Hemagglutinin and neuraminidase antibodies are induced in an age- and subtype-dependent manner after influenza virus infection.

Sook-San Wong¹,²,³, Ben Waite⁴, Jacqui Ralston⁴, Tim Wood⁴, G Edwin Reynolds⁵, Ruth Seeds⁴,⁸, E. Claire Newbern⁴, Mark G. Thompson⁶, Q. Sue Huang⁴, Richard J. Webby³,⁹, and the SHIVERS Investigation Team⁷.

¹State Key Laboratory of Respiratory Disease, Guangzhou Medical University, 195 Dongfengxi Road, Guangzhou 510182, People's Republic of China
²School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong SAR, People's Republic of China.
³Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, TN 38105, USA
⁴Institute of Environmental Science and Research Ltd, NCBID Wallaceville, 66 Ward Street, Upper Hutt 5018, New Zealand
⁵Immunisation Advisory Centre (IMAC), University Services, University of Auckland, Auckland, New Zealand
⁶Current Affiliation: Auckland’s Regional Public Health Service, Auckland, New Zealand
⁷SHIVERS Science Management Team: Nikki Turner, Michael Baker, Cameron Grant, Colin McArthur, Sally Roberts, Adrian Trenholme, Conroy Wong, Susan Taylor, Diane Gross, Jazmin Duque; Marc-Alain Widdowson; The research nurses at Auckland District Health Board

SHIVERS investigation team:
(ADHB): Bhamita Chand, Kathryn Haven, Pamela Muponisi, Debbie Aley, Claire Sherring, Miriam Rea, Judith Barry, Tracey Bushell, Julianne Brewer, Catherine McClymont;
The research nurses at Counties Manukau District Health Board (CMDHB): Shirley Lawrence, Emma Collis, Amanda Retter, Shona Chamberlin, Reniza Ongcoy, Kirstin Davey, Emilina Jasmat, Maree Dickson, Annette Western, Olive Lai, Sheila Fowlie, Faasoa Aupa’au, Louise Robertson;
The WHO National Influenza Centre, Institute of Environmental Science and Research (ESR):
Angela Todd, Lauren Jelley, Judy Bocacao, Wendy Gunn, Pam Kawakami, Sue Walker, Robyn Madge, Amanda des Barres;
The ADHB Laboratory: Fahimeh Rahnama;
The CMDHB Laboratory: Helen Qiao, Fifi Tse, Mahtab Zibaei, Tirzah Korrapadu, Louise Optland, Cecilia Dela Cruz.
Labtests Laboratory in Auckland; and sentinel general practices in Auckland.
*Current affiliation: Ministry of Primary Industry, 66 Ward Street, Upper Hutt 5018, New Zealand

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#Address correspondence to Richard Webby, richard.webby@stjude.org

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Abstract.

Despite evidence that antibodies targeting the influenza virus neuraminidase (NA) protein can be protective and are broadly cross-reactive, the immune response to NA during infection is poorly understood compared to the response to hemagglutinin (HA) protein. As such, we compared the antibody profile to HA and NA in two naturally-infected human cohorts in Auckland, New Zealand; a serosurvey cohort, consisting of pre- and post-influenza season sera from PCR-confirmed influenza cases (n=50), and an immunology cohort, consisting of paired sera collected after PCR-confirmation of infection (n=94). The induction of both HA and NA-antibodies in these cohorts was influenced by age and subtype. Seroconversion to HA was more frequent in those < 20 years old (yo) for influenza A (Serosurvey, p=0.01, Immunology, p=0.02), but not influenza B virus infection. Seroconversion to NA was not influenced by age or virus type.

Adults ≥ 20 yo infected with influenza A viruses were more likely to show NA-only seroconversion compared to children (56% vs 14% [5 – 19 yo] and 0% [0 – 4 yo] respectively).

Conversely, children infected with influenza B viruses were more likely than adults to show NA-only seroconversion (88% [0 – 4 yo] and 75% [5 – 19 yo] vs 40% [≥ 20 yo]). These data indicate a potential role for immunological memory in the dynamics of HA and NA-antibody responses. A better mechanistic understanding of this phenomenon will be critical for any future vaccines aimed at eliciting NA immunity.

Importance.

Data on the immunologic responses to neuraminidase (NA) is lacking when compared to what is available on hemagglutinin (HA) responses, despite growing evidence that NA-immunity can be protective and broadly cross-reactive. Understanding these NA responses during natural
infection is key to exploiting these properties for improving influenza vaccines. Using two community-acquired influenza cohorts, we showed that the induction of both HA and NA-antibody after infection is influenced by age and subtypes. Such response dynamics suggests the influence of immunological memory and understanding how this process is regulated will be critical to any vaccine effort targeting NA-immunity.

Key Words: Influenza, antibody, hemagglutination-inhibition, neuraminidase-inhibition, serology
Introduction

Neuraminidase (NA) is the second most abundant glycoprotein on the surface of the influenza virus. It functions as a sialidase to facilitate virus trafficking through host mucosal barriers as well as egress from infected cells. Inhibition of its activity has been the cornerstone of currently recommended influenza antivirals ([https://www.cdc.gov/mmwr/pdf/rr/rr6001.pdf](https://www.cdc.gov/mmwr/pdf/rr/rr6001.pdf)). Although immunity to NA limits disease severity (1-4), relatively little is known about population immunity to the protein or the robustness of the antibody response against it following infection. These facets of NA immunity have been identified as crucial knowledge gaps in the campaign to develop better seasonal influenza vaccines (5-7).

The paucity of data on NA-immunity has historically been impeded by the lack of an appropriate and scalable assay. The traditional NA-inhibition (NAI) assay that was based on the enzymatic cleavage of sialic acids on the substrate fetuin was laborious and required the use of hazardous chemicals such as arsenite and thiobarbituric acid (8). However, the development and validation of an enzyme-linked lectin assay (ELLA) has enabled rapid and high-throughput testing of NAI antibodies (9, 10), leading to renewed interest in evaluating the role of NA antibodies during influenza virus infection and vaccination (1, 2, 4, 11, 12). The ELLA assay has shown high subtype-specificity and reproducibility when tested with antigen-specific ferret antisera (9) and higher sensitivity compared to the traditional NAI assay (13).

