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# Serum antibody response to *Moraxella catarrhalis* proteins OMP CD, OppA, Msp22, Hag, and PilA2 after nasopharyngeal colonization and acute otitis media in children

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## Abstract

**Background—**There is no licensed vaccine for *Moraxella catarrhalis* (*Mcat*), which is a prominent bacterium causing acute otitis media (AOM) in children and lower respiratory tract infections in adults. Nasopharyngeal (NP) colonization caused by respiratory bacteria results in natural immunization of the host. To identify *Mcat* antigens as vaccine candidates, we evaluated the development of naturally induced antibodies to 5 *Mcat* surface proteins in children 6–30 months of age during *Mcat* NP colonization and AOM.

**Methods—**Human serum IgG against the recombinant *Mcat* proteins, outer membrane protein (OMP) CD, oligopeptide permease (Opp)A, hemagglutinin (Hag), Moraxella surface protein (Msp)22, and PilA clade 2 (PilA2) was quantitated by using an ELISA assay.

**Results—**There were 223 *Mcat* NP colonization episodes documented in 111 (60%) of 184 children in the study. Thirty five *Mcat* AOM episodes occurred in 30 (16%) of 184 children. All 5

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*Authors' contribution*: DR and MEP conceived and designed the study. DR, MEP, TFM, ERL, AAC, NLM and JRC provided materials. DR performed experiments. DR and ALA conducted statistics. DR and MEP wrote the manuscript. All authors have approved the final article.

*Conflict of interest*: Timothy F. Murphy has patents for vaccines for *M. catarrhalis*. No other potential conflicts of interest were disclosed.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.09.023.

*Mcat* candidate vaccine antigens evaluated stimulated a significant rise in serum IgG levles over time from 6 to 36 months of age ($P < 0.001$), with a rank order as follows: Msp22 = OppA > OMP CD = Hag = PilA2. Children with no detectable *Mcat* NP colonization showed a higher serum IgG level against OppA, Hag, and Msp22 compared to those with *Mcat* NP colonization ($P < 0.05$). Individual data showed that some children responded to AOM with an antibody increase to one or more of the studied *Mcat* proteins but some children failed to respond.

**Conclusions—**Serum antibody to *Mcat* candidate vaccine proteins OMP CD, OppA, Msp22, Hag, and PilA2 increased with age in naturally immunized children age 6–30 months following *Mcat* NP colonization and AOM. High antibody levels against OppA, Msp22, and Hag correlated with reduced carriage. The results support further investigation of these vaccine candidates in protecting against *Mcat* colonization and infection.

### Keywords

Nasopharyngeal colonization; Acute otitis media; Immunogenicity; Recombinant proteins; Antigen; Natural immunization; Carriage; Vaccine

## 1. Introduction

*Moraxella catarrhalis* (*Mcat*) is a Gram-negative diplococcus that commonly colonizes mucous membranes of the human nasopharynx [1]. *Mcat* is also a transmittable pathogen responsible for various respiratory infections in children and adults resulting in a significant medical and economic burden worldwide [1–3]. Our recent studies revealed that *Mcat* now has overtaken *Streptococcus pneumoniae* (*Spn*) and non-typeable *Haemophilus influenzae* (NTHi) as the most frequent cause of episodic and recurrent acute otitis media (AOM) in children [4]. AOM and all respiratory bacterial infections begin pathogenesis with nasopharyngeal (NP) colonization. However, colonization is mostly asymptomatic; only when the condition of the host is altered will *Mcat* invade the middle ear, causing AOM or the bronchi and lungs, causing acute exacerbations of chronic bronchitis in adults.

