Vaccine
Seroprevalence of IgG and IgM antibodies to Haemophilus influenzae type a in Canadian children

Seroprevalence of IgG and IgM antibodies to *Haemophilus influenzae* type a in Canadian children

Brenda Huska[a#], Chelsea Kubinec[a#], Manish Sadarangani[b,c], Marina Ulanova[a*] for the Canadian Immunization Research Network Investigators

[a]Northern Ontario School of Medicine, Thunder Bay, ON, Canada
[b]Vaccine Evaluation Center, BC Children's Hospital Research Institute, Vancouver, BC, Canada
[c]Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada

[#]Contributed equally

*Corresponding author: Marina Ulanova, Division of Medical Sciences, Northern Ontario School of Medicine, Lakehead University, 955 Oliver Road, Thunder Bay, ON, P7B 5E1, Canada
Email address: mulanova@nosm.ca

Seroprevalence of IgG and IgM antibodies to *Haemophilus influenzae* type a in Canadian children

Brenda Huska[a#], Chelsea Kubinec[a#], Manish Sadarangani[b,c], Marina Ulanova[a*] for the Canadian Immunization Research Network Investigators

[a]Northern Ontario School of Medicine, Thunder Bay, ON, Canada

[b]Vaccine Evaluation Center, BC Children's Hospital Research Institute, Vancouver, BC, Canada

[c]Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada

[#]Contributed equally

*Corresponding author: Marina Ulanova, Division of Medical Sciences, Northern Ontario School of Medicine, Lakehead University, 955 Oliver Road, Thunder Bay, ON, P7B 5E1, Canada

Email address: mulanova@nosm.ca

ABSTRACT

Background

Over the last 2 decades, *Haemophilus influenzae* type a (Hia) has emerged as a significant cause of invasive disease in some geographic regions and populations. Recognition of the importance of Hia in the etiology of serious disease, particularly in young children, prompted the development of a new protein-capsular polysaccharide conjugate vaccine, similar in design to a vaccine against *H. influenzae* type b. At present, understanding of Hia immunology is incomplete; the immunological correlate of protection against invasive disease is unknown.

Methods

Our objective was to study Hia antibody in children of various ages residing in a Canadian province with low incidence rates of invasive disease. The enzyme-linked immunosorbent assays were performed to quantify plasma IgG and IgM specific to Hia capsular polysaccharide in 133 children (3 months to 16 years).

Results

Both anti-Hia IgG and IgM concentrations increased with age and were significantly higher in older children; a positive correlation between age and concentrations of Hia antibody was found. IgM antibody concentrations were significantly higher than IgG, with mean IgM concentrations over 10 times larger than IgG across all age groups.

Conclusions

The steady rise of naturally acquired, Hia-specific IgG and IgM concentrations in a pediatric population with low incidence rates of invasive Hia disease suggests the exposure to some cross-reactive environmental antigens as a major source of the antibody. However, the carriage rates of Hia in the region are unknown and further seroepidemiological studies are warranted. Although natural antibody may protect certain population groups against invasive disease, immunization of younger children will be essential to prevent serious infections if Hia continues to spread across North America.

Keywords:

Haemophilus influenzae type a; seroprevalence; natural antibody; children

1. INTRODUCTION

The Gram-negative coccobacillus *Haemophilus influenzae* frequently colonizes the nasopharynx of healthy individuals and can cause both local and systemic infections including otitis media, pneumonia, epiglottitis, meningitis, and sepsis [1]. *H. influenzae* is categorized based on the presence or absence of a polysaccharide capsule. There are six distinct serotypes of encapsulated *H. influenzae* (a-f); non-encapsulated strains are termed non-typeable (NTHi) [1]. The polysaccharide capsule is the major virulence factor of *H. influenzae* as it prevents complement deposition and phagocytosis required for bacterial clearance; anti-capsular antibodies are essential in immune defenses against the infection [1,2]. Among encapsulated *H. influenzae*, *H. influenzae* serotype b (Hib) is the most virulent, followed by serotype a (Hia) [3]. Prior to introduction of the Hib conjugate vaccine in late 1980s, Hib was the major cause of serious invasive diseases in children worldwide. The global weighted average incidence of meningitis caused by Hib was 57/100,000 in children <5 years of age, with much higher rates reported from North American and Australian Indigenous populations [4]. Pediatric immunization against Hib resulted in the dramatic reduction of Hib invasive disease in all the countries where it has been used [4]. However, over the last 20 years, invasive Hia disease, which shows remarkable similarities in epidemiology, clinical presentation, severity, and fatality rates to those caused by Hib, has emerged in some geographic areas including Alaska and Northern Canada [5]. The highest incidence rates of invasive Hia disease were reported from Alaska Native and Canadian Inuit children < 1 year of age, i.e. as high as 82/100,000 in Alaska [6], and 300/100,000 in Nunavut and Nunavik, the regions of Northern Canada [7,8]. Although data on epidemiology of invasive Hia disease in different parts of Canada are incomplete, it appears that the disease is much less common in some regions outside of the Arctic, such as the province of British Columbia. Between January 1, 2018 and December 31, 2019, a total of 14 cases of invasive Hia disease were reported from this province (0.1/100,000 population per year) [9,10].

The recognition of significance of Hia as a cause of serious invasive disease prompted the development of a new vaccine in Canada that has been recently investigated pre-clinically [11]. This vaccine was formulated on the basis of the same principles as vaccine against Hib, i.e. using Hia capsular polysaccharide as an

77 antigen conjugated to a protein carrier to make it immunogenic in young children who are yet unable to
78 develop antibody response to pure polysaccharide antigens [12]. To determine target population groups for
79 immunization against this infection, understanding of Hia immunity and defining immunological correlates of
80 protection are essential.
81
82 The reason for increased susceptibility to invasive Hia disease in certain population groups is currently
83 unknown [13]. Whereas the highest rates of invasive Hia disease have been reported from young Indigenous
84 children, immunocompetent adults do not typically develop invasive Hia disease [5, 14-16]. We have
85 recently established that healthy Indigenous adults possess naturally acquired functionally active antibodies
86 against Hia suggesting that certain level of natural immunity may protect against invasive Hia disease
87 [17,18]. We hypothesized that antibodies specific to Hia capsular polysaccharide develop with age due to
88 exposure to the pathogen and/or certain cross-reactive antigens. Our objective was to study plasma antibody
89 concentrations in children of various ages in a population with low incidence of invasive Hia disease.
90
91 2. METHODS
92
93 **2.1. Plasma samples**
94 Pediatric plasma samples were collected at BC Children's Hospital Biobank (Vancouver, British Columbia,
95 Canada) between October 2015 and May 2019. The ages of the children ranged from 3 months to 16 years
96 (Table 1). The study was approved by the University of British Columbia – Children's & Women's Health
97 Centre of BC and Lakehead University Research Ethics Boards. The samples were shipped frozen and
98 stored at -80°C until used.
99
100 **2.2. Quantification of *Haemophilus influenzae* type a (Hia) specific IgG and IgM in plasma**
101 To quantify IgG and IgM specific to Hia capsular polysaccharide, we used a previously described enzyme-
102 linked immunosorbent assay (ELISA) [17]. Antibody concentrations were determined using our internal
103 standard that was cross standardized to the Hia reference serum provided by the Centers for Disease
104 Control and Prevention. The internal standard contained 1.25 µg/mL anti-Hia IgG and 2.09 µg/mL anti-Hia

IgM. Absorbance measurements were made with OD_{450}, and OD_{630} as reference, and a log-log linear regression analysis was used to determine antibody concentration in µg/mL. The lower limit of detection was 0.04 µg/mL for IgM and 0.02 µg/mL for IgG; for statistical purposes, samples with values below detection were assigned a value ½ the lower limit. All samples were tested in duplicate.

2.3. Statistical analysis

The data were $\log_{10}$ transformed before analysis. Statistical analysis was performed using Graph-Pad Prism 6 (GraphPad Prism Software Inc., San Diego, CA). Geometric mean antibody concentrations (GMC) with 95% confidence intervals (CI) were calculated. Comparison of continuous variables was conducted by one-way ANOVA, Student's *t*-test, or Mann-Whitney U test depending on data distribution and number of groups; Fishers' exact test was used to compare categorical variables. To study association of antibody concentrations with age, linear regression and Spearman correlation analyses were performed. A cut-off 0.1 µg/mL of Hia-specific IgG was used for analysis as this concentration was previously found to correspond to the lowest IgG level associated with a measurable titre of functionally active antibody [19]. Significance was determined at $p < 0.05$.

3. RESULTS

For the study of seroprevalence of IgG and IgM antibodies specific to Hia capsular polysaccharide in a pediatric population undergoing diagnostic testing, plasma samples of 133 children aged between 3 months and 16 years were analyzed (Table 1). The majority of children attended the Otolaryngology Department for tonsillectomy (n = 93, 70%) and the remaining were assessed at the Neurology Department. There were 6 children < 1 year of age; most samples were collected from children ≥ 5 years of age.

The concentrations of Hia-specific IgG and IgM were high enough for detection with ELISA in the majority of plasma samples, with only 2 samples for IgG (both age 2 years) and 5 samples for IgM (children aged 3 months, 5 months, 10 months, 2 years, and 8 years) falling below detection limits. IgM antibody concentrations (GMC: 2.17, CI: 1.57, 3.01 µg/mL) were significantly higher than IgG (GMC: 0.22, CI: 0.17, 0.28 µg/mL) for the total sample population ($p < 0.0001$, Fig. 1), as well as across all the age groups, with

mean IgM/IgG ratio being 11.23 for the total sample population, from 6.9 to 16.9 for children of different ages (Table 2).