In this study, we explored the antibody response to HA and NA following community-acquired influenza virus infection in two cohorts enrolled through the Southern Hemisphere Influenza Vaccine Effectiveness Research Surveillance (SHIVERS) study based in Auckland, New
Zealand (14). The cohorts utilized distinct sampling protocols that were representative of epidemiological and clinical-based studies. We examined the seroconversion frequencies to HA using the standard hemagglutination-inhibition (HAI) assay and NA using ELLA, in individuals with PCR-confirmed influenza virus infections. Since recent studies suggest that the HAI-assay may have limited sensitivity in detecting recent influenza virus infections (15-17), we further examined the added benefit of using NAI-antibody responses as an alternative serologic marker of infection. Data from this study suggests that the dynamics of antibody responses to both HA and NA after infection is influenced by age and subtype and that the use of the ELLA assay to detect NAI-antibodies after infection can circumvent some of the current limitations associated with HAI-assays.

Methods

Ethics Approval

This study received ethics approval from the New Zealand Northern (A) Health & Disability Ethics Committee under references NTX/11/11/102/AM02, AM05, AM06, AM13, and AM14.

Study Design

The sampling designs for this study are summarized in Figure 1. For the serosurvey cohort, randomly selected community participants enrolled at SHIVERS participating general practices (GPs) provided pre-influenza season sera in March to June and post-season sera in October to November of 2015. During the influenza season (May to September), nasopharyngeal swabs from 209 unvaccinated participants with influenza-like illness (ILI) (defined as cough and fever with onset ≤10 days) were tested by PCR for evidence of influenza virus infection. Fifty-one
infections (24% positivity) were identified and the sera from these individuals were analyzed for this study. The immunology cohort was recruited in the 2013 and 2015 seasons and consisted of 94 patients presenting to GP clinics (N=29) with acute respiratory illness (ARI) or to hospitals with severe acute respiratory illness (SARI) (N=65) based on the World Health Organization disease classification (18). For this cohort, the first blood draw occurred following a positive influenza PCR test and the second occurred at least 14 days later. Study data were collected and managed using Research Electronic Data Capture (REDCap) tools (19) hosted at the Institute for Environmental Science and Research (ESR). To examine the specificity of the assays, a panel comprising of 47 paired sera, collected approximately 14 days apart during the summer of 2016 for arbovirus/legionella testing at ESR, were also evaluated.

PCR Diagnosis

Respiratory samples were tested and subtyped using the United States Centers for Disease Control and Prevention real-time RT-PCR protocol (16) or the AusDiagnostics PCR protocol (20), as previously described (21-23).

Serologic Testing

Serologic testing was performed at the National Influenza Center at ESR, New Zealand. Receptor-destroying enzyme (RDE)–treated sera were tested against the strains included in the Southern Hemisphere vaccine for the relevant enrollment year (A/H3, A/H1 and the two B-lineages; Table 1) according to standard practice (24). The ELLA assay was used to test for NA-specific antibodies as previously described (9, 11, 25). Recombinant influenza A viruses (IAVs) composed of NA from the viruses to be tested and a mismatched HA from A/Teal/Hong
Kong/W312/1997 (H6N1), were generated using the reverse-genetics (rg) method and used as antigens. The NAs used in recombinant viruses were: N1 from A/California/04/2009 (H1N1) and the N2 from A/Victoria/361/2011 (H3N2) and A/ Switzerland/9715293/2013 (H3N2). Wild type viruses B/Brisbane/60/2008 (Victoria-lineage, Vic) or B/Phuket/3073/2013 (Yamagata-lineage, Yam) (9) were used in the influenza B virus (IBV) ELLA assays. Serum samples were tested at a starting dilution of 1:10. For the summer control cohort, sera from those individuals were tested against all four influenza viruses and the percentage of seroconversion events against IAV (A/H3 and A/H1) and IBV (B/Yam and B/Vic) were reported.

**Statistical Analysis**

Seroconversion was defined as a 4-fold increase between the first and second HAI or NAI titer against the subtype of the infecting virus. Additionally, if the first titer was below the detection threshold (<10), the second titer had to be ≥40. IAV cases that were not subtyped were assumed to be A/H3N2, as it was the predominant subtype circulating in both seasons of the study. Non-lineage-typed IBVs were assumed to be the B-antigens with the highest increase in titer. Geometric mean titers (GMTs) were reported as the back-transformed average of the log₂-antibody titers and the geometric mean fold-change (GMFC) were reported as the back-transformed average of the differences between the first and second log₂-antibody titer (25). Age-specific effects on the antibody responses were analyzed by Analysis of Variance (ANOVA) tests.

Kappa (κ) statistics were used to describe the level of agreement between PCR diagnosis and seroconversion status by HAI only, NAI only, HAI and NAI, and HAI or NAI. The strength of
agreement between the two assays was categorized based on the Landis and Koch Kappa Benchmark Scale [33]. Test sensitivity and specificity scores were also calculated for each assay or combination of assays. For the serosurvey cohort, the negative cases were the ILI-individuals that were PCR-negative during the season (N=209). For the immunology cohort, the negative cases were derived from the summer cohort. For IAV, we considered PCR-subtype positivity as the gold standard outcome, and the homologous subtype seroconversion event as the test to ascertain agreement. For IBV, we only considered PCR-type and any flu B serotype seroconversion for agreement. All calculations were generated by using the R package epiR (26, 27).

Results

Cohort Characteristics

Table 2 describes the characteristics of the study participants and sampling details in the two cohorts of this study. The 50 PCR-confirmed influenza positive serosurvey cohort participants (one participant was infected with both IAV and IBV) were relatively equally distributed across the three age groups (< 5 years old (yo), 5 to 19 yo and ≥20 yo). Twenty-one (42%) participants identified as Maori (n=7), Pacific (n=5) or of Asian (n=9) descent and the remaining participants were of European descent (classified as others) (Table 2). There were equal proportions of IAV (25/51, 49%) and IBV (26/51, 51%), and both B-lineages were equally represented (B/Vic: 15/51, 29%; B/Yam: 11/51, 22%). The median time from pre-season serum collection to symptom onset was 134 days (approximately 4 months, range: 22 to 193 days). The median interval between the pre-season and post-season sera collections was approximately 7 months (218 days, range: 132 to 274 days).
In the immunology cohort, 82 of 94 (87%) paired sera were collected from adults (>20 yo). Forty-six (49%) participants identified as Pacific (n=23), Maori (n=15), or of Asian (n=8) descent, and the remaining 47 were of European descent. Most infections were due to IAVs (74%) or IBVs of Yamagata lineage (20%). Because PCR testing and serum collection was conducted only following presentation with symptoms, the median time from symptom onset to first serum collection was 12 days (range 1 to 28).