*Mcat* vaccine development is currently moving from antigen identification to clinical trial. A number of potential vaccine antigens of *Mcat* have shown significant immunogenicity and protective effectiveness in various animal models [5–7]. Several prior studies have detected antibody responses to *Mcat* proteins in humans [5–11]. Some *Mcat* proteins have been eliminated as vaccine candidates due to surface epitope heterogeneity or variable expression. Desirable *Mcat* candidate antigens should be conserved among strains and immunogenic in children and adults. In the work reported here, we studied 5 *Mcat* protein vaccine candidates: outer membrane protein (OMP) CD, oligopeptide permease A (OppA), a non-lipidated form of Msp22 which we named Msp22NL, a truncated form of MID/Hag (Hag5–9), and PilA clade 2 (PilA2). OMP CD is a porin and adhesin and is highly conserved with exposed epitopes on the bacterial surface [12]. OppA is an oligopeptide binding protein which is located on the surface of *Mcat* and is involved in a number of functions of bacterial physiology including nutrient acquisition and persistence in the respiratory tract [13,14]. Msp22 is a putative outer membrane lipoprotein which may be involved in the transport of divalent cations across the outer membrane [15]. MID/Hag is an autotransporter outer membrane adhesin protein and hemagglutinin. It contains regions of highly conserved and

moderately conserved domains [16]. PilA2 is the major pilin subunit that is conserved and essential for genetic transformation, adherence to eukaryotic cells and biofilm formation [17].

For *Mcat* vaccine development it is important to know whether a target antigen is immunogenic in the human host in the age time frame when vaccination is anticipated. The results from that knowledge would be to expect natural priming and boosting of vaccine responses caused by natural colonization. Therefore, we examined the antibody responses in young children after natural *Mcat* exposure by asymptomatic NP colonization and after a local infection, AOM. To our knowledge, this is the first study to prospectively compare the development of naturally induced antibodies to these 5 *Mcat* OMPs simultaneously in a single cohort of children 6–30 months of age during NP colonization and AOM. Specifically, we compared: (1) Changes of serum IgG antibodies to proteins OMP CD, OppA, Msp22, Hag, and PilA2 in children when their age increased from 6 to 30 months old; (2) Differences in antibody levels between children with NP colonization of *Mcat* and those with no NP colonization of *Mcat* at age 6–30 months old; (3) Differences in antibody levels during acute onset of AOM versus convalescence; (4) Variations in individual antibody responses following AOM.

## 2. Materials and methods

### 2.1. Subjects and sampling

**2.1.1. Patient population—**The samples collected and analyzed were obtained during a prospective study supported by the National Institute of Deafness and Communication Disorders, as previously described [18,19]. Healthy children without previous episodes of AOM were enrolled at 6 months of age from a middle class, suburban socio-demographic pediatric practice in Rochester, NY (Legacy Pediatrics) during June, 2008 to March, 2014. For this study we assessed a total of 184 children followed prospectively until 30 months of age. Serum samples, and NP and oropharyngeal (OP) cultures were obtained 7 times during the study period at 6, 9, 12, 15, 18, 24, and 30 months of age. During the study period whenever children in this group experienced an AOM episode a confirmatory tympanocentesis was performed and MEF samples microbiologically assessed; plus serum, NP, and OP cultures were obtained. The study was approved by the Rochester General Hospital Research Subjects Review Boards and written informed consent was obtained for participation and all procedures.

**2.1.2. Sample collection—**Serum, NP, OP and middle ear fluid (MEF) sampling was conducted as previously described [18].

**2.1.3. Microbiology—**Bacteria were isolated as previously described [18].

### 2.2 Mcat protein expression and purification

Recombinant *Mcat* proteins OMP CD [12], OppA [14], Hag5–9 (truncated Hag protein) [16], and PilA2 [17] were expressed and purified as previously described. We designed and constructed an expression plasmid for the gene of non-lipidated Msp22 which we named

Msp22NL in a vector, pET303 with His-tag at its C-terminal. The sequence of the cloned *msp22nl* was verified by DNA sequencing. The plasmid was transformed into *E. coli* expression strain one shot BL21 (DE3) (Life Technologies, Grand Island, NY) and the Msp22NL was then expressed and purified by standard methods [14]. All the protein antigens and their purity were characterized by using SDS-PAGE along with Western blot (Fig. 1S).