The concentrations of both IgG and IgM specific to Hia increased with age, with statistically significantly higher GMC found in children ≥ 5 years of age compared to younger children, i.e. IgG, 0.32, CI 0.24, 0.42 *vs*. 0.10, CI 0.06, 0.15 µg/mL; IgM, 3.35, CI 2.81, 3.99 *vs*. 1.25, CI 0.72, 2.14 µg/mL, correspondingly ($p<0.0001$ between 0-4 and 5-16 years old for both). Linear regression analysis showed significant association of both IgG and IgM Hia-specific antibody concentrations with age (Fig. 2 - 3). There was a positive, but moderate, correlation between age and concentrations of Hia-specific IgG ($r = 0.37$, $p<0.0001$) and IgM ($r = 0.33$, $p=0.0001$).

Concentrations of Hia-specific IgG exceeded 0.1 µg/mL in 78 samples (64%), with a lower proportion of samples above this cut-off among younger children (21% in ages 0-4 and 79% in ages 5-16, $p <0.001$). No difference in IgG antibody concentrations between males and females was found. GMC of IgM were higher in females than males: for ages 0-4, 1.38 *vs.* 1.09 µg/mL ($p=0.8$), for ages 5-16, 4.48 *vs*. 2.72 µg/mL ($p=0.003$).

4. DISCUSSION

In this study, we detected IgG and IgM specific to Hia capsular polysaccharide in 131/133 and 128/133 pediatric plasma samples, respectively. Concentrations of Hia-specific IgG and IgM steadily increased with age in a sample of pediatric population with low incidence rates of invasive Hia disease, with significantly higher antibody values in children of 5 years and older as compared to the younger ones. We also found a greater prevalence of IgM over IgG antibodies across all the ages; in average, concentrations of Hia-specific IgM were 11-fold higher than IgG.

The current understanding of Hia immunology is very limited; the data on concentrations of natural IgG antibodies specific to Hia capsular polysaccharide in Alaskan children have only been recently published [20]. Comparably to our data, in Alaska Native children, IgG antibody levels significantly increased with age,

161 despite the differences in Hia disease prevalence in Alaska and our region of interest. Over a period of 12
162 years (2007-2018), a total of 47 Hia isolates were collected from disease cases in British Columbia, a region
163 of Western Canada bordering Alaska [21]. Considering that the population of British Columbia is roughly 5
164 million people, an average annual incidence rate of invasive Hia disease was 0.08/100,000 population. In
165 comparison, an average annual incidence of invasive Hia disease in Alaska in 2008-2017 was 0.68/100,000
166 population, with much higher numbers in young Alaska Native children (27.74/100,000 population < 5 years
167 of age) [6]. In Alaska, invasive Hia disease has been present since 2002 and incidence rates significantly
168 increased since then [15,16, 22]. As reported by McClure et al. [20], 82.4% of children <10 years of age in
169 Yukon-Kuskokwim Delta (where most cases of invasive Hia disease occurred) and 86.8% in Anchorage (with
170 much lower incidence of disease) had concentrations of Hia-specific IgG > 0.1 μg/mL, a concentration
171 previously found to correspond to the lowest IgG level associated with a measurable functionally active
172 antibody [19]. In our study, we found a lower proportion of children <10 years of age with Hia-specific IgG >
173 0.1 μg/mL, i.e. 66%. This discrepancy may be explained by a lower circulation of Hia in British Columbia
174 compared to Alaska. However, while the incidence of Hia invasive disease in our region of interest is much
175 lower than that in Alaska, the carriage rates of Hia are unknown.
176
177 Age-dependent rise in natural Hia antibodies in children is reminiscent of historical data on immunology of
178 Hib collected in the pre-Hib vaccine era. There are obvious similarities in the natural histories of Hia and Hib
179 infections. Both Hia and Hib have polysaccharide capsules acting as major virulence factors; both can be
180 carried asymptomatically in the nasopharynx by healthy individuals and cause invasive disease
181 predominantly in children < 2 years of age while older children and immunocompetent adults are relatively
182 resistant to the infection. In the pre-Hib vaccine era, protective immunity against Hib gradually developed in
183 children after 2 years of age; an increase in antibody titres with age correlated with a decrease in the
184 incidence of invasive disease (reviewed by [23]). However, McClure et al. [20] detected Hia-specific IgG in
185 both Alaska Native and non-Native population groups residing in regions with different rates of invasive
186 disease, with no significant differences in antibody concentrations by the race, study region (the Yukon-
187 Kuskokwim Delta versus Anchorage), or time of sample collection (the 1980s and 1990s when no Hia
188 disease was present, and after the emergence of the disease in 2000s). Our earlier studies also showed that

concentrations of Hia-specific IgG did not differ between Canadian Indigenous adults living in the regions with high versus low incidence of invasive Hia disease [18]. These observations illustrate the basic principle of epidemiology, i.e. the probability of the development of infectious disease depends both on the exposure to infectious agent and presence or absence of protective immunity. In regions with higher colonization rates and presence of factors facilitating the transmission of the infectious agent, such as overcrowding, lack of clean water, and indoor air pollution, higher antibody levels would be required to prevent the disease. Such environmental factors can explain the differences in rates of invasive disease between different populations [20]. Although no data on Hia colonization in Canada have yet been published, in Northwestern Ontario First Nations communities, over 8% of healthy children aged 3-5 years carried Hia in the nasopharynx (M. Ulanova, unpublished observations). In this region of Central Canada, where the annual average incidence rates of invasive Hia disease were 7/100,000 in 2004-2008 and 3.1/100,000 in 2010-2015 [24-27], Hia-specific antibodies were detected in all healthy Indigenous adults (median age 36.5 years), with geometrical mean IgG and IgM concentrations of 1.47 and 2.09 μg/mL, correspondingly [18]. Because healthy adults do not typically develop invasive Hia disease, these antibody concentrations are likely above the protective level. However, to gain better understanding of the immunological correlate of protection against invasive disease, more seroepidemiological studies will be needed, including studies of Hia colonization in different age groups and populations with high versus low incidence of invasive disease. With regards to our current data, lower incidence of Hia invasive disease within the population of interest may reflect lower carriage rates rather than protection from naturally acquired antibodies.

While we have previously collected data on natural immunity against Hia in both healthy and immunocompromized adults [17,18,28], serological studies in healthy children represent a significant challenge. In our study, the sample population included pediatric patients undergoing diagnostic testing in a large hospital in British Columbia that may not accurately represent the general pediatric population. Because the majority of children in our sample attended the Otolaryngology Department for tonsillectomy, they might have been potentially higher exposed to respiratory pathogens, including *Haemopilus influenzae*, and consequently developed higher antibody levels than other children. As we found clear prevalence of IgM over IgG among anti-Hia antibody in our sample, a recent exposure to Hia might potentially account for these

observations. However, in our previous studies of natural antibodies in healthy Indigenous and non-Indigenous adults, we also observed clear predominance of Hia-specific IgM over IgG although the opposite was true in case of naturally acquired antibodies specific to Hib [17,18]. To interpret these findings, it is important to consider the dominant role of IgM in the natural antibody repertoire [29-31].

While most seroprevalence studies traditionally focus on IgG antibody, the role of IgM in protection against invasive bacterial disease is indispensable. IgM has superior capacity to activate the complement and is able to greatly contribute to the bactericidal and opsonizing effects of antibody specific to encapsulated bacteria, such as Hia [2,29]. In addition, IgM produced by a particular subset of B cells (B-1) represents a major part of the natural antibody repertoire; while developing with age without a defined antigenic stimulation, the natural IgM antibody provides powerful defense against various pathogens [30,31]. Although the origin of natural anti-Hia antibodies in a pediatric population with low disease prevalence remains uncertain, our data suggest a potential role of immunization with cross-reactive antigens present in the environment. Indeed, serological cross-reactivity between Hia capsular polysaccharide and polysaccharide antigens of other bacteria, including *Streptococcus pneumoniae* serotype 6B, was documented [32]. By analogue, the role of cross-reactive antigens of commensal bacteria, in particular *Escherichia coli* K100, in the development of natural antibody against Hib in the pre-vaccine era was demonstrated [33]. Back in the 1980s, it was found that acquisition of natural anti-capsular antibody to Hib by Alaskan Eskimos was associated with pharyngeal carriage of Hib [34]. However, while Hib carriage was relatively uncommon (6.8% of all tested individuals), a clear positive association between antibody levels and age was observed: GMC of anti-Hib antibody increased in children between 6 months and 10 years of age [36], suggesting the effect of antigenic stimulation unrelated to Hib. With regards to our findings, as Hia carriage data in the region of interest are unavailable, the possibility of asymptomatic Hia colonization stimulating the development of antibodies in this population remains open. However, direct exposure to *H. influenzae* may not completely account for an increased antibody concentration with age; additional factors, such as cross-reactive antigens, can also be responsible for this. Overall, more seroepidemiological studies are required to elucidate the origin of natural Hia antibodies.

