > 10 days and unpaired T-test for other comparisons).



***M. pneumoniae*-specific antibodies in nasal secretions block the adhesion of *M. pneumoniae* to respiratory epithelial cells.**
Adhesion of *M. pneumoniae* to A549 epithelial cells in vitro when cultured in the presence or absence of *M. pneumoniae*-specific antibodies containing nasal lavages from both cohort 1 and 2 (n=33). Number of adhering bacteria (expressed as CFU) in relation to (A) *M. pneumoniae*-specific IgA levels or (B) *M. pneumoniae*- specific IgG levels in nasal secretions (Spearman's rank correlation coefficient).

Supplemental information

Detection of nasal lavage *M. pneumoniae*-specific and total Immunoglobulin levels

Maxisorp 96-wells plates (Corning Costar, Corning, NY, USA) were coated overnight with *M. pneumoniae* M129 (ATCC 29342) cell lysate for the detection of *M. pneumoniae*-specific antibodies and coated with an anti-human universal immunoglobulin (SouthernBiotech, Birmingham, AL, USA) for the detection of total antibodies levels. After blocking with PBS/BSA, nasal lavage samples diluted in 0,1% BSA/PBS were added and incubated overnight. Standards for *M. pneumoniae*-specific antibodies were created by making a twofold dilution series of *M. pneumoniae*-IgA, IgG and IgM positive controls (Virion\Serion GmbH, Wurzburg, Germany). Undiluted positive controls were set to 100 Arbitrary Units. To create standards for total IgA, IgG and IgM we used purified IgA (InvivoGen, San Diego, CA, USA), purified IgG and purified IgM (Sigma-Aldrich, St. Louis, MO, USA). For the detection of specific isotypes we used goat anti-human IgA-Alkaline Phosphatase, goat anti-human IgM Horse radish peroxidase and goat anti-human IgG-Alkaline phosphatase (Sigma). 3,3',5,5'-Tetramethylbenzidine (TMB) or para-Nitrophenylphosphate (Sigma-Aldrich) was used as a substrate. Optical density was measured at 450 nm or 405 nm respectively using a microplate reader (SpectraMax iD3, Molecular Devices, San José, USA). Antibody levels were normalized to nasal lavage total protein to account for sampling variation. Total protein levels were measured using CBQCA Protein Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA).

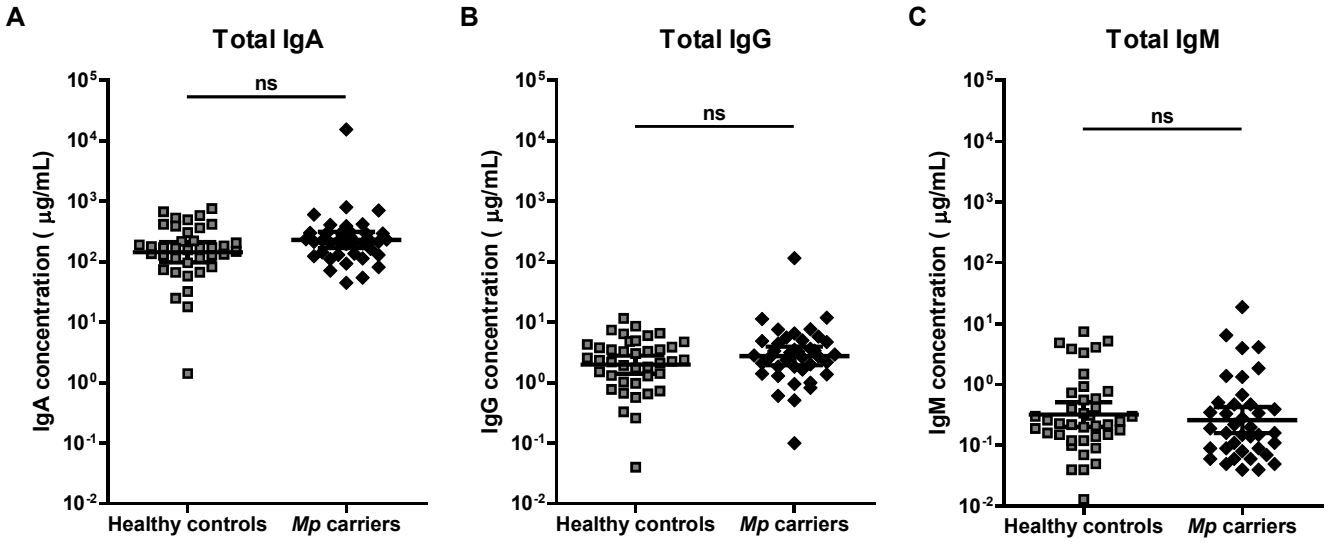
Functional antibody assay

To assess a potential inhibitory effect of nasal lavage antibodies on *M. pneumoniae* epithelial adhesion, A549 respiratory epithelial cells were cultured in 24-wells plates until confluent (Greiner Bio-one International, GmbH, Kremsmünster, Austria). Medium was replaced with nasal lavage which was