**HAI and NAI-antibody responses in the serosurvey cohort**

The serosurvey cohort allowed us to collect true baseline samples prior to substantial community influenza activity. HAI and NAI-antibody titers were determined on pre- and post-season serum samples from individuals with laboratory-confirmed influenza during the season. For participants with IAV infections (Table 3), the baseline HAI geometric mean titers (GMT) were low in this cohort across all age groups whereas there were relatively small increases in baseline NAI GMTs with increasing age [GMT (95% CI) for 0-4, 5-19 and ≥20 yo: 5 (5, 5), 24 (15, 40) and 13 (7, 24), respectively, p=0.0005]. The Geometric Mean Fold Change (GMFC) between the pre- and post-season sera decreased with increasing age for HAI [GMFC (95% CI) for 0-4, 5-19, ≥20 yo: 22 (14, 35), 10 (8, 12), 4 (3, 6), respectively, p=0.0005] but not for NAI. There were no age-dependent differences on the NAI GMFC responses. Baseline titers did not appear to influence the magnitude of fold-change for HAI- or NAI-antibody titers (Figure 2A and 2B).

We next looked at seroconversion events detected by each assay, as determined by a four-fold or greater rise in titer and meeting the minimum threshold titer of 40. The frequency of HAI-seroconversion in IAV were lowest in adults (44% [95% CI: 4% - 85%]) compared to children.
(86% [95% CI: 51% - 121%]) and young children (100%) (p =0.0133). In contrast, the frequencies of NAI-seroconversion ranged from 67% to 100% and were not statistically different by age.

For IBV, the baseline GMT increased significantly with age for NAI [GMT (95% CI) for 0-4, 5-19, ≥20 yo: 11 (7 - 18), 31 (11 - 86), 149 (64 - 348) respectively, p <0.001] but not for HAI, which were relatively low in all age groups. HAI-seroconversion to IBV were more frequent in adults compare to younger participants [% Seroconversion (95% CI) for 0-4, 5-19, ≥20 yo: 13 (-17, 42), 25 (-14, 64), 60 (23, 97) respectively] although this had weak statistical support (p=0.09). NAI-seroconversion was detected in 100% of the IBV-infected individuals, with children having a larger GMFC compared to adults. Although the magnitude of the response was greater in those with low baseline NAI-titers (< 40), individuals with high baseline titers (between 160-640) were still capable of mounting at least a 4-fold increase in titer (Figure 2C and 2D).

We next looked at seroconversion events to HA and NA at an individual level. In the serosurvey cohort, concurrent HAI and NAI-seroconversions for IAV occurred in 67% (6/9), 86% (6/7) and 33% (3/9) of 0-4, 5-19, ≥20 yo (Figure 3) respectively. HAI-seroconversions only (no NAI-seroconversions) occurred in 16% (4/25) (3 in 0-4 yo, 1 in ≥20 yo) of the PCR-positive cases. NAI-only seroconversions (no HAI-seroconversions) occurred in 24% (6/25) of individuals, mostly in adults [0%, 14% (1/7) and 56% (5/9) in the respective age groups]. In contrast to the trend observed for IAV, HAI and NAI-seroconversions in IBV infected individuals occurred in 12.5% (1/8), 25% (2/8) and 60% (6/10) of 0-4, 5-19, ≥20 yo respectively. Additionally, 17/26 of
the IBV-infected individuals that did not show HAI-seroconversion, seroconverted to NA (88% (7/8), 75% (6/8) and 40% (4/10) in the 0-4, 5-19, ≥20 yo respectively). No HAI-only seroconversions were detected.

The challenge of conducting immunologic studies in medical care facilities is that infections, and corresponding immune responses, are often well underway by the time patients present. Therefore, true baseline blood draws are difficult to obtain. This was likely the case in our immunology cohort, since the baseline antibody GMT to HA and NA were higher compared to the serosurvey cohort across all age-groups (Table 4). Within this cohort there were no statistical differences in the IAV baseline GMT for HAI or NAI amongst the different age groups but there were age-specific differences in the GMFC responses (HAI GMFC, p=0.0014, NAI GMFC, p=0.067). Those <5 yo showed the highest GMFC in both assays. The frequency of HAI-seroconversions were highest amongst young children (0-4 yo) (83% [95% CI: 41-126%]) compared to adults (≥20 yo) (34% [95% CI: 22-46%]) while NAI-seroconversions did not show any age-dependent trends. Unlike the serosurvey cohort, a large percentage of IAV cases across a broad-range of baseline HAI and NAI-titers failed to mount at least a four-fold increase in antibody titers (Figure 4A and 4B). An exception was the NAI response against IBV, where non-responders (FC ≤ 2) had high-baseline (> 320) NAI-titers (Figure 4C and 4D).

Concurrent HAI and NAI-seroconversions were marginally more frequent in those < 5 yo (3/6, 50%) compared to adults (9/59, 15%) (p=0.07, Fischer exact test) (Figure 5). HAI-only
seroconversion only was observed in 13/69 (19%) of individuals, while NAI-only seroconversions were only observed in 6/69 (9%) participants.

Age-dependent observations were limited in IBV cases due to low enrollment in the <20 yo age bracket. In those ≥20 yo, 39% (95% CI: 18-61%) and 35% (95% CI: 14-56%) seroconverted by HAI and NAI-assays, respectively. Concurrent HAI and NAI-seroconversions were detected in only 6/25 (24%) individuals while HAI or NAI-only seroconversion occurred in 3/25 (12%) individuals, respectively.

NAI-antibodies as serologic markers of influenza virus infections

Given the poor detection of HAI-seroconversion in certain age groups, we asked if the NAI-antibody responses could be used as an alternative serological marker to detect recent infections. We first determined the overall seroconversion frequencies in both cohorts by either assay or both assays and we then evaluated the performance of these assays using a statistical metric.