This study has several limitations. Complete data on Hia prevalence in the region are unavailable; information on Hia epidemiology is limited by cases of invasive disease, which is reportable in Canada. The most recent publication by Tsang et al. [21] described 47 isolates of Hia from British Columbia submitted to the National Microbiology Laboratory (Winnipeg, Manitoba) during 2007-2018. In addition, 29 cases of invasive Hia disease were identified between 1996 and 2008, based on serotyping of isolates submitted to BC Public Health Microbiology and Reference Laboratory (Dr. Linda Hoang, personal communication). However, etiology of non-invasive disease, such as non-bacteremic pneumonia or otitis media, is rarely documented. As there is a potential presence of Hia carriage in the population, a study of nasopharyngeal colonization in children of various ages will be important. In addition, our results are based on the analysis of antibodies from residual plasma samples in children undergoing diagnostic blood work rather than samples from healthy children because blood collection from healthy children for research purposes is not feasible for ethical reasons. Because the sample was determined by the availability of plasma specimens in the Biobank, most were from children ≥ 5 years of age, and fewer from younger children. We avoided using samples from patients who were potentially immunocompromised (e.g., children admitted to the oncology department); these samples were not included in our study. Most samples were from children scheduled for tonsillectomy; others were from children undergoing diagnostic testing for the neurology department. As discussed above, the prevalence of children with recurrent tonsillitis or hypertrophy of tonsils in our sample might account for high antibody concentrations in those who had potentially been exposed to Hia although no statistical differences in antibody levels between children attending Otolaryngology and Neurology Departments were noted (data not shown). Information on demographics and medical history collected at the Biobank database was limited. In particular, because information on ethnicity is not routinely gathered in the Canadian healthcare system, we could not include ethnicity in the demographic analysis.

5. CONCLUSIONS

Our data suggest that the development of natural immunity against Hia in a region without high incidence of invasive Hia disease may reflect accumulation of cross-reactive antibodies acquired through exposure to other common bacterial and/or environmental polysaccharide antigens. The prevalence of IgM suggests that

these anti-Hia antibodies are part of the natural antibody repertoire. Regardless of the antibody origin, these antibodies may be protective against invasive disease; however, an accurate protective threshold needs to be defined by extended seroepidemiological studies including regions with high incidence of Hia disease. Although natural antibodies may be protective in certain population groups, immunization of younger children will be essential to prevent serious invasive disease, especially if Hia continues to spread across North America.

FUNDING

This work was supported by the Canadian Immunization Research Network (CT20ON31) and the Northern Ontario School of Medicine Summer Medical Student Research Award to Chelsea Kubinec.

ACKNOWLEDGEMENTS

We are grateful to the personnel at the BC Children's Hospital Biobank for providing plasma samples for analysis and reviewing health charts of study participants and to Dr. Linda Hoang (BC Public Health Microbiology and Reference Laboratory) for sharing unpublished data. We thank Dr. Andrew Cox (National Research Council Canada) for providing the Hia capsular polysaccharide and Dr. Karen Rudolph (Arctic Investigation Program, Centers for Disease Control and Prevention) for donating the reference serum. Technical help of Ms. Angele Desbiens for optimizing ELISA is greatly appreciated.

REFERENCES

[1] Murphy TF. Haemophilus Species, Including H. influenzae and H. ducreyi (Chancroid). Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th ed., Philadelphia, PA: Elsevier/Saunders; 2015, p. 2575–83.

[2] Schreiber JR, Barrus V, Cates KL, Siber GR. Functional Characterization of Human IgG, IgM, and IgA Antibody Directed to the Capsule of Haemophilus influenzae Type b. J Infect Dis 1986;153:8–16. https://doi.org/10.1093/infdis/153.1.8.

[3] Zwahlen A, Kroll JS, Rubin LG, Moxon ER. The molecular basis of pathogenicity in Haemophilus influenzae: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus cap. Microb Pathog 1989;7:225–35. https://doi.org/10.1016/0882-4010(89)90058-2.

[4] Peltola H. Worldwide Haemophilus influenzae Type b Disease at the Beginning of the 21st Century: Global Analysis of the Disease Burden 25 Years after the Use of the Polysaccharide Vaccine and a Decade after the Advent of Conjugates. Clin Microbiol Rev 2000;13:302–17. https://doi.org/10.1128/CMR.13.2.302-317.2000.

[5] Ulanova M, Tsang RSW. Haemophilus influenzae serotype a as a cause of serious invasive infections. Lancet Infect Dis 2014;14:70–82. https://doi.org/10.1016/S1473-3099(13)70170-1.

[6] Soeters HM, Oliver SE, Plumb ID, Blain AE, Zulz T, Simons BC, et al. Epidemiology of Invasive Haemophilus influenzae Serotype a Disease—United States, 2008–2017. Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa875.

[7] Boisvert A-A, Moore D. Invasive Disease due to *Haemophilus influenzae* Type A in Children in Canada'S North: A Priority for Prevention. Can J Infect Dis Med Microbiol 2015;26:291–2. https://doi.org/10.1155/2015/613820.

[8] Tsang RSW, Li YA, Mullen A, Baikie M, Whyte K, Shuel M, et al. Laboratory characterization of invasive *Haemophilus influenzae* isolates from Nunavut, Canada, 2000–2012. Int J Circumpolar Health 2016;75:29798. https://doi.org/10.3402/ijch.v75.29798.

[9] http://www.bccdc.ca/resource-gallery/Documents/VPD+Invasive%20Bacterial%20Q4%202018%20Surveillance%20Report.pdf (assessed February 3, 2021).

[10] http://www.bccdc.ca/resource-gallery/Documents/Statistics%20and%20Research/Statistics%20and%20Reports/Immunization/Coverage/VPD+iGAS%20Q4%202019%20Surveillance%20Report.pdf (assessed February 3, 2021).

[11] Cox A, Barreto L, Ulanova M, Bruce M, Tsang R. Developing a vaccine for Haemophilus influenzae serotype a: Proceedings of a workshop. Canada Communicable Disease Report 2017;43:89–95. https://doi.org/10.14745/ccdr.v43i05a02.

[12] Cox AD, Williams D, Cairns C, St. Michael F, Fleming P, Vinogradov E, et al. Investigating the candidacy of a capsular polysaccharide-based glycoconjugate as a vaccine to combat Haemophilus influenzae type a disease: A solution for an unmet public health need. Vaccine 2017;35:6129–36. https://doi.org/10.1016/j.vaccine.2017.09.055.

[13] Ulanova M. Invasive Haemophilus influenzae Serotype a Disease in the H. influenzae Serotype b Conjugate Vaccine Era: Where Are We Going? Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa868.

[14] McConnell A, Tan B, Scheifele D, Halperin S, Vaudry W, Law B, et al. Invasive Infections Caused by Haemophilus influenzae Serotypes in Twelve Canadian IMPACT Centers, 1996–2001: Pediatr Infect Dis J 2007;26:1025–31. https://doi.org/10.1097/INF.0b013e31812f4f5b.

[15] Bruce MG, Zulz T, DeByle C, Singleton R, Hurlburt D, Bruden D, et al. Haemophilus influenzae Serotype a Invasive Disease, Alaska, USA, 1983–2011. Emerg Infect Dis 2013;19:932–7. https://doi.org/10.3201/eid1906.121805.

[16] Plumb ID, Lecy KD, Singleton R, Engel MC, Hirschfeld M, Keck JW, et al. Invasive Haemophilus influenzae Serotype a Infection in Children: Clinical Description of an Emerging Pathogen—Alaska, 2002–2014. Pediatr Infect Dis J 2018;37:298–303. https://doi.org/10.1097/INF.0000000000001764.

[17] Nix EB, Williams K, Cox AD, St. Michael F, Romero-Steiner S, Schmidt DS, et al. Naturally Acquired Antibodies against *Haemophilus influenzae* Type a in Aboriginal Adults, Canada. Emerg Infect Dis 2015;21:273–9. https://doi.org/10.3201/eid2102.140722.

[18] Nix EB, Choi J, Anthes C, Gaultier GN, Thorgrimson J, Cox AD, et al. Characterization of natural bactericidal antibody against Haemophilus influenzae type a in Canadian First Nations: A Canadian Immunization Research Network (CIRN) Clinical Trials Network (CTN) study. PLOS ONE 2018;13:e0201282. https://doi.org/10.1371/journal.pone.0201282.

[19] Schmidt DS, Bieging KT, Gomez-de-León P, Villaseñor-Sierra A, Inostroza J, Robbins JB, et al. Measurement of Haemophilus Influenzae Type A Capsular Polysaccharide Antibodies in Cord Blood Sera: Pediatr Infect Dis J 2012;31:876–8. https://doi.org/10.1097/INF.0b013e31825ab166.

[20] McClure M, Miernyk K, Bruden D, Rudolph K, Hennessy TW, Bruce MG, et al. Presence of Antibodies Against Haemophilus influenzae Serotype a in Alaska Before and After the Emergence of Invasive Infections. J Infect Dis. 2021 Feb 3;223(2):326-332. doi: 10.1093/infdis/jiaa369.PMID: 32594132

[21] Tsang RSW, Shuel M, Ahmad T, Hayden K, Knox N, Van Domselaar G, et al. Whole genome sequencing to study the phylogenetic structure of serotype a *Haemophilus influenzae* recovered from patients in Canada. Can J Microbiol 2020;66:99–110. https://doi.org/10.1139/cjm-2019-0406.

[22] Bruce MG, Deeks SL, Zulz T, Navarro C, Palacios C, Case C, et al. Epidemiology of *Haemophilus influenzae* Serotype a, North American Arctic, 2000–2005. Emerg Infect Dis 2008;14:48–55. https://doi.org/10.3201/eid1401.070822.