### 2.3. Enzyme-linked immunosorbent assay

Protein-specific antibody concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using purified recombinant proteins. Ninety six-well Nunc MaxiSorp plates were coated with 1 μg/mL of individual proteins (100 μL/well) in phosphate-buffered saline (PBS, pH 7.4) and incubated at 37 °C for 1 h. After five washes, the plates were blocked with 10% fetal bovine serum (FBS) in PBS (pH 7.4) at 37 °C for 1 h (200 μl per well). After washing, 100 μl of serum 2-fold serially diluted at a starting dilution of 1:50 (in PBS–10% FBS) was added to each well. Human serum IgGs, Carimune (CSL Behring AG, Bern, Switzerland) and Gammagard (Baxter, Deerfield, IL) were used as references and in-house control sera with high and low titers were run on each plate. The plates were incubated at room temperature for 1 h followed by the addition of affinity purified goat anti-human IgG antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX) as a secondary antibody. The reaction products were developed with TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD), stopped by addition of 1.0 M phosphoric acid and read by a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA) using a 450-nm filter.

To provide quantitative results on antibody concentrations, the level of the specific antibody present in the unknown sample was determined by comparison to an internal reference serum (Carimune for OMP CD and Gammagard for OppA, Msp22, Hag, and PilA2). The levels of IgG in the reference serum were quantitatively measured by using a human IgG ELISA quantitation kit (Bethyl laboratories). A four-parameter logistic-log function was used to form the reference and sample curves. This ELISA was fully validated according to International Conference on Harmonisation (ICH) Guidance. The assay lower limit of detection was 69 ng/mL for OMP CD, 171 ng/mL for OppA, 222 ng/mL for Msp22NL, 191 ng/mL for Hag5–9, and 47 ng/mL for PilA2. The inter-assay coefficient of variation was ≤30% for all antigens and secondary antibody combinations.

### 2.4. Statistical analysis

Antibody concentrations were transformed using the Box–Cox power transformation method. This represents a family of transformations of which the log transformation is a special case. Linear models including transformed antibody concentrations as response, age number and colonization indicator as predictors were used for comparison between colonized and non-colonized groups, and for analyzing the slope of age-dependent change of serum IgG concentrations. Age numbers were log-transformed and nonlinear relationships were captured by including a quadratic age term. In order to control for subject-level dependence induced by repeated measures, a bootstrap procedure was used to estimate statistical significance, using subject-level resampling. One-way analysis of variance

(ANOVA) was used to compare the difference of antibody concentrations among all antigens. A non-parametric Wilcoxon matched-pairs test was employed to compare the difference between AOM and convalescence groups.

## 3. Results

### 3.1. NP colonization and AOM events

A total of 570 visits among 184 children were studied. There were 223 *Mcat* OP/NP colonization episodes from one or more of the seven sampling visits documented in 111 (60%) of 184 children. Thirty five *Mcat* AOM episodes occurred in 30 (16%) of 184 children. Because the study design called for NP/OP sampling at 7 specific times separated by 3–6 mo, some *Mcat* colonization events were not detected by culture but most likely occurred as reflected in significant rises in specific antibody to one or more of the *Mcat* antigens studied.

### 3.2. Natural acquisition of serum antibody to OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 over time

Fig. 1 shows the geometric mean concentration of serum antibodies to OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 at 6, 9, 12, 15, 18, 24 and 30 mo of age corresponding to visits 1–7 for the study cohort. There is a significant antibody concentration increase over time for all 5 proteins (Fig. 1 and Table 1S, $P < 0.001$). The estimated gradients of serum IgG increase across each time point are shown in the supplemental Table 1 (Table 1S). There is a clear age-dependent elevation of antibody level and a decline of the gradient of antibody increase against each antigen from age 6 to 30 mo in the children (Table 1S). Msp22NL and OppA had a significantly higher level of IgG responses than OMP CD, Hag5–9, and PilA2 at each age time point ($P < 0.05$) except that OppA did not show significant difference from OMP CD in IgG response at age of 24 mo (Fig. 1). There is a much similar level of serum IgG response to OMP CD, Hag5–9, and PilA2 at each age time point ($P > 0.05$). Overall, IgG antibody concentrations to the five proteins were significantly different ($P < 0.0001$) among the study children with a rank order: Msp22 = OppA > OMP CD = Hag5–9 = PilA2 (Fig. 1).