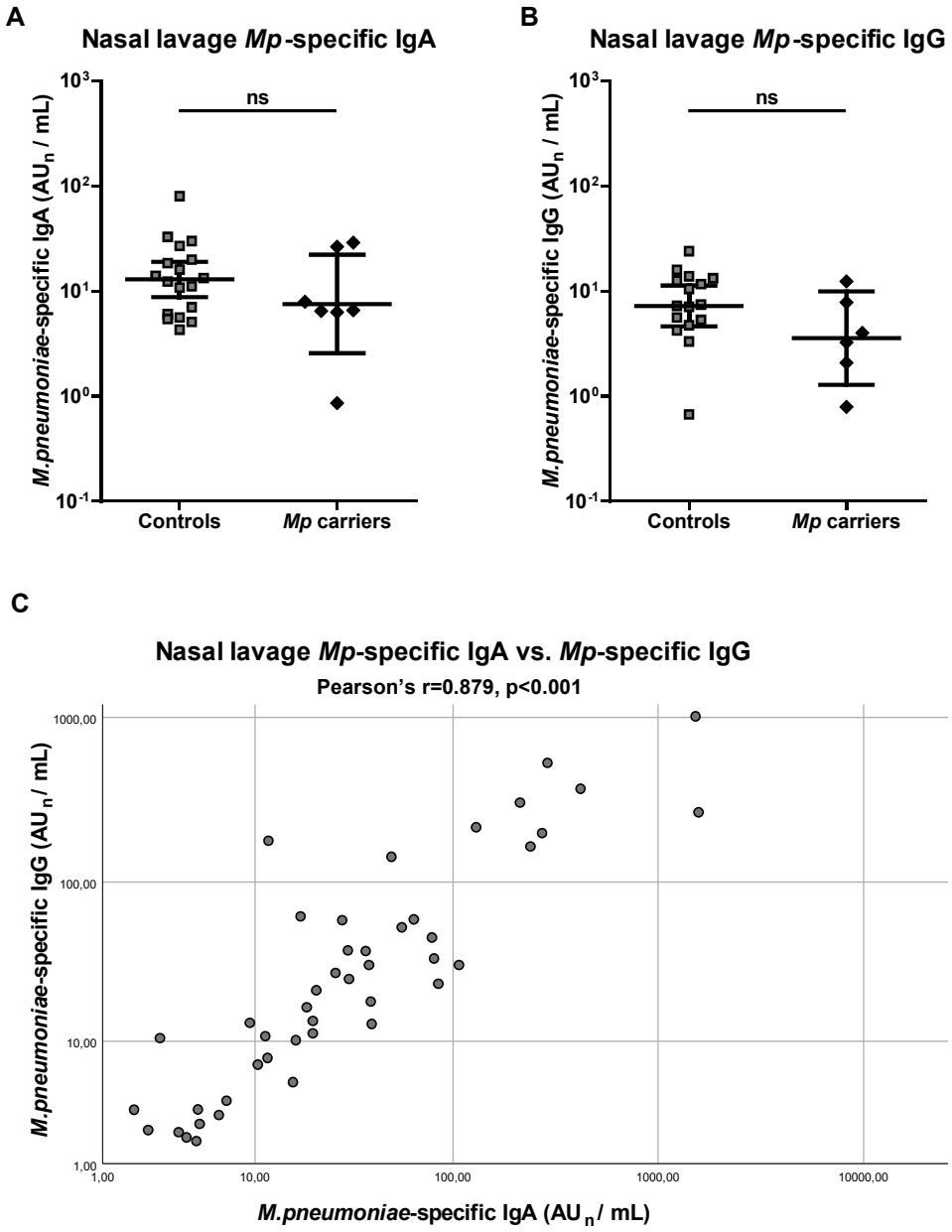
diluted 10-fold in RPMI 1640 containing L-glutamine (Gibco, Carlsbad, USA) and subsequently, 10^9 Colony Forming Units (CFUs) per well of *M. pneumoniae* strain M129 were added. After 4h incubation, unbound bacteria were washed off and epithelial cells were then lysed by passing through a 25G needle in deionized water leaving adhering bacteria intact. The resulting suspension was plated out on PPLO agar plates and CFUs were counted.