[23] Kelly DF, Moxon ER, Pollard AJ. Haemophilus influenzae type b conjugate vaccines. Immunology 2004;113:163–74. https://doi.org/10.1111/j.1365-2567.2004.01971.x.

[24] Kelly L, Tsang RSW, Morgan A, Jamieson FB, Ulanova M. Invasive disease caused by Haemophilus influenzae type a in Northern Ontario First Nations communities. J Med Microbiol 2011;60:384–90. https://doi.org/10.1099/jmm.0.026914-0.

[25] Eton V, Schroeter A, Kelly L, Kirlew M, Tsang RSW, Ulanova M. Epidemiology of invasive pneumococcal and Haemophilus influenzae diseases in Northwestern Ontario, Canada, 2010–2015. Int J Infect Dis 2017;65:27–33. https://doi.org/10.1016/j.ijid.2017.09.016.

[26] Brown VM, Madden S, Kelly L, Jamieson FB, Tsang RSW, Ulanova M. Invasive *Haemophilus influenzae* Disease Caused by Non–Type b Strains in Northwestern Ontario, Canada, 2002–2008. Clin Infect Dis 2009;49:1240–3. https://doi.org/10.1086/605671.

[27] Sadeghi-Aval P, Tsang RS, Jamieson FB, Ulanova M. Emergence of Non-Serotype b Encapsulated *Haemophilus influenzae* as a Cause of Pediatric Meningitis in Northwestern Ontario. Can J Infect Dis Med Microbiol 2013;24:13–6. https://doi.org/10.1155/2013/828730.

[28] Gaultier GN, McCready W, Ulanova M. Natural immunity against Haemophilus influenzae type a and B-cell subpopulations in adult patients with severe chronic kidney disease. Vaccine 2019;37:3677–84. https://doi.org/10.1016/j.vaccine.2019.05.036.

[29] Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A Critical Role of Natural Immunoglobulin M in Immediate Defense Against Systemic Bacterial Infection. J Exp Med 1998;188:2381–6. https://doi.org/10.1084/jem.188.12.2381.

411 [30] Baumgarth N, Tung JW, Herzenberg LA. Inherent specificities in natural antibodies: a key to immune
412 defense against pathogen invasion. Springer Seminars in Immunopathology 2005;26:347–62.
413 https://doi.org/10.1007/s00281-004-0182-2.
414
415 [31] Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and
416 bacterial distribution and disease by natural antibodies. Science. 1999;286(5447):2156-9.
417
418 [32] Lagergård T, Branefors P. Nature of cross-reactivity between Haemophilus influenzae types a and b and
419 Streptococcus pneumoniae types 6A and 6B. Acta Pathol Microbiol Immunol Scand C 1983;91:371–6.
420
421 [33] Schneerson R, Robbins JB. Induction of Serum *Haemophilus influenzae* Type B Capsular Antibodies in
422 Adult Volunteers Fed Cross-Reacting *Escherichia coli* 075:K100:H5. New England J Med 1975;292:1093–6.
423 https://doi.org/10.1056/NEJM197505222922103.
424
425 [34] Hall DB, Lum MKW, Knutson LR, Heyward WL, Ward JI. Pharyngeal carriage and acquisition of
426 anticapsular antibody to Haemophilus influenzae type b in a high-risk population in Southwestern Alaska. Am
427 J Epidemiol 1987;126:1190–7. https://doi.org/10.1093/oxfordjournals.aje.a114758.

Table 1: Demographics of sample population

Group	*n*	Age (years)		Department	
		Mean ± SD	No. (%) Female	Otolaryngology	Neurology
<5 years old	40	2.69 ± 1.23	13 (33%)	16 (40%)	24 (60%)
5-16 years old	93	8.49 ± 2.90	38* (41%)	77 (83%)	16 (17%)
All	133	6.75 ± 3.67	51* (38%)	93 (70%)	40 (30%)

*One unknown

443 **Table 2:** Geometrical mean IgM and IgG antibody concentrations against *Haemophilus influenzae*
444 type a in children of ages 0-2, 3-4, 5-6, 7-8, 9-10, 11-12, and 13-16 years.
445
446

Age (yrs)	*n*	IgM Antibody GMC, 95% CI (µg/ml)	IgG Antibody GMC, 95% CI (µg/ml)	P	IgM/IgG ratio 95% CI
0-2	15	0.47 [0.12-1.79]	0.07 [0.03-0.14]	0.0395	6.9 [1.7-28.6]
3-4	25	2.04 [1.58-2.64]	0.12 [0.07-0.20]	<0.0001	16.9 [9.5-29.8]
5-6	29	3.16 [2.47-4.05]	0.23 [0.14-0.39]	<0.0001	13.6 [7.5-24.6]
7-8	27	3.05 [1.92-4.86]	0.38 [0.22-0.66]	<0.0001	7.7 [4.5-17.2]
9-10	11	4.05 [2.47-6.64]	0.34 [0.20-0.58]	<0.0001	12.0 [5.7-25.4]
11-12	14	4.56 [3.10-6.70]	0.48 [0.21-1.08]	<0.0001	11.9 [5.2-27.2]
13-16	12	2.84 [1.83-4.41]	0.26 [0.11-0.61]	0.0001	10.9 [4.1-28.7]

Figure Legends

Figure 1.

Geometrical mean IgM and IgG antibody concentrations specific to *Haemophilus influenzae* type a in children of all ages [95% Confidence Intervals], µg/ml. The solid lines represent the geometric mean concentrations (GMC): IgM: 2.17 [1.57-3.01]; IgG, 0.22 [0.17-0.28]. ****p < 0.0001, Mann-Whitney U test.

Figure 2.

Linear regression analysis of association of IgG antibody concentrations specific to *Haemophilus influenzae* type a with age; p=0.03, slope=0.07.

Figure 3.

Linear regression analysis of association of IgM antibody concentrations specific to *Haemophilus influenzae* type a with age; p=0.001, slope=0.20.

Seroprevalence of IgG and IgM antibodies to *Haemophilus influenzae* type a in Canadian children

Brenda Huska[a#], Chelsea Kubinec[a#], Manish Sadarangani[b,c], Marina Ulanova[a*] for the Canadian Immunization Research Network Investigators

[a]Northern Ontario School of Medicine, Thunder Bay, ON, Canada

[b]Vaccine Evaluation Center, BC Children's Hospital Research Institute, Vancouver, BC, Canada

[c]Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada

[#]Contributed equally

*Corresponding author: Marina Ulanova, Division of Medical Sciences, Northern Ontario School of Medicine, Lakehead University, 955 Oliver Road, Thunder Bay, ON, P7B 5E1, Canada

Email address: mulanova@nosm.ca

ABSTRACT

Background

Over the last 2 decades, *Haemophilus influenzae* type a (Hia) has emerged as a significant cause of invasive disease in some geographic regions and populations. Recognition of the importance of Hia in the etiology of serious disease, particularly in young children, prompted the development of a new protein-capsular polysaccharide conjugate vaccine, similar in design to a vaccine against *H. influenzae* type b. At present, understanding of Hia immunology is incomplete; the immunological correlate of protection against invasive disease is unknown.

Methods

Our objective was to study Hia antibody in children of various ages residing in a Canadian province with low incidence rates of invasive disease. The enzyme-linked immunosorbent assays were performed to quantify plasma IgG and IgM specific to Hia capsular polysaccharide in 133 children (3 months to 16 years).

Results

Both anti-Hia IgG and IgM concentrations increased with age and were significantly higher in older children; a positive correlation between age and concentrations of Hia antibody was found. IgM antibody concentrations were significantly higher than IgG, with mean IgM concentrations over 10 times larger than IgG across all age groups.

Conclusions

The steady rise of naturally acquired, Hia-specific IgG and IgM concentrations in a pediatric population with low incidence rates of invasive Hia disease suggests the exposure to some cross-reactive environmental antigens as a major source of the antibody. However, the carriage rates of Hia in the region are unknown and further seroepidemiological studies are warranted. Although natural antibody may protect certain population groups against invasive disease, immunization of younger children will be essential to prevent serious infections if Hia continues to spread across North America.

Keywords:

Haemophilus influenzae type a; seroprevalence; natural antibody; children

1. INTRODUCTION

The Gram-negative coccobacillus *Haemophilus influenzae* frequently colonizes the nasopharynx of healthy individuals and can cause both local and systemic infections including otitis media, pneumonia, epiglottitis, meningitis, and sepsis [1]. *H. influenzae* is categorized based on the presence or absence of a polysaccharide capsule. There are six distinct serotypes of encapsulated *H. influenzae* (a-f); non-encapsulated strains are termed non-typeable (NTHi) [1]. The polysaccharide capsule is the major virulence factor of *H. influenzae* as it prevents complement deposition and phagocytosis required for bacterial clearance; anti-capsular antibodies are essential in immune defenses against the infection [1,2]. Among encapsulated *H. influenzae*, *H. influenzae* serotype b (Hib) is the most virulent, followed by serotype a (Hia) [3]. Prior to introduction of the Hib conjugate vaccine in late 1980s, Hib was the major cause of serious invasive diseases in children worldwide. The global weighted average incidence of meningitis caused by Hib was 57/100,000 in children <5 years of age, with much higher rates reported from North American and Australian Indigenous populations [4]. Pediatric immunization against Hib resulted in the dramatic reduction of Hib invasive disease in all the countries where it has been used [4]. However, over the last 20 years, invasive Hia disease, which shows remarkable similarities in epidemiology, clinical presentation, severity, and fatality rates to those caused by Hib, has emerged in some geographic areas including Alaska and Northern Canada [5]. The highest incidence rates of invasive Hia disease were reported from Alaska Native and Canadian Inuit children < 1 year of age, i.e. as high as 82/100,000 in Alaska [6], and 300/100,000 in Nunavut and Nunavik, the regions of Northern Canada [7,8]. Although data on epidemiology of invasive Hia disease in different parts of Canada are incomplete, it appears that the disease is much less common in some regions outside of the Arctic, such as the province of British Columbia. Between January 1, 2018 and December 31, 2019, a total of 14 cases of invasive Hia disease were reported from this province (0.1/100,000 population per year) [9,10].