In the serosurvey cohort, the overall (IAV and IBV) frequency of HAI-or NAI-seroconversion events was 55% (95% CI: 41%-69%, N=28) and 92% (95% CI: 85% - 99%, N=47), respectively. In the immunology cohort, the overall frequency of HAI-or NAI-seroconversion events was 36% (95% CI: 26%-46%, N=34) and 28% (95% CI: 19%-37%, N=27), respectively. Using either assay to define seroconversions captured 100% and 46% of all the infected cases in the serosurvey and immunology cohorts, respectively. Thus, including NAI-seroconversions can improve the accuracy of serologic detection of recent influenza virus infections.
Interassay diagnostic agreement with PCR-positivity and sensitivity and specificity of the 
serologic assays

We used κ-statistics to evaluate the level of agreement between PCR-subtype positivity and 
serologic seroconversions by each assay when used alone, either-or, and in combination (Table 
5). Higher κ-scores (scaled from 0 to 1) indicate a better agreement between the two methods 
(28).

κ-scores calculated for IAV cases for PCR vs HAI-alone and PCR vs NAI-alone in both cohorts 
were comparable [serosurvey κ (95% CI): 0.48 (0.33 - 0.64) vs 0.47 (0.32 - 0.61); immunology κ 
(95% CI): 0.27 (0.14 - 0.41) vs 0.24 (0.12 - 0.35)]. κ-scores calculated for IBV cases for PCR vs 
NAI [κ (95% CI): 0.56 (0.43 - 0.69)] were higher compared to PCR vs HAI [κ (95% CI): 0.24 
(0.06 - 0.42)] in the serosurvey cohort, but not the immunology cohort. However, κ-scores were 
the highest for PCR vs HAI or NAI (IAV= 0.33 to 0.48, IBV= 0.53 to 0.60). Thus, HAI and 
NAI-assays have comparable sensitivity for IAV, but NAI-assay was more sensitive for IBV 
[sensitivity score (95%CI): 1.00 (0.81, 1.00)] compared to HAI [sensitivity score (95%CI): 0.35 
(0.17, 0.56)]. All assays had high specificity scores (range 0.81 to 1), although unsurprisingly, 
the combination of HAI and NAI assays offered the highest specificity (0.91 to 1).

The HAI and NAI assays also detected seroconversions against H1N1 strain in 4% and 20% 
respectively, in the serosurvey and 1% and 13% respectively, in the immunology cohorts (Table 
6).
Specificity of assay in control (summer) cohort

To further examine the specificity of the assays, we tested paired sera collected for diagnosis of non-respiratory illnesses during the months when influenza activity was not epidemic. Our rationale for this was that any seroconversions detected were more likely due to assay complications rather than true infections. The percentages reported here represent seroconversion events against either IAVs (H1 and H3 subtypes) or IBVs (Yamagata and Victoria lineages) (Table 7). As a group, no significant rise in GMFC was detected for any virus. The frequency of a HAI-seroconversion event against IAVs, was 3% (95% CI: -1-6%) (3/94). All of these seroconversion events were directed against H3. The frequency of NAI-seroconversion against IAVs was 4% (95% CI: 0 - 8%, 4/94). The frequency of NAI-seroconversion against IBV was 2% (95% CI: 0 - 5%, 2/94) and no HAI-seroconversion events were detected. Notably, 3 of the 5 individuals seroconverted to multiple antigens. Two individuals seroconverted to both N1 and N2, and one individual seroconverted to 3 antigens; H3 and NA to both B lineages (Table 8). No individual seroconverted to both HA and NA simultaneously.

Discussion

Our study evaluated the induction of HA and NA-antibodies in individuals with community-acquired influenza through two cohorts with distinct sampling design. We found that the dynamics of HA and NA-antibody responses were age and virus-dependent after influenza virus infection. HAI-seroconversion events in IAV cases were highest in young children (≤ 5 yo) but then decreased with increasing age. These young children, who are likely experiencing their first influenza virus infection, were also more likely to seroconvert to both HA and NA or show an HA-dominant antibody response. The latter finding is consistent with the expectation of HA
being immunodominant compared to NA in HA-naïve individuals (29, 30). The age-dependent increase in baseline NAI-but not HAI titers in our cohorts suggests either an age-associated shift in antibody immunodominance or, perhaps more likely, a closer match between the NA-antigen used in our assay to the prior circulating strain (i.e., a less drifted NA-antigen) (12). This could explain the NA-only seroconversions seen in IAV-infected adults. The presence of NA-specific memory B-cells could favor the NA-specific antibody responses over HA in these adults, a model previously proposed to explain the “damping” of NA-antibody responses in HA-primed individuals (31-33). Several studies have reported that the elderly subjects may have a more NA-biased antibody response compared to the younger subjects and that evidence of original antigenic sin can be observed for NA-antibody responses as well (12, 34, 35). Based on these observations, it appears that, NA-antibodies, to strains encountered early in life may persist and like HA-antibodies, can be “back-boosted” (36). It is therefore likely that the relative conservation of this antigen can further contribute to boost the preexisting NA-antibody response in the adults.

Compared to the NAI-assay, the HAI-assay was surprisingly insensitive in detecting seroconversions against IBVs. This was also observed in our larger serosurvey cohort comprising of the PCR-negative cases (N=701), where the proportion of IBV NAI-seroconverters was higher than the HAI-seroconverters, particularly in the young children (37). This is in contrast to the findings by Rajendran et al., where they reported lower post-vaccination NAI-titers to IBV in children compared to adults (12), suggesting that, like IAV, there are differences in the NA-antibody response after IBV infection versus vaccination (38). Whether the reduced IBV HAI-sensitivity seen in our study was due to technical reasons (i.e mismatched
antigen, or influence of egg-adapted antigens) or antigenic competition, we found that the NAI-assay compensated for these limitations, as 100% of the IBV cases were captured.

That ELLA was particularly sensitive for IBV suggests that NA-antibodies against IBVs could be more broadly cross-reactive than HA, as had been reported recently for IAV (38). However, we cannot exclude the possibility of non-specific inhibition of NA-activity by HA-antibodies, particularly those that bind outside of the globular head which were not detected by HAI-assays (since most of our participants had very low pre-existing HAI-titers to IBVs) (39, 40). How much of these antibodies are present and thus, the extent of interference, in a polyclonal serum, is unknown.