### 3.3. Comparison of serum IgG levels to OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 in NP colonized versus non-colonized children at various ages

We compared the level of antibody to OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 between 27, 30, 39, 36, 33, 31 and 27 children who were culture-positive for *Mcat* at age 6, 9, 12, 15, 18, 24 and 30 mo, respectively, and 29, 37, 37, 39, 38, 42 and 31 children of the same age who were not culture-positive. Children with detected colonization revealed a significant lower serum IgG to OppA (Fig. 2B, $P = 0.036$), Msp22NL (Fig. 2C, $P = 0.034$), and Hag5–9 (Fig. 2D, $P = 0.029$), respectively, than children without detected colonization over time. Differently, serum antibody to OMP CD (Fig. 2A, $P = 0.146$) and PilA2 (Fig. 2E, $P = 0.358$), did not exhibit significant difference between children with detected colonization and children without detected colonization over time.

### 3.4. Comparison of acute and convalescent serum IgG levels to OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 in children with Mcat-caused AOM

The mean concentrations of IgG antibody in acute and convalescence sera of AOM children is shown in Table 2S. Serum IgG to OMP CD in the convalescence phase was significantly higher than that in the acute phase ($P < 0.05$) whereas no difference was found in antibodies to other proteins between onset of AOM and convalescence phases. Fig. 3 displays individual responses of children to all 5 proteins at the onset of and after an AOM. When we examined the individual responses for the 32–35 paired sera available we observed that 43–64% of children showed a rising antibody response to each protein. Less than 2% had no change in antibody level between acute and convalescent sera, and 36–55% had a falling antibody level (Fig. 3).

## 4. Discussion

Sustained efforts have been made to identify and characterize efficacious vaccine antigens to prevent *Mcat*-caused respiratory infections especially AOM in children and acute exacerbations of chronic bronchitis in adults. It is important to know the natural immune responses to these *Mcat* antigens in the targeted vaccine population before moving into clinical trials. Here we have characterized the serum antibody response to 5 *Mcat* proteins mounted by children who experienced *Mcat* NP colonization and AOM. We found a rise in the serum levels of OMP CD, OppA, Msp22, Hag, and PilA2-specific IgG antibodies in children as they increased in age from 6 to 30 months old. The rank order of antibody concentration was highest to Msp22 followed by OppA, then OMP CD, then Hag5–9 and last PilA2. Serum antibody levels following detected NP colonization without progression to AOM revealed a similar magnitude and rank order as those in NP colonization associated with AOM caused by *Mcat*.

The continuous increase of naturally acquired serum antibody in children over time to vaccine candidate antigen proteins OMP CD, OppA, Msp22, Hag, and PilA2 demonstrates that the 5 proteins are immunogenic in young children from 6 to 30 months of age. Such an observation is strongly supportive of the potential of these antigens to be useful in a vaccine against *Mcat* infection in children. Differently, Verhaegh and co-workers did not find a significant increase of serum antibody to *Mcat* protein $Hag^{385–863}$ (Hag5–9) in their studies on naturally induced antibody response in children age 6 mo to 2 years old whereas our study identified a rise [9]. The divergent socioeconomic background, geographic environment and other factors may account for the different antibody responses between our subject population and theirs. However, they did find an increase of serum antibodies to some other *Mcat* proteins $MID^{764–913}$, $MID^{962–1200}$, $UspA^{1557–704}$ and $UspA2^{165–318}$ in the same cohort, suggesting varied immune activity among different antigens and even different immune epitopes of the same antigen [9].