The recognition of significance of Hia as a cause of serious invasive disease prompted the development of a new vaccine in Canada that has been recently investigated pre-clinically [11]. This vaccine was formulated on the basis of the same principles as vaccine against Hib, i.e. using Hia capsular polysaccharide as an

77 antigen conjugated to a protein carrier to make it immunogenic in young children who are yet unable to
78 develop antibody response to pure polysaccharide antigens [12]. To determine target population groups for
79 immunization against this infection, understanding of Hia immunity and defining immunological correlates of
80 protection are essential.
81
82 The reason for increased susceptibility to invasive Hia disease in certain population groups is currently
83 unknown [13]. Whereas the highest rates of invasive Hia disease have been reported from young Indigenous
84 children, immunocompetent adults do not typically develop invasive Hia disease [5, 14-16]. We have
85 recently established that healthy Indigenous adults possess naturally acquired functionally active antibodies
86 against Hia suggesting that certain level of natural immunity may protect against invasive Hia disease
87 [17,18]. We hypothesized that antibodies specific to Hia capsular polysaccharide develop with age due to
88 exposure to the pathogen and/or certain cross-reactive antigens. Our objective was to study plasma antibody
89 concentrations in children of various ages in a population with low incidence of invasive Hia disease.
90
91 2. METHODS
92
93 **2.1. Plasma samples**
94 Pediatric plasma samples were collected at BC Children's Hospital Biobank (Vancouver, British Columbia,
95 Canada) between October 2015 and May 2019. The ages of the children ranged from 3 months to 16 years
96 (Table 1). The study was approved by the University of British Columbia – Children's & Women's Health
97 Centre of BC and Lakehead University Research Ethics Boards. The samples were shipped frozen and
98 stored at -80°C until used.
99
100 **2.2. Quantification of *Haemophilus influenzae* type a (Hia) specific IgG and IgM in plasma**
101 To quantify IgG and IgM specific to Hia capsular polysaccharide, we used a previously described enzyme-
102 linked immunosorbent assay (ELISA) [17]. Antibody concentrations were determined using our internal
103 standard that was cross standardized to the Hia reference serum provided by the Centers for Disease
104 Control and Prevention. The internal standard contained 1.25 µg/mL anti-Hia IgG and 2.09 µg/mL anti-Hia

IgM. Absorbance measurements were made with OD_{450}, and OD_{630} as reference, and a log-log linear regression analysis was used to determine antibody concentration in µg/mL. The lower limit of detection was 0.04 µg/mL for IgM and 0.02 µg/mL for IgG; for statistical purposes, samples with values below detection were assigned a value ½ the lower limit. All samples were tested in duplicate.

2.3. Statistical analysis

The data were $\log_{10}$ transformed before analysis. Statistical analysis was performed using Graph-Pad Prism 6 (GraphPad Prism Software Inc., San Diego, CA). Geometric mean antibody concentrations (GMC) with 95% confidence intervals (CI) were calculated. Comparison of continuous variables was conducted by one-way ANOVA, Student's *t*-test, or Mann-Whitney U test depending on data distribution and number of groups; Fishers' exact test was used to compare categorical variables. To study association of antibody concentrations with age, linear regression and Spearman correlation analyses were performed. A cut-off 0.1 µg/mL of Hia-specific IgG was used for analysis as this concentration was previously found to correspond to the lowest IgG level associated with a measurable titre of functionally active antibody [19]. Significance was determined at $p < 0.05$.

3. RESULTS

For the study of seroprevalence of IgG and IgM antibodies specific to Hia capsular polysaccharide in a pediatric population undergoing diagnostic testing, plasma samples of 133 children aged between 3 months and 16 years were analyzed (Table 1). The majority of children attended the Otolaryngology Department for tonsillectomy (n = 93, 70%) and the remaining were assessed at the Neurology Department. There were 6 children < 1 year of age; most samples were collected from children ≥ 5 years of age.

The concentrations of Hia-specific IgG and IgM were high enough for detection with ELISA in the majority of plasma samples, with only 2 samples for IgG (both age 2 years) and 5 samples for IgM (children aged 3 months, 5 months, 10 months, 2 years, and 8 years) falling below detection limits. IgM antibody concentrations (GMC: 2.17, CI: 1.57, 3.01 µg/mL) were significantly higher than IgG (GMC: 0.22, CI: 0.17, 0.28 µg/mL) for the total sample population ($p < 0.0001$, Fig. 1), as well as across all the age groups, with

mean IgM/IgG ratio being 11.23 for the total sample population, from 6.9 to 16.9 for children of different ages (Table 2).

The concentrations of both IgG and IgM specific to Hia increased with age, with statistically significantly higher GMC found in children ≥ 5 years of age compared to younger children, i.e. IgG, 0.32, CI 0.24, 0.42 *vs.* 0.10, CI 0.06, 0.15 µg/mL; IgM, 3.35, CI 2.81, 3.99 *vs*. 1.25, CI 0.72, 2.14 µg/mL, correspondingly (p<0.0001 between 0-4 and 5-16 years old for both). Linear regression analysis showed significant association of both IgG and IgM Hia-specific antibody concentrations with age (Fig. 2 - 3). There was a positive, but moderate, correlation between age and concentrations of Hia-specific IgG (r = 0.37, p<0.0001) and IgM (r = 0.33, p=0.0001).

Concentrations of Hia-specific IgG exceeded 0.1 µg/mL in 78 samples (64%), with a lower proportion of samples above this cut-off among younger children (21% in ages 0-4 and 79% in ages 5-16, p <0.001). No difference in IgG antibody concentrations between males and females was found. GMC of IgM were higher in females than males: for ages 0-4, 1.38 *vs.* 1.09 µg/mL (p=0.8), for ages 5-16, 4.48 *vs*. 2.72 µg/mL (p=0.003).

4. DISCUSSION

In this study, we detected IgG and IgM specific to Hia capsular polysaccharide in 131/133 and 128/133 pediatric plasma samples, respectively. Concentrations of Hia-specific IgG and IgM steadily increased with age in a sample of pediatric population with low incidence rates of invasive Hia disease, with significantly higher antibody values in children of 5 years and older as compared to the younger ones. We also found a greater prevalence of IgM over IgG antibodies across all the ages; in average, concentrations of Hia-specific IgM were 11-fold higher than IgG.

The current understanding of Hia immunology is very limited; the data on concentrations of natural IgG antibodies specific to Hia capsular polysaccharide in Alaskan children have only been recently published [20]. Comparably to our data, in Alaska Native children, IgG antibody levels significantly increased with age,

despite the differences in Hia disease prevalence in Alaska and our region of interest. Over a period of 12 years (2007-2018), a total of 47 Hia isolates were collected from disease cases in British Columbia, a region of Western Canada bordering Alaska [21]. Considering that the population of British Columbia is roughly 5 million people, an average annual incidence rate of invasive Hia disease was 0.08/100,000 population. In comparison, an average annual incidence of invasive Hia disease in Alaska in 2008-2017 was 0.68/100,000 population, with much higher numbers in young Alaska Native children (27.74/100,000 population < 5 years of age) [6]. In Alaska, invasive Hia disease has been present since 2002 and incidence rates significantly increased since then [15,16, 22]. As reported by McClure et al. [20], 82.4% of children <10 years of age in Yukon-Kuskokwim Delta (where most cases of invasive Hia disease occurred) and 86.8% in Anchorage (with much lower incidence of disease) had concentrations of Hia-specific IgG > 0.1 µg/mL, a concentration previously found to correspond to the lowest IgG level associated with a measurable functionally active antibody [19]. In our study, we found a lower proportion of children <10 years of age with Hia-specific IgG > 0.1 µg/mL, i.e. 66%. This discrepancy may be explained by a lower circulation of Hia in British Columbia compared to Alaska. However, while the incidence of Hia invasive disease in our region of interest is much lower than that in Alaska, the carriage rates of Hia are unknown.