The two sampling designs used in the serosurvey and immunology cohort are typical of most seroepidemiology and clinical studies. The serosurvey cohort utilized a pre- and post- influenza season sampling design. This allowed for baseline sera to be collected prior to the actual infection, although with such a design a large initial cohort is required in order to capture sufficient infected cases during the influenza season. In contrast, the immunology cohort sampled individuals that actively sought healthcare. Correspondingly, there was a large variability in the time of the first sera sampled after symptom onset in this cohort, attributed in part, to the logistical delay in sampling the ILI-cases that present to the GPs across Auckland. This likely contributed to the high baseline titers and thus decreasing the likelihood of meeting the seroconversion criteria (41). Indeed, when we examined in detail the time of sampling when broken down by age (Table 9), we found that the older children group (5 to 19 y.o), which had the highest baseline HAI-titer in the immunology cohort, was also the group that had the largest
time interval between symptom onset and first sera collection. This is likely associated with health-seeking behavior as older children are less likely to present with severe influenza. We attempted to correct for the variable sampling time in our analysis by excluding the outliers (those sampled < 14 days or > 20 days), but this resulted in the loss of statistical power without affecting the population average (data not shown). Hence, we kept the original analysis for this study. Nevertheless, despite the difference detected in the frequency of seroconversions, data from both cohorts, at least for IAV cases, suggested that adults were more likely than children to show discordant HA- and NA-antibody induction, confirming previous observations (42, 43).

Despite H3N2 being the dominant circulating strain during our study, some seroconversions to H1N1 were also detected in our two cohorts. Seroconversions to NA were more frequent compared to HA, potentially due to the higher cross-reactivity of NAI-antibodies or co-infection events during the influenza season. For this reason, we used the summer cohort (where sera were collected for non-respiratory disease testing when community influenza activity is low) as a proxy for an “influenza-negative” population to evaluate the specificity of these assays. Since three of the five paired sera showed seroconversion to multiple antigens (Table 8), it is likely that these are false-positive events. With the caveat that we could not discount the possibility of any “out-of-season” influenza cases, the false-positive frequencies were between 0 and 4% of the total reactions tested and appeared to be comparable between HAI and NAI-assays.

Principally, we found that including the NAI-assay improved the serological detection of influenza cases in both our cohorts, although we acknowledge that the detection sensitivities of HAI and NAI-assays reported here may vary during different influenza seasons with different
antigens. With further validation across different influenza seasons and antigens, including the NAI-assay as a hierarchical or targeted testing approach can increase the power and accuracy of a seroepidemiological study and will circumvent some of the limitations and variable robustness associated with HAI-assay.

Overall, our findings highlight the age and subtype-dependent nature of HA and NA-antibody responses after PCR-confirmed influenza virus infections. The differences in the antibody response to HA and NA that we observed between adults and children suggests a possible influence of immunological memory on the recall response. This is an important consideration for any vaccination strategy aimed at eliciting NA-immunity as it suggests that the antibody response may differ with different age groups. The mechanism underlying such response dynamics should be further investigated.
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Conflict of Interests

None of the authors report conflicts of interests.

Contact Information for corresponding author:

Richard J. Webby
Department of Infectious Diseases, MS330
St. Jude Children’s Research Hospital
List of Figures.

Figure 1. Sampling and recruitment of individuals in each study cohort.

Figure 2. Relationship between pre-season Hemagglutination-Inhibition (HAI) (A, C) and Neuraminidase-Inhibition (NAI)-antibody titer (B, D) with titer fold-increase in post-season sera in the Serosurvey cohort. Figure (A, B) is shown for A/H3N2 and (C, D) is for influenza B cases. X-axes indicates the baseline HA or NA titer and Y-axis indicates the percentage of responders showing the antibody fold-increase according to the colors indicated in the legend. Legend indicates the range of fold-change in titers: grey indicate titer-changes of less than 2-fold while red, orange and yellow indicate titer-changes greater than 4-fold.

Figure 3. Percentage of individuals that seroconverted by hemagglutination-inhibition assay (HAI) only, neuraminidase-inhibition assay (NAI) only, or both HAI and NAI in the PCR confirmed influenza cases in the Serosurvey cohort. Error bars represents 95% confidence intervals.

Figure 4. Relationship between baseline first Hemagglutination-Inhibition (HAI) (A, C) and Neuraminidase-Inhibition (NAI)-titer (B, D) with the antibody titer fold-increase in the second sera in the Immunology cohort. Figure (A, B) is shown for A/H3N2 and (C, D) is for influenza B cases. X-axes indicates the baseline HA or NA titer and Y-axis indicates the percentage of responders showing the antibody fold-increase according to the colors indicated in the legend. Legend indicates the range of fold-change in titers: grey indicate titer-changes of less than 2-fold while red, orange and yellow indicate titer-changes greater than 4-fold.

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Table 8. Details of seroconversion events (highlighted in bold) detected in the five individuals from the summer cohort.

Table 9. Details on the time interval between the first sera collection from symptom onset or swab specimen and time between sera collection, as stratified by age and ethnicity in the Serosurvey and Immunology cohort.
Table 1. List of virus strains tested for each cohort.

| Year | Subtype       | Virus strain                  | Cohorts                      |
|------|---------------|-------------------------------|------------------------------|
| 2013 | H1N1          | A/California/7/09             |                              |
|      | H3N2          | A/Victoria/361/2011           | Immunology                   |
|      | B (Victoria)  | B/Brisbane/60/2008            |                              |
|      | B (Yamagata)  | B/Massachusetts/02/2012       |                              |
| 2015 | H1N1          | A/California/7/09             |                              |
|      | H3N2          | A/Switzerland/9715293/2013    | Serosurvey, Immunology and   |
|      | B (Victoria)  | B/Brisbane/60/2008            | Summer                       |
|      | B (Yamagata)  | B/Phuket/3073/2013            |                              |
Table 2. Characteristics of participants, influenza infections, and timing of serum collections in the Serosurvey, Immunology and Summer Cohorts.