We found that serum antibodies to OppA, Msp22NL, and Hag5–9 in children with *Mcat* NP colonization are lower than those in children without *Mcat* colonization identified by culture over age 6–30 mo. High antibody responses correlate with low carriage, suggesting a potential protection of naturally acquired *Mcat* protein-specific serum antibody from bacterial NP colonization. Verhaegh and co-workers had similar findings that the serum IgG

response to $MID^{962–1200}$ was higher in the 2 year old children without *Mcat* colonization than that in the children with *Mcat* NP colonization [9]. We did not observe a correlation of colonization events with antibody levels for OMP CD and PilA2 indicating a different natural immune response developed compared to other three *Mcat* proteins. Similarly, Verhaegh and co-workers also did not find a difference of the serum antibodies to most of the tested *Mcat* antigens between children with *Mcat* colonization and children with no *Mcat* colonization over age 6 mo to 2 years old. Age of the child and preexisting antibody levels are important covariates in predicting an antibody response to NP colonization. This may prove true also for vaccination. Our prior studies showed varied serum IgG responses during NP colonization of the other two otopathogens, *Spn* or NTHi versus non-NP colonization in children age 6–30 mo old. *Spn* and NTHi also display differences from *Mcat* in producing anti-infection acute responses in our prior observations [20]. Thus, different pathogens may evoke varied antibody responses in relation to NP colonization in children as we observed in this study.

No elevation of antibodies to *Mcat* proteins except OMP CD was observed 3 weeks after an AOM event in our study cohort. Hence, *Mcat* AOM events did not have a correlation with antibody response to most *Mcat* proteins tested. Our prior studies on antibody responses to protein components of AOM pathogens *Spn* and NTHi did not reveal a difference between AOM and the following convalescence phase as well. NP colonization appears to be the immunizing event whereas AOM infection neither boosts nor suppresses antibody levels. Individual subject data suggests that changes in antibody level between acute onset of AOM and convalescence reflect the timing of prior NP colonization and associated antibody response rather than a response to the AOM infection per se. OMP CD may be an exception to the general observation regarding NP colonization response and AOM response since an increase of serum antibodies to OMP CD after AOM suggests OMP CD is different from other proteins in eliciting antibody responses to AOM. Further studies are proposed to disclose the immune features of OMP CD contributing to these discrepancies. In another study, Verhaegh and co-workers did not find a correlation between the serum and MEF antibody levels to ten recombinant *Mcat* proteins or *Mcat* NP colonization and AOM events in children under 5 years old during recurrent AOM and chronic otitis media with effusion [10]. Our observations and those of others indicate that there is a complicated interplay between the host antibody immune response and *Mcat* acute infections. Maternal antibodies play a key role in immune defense against infectious diseases during the first 6 mo of postnatal life. However, these antibodies wane between 6 and 9 months after birth and are largely replaced by antibodies produced by the child. We observed a higher level of serum IgG to rPilA2 at 6 mo than 9 mo of age in the children, which suggests that maternal antibodies to PilA2 in the children were still declining during that time frame. In contrast, antibody levels to OMP CD, OppA, Hag and Msp22 rose from 6 mo to 9 mo of age, clearly indicating an active antibody response.

Our study has limitations. The study subjects were enrolled from a predominantly middle class socioeconomic population in a developed country and may not be generalizable to children in other parts of the world especially those developing countries with lower socioeconomic status where the NP colonization frequency and bacterial load may be

higher. *Mcat* is a mucosal pathogen and mucosal antibody response is an important portion of the host immune defense. Further studies on mucosal antibody responses to the 5 *Mcat* proteins during *Mcat* colonization and AOM have been planned to better understand the naturally induced antibody responses in the same child population, similar to our prior work with *Spn* candidate vaccine antigens [21].

