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ABSTRACT

Introduction: Haemagglutination-inhibition (HI) antibody titer is a correlate of protection against influenza; its persistence after infection or vaccination is important to determining susceptibility to subsequent infection. Few studies, however, have reported longitudinal data regarding the magnitude and duration of HI protection following natural seasonal influenza A infection.

Methods: Using French influenza cohort study data collected from 2008 to 2010, we investigated persistence of serological protection among subjects according to influenza-like illness (ILI) and laboratory-confirmed seasonal 2007 influenza A(H1N1) infection status at inclusion in 2008 (ILI-A(H1N1) positive, ILI-A(H1N1) negative, or no-ILI). Antibody titers against seasonal 2007 A(H1N1) were determined using the HI technique for sera. Regression models for interval-censored data were used to estimate geometric mean titers (GMT) for HI assays. A logistic regression model adjusted for age group (subjects <30, 30–50 and >50 years old) was used to quantify the association between HI titer and protection against infection.

Results: Based on 310 total subjects, influenza A(H1N1) infection was confirmed in 39 of 115 ILI subjects at inclusion. GMT associated with 50% probability of protection among ILI subjects decreased with age group (subjects <30 yo: GMT of 40.8 was associated with 50% [95CI: 29.3; 70.7%] probability of protection, subjects 30–50 yo: 26.8 [95CI: 34.4%; 65.6%] and subjects >50 yo: 8.9 [95CI: 15.3%; 84.7%]). GMT declined after the first annual study visit among ILI-A(H1N1) positive subjects but remained higher compared to inclusion at the 2010 study visit (41.5 [95CI: 34.8; 49.5], $p = 0.0157$). GMT remained stable among ILI-A(H1N1) negative subjects ($p = 0.7502$), but decreased among no-ILI subjects ($p < 0.0001$).

Conclusion: Our results confirm the positive relationship between HI titer and probability of protection among naturally infected subjects, and provides evidence that protection associated with HI titer varies with age. This longitudinal analysis suggests the rise in HI titers following seasonal 2007 influenza A(H1N1) infection may persist into subsequent influenza seasons.

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1. Introduction

Influenza represents an important public health burden; a lifetime of exposure to numerous influenza viruses results in an estimated 10 infections if never vaccinated [1]. Cohort studies have provided valuable information concerning the epidemiology of influenza [2–7], however, specific knowledge regarding the persistence of the immune response is limited [8].

Exposure to the influenza virus activates an immune response that includes the production of virus-specific antibodies. The concentration of antibodies targeting two virus proteins, haemagglutinin (HA) and neuraminidase (NA), provides an indication of

the level of protection an individual has against infection, and are therefore considered "correlates of protection" [9,10]. In the 1970s, it was demonstrated that haemagglutination-inhibiting (HI) antibody titers of 40 were associated with 50% clinical protection in the event of influenza A2 or B exposure [11]. Although widely accepted and used as a major endpoint in vaccine efficacy studies [12,13], few studies have been performed to validate this threshold among naturally infected subjects [14] or considered age-related differences in protection. Further to this, little is known regarding how protection changes over time.

In this study we aimed to confirm the association between HI titer and protection against natural influenza infection, investigate age-related differences in protection, and to study the persistence of the antibody response. Evaluation of HI titer durability following infection is of interest to discerning susceptibility of individuals to recurrent infections, and may have important implications on population immunity.

2. Methods

2.1. Patient recruitment/participation

Data from the FLUREC study, a French cohort designed to study recurrent influenza infection, was used in this study. Thirty-six French metropolitan general practitioners (GP) participating in the Sentinelles network recruited subjects during GP visits. Subject recruitment was stratified by reason for medical visit (influenza-like illness (ILI) or no-ILI related illness) and age (10-year age groups). An ILI was defined as a sudden onset of fever (38 °C) accompanied by muscle soreness and cough. Subjects performed an inclusion visit and a total of 3 annual study visits throughout the follow-up period. More details regarding study design and subject recruitment can be found elsewhere [15].