| Characteristics          | Serosurvey cohort (n=50) | Immunology cohort (n=94) | Summer cohort (n=47) |
|--------------------------|--------------------------|--------------------------|----------------------|
|                         | n (%)                    | n (%)                    | n (%)                |
| Age (years)              |                          |                          |                      |
| 0-4                      | 16 (32)                  | 7 (7)                    | 0 (0)                |
| 5-19                     | 15 (30)                  | 5 (5)                    | 1 (2)                |
| 20+                      | 19 (38)                  | 82 (87)                  | 46 (98)              |
| Sex                      |                          |                          |                      |
| Male                     | 16 (32)                  | 44 (47)                  |                      |
| Female                   | 34 (68)                  | 50 (53)                  |                      |
| Ethnicity                |                          |                          |                      |
| Maori                    | 7 (14)                   | 15 (16)                  |                      |
| Pacific                  | 5 (10)                   | 23 (25)                  |                      |
| Asian                    | 9 (18)                   | 8 (9)                    |                      |
| European-descent / Other | 29 (58)                  | 47 (51)                  |                      |
| Infection subtype        |                          |                          |                      |
| A (Not subtyped)         | 10 (20)                  | 15 (16)                  |                      |
| A (H3)                   | 15 (29)                  | 53 (56)                  |                      |
| A (H1)                   | 0 (0)                    | 1 (1)                    |                      |
| B (Not Subtyped)         | 0 (0)                    | 2 (2)                    |                      |
| B (Victoria)             | 15 (29)                  | 4 (4)                    |                      |
| B (Yamagata)             | 11 (22)                  | 19 (20)                  |                      |
| Time from first sera collection to symptom onset, median no. of days (range) | 134 (22, 193) | n=39 | |
| Time from symptom onset to first sera collection, median no. of days (range) | 12 (1,28) | n=58 | |
| Time from first sera collection to swab specimen, median no. of days (range) | 141 (28, 202) | | |
| Time from swab specimen to first sera collection, median no. of days (range) | 6 (0, 23) | n=93 | |
| Time between sera collections, median no. of days (range) | 218 (132, 274) | 16 (5, 27) | n=93 |

*a Number of PCR-confirmed influenza cases, b N=50 individuals, 1 was positive for influenza A and B, c Ethnicity data was only available for 93 participants
Table 3. Hemagglutination-inhibition (HAI) and neuraminidase-inhibition (NAI) antibody responses to influenza A and B in those with PCR-confirmed influenza in the Serosurvey cohort.

| Age (years) | Antibody titer | Influenza A | | | | Influenza B | | | |
|-------------|----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|              | n    | HAI (95%CI) | P for age | NAI (95%CI) | P for age | by HAI or NAI | n   | HAI 95%CI | P for age | NAI 95%CI | P for age | by HAI or NAI |
| 0-4         | GMT 1a | 7   | (5, 9) | 5 (5, 5) | 8 (5, 13) | 11 (7, 18) | 14 (97, 226) | 59 (31, 112) | 15 (9, 27) | 761 (435, 1332) |
|             | GMFCb | 22  | (14, 35) | 12 (10, 14) | 8 (6, 4) | 70 (68, 72) | 22 (14, 35) | 12 (10, 14) | 8 (6, 4) | 70 (68, 72) |
|             | % Seroconvertc | 100 | (100, 100) | 67 (28, 105) | 100 | 100 | 100 | 100 | 100 | 100 |
| 5-19        | GMT 1a | 14  | (6, 31) | 24 (15, 40) | 5 (5, 5) | 1874d | 31 (11, 86) | 0.0074de | 1522 (683, 3393) | 0.0520ef |
|             | GMFCb | 131 | (46, 378) | 238 (124, 457) | 22 (11, 44) | 3888g | 49 (47, 51) | 0.0251fh | 49 (47, 51) | 0.0251fh |
|             | % Seroconvertc | 86  | (51, 121) | 100 (100, 100) | 100 | 100 | 100 | 100 | 100 | 100 |
| 20+         | GMT 1a | 12  | (7, 19) | 13 (7, 24) | 6 (5, 8) | 149 (64, 348) | 50 (23, 110) | 101 (53, 191) | 33 (17, 64) | 2744 (1383, 5445) |
|             | GMFCb | 4    | (3, 6) | 8 (6, 10) | 5 (3, 7) | 18 (17, 20) | 4 (3, 6) | 8 (6, 10) | 5 (3, 7) | 18 (17, 20) |
|             | % Seroconvertc | 44  | (4, 85) | 89 (63, 115) | 100 | 100 | 100 | 100 | 100 | 100 |

a GMT: Geometric Mean Titer
b GMFC: Geometric Mean Fold-Change
c Percentage of individuals that seroconverted, defined by 4-fold rise in antibody titer in the specified assay between first and second sera
d Age vs. GMT 1 by analysis of variance (ANOVA)
e Age vs GMT 2 by analysis of variance (ANOVA)

488
489
490
491
492
493
Age vs GMFC by analysis of variance (ANOVA)

Age vs % Seroconvert by analysis of variance (ANOVA)
Table 4. Hemagglutination-inhibition (HAI) and neuraminidase-inhibition (NAI) antibody responses to influenza A and B in those with PCR-confirmed influenza in the Immunology cohort.

| Age (years) | Antibody titer | Influenza A | | | Influenza B | |
|-------------|----------------|-------------|----------------|----------------|----------------|----------------|----------------|
|             |                | n | HAI | 95%CI | P for age | NA | 95%CI | P for age | by HAI or NAI | n | HAI | 95%CI | P for age | by HAI or NAI |
| 0-4         | GMT 1a         | 6 | 1220 (401, 3714) | 5 (1, 20) | 10 | (N/A) | 5 | (N/A) | 10 | (N/A) | 160 | (N/A) | 32 | (N/A) |
|             | GMFCb          |   | 83 | (41, 126) | 50 | (-8, 108) | 83 | (N/A) | 100 | (N/A) | 100 | (N/A) | 100 | (N/A) |
| 5-19        | GMT 1a         | 4 | 226 (153, 335) | 1 (1, 1) | 1 | (N/A) | 1 | (N/A) | 1 | (N/A) | 32 | (N/A) | 32 | (N/A) |
|             | GMFCb          |   | 1 | (1, 1) | 0.0014d | 1 | (1, 1) | 0.0673d | 1 | (N/A) | 1 | (N/A) | 1 | (N/A) |
|             | % Seroconvertc |   | 83 | (N/A) | 0 | (N/A) | 0 | (N/A) | 0 | (N/A) | 0 | (N/A) | 0 | (N/A) |
| 20+         | GMT 1a         | 59 | 144 (91, 227) | 2 (2, 3) | 23 | (1, 2) | 3 | (2, 5) | 3 | (1, 5) | 32 | (1, 5) | 32 | (1, 5) |
|             | GMFCb          |   | 59 | (22, 46) | 25 | (14, 37) | 44 | (18, 61) | 35 | (14, 56) | 48 | (14, 56) | 48 | (14, 56) |