In conclusion, we found increasing levels of IgG antibodies to 5 studied *Mcat* vaccine candidate proteins in children from 6 to 30 mo old. High antibody levels to OppA, Msp22NL, and Hag5–9 correlated with reduced *Mcat* carriage. The results support further studies on these proteins as vaccine antigens against *Mcat* colonization and infection in children.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **Mcat** | Moraxella catarrhalis |
| **AOM** | acute otitis media |
| **NP** | nasopharyngeal |
| **OP** | oropharyngeal |
| **OMP** | outer membrane protein |
| **Spn** | Streptococcus pneumoniae |
| **NTHi** | non-typeable *Haemophilus influenzae* |
| **OppA** | oligopeptide permease A |
| **Msp** | Moraxella surface protein |
| **MID** | Moraxella IgD-binding protein |
| **Hag** | hemagglutinin |
| **PilA2** | PilA clade 2 |
| **mo** | months |
| **MEF** | middle ear fluid |
| **E. coli** | Escherichia coli |

| | |
|---|---|
| **ELISA** | enzyme-linked immunosorbent assay |
| **FBS** | fetal bovine serum |
| **ICH** | International Conference on Harmonisation |
| **ANOVA** | analysis of variance |
| **GAM** | generalized additive model |

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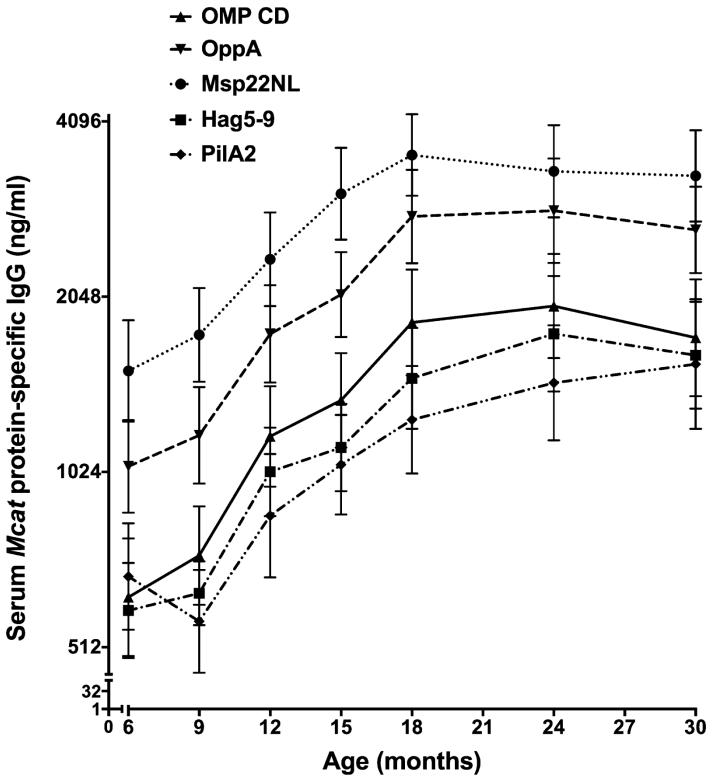


**Fig. 1.**

Serum IgG antibody levels to *Mcat* proteins OMP CD, OppA, Msp22NL, Hag5–9, and PilA2 in healthy children increased with age. Plots of the geometric mean concentrations (ng/ml) with the 95% confidence interval during 7 sampling visits at 6, 9, 12, 15, 18, 24, 30 mo of age. The *y*-axis is presented as log2 scale. The numbers of sera included at each time point were 56, 67, 76, 75, 71, 73, 58, respectively.