Age-dependent rise in natural Hia antibodies in children is reminiscent of historical data on immunology of Hib collected in the pre-Hib vaccine era. There are obvious similarities in the natural histories of Hia and Hib infections. Both Hia and Hib have polysaccharide capsules acting as major virulence factors; both can be carried asymptomatically in the nasopharynx by healthy individuals and cause invasive disease predominantly in children < 2 years of age while older children and immunocompetent adults are relatively resistant to the infection. In the pre-Hib vaccine era, protective immunity against Hib gradually developed in children after 2 years of age; an increase in antibody titres with age correlated with a decrease in the incidence of invasive disease (reviewed by [23]). However, McClure et al. [20] detected Hia-specific IgG in both Alaska Native and non-Native population groups residing in regions with different rates of invasive disease, with no significant differences in antibody concentrations by the race, study region (the Yukon-Kuskokwim Delta versus Anchorage), or time of sample collection (the 1980s and 1990s when no Hia disease was present, and after the emergence of the disease in 2000s). Our earlier studies also showed that

concentrations of Hia-specific IgG did not differ between Canadian Indigenous adults living in the regions with high versus low incidence of invasive Hia disease [18]. These observations illustrate the basic principle of epidemiology, i.e. the probability of the development of infectious disease depends both on the exposure to infectious agent and presence or absence of protective immunity. In regions with higher colonization rates and presence of factors facilitating the transmission of the infectious agent, such as overcrowding, lack of clean water, and indoor air pollution, higher antibody levels would be required to prevent the disease. Such environmental factors can explain the differences in rates of invasive disease between different populations [20]. Although no data on Hia colonization in Canada have yet been published, in Northwestern Ontario First Nations communities, over 8% of healthy children aged 3-5 years carried Hia in the nasopharynx (M. Ulanova, unpublished observations). In this region of Central Canada, where the annual average incidence rates of invasive Hia disease were 7/100,000 in 2004-2008 and 3.1/100,000 in 2010-2015 [24-27], Hia-specific antibodies were detected in all healthy Indigenous adults (median age 36.5 years), with geometrical mean IgG and IgM concentrations of 1.47 and 2.09 μg/mL, correspondingly [18]. Because healthy adults do not typically develop invasive Hia disease, these antibody concentrations are likely above the protective level. However, to gain better understanding of the immunological correlate of protection against invasive disease, more seroepidemiological studies will be needed, including studies of Hia colonization in different age groups and populations with high versus low incidence of invasive disease. With regards to our current data, lower incidence of Hia invasive disease within the population of interest may reflect lower carriage rates rather than protection from naturally acquired antibodies.

While we have previously collected data on natural immunity against Hia in both healthy and immunocompromized adults [17,18,28], serological studies in healthy children represent a significant challenge. In our study, the sample population included pediatric patients undergoing diagnostic testing in a large hospital in British Columbia that may not accurately represent the general pediatric population. Because the majority of children in our sample attended the Otolaryngology Department for tonsillectomy, they might have been potentially higher exposed to respiratory pathogens, including *Haemopilus influenzae*, and consequently developed higher antibody levels than other children. As we found clear prevalence of IgM over IgG among anti-Hia antibody in our sample, a recent exposure to Hia might potentially account for these

observations. However, in our previous studies of natural antibodies in healthy Indigenous and non-Indigenous adults, we also observed clear predominance of Hia-specific IgM over IgG although the opposite was true in case of naturally acquired antibodies specific to Hib [17,18]. To interpret these findings, it is important to consider the dominant role of IgM in the natural antibody repertoire [29-31].

While most seroprevalence studies traditionally focus on IgG antibody, the role of IgM in protection against invasive bacterial disease is indispensable. IgM has superior capacity to activate the complement and is able to greatly contribute to the bactericidal and opsonizing effects of antibody specific to encapsulated bacteria, such as Hia [2,29]. In addition, IgM produced by a particular subset of B cells (B-1) represents a major part of the natural antibody repertoire; while developing with age without a defined antigenic stimulation, the natural IgM antibody provides powerful defense against various pathogens [30,31]. Although the origin of natural anti-Hia antibodies in a pediatric population with low disease prevalence remains uncertain, our data suggest a potential role of immunization with cross-reactive antigens present in the environment. Indeed, serological cross-reactivity between Hia capsular polysaccharide and polysaccharide antigens of other bacteria, including *Streptococcus pneumoniae* serotype 6B, was documented [32]. By analogue, the role of cross-reactive antigens of commensal bacteria, in particular *Escherichia coli* K100, in the development of natural antibody against Hib in the pre-vaccine era was demonstrated [33]. Back in the 1980s, it was found that acquisition of natural anti-capsular antibody to Hib by Alaskan Eskimos was associated with pharyngeal carriage of Hib [34]. However, while Hib carriage was relatively uncommon (6.8% of all tested individuals), a clear positive association between antibody levels and age was observed: GMC of anti-Hib antibody increased in children between 6 months and 10 years of age [36], suggesting the effect of antigenic stimulation unrelated to Hib. With regards to our findings, as Hia carriage data in the region of interest are unavailable, the possibility of asymptomatic Hia colonization stimulating the development of antibodies in this population remains open. However, direct exposure to *H. influenzae* may not completely account for an increased antibody concentration with age; additional factors, such as cross-reactive antigens, can also be responsible for this. Overall, more seroepidemiological studies are required to elucidate the origin of natural Hia antibodies.

This study has several limitations. Complete data on Hia prevalence in the region are unavailable; information on Hia epidemiology is limited by cases of invasive disease, which is reportable in Canada. The most recent publication by Tsang et al. [21] described 47 isolates of Hia from British Columbia submitted to the National Microbiology Laboratory (Winnipeg, Manitoba) during 2007-2018. In addition, 29 cases of invasive Hia disease were identified between 1996 and 2008, based on serotyping of isolates submitted to BC Public Health Microbiology and Reference Laboratory (Dr. Linda Hoang, personal communication). However, etiology of non-invasive disease, such as non-bacteremic pneumonia or otitis media, is rarely documented. As there is a potential presence of Hia carriage in the population, a study of nasopharyngeal colonization in children of various ages will be important. In addition, our results are based on the analysis of antibodies from residual plasma samples in children undergoing diagnostic blood work rather than samples from healthy children because blood collection from healthy children for research purposes is not feasible for ethical reasons. Because the sample was determined by the availability of plasma specimens in the Biobank, most were from children ≥ 5 years of age, and fewer from younger children. We avoided using samples from patients who were potentially immunocompromised (e.g., children admitted to the oncology department); these samples were not included in our study. Most samples were from children scheduled for tonsillectomy; others were from children undergoing diagnostic testing for the neurology department. As discussed above, the prevalence of children with recurrent tonsillitis or hypertrophy of tonsils in our sample might account for high antibody concentrations in those who had potentially been exposed to Hia although no statistical differences in antibody levels between children attending Otolaryngology and Neurology Departments were noted (data not shown). Information on demographics and medical history collected at the Biobank database was limited. In particular, because information on ethnicity is not routinely gathered in the Canadian healthcare system, we could not include ethnicity in the demographic analysis.

5. CONCLUSIONS

Our data suggest that the development of natural immunity against Hia in a region without high incidence of invasive Hia disease may reflect accumulation of cross-reactive antibodies acquired through exposure to other common bacterial and/or environmental polysaccharide antigens. The prevalence of IgM suggests that

these anti-Hia antibodies are part of the natural antibody repertoire. Regardless of the antibody origin, these antibodies may be protective against invasive disease; however, an accurate protective threshold needs to be defined by extended seroepidemiological studies including regions with high incidence of Hia disease. Although natural antibodies may be protective in certain population groups, immunization of younger children will be essential to prevent serious invasive disease, especially if Hia continues to spread across North America.

FUNDING

This work was supported by the Canadian Immunization Research Network (CT20ON31) and the Northern Ontario School of Medicine Summer Medical Student Research Award to Chelsea Kubinec.

ACKNOWLEDGEMENTS

We are grateful to the personnel at the BC Children's Hospital Biobank for providing plasma samples for analysis and reviewing health charts of study participants and to Dr. Linda Hoang (BC Public Health Microbiology and Reference Laboratory) for sharing unpublished data. We thank Dr. Andrew Cox (National Research Council Canada) for providing the Hia capsular polysaccharide and Dr. Karen Rudolph (Arctic Investigation Program, Centers for Disease Control and Prevention) for donating the reference serum. Technical help of Ms. Angele Desbiens for optimizing ELISA is greatly appreciated.

REFERENCES

[1] Murphy TF. Haemophilus Species, Including H. influenzae and H. ducreyi (Chancroid). Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th ed., Philadelphia, PA: Elsevier/Saunders; 2015, p. 2575–83.

[2] Schreiber JR, Barrus V, Cates KL, Siber GR. Functional Characterization of Human IgG, IgM, and IgA Antibody Directed to the Capsule of Haemophilus influenzae Type b. J Infect Dis 1986;153:8–16. https://doi.org/10.1093/infdis/153.1.8.

[3] Zwahlen A, Kroll JS, Rubin LG, Moxon ER. The molecular basis of pathogenicity in Haemophilus influenzae: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus cap. Microb Pathog 1989;7:225–35. https://doi.org/10.1016/0882-4010(89)90058-2.

[4] Peltola H. Worldwide Haemophilus influenzae Type b Disease at the Beginning of the 21st Century: Global Analysis of the Disease Burden 25 Years after the Use of the Polysaccharide Vaccine and a Decade after the Advent of Conjugates. Clin Microbiol Rev 2000;13:302–17. https://doi.org/10.1128/CMR.13.2.302-317.2000.

[5] Ulanova M, Tsang RSW. Haemophilus influenzae serotype a as a cause of serious invasive infections. Lancet Infect Dis 2014;14:70–82. https://doi.org/10.1016/S1473-3099(13)70170-1.

[6] Soeters HM, Oliver SE, Plumb ID, Blain AE, Zulz T, Simons BC, et al. Epidemiology of Invasive Haemophilus influenzae Serotype a Disease—United States, 2008–2017. Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa875.