a GMT: Geometric Mean Titer
b GMFC: Geometric Mean Fold-Change
c Percentage of individuals that seroconverted, as defined by 4-fold rise in antibody titer by the specified assay between first and second sera
d Age vs. GMT 1 by analysis of variance (ANOVA)
e Age vs GMT 2 by analysis of variance (ANOVA)
f Age vs GMFC by analysis of variance (ANOVA)
g Age vs % Seroconvert by analysis of variance (ANOVA)
| Cohort          | Assay | 48 (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) |
|-----------------|-------|-------------|----------------------|---------------------|
| Serosurvey      | HAI   | 0.48 (0.33, 0.64) | 0.76 (0.55, 0.91) | 0.87 (0.81, 0.92) |
|                 | NAI   | 0.47 (0.32, 0.61) | 0.84 (0.64, 0.95) | 0.84 (0.77, 0.89) |
| Immunology      | HAI   | 0.27 (0.14, 0.41) | 0.35 (0.24, 0.48) | 0.92 (0.83, 0.97) |
|                 | NAI   | 0.24 (0.12, 0.35) | 0.26 (0.17, 0.39) | 0.97 (0.90, 1.00) |
|                 | HAI or NAI | 0.18 (0.08, 0.27) | 0.18 (0.09, 0.29) | 1.00 (0.93, 1.00) |

Strength of agreement based on Landis and Koch's scale [28]: < 0.2 = poor or slight, ≥ 0.2 to < 0.4 = fair, 0.4 to < 0.6 = moderate, 0.6 to < 0.8 = substantial, 0.8 to 1 = almost perfect to perfect.
Table 6. Hemagglutination-inhibition (HAI) and neuraminidase-inhibition (NAI) antibody responses to influenza A (H1N1) in the Serosurvey and Immunology cohort.

| Age (years) | Antibody titer | Serosurvey cohort | Immunology cohort |
|-------------|----------------|-------------------|-------------------|
|             | n | HAI | 95% CI | NAI | 95% CI | by HAI/NAI (%) | n | HAI | 95% CI | NAI | 95% CI | by HAI/NAI (%) |
| **Influenza A virus** | | | | | | | | | | | | |
| 0-4 | | | | | | | | | | | | |
| GMT 1<sup>a</sup> | 9 | 29 | (-58, 117) | 17 | (-54, 88) | 6 | 10 | (10, 10) | 5 | (5, 5) | 30 |
| GM 2 | 9 | 37 | (-48, 122) | 25 | (-109, 160) | 6 | 14 | (-9, 37) | 9 | (-42, 59) | |
| GMF<sup>b</sup> | 1 | 1 (11) | 1 (11) | 22 | (-0.02, 3) | 2 | 1 (17) | 1 (17) | 2 | (-0.8, 4) | 2 | (-8, 12) |
| N (% SC<sup>c</sup>) | | | | | | | | | | | | |
| 20+ | | | | | | | | | | | | |
| GMT 1<sup>a</sup> | 9 | 23 | (-5, 32) | 108 | (16, 200) | 6 | 8 | (6, 11) | 57 | (34, 79) | 0 |
| GM 2 | 7 | 24 | (-17, 66) | 160 | (6, 314) | 4 | 7 | (4, 10) | 40 | (15, 64) | |
| GMF<sup>b</sup> | 0 | 0 | 0 | 11 | 1 | 1 (0.2, 2) | 1 | 1 | (1, 1) | 0 | |
| N (% SC<sup>c</sup>) | | | | | | | | | | | | |
| **Influenza B virus** | | | | | | | | | | | | |
| 0-4 | | | | | | | | | | | | |
| GMT 1<sup>a</sup> | 7 | 52 | (-3, 144) | 57 | (-17, 130) | 10 | 10 | (N/A) | 5 | (N/A) | 30 |
| GM 2 | 8 | 62 | (-27, 151) | 80 | (-70, 230) | 10 | 10 | (N/A) | 5 | (N/A) | |
| GMF<sup>b</sup> | 1 | 1 (11) | 1 (11) | 13 | 1 (0.3, 3) | 1 | 1 | (N/A) | 1 | (N/A) | 0 |
| N (% SC<sup>c</sup>) | | | | | | | | | | | | |
| 20+ | | | | | | | | | | | | |
| GMT 1<sup>a</sup> | 10 | 49 | (-12, 111) | 121 | (-124, 366) | 11 | 11 | (1, 21) | 45 | (82, 911) | |
| GM 2 | 53 | 57 | (-34, 148) | 269 | (-121, 659) | 5 | 5 | (N/A) | 40 | (N/A) | 0 |
| GMF<sup>b</sup> | 1 | 1 (11) | 1 (11) | 23 | 1 (1, 1) | 1 | 1 | (1, 1) | 0 | (N/A) | |
| N (% SC<sup>c</sup>) | | | | | | | | | | | | |

<sup>a</sup>GMT: Geometric Mean Titer  
<sup>b</sup>GMFC: Geometric Mean Fold-Change  
<sup>c</sup>Percentage of individuals that seroconverted, as defined by 4-fold rise in antibody titer by the specified assay between first and second sera.
Table 7. Hemagglutination-inhibition (HAI) and neuraminidase-inhibition (NAI) antibody responses to influenza A (A/H3 and A/H1pdm09) and B (B/Yam and B/Vic) in the non-influenza season (summer) cohort.

| Type               | Total reactions<sup>a</sup> | Antibody titer | HAI   | 95% CI | NAI   | 95% CI |
|--------------------|-----------------------------|----------------|-------|--------|-------|--------|
|                    |                             |                |       |        |       |        |
| Influenza A (H1 & H3) |                             |                |       |        |       |        |
| First sera GMT     | 17                          | 17             | (14, 22) | 39     | (28, 54) |
| Second sera GMT    | 18                          | 18             | (15, 23) | 43     | (31, 60) |
| GMFC               | 1                           | 1              | (0, 2)   | 1      | (0, 2)   |
| Seroconversion (%)<sup>c</sup> | 3                           | 3              | (-1, 6) | 4      | (0, 8)   |
| No. of individuals that seroconverted | 3                           | 2              |       |        |       |        |
| Influenza B (Yam & Vic) | 94                          |                |       |        |       |        |
| First sera GMT     | 11                          | 11             | (9, 13) | 905    | (707, 1159) |
| Second sera GMT    | 11                          | 11             | (9, 14) | 967    | (767, 1220) |
| GMFC               | 1                           | 1              | (0, 2)   | 1      | (0, 2)   |
| Seroconversion (%)<sup>c</sup> | 0                           | 0              | (-1, 6) | 2      | (0, 5)   |
| No. of individuals that seroconverted | 0                           | 1              |       |        |       |        |

<sup>a</sup> Summer in the Southern Hemisphere: December 2015 to March 2016

<sup>b</sup> 47 paired samples were tested against two influenza A or two influenza B strains, making a total of 94 reactions for each virus type.