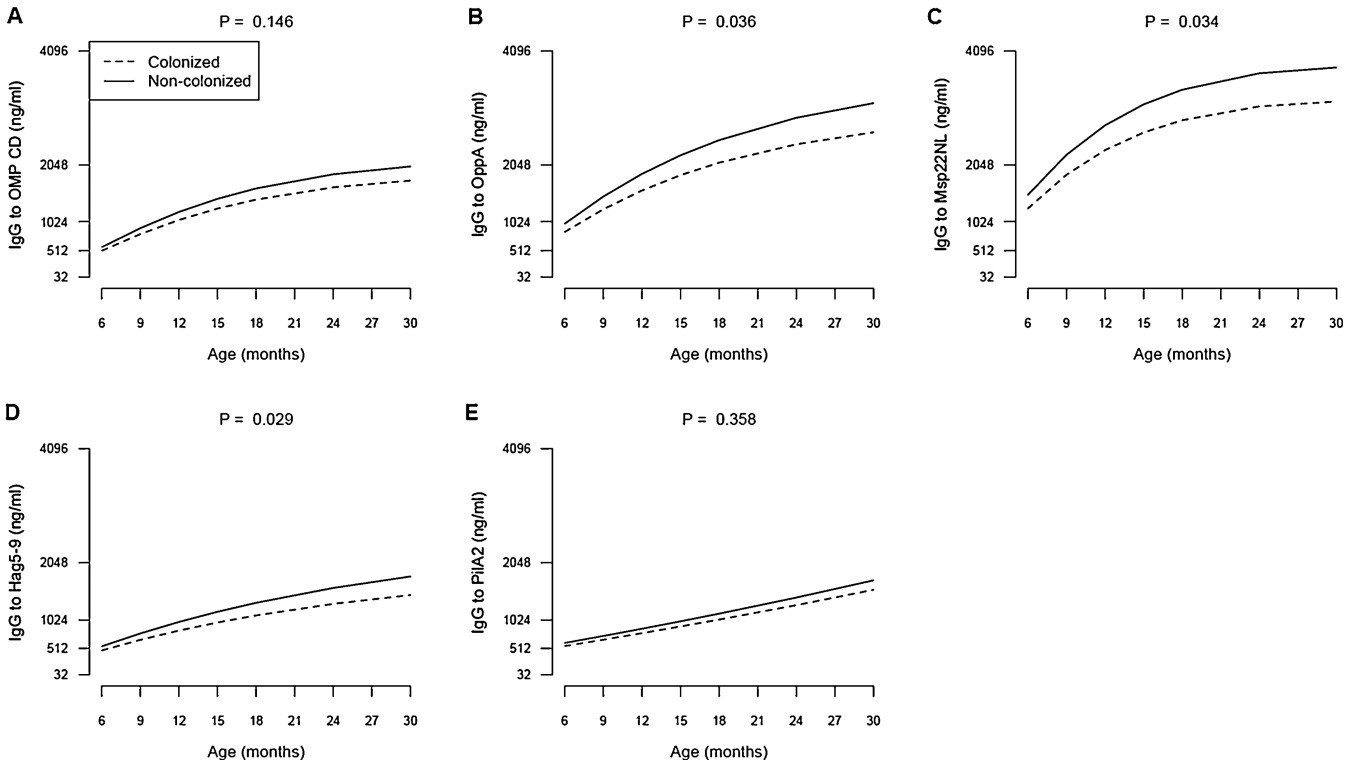


**Fig. 2.**

Comparison of serum IgG antibody concentration (ng/ml) to *Mcat* proteins OMP CD (A), OppA (B), Msp22NL (C), Hag5–9 (D), and PilA2 (E) in *Mcat* NP colonized (broken line) and non-colonized (solid line) healthy children from 6 to 30 mo of age. Serum anti-*Mcat* protein specific IgG antibody concentrations were determined with a quantitative ELISA and then power transformed using the Box–Cox method. The numbers of sera included at each time point were 27, 30, 39, 36, 33, 31, 27 for *Mcat*-colonized children and 29, 37, 37, 39, 38, 42, 31 for non-colonized children. Linear models including transformed antibody concentrations as response, age number and colonization indicator as predictors were used for comparison between colonized and non-colonized groups. Age numbers were log-transformed and nonlinear relationships were captured by including a quadratic age term. In order to control for subject-level dependence induced by repeated measures, a bootstrap procedure was used to estimate statistical significance, using subject-level resampling. The *P*-values correspond to the regression coefficient (beta not equal to 0) associated with the group factor (group main effect). $P < 0.05$ was considered significant.