[7] Boisvert A-A, Moore D. Invasive Disease due to *Haemophilus influenzae* Type A in Children in Canada'S North: A Priority for Prevention. Can J Infect Dis Med Microbiol 2015;26:291–2. https://doi.org/10.1155/2015/613820.

[8] Tsang RSW, Li YA, Mullen A, Baikie M, Whyte K, Shuel M, et al. Laboratory characterization of invasive *Haemophilus influenzae* isolates from Nunavut, Canada, 2000–2012. Int J Circumpolar Health 2016;75:29798. https://doi.org/10.3402/ijch.v75.29798.

[9] http://www.bccdc.ca/resource-gallery/Documents/VPD+Invasive%20Bacterial%20Q4%202018%20Surveillance%20Report.pdf (assessed February 3, 2021).

[10] http://www.bccdc.ca/resource-gallery/Documents/Statistics%20and%20Research/Statistics%20and%20Reports/Immunization/Coverage/VPD+iGAS%20Q4%202019%20Surveillance%20Report.pdf (assessed February 3, 2021).

[11] Cox A, Barreto L, Ulanova M, Bruce M, Tsang R. Developing a vaccine for Haemophilus influenzae serotype a: Proceedings of a workshop. Canada Communicable Disease Report 2017;43:89–95. https://doi.org/10.14745/ccdr.v43i05a02.

[12] Cox AD, Williams D, Cairns C, St. Michael F, Fleming P, Vinogradov E, et al. Investigating the candidacy of a capsular polysaccharide-based glycoconjugate as a vaccine to combat Haemophilus influenzae type a disease: A solution for an unmet public health need. Vaccine 2017;35:6129–36. https://doi.org/10.1016/j.vaccine.2017.09.055.

[13] Ulanova M. Invasive Haemophilus influenzae Serotype a Disease in the H. influenzae Serotype b Conjugate Vaccine Era: Where Are We Going? Clin Infect Dis 2020. https://doi.org/10.1093/cid/ciaa868.

[14] McConnell A, Tan B, Scheifele D, Halperin S, Vaudry W, Law B, et al. Invasive Infections Caused by Haemophilus influenzae Serotypes in Twelve Canadian IMPACT Centers, 1996–2001: Pediatr Infect Dis J 2007;26:1025–31. https://doi.org/10.1097/INF.0b013e31812f4f5b.

[15] Bruce MG, Zulz T, DeByle C, Singleton R, Hurlburt D, Bruden D, et al. Haemophilus influenzae Serotype a Invasive Disease, Alaska, USA, 1983–2011. Emerg Infect Dis 2013;19:932–7. https://doi.org/10.3201/eid1906.121805.

[16] Plumb ID, Lecy KD, Singleton R, Engel MC, Hirschfeld M, Keck JW, et al. Invasive Haemophilus influenzae Serotype a Infection in Children: Clinical Description of an Emerging Pathogen—Alaska, 2002–2014. Pediatr Infect Dis J 2018;37:298–303. https://doi.org/10.1097/INF.0000000000001764.

[17] Nix EB, Williams K, Cox AD, St. Michael F, Romero-Steiner S, Schmidt DS, et al. Naturally Acquired Antibodies against *Haemophilus influenzae* Type a in Aboriginal Adults, Canada. Emerg Infect Dis 2015;21:273–9. https://doi.org/10.3201/eid2102.140722.

[18] Nix EB, Choi J, Anthes C, Gaultier GN, Thorgrimson J, Cox AD, et al. Characterization of natural bactericidal antibody against Haemophilus influenzae type a in Canadian First Nations: A Canadian Immunization Research Network (CIRN) Clinical Trials Network (CTN) study. PLOS ONE 2018;13:e0201282. https://doi.org/10.1371/journal.pone.0201282.

[19] Schmidt DS, Bieging KT, Gomez-de-León P, Villaseñor-Sierra A, Inostroza J, Robbins JB, et al. Measurement of Haemophilus Influenzae Type A Capsular Polysaccharide Antibodies in Cord Blood Sera: Pediatr Infect Dis J 2012;31:876–8. https://doi.org/10.1097/INF.0b013e31825ab166.

[20] McClure M, Miernyk K, Bruden D, Rudolph K, Hennessy TW, Bruce MG, et al. Presence of Antibodies Against Haemophilus influenzae Serotype a in Alaska Before and After the Emergence of Invasive Infections. J Infect Dis. 2021 Feb 3;223(2):326-332. doi: 10.1093/infdis/jiaa369.PMID: 32594132

[21] Tsang RSW, Shuel M, Ahmad T, Hayden K, Knox N, Van Domselaar G, et al. Whole genome sequencing to study the phylogenetic structure of serotype a *Haemophilus influenzae* recovered from patients in Canada. Can J Microbiol 2020;66:99–110. https://doi.org/10.1139/cjm-2019-0406.

[22] Bruce MG, Deeks SL, Zulz T, Navarro C, Palacios C, Case C, et al. Epidemiology of *Haemophilus influenzae* Serotype a, North American Arctic, 2000–2005. Emerg Infect Dis 2008;14:48–55. https://doi.org/10.3201/eid1401.070822.

[23] Kelly DF, Moxon ER, Pollard AJ. Haemophilus influenzae type b conjugate vaccines. Immunology 2004;113:163–74. https://doi.org/10.1111/j.1365-2567.2004.01971.x.

[24] Kelly L, Tsang RSW, Morgan A, Jamieson FB, Ulanova M. Invasive disease caused by Haemophilus influenzae type a in Northern Ontario First Nations communities. J Med Microbiol 2011;60:384–90. https://doi.org/10.1099/jmm.0.026914-0.

[25] Eton V, Schroeter A, Kelly L, Kirlew M, Tsang RSW, Ulanova M. Epidemiology of invasive pneumococcal and Haemophilus influenzae diseases in Northwestern Ontario, Canada, 2010–2015. Int J Infect Dis 2017;65:27–33. https://doi.org/10.1016/j.ijid.2017.09.016.

[26] Brown VM, Madden S, Kelly L, Jamieson FB, Tsang RSW, Ulanova M. Invasive *Haemophilus influenzae* Disease Caused by Non–Type b Strains in Northwestern Ontario, Canada, 2002–2008. Clin Infect Dis 2009;49:1240–3. https://doi.org/10.1086/605671.

[27] Sadeghi-Aval P, Tsang RS, Jamieson FB, Ulanova M. Emergence of Non-Serotype b Encapsulated *Haemophilus influenzae* as a Cause of Pediatric Meningitis in Northwestern Ontario. Can J Infect Dis Med Microbiol 2013;24:13–6. https://doi.org/10.1155/2013/828730.

[28] Gaultier GN, McCready W, Ulanova M. Natural immunity against Haemophilus influenzae type a and B-cell subpopulations in adult patients with severe chronic kidney disease. Vaccine 2019;37:3677–84. https://doi.org/10.1016/j.vaccine.2019.05.036.

[29] Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A Critical Role of Natural Immunoglobulin M in Immediate Defense Against Systemic Bacterial Infection. J Exp Med 1998;188:2381–6. https://doi.org/10.1084/jem.188.12.2381.

411 [30] Baumgarth N, Tung JW, Herzenberg LA. Inherent specificities in natural antibodies: a key to immune
412 defense against pathogen invasion. Springer Seminars in Immunopathology 2005;26:347–62.
413 https://doi.org/10.1007/s00281-004-0182-2.
414
415 [31] Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and
416 bacterial distribution and disease by natural antibodies. Science. 1999;286(5447):2156-9.
417
418 [32] Lagergård T, Branefors P. Nature of cross-reactivity between Haemophilus influenzae types a and b and
419 Streptococcus pneumoniae types 6A and 6B. Acta Pathol Microbiol Immunol Scand C 1983;91:371–6.
420
421 [33] Schneerson R, Robbins JB. Induction of Serum *Haemophilus influenzae* Type B Capsular Antibodies in
422 Adult Volunteers Fed Cross-Reacting *Escherichia coli* 075:K100:H5. New England J Med 1975;292:1093–6.
423 https://doi.org/10.1056/NEJM197505222922103.
424
425 [34] Hall DB, Lum MKW, Knutson LR, Heyward WL, Ward JI. Pharyngeal carriage and acquisition of
426 anticapsular antibody to Haemophilus influenzae type b in a high-risk population in Southwestern Alaska. Am
427 J Epidemiol 1987;126:1190–7. https://doi.org/10.1093/oxfordjournals.aje.a114758.

Table 1: Demographics of sample population

Group	*n*	Age (years) Mean ± SD	No. (%) Female	Department Otolaryngology	Department Neurology
<5 years old	40	2.69 ± 1.23	13 (33%)	16 (40%)	24 (60%)
5-16 years old	93	8.49 ± 2.90	38* (41%)	77 (83%)	16 (17%)
All	133	6.75 ± 3.67	51* (38%)	93 (70%)	40 (30%)

*One unknown

Table 2: Geometrical mean IgM and IgG antibody concentrations against *Haemophilus influenzae* type a in children of ages 0-2, 3-4, 5-6, 7-8, 9-10, 11-12, and 13-16 years.