<sup>c</sup> Percentage of reactions that showed a 4-fold rise in antibody titer by the specified assay between first and second sera.
Table 8. Details of seroconversion events (highlighted in bold) detected in the five individuals from the summer cohort.

| Antigen                  | Sera     | Individual | 1   | 2   | 3   | 4   | 5   |
|--------------------------|----------|------------|-----|-----|-----|-----|-----|
| **IAV_a_H1**             | First    | 40         | 5   | 5   | 10  | 5   | 5   |
|                          | Second   | 40         | 5   | 20  | 10  | 5   | 5   |
| **IAV_H3**               | First    | 10         | 10  | 5   | 5   | 10  |
|                          | Second   | 40         | 10  | 10  | 5   | 40  |
| **IAV_N1**               | First    | 80         | 20  | 10  | 10  | 10  | 320 |
|                          | Second   | 160        | 80  | 320 | 80  | 320 |
| **IAV_N2**               | First    | 40         | 10  | 10  | 10  | 20  |
|                          | Second   | 40         | 80  | 40  | 40  | 40  |
| **IBV (Victoria)_HA**    | First    | 5          | 80  | 5   | 5   | 5   |
|                          | Second   | 5          | 80  | 5   | 5   | 5   |
| **IBV (Victoria)_NA**    | First    | 640        | 2560| 40  | 320 | 640 |
|                          | Second   | 640        | 2560| 160 | 320 | 640 |
| **IBV (Yamagata)_HA**    | First    | 5          | 80  | 5   | 5   | 5   |
|                          | Second   | 5          | 80  | 5   | 5   | 40  |
| **IBV (Yamagata)_NA**    | First    | 640        | 2560| 40  | 320 | 320 |
|                          | Second   | 640        | 2560| 160 | 320 | 2560|

- influenza A virus
- influenza B virus
Table 9. Details on the time interval between the first sera collection from symptom onset or swab specimen and time between sera collection, as stratified by age and ethnicity in the Serosurvey and Immunology cohort.

| Characteristics | Serosurvey cohort (n=50) | Immunology cohort (n=94) |
|-----------------|--------------------------|--------------------------|
|                 | n (%)                    | Time from first sera collection to symptom onset, median no. of days (range) | Time from first sera collection to swab specimen, median no. of days (range) | Time between sera collections, median no. of days (range) | n (%)                    | Time from symptom onset to first sera collection, median no. of days (range) | Time from swab specimen to first sera collection, median no. of days (range) | Time between sera collections, median no. of days (range) |
| Age (years)     |                          |                          |                          |                          |                          |                          |                          |                          |
| 0-4             | 16 (32)                  | 128.5 (53, 180)          | 134.5 (63, 188)          | 220 (132, 259)          | 7 (7)                   | 7 (1, 14)                 | 2 (0, 6)                  | 17 (14, 24)                 |
| 5-19            | 15 (30)                  | 142.5 (22, 193)          | 147 (28, 176)            | 215 (133, 249)         | 5 (5)                   | 17 (10, 28)               | 12 (7, 23)                | 14 (12, 19)                 |
| 20+             | 19 (38)                  | 137.5 (29, 170)          | 144.5 (35, 178)          | 216 (144, 274)         | 82 (87)                 | 12 (1, 26)                | 6 (0, 19)                 | 16 (5, 27)                 |
| Sex             |                          |                          |                          |                          |                          |                          |                          |                          |
| Male            | 16 (32)                  | 113 (29, 193)            | 118 (35, 185)            | 216.5 (139, 274)       | 44 (47)                 | 12 (1, 28)                | 5 (0, 23)                 | 17 (5, 27)                 |
| Female          | 34 (68)                  | 141 (22, 180)            | 148 (28, 188)            | 218.5 (132, 259)       | 50 (53)                 | 13.5 (1, 26)              | 6 (0, 15)                 | 14 (12, 24)                |
| Ethnicity       |                          |                          |                          |                          |                          |                          |                          |                          |
| Maori           | 7 (14)                   | 137.5 (105, 165)         | 148.5 (113, 169)         | 232 (207, 259)         | 15 (16)                 | 12 (1, 18)                | 5 (0, 13)                 | 16 (12, 24)                |
| Pacific         | 5 (10)                   | 79.5 (22, 113)           | 87.5 (28, 118)           | 210 (132, 274)         | 23 (25)                 | 11 (5, 14)                | 6 (1, 10)                 | 15 (14, 27)                |
| Asian           | 9 (18)                   | 141.5 (109, 163)         | 148 (115, 167)           | 214 (144, 250)         | 8 (9)                   | 16 (12, 17)               | 5.5 (2, 12)               | 16 (14, 21)                |
| European-descent / Other | 29 (58) | 138.5 (29, 193) | 144.5 (35, 188) | 219 (139, 249) | 47 (51) | 12.5 (1, 28) | 6 (0, 23) | 16 (5, 24) |
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SHIVERS

Serosurvey cohort 2015
n=911

Pre-season (first bleed)
March – June 2015

Swabs from ILI patients
n=209

PCR-confirmed influenza positive
n=50 (51 infections)

Post-season bleed
October - November 2015

Immunology cohort 2013 and 2015
n=94

ILI-symptoms, PCR-confirmed influenza positive

First bleed

Second bleed
14 days post first bleed

Summer cohort
n=47

Paired sera collected 2 weeks apart for arbovirus/legionella testing
September - December 2016