2.2. Data collection

Study data was collected and entered on electronic case report forms by GPs. Data regarding medical history (including chronic conditions, influenza episodes and/or influenza vaccination in the past two years) and sociodemographic information were collected at inclusion for all subjects. Symptoms data and duration were collected for subjects reporting an ILI-related illness at inclusion. During annual study visits, information regarding clinical history (ILI-related illnesses, vaccination status) was documented. Nasal swabs (VIROCULT®, KITVIA, Labarthe Inard, France) were collected at inclusion for ILI subjects. Serological blood samples were collected for ILI subjects at inclusion, and for all study subjects at the annual visits. Nasal swabs and blood samples were collected in the event of an ILI episode during study follow-up at a dedicated study visit.

2.3. Surveillance data

Influenza A/Brisbane/59/2007(H1N1) (62%), A/Brisbane/10/2007(H3N2) (2%) and influenza B/Florida/4/2006 (36%) circulated during the 2007–2008 seasonal epidemic which started 7 January and ended on 9 March 2008 [16]. The 2008–2009 influenza epidemic began on 15 December 2008 and ended on 22 February 2009, and was characterized by the predominant circulation of the influenza A/Brisbane/10/2007(H3N2) (85–88%) compared to influenza B (10–12%) (B/Victoria/2/87 lineage and B/Yamagata/16/88 lineage were detected in Europe) [17,18]. The 2009–2010 A(H1N1) pandemic began in France on 5 October 2009 and ended on 11 January 2010; influenza A/California/7/2009 (H1N1pdm09) represented 95% of the viruses circulating during this season [19].

The 2007–2008 seasonal influenza vaccine was composed of the A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004 strains [20]. Seasonal vaccines for 2008–2009 and 2009–2010 influenza seasons contained the A/Brisbane/59/2007 (H1N1) strain.

2.4. Laboratory procedures

2.4.1. RT-PCR

Nasopharyngeal swabs collected at inclusion were tested for the presence of the seasonal 2007 A(H1N1) virus using one step real time RT-PCR; procedures have been previously described [15].

2.4.2. Haemagglutination inhibition (HI) titer

HI titers against seasonal A(H1N1) were determined using the haemagglutination-inhibiting technique for serological samples collected at inclusion and annual study visits using an A/Paris/6/2007 (H1N1)-like strain. Serial two-fold dilutions (1/10–1/1280) of heat-inactivated sera were used in these experiments. Serum HI titer was determined to be the reciprocal of the highest serial dilution at which complete inhibition of haemagglutination occurred in two independent readings [21]. Two controls were present on each plate; serum HI titer readings were adjusted according to control results. Samples for which HI titer was undetectable or highly elevated were double verified. Intralaboratory variation assessments were performed to evaluate the proportion of serum samples in which a >2-fold and >4-fold difference was identified in replicate assays [22]; all samples were found to be within a 2-fold range.

2.5. Statistical methods

Infection was defined as laboratory-confirmed seasonal 2007 A(H1N1) on a nasopharyngeal swab collected at inclusion. Three groups were defined according to presence of ILI and infection status at inclusion: ILI-A(H1N1) positive, ILI-A(H1N1) negative, and no-ILI. Analyses were restricted to subjects whose infection status could be determined at inclusion and samples for which all serological and vaccination data was complete. We selected ILI subjects with an inclusion blood sample collected before the end of the 2007–2008 seasonal epidemic and, to limit the possibility of elevated titers associated with infection among ILI subjects, within 5 days of the inclusion visit. Subjects reporting seasonal (2008–2009, 2009–2010) or A(H1N1)pdm09 (pandemic) vaccination during study follow-up were censored at the given study visit date. *p* Values <0.05 were determined to be significant.

Differences in baseline characteristics between ILI and no-ILI subjects were studied using the Wilcoxon rank-sum test for continuous covariates and