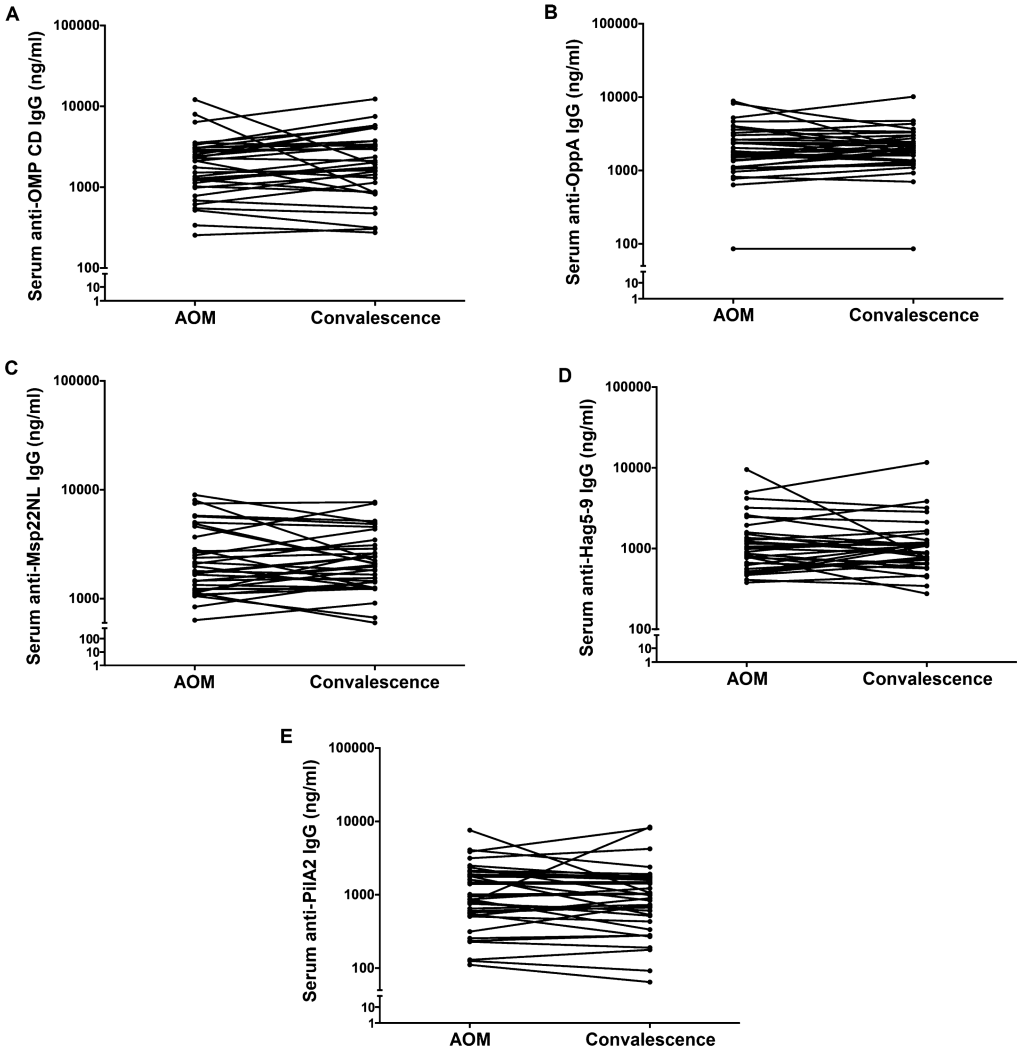


**Fig. 3.**

Individual serum IgG antibody levels (ng/ml) to *Mcat* proteins OMP CD (A, *n* = 34), OppA (B, *n* = 34), Msp22NL (C, *n* = 34), Hag5–9 (D, *n* = 32), and PilA2 (E, *n* = 35) in acute and convalescent sera of children with *Mcat* AOM.