Additional information on statistical analysis

Nasal lavage *M. pneumoniae*-specific and total antibody levels were measured in quadruplicate, whereas other measurements were performed in duplicate. Technical duplicates were averaged before data analysis. *M. pneumoniae*-specific and total antibody levels were assumed to be log-normally distributed and log(10)-transformed before statistical hypothesis testing. *M. pneumoniae* bacterial load, number of Antibody Secreting Cells and number of adhering bacteria in our adhesion assay were not assumed to be (log-)normally distributed and analysed using non-parametric tests.



***M. pneumoniae* upper respiratory tract colonization does not increase total antibody levels**
(A-C) Cohort 1 nasal lavage samples of age- and season-matched *M. pneumoniae* carriers (n=39/40) and healthy controls (n=40/40) (A) Levels of total IgA (B) Levels of total IgG (C) Levels of total IgM. Lines and error bars represent geometric means with 95% CI. Ns for non-significant (Paired T-test).



***M. pneumoniae*-specific antibody levels are not different between cohort 2 *M. pneumoniae* carriers and healthy controls.**
(A-B) Cohort 2 nasal lavage samples of *M. pneumoniae* carriers (n=6/8) and healthy controls (n=16-18/21) (A) Levels of *M. pneumoniae*-specific IgA (B) Levels of *M. pneumoniae*-specific IgG. (C) Nasal lavage levels of specific IgA and IgG of cohort 2 *M. pneumoniae* CAP patients. (n=45) Lines and error bars represent geometric means with 95% CI. Ns for non-significant (T-test).

	Non-carrier controls (n=21)	*M. pneumoniae* carriers[a] (n=8)	*p*-value
Sex Male Female	 15 6	 7 1	Ns[b]
Age[c]	6,2 (3,2-14,1)	5,0 (2,7-7,6)	Ns[d]
Year 2016 2017	 20 1	 6 2	Ns[b]
URT *M. pneumoniae* load[e]	0 (0-0)	663 (386-1271)	<0.001[f]

Supplemental Table 1. Descriptive statistics of cohort 2: Controls vs. *M. pneumoniae* carriers

a) *M. pneumoniae* carriers defined as *M. pneumoniae* qPCR positive on URT sample, b) chi-squared test, c) Mean age (range) in years, d) t-test with Welch's correction, e) median (Q1-Q3) upper respiratory tract *M. pneumoniae* load, copy numbers/mL, f) independent samples Mann-Whitney U-test

A. Mucosal IgA antibodies against *M. pneumoniae* proteins

	Non-carriers					*M. pneumoniae* carriers				
Patients:	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**
P1$_{1287-1518}$	-	-	-	-	-	-	-	-	-	+
P1$_{694-787}$	-	+	+	+	+	-	++	+	+	+
P30$_{17-71}$	-	-	-	-	-	-	+	-	-	-
P30$_{98-274}$	-	-	-	+	-	-	-	+	-	-
P116	-	+	+	+	+	+	+	+	-	+

B. Mucosal IgG antibodies against *M. pneumoniae* proteins

	Non-carriers					*M. pneumoniae* carriers					Non-*M. pneumoniae* CAP				
Patients:	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**	**#12**	**#13**	**#14**	**#15**
P1$_{1287-1518}$	-	-	+	+	-	-	+	+	-	+	-	-	-	-	-
P1$_{694-787}$	-	-	-	++	++	-	-	-	-	+++	-	-	-	-	-
P30$_{17-71}$	-	-	++	+	+	-	-	-	-	+	-	-	-	-	-
P30$_{98-274}$	+	+	+++	++	+	+	+	-	+	++	-	+	+	+	+
P116	-	+	+++	+	++	-	+	+	-	+++	-	-	+	-	+

Supplemental Table 2 Nasal lavage specific IgA and IgG antibody levels against *M. pneumoniae* protein fragments P1, P30 and P116 in individual *M. pneumoniae* carriers and non-carriers and non-*M. pneumoniae* CAP patients. Below detection limit: (-), 1-1.5x detection limit: (+), 1.5-2x detection limit: (++), >2x detection limit: (+++)