Age (yrs)	*n*	IgM Antibody GMC, 95% CI (µg/ml)	IgG Antibody GMC, 95% CI (µg/ml)	P	IgM/IgG ratio 95% CI
0-2	15	0.47 [0.12-1.79]	0.07 [0.03-0.14]	0.0395	6.9 [1.7-28.6]
3-4	25	2.04 [1.58-2.64]	0.12 [0.07-0.20]	<0.0001	16.9 [9.5-29.8]
5-6	29	3.16 [2.47-4.05]	0.23 [0.14-0.39]	<0.0001	13.6 [7.5-24.6]
7-8	27	3.05 [1.92-4.86]	0.38 [0.22-0.66]	<0.0001	7.7 [4.5-17.2]
9-10	11	4.05 [2.47-6.64]	0.34 [0.20-0.58]	<0.0001	12.0 [5.7-25.4]
11-12	14	4.56 [3.10-6.70]	0.48 [0.21-1.08]	<0.0001	11.9 [5.2-27.2]
13-16	12	2.84 [1.83-4.41]	0.26 [0.11-0.61]	0.0001	10.9 [4.1-28.7]

Figure Legends

Figure 1.

Geometrical mean IgM and IgG antibody concentrations specific to *Haemophilus influenzae* type a in children of all ages [95% Confidence Intervals], µg/ml. The solid lines represent the geometric mean concentrations (GMC): IgM: 2.17 [1.57-3.01]; IgG, 0.22 [0.17-0.28]. ****p < 0.0001, Mann-Whitney U test.

Figure 2.

Linear regression analysis of association of IgG antibody concentrations specific to *Haemophilus influenzae* type a with age; p=0.03, slope=0.07.

Figure 3.

Linear regression analysis of association of IgM antibody concentrations specific to *Haemophilus influenzae* type a with age; p=0.001, slope=0.20.

Figures

Click here to access/download;Figures;New Figure 1 (1).jpg

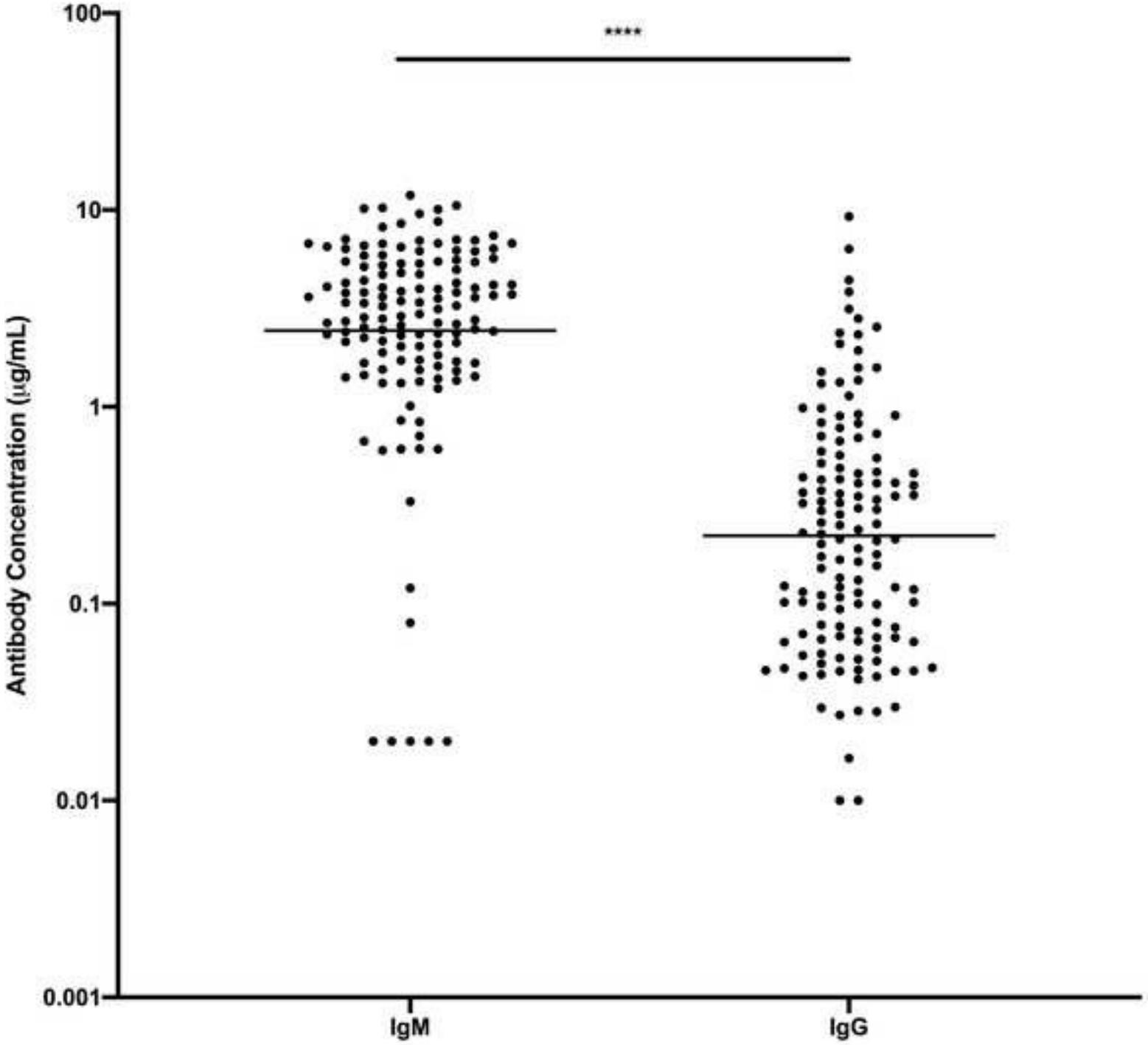

Figure 2

Click here to access/download;Figures;New Figure 2.jpg

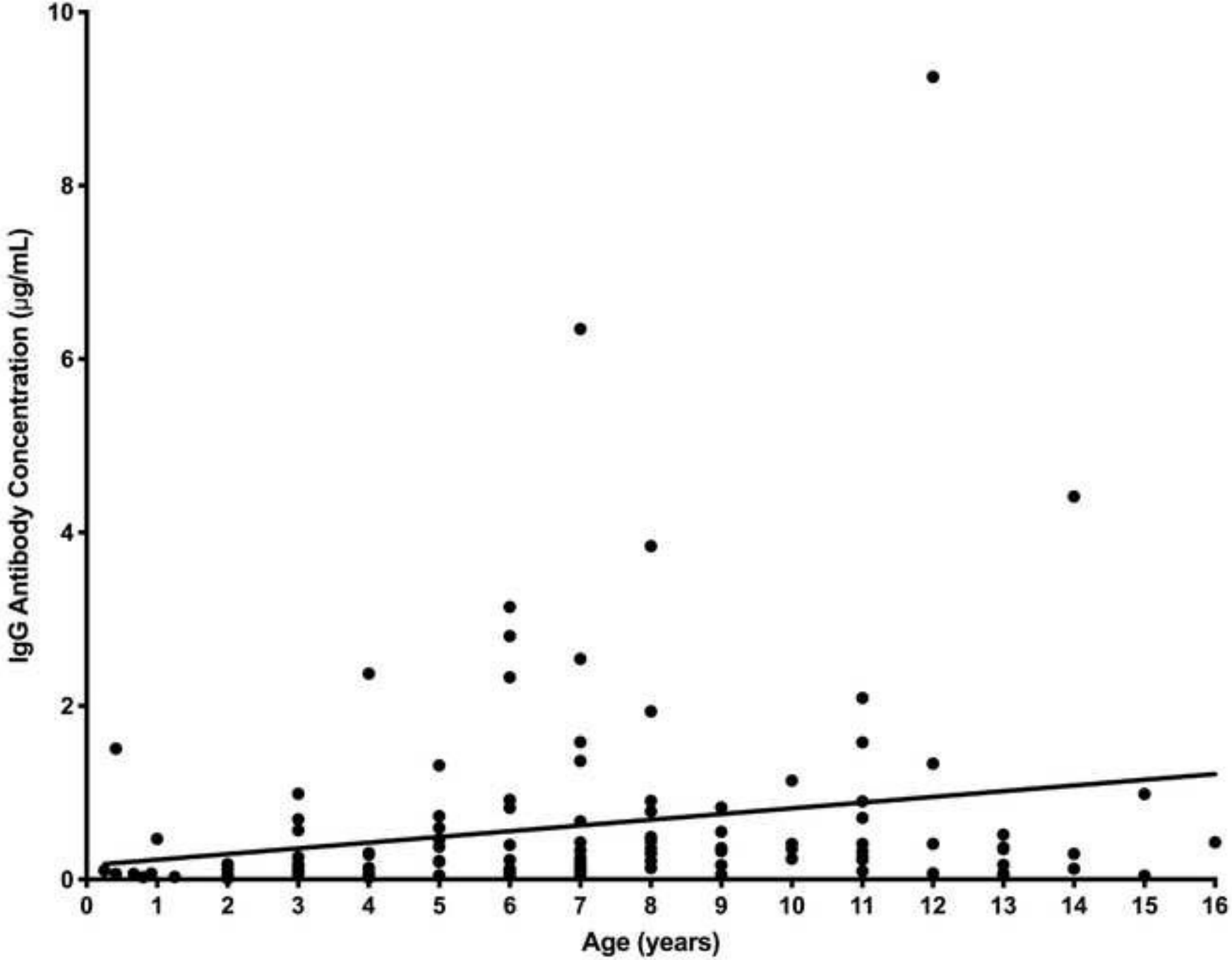

Figure 3

Click here to access/download;Figures;New Figure 3.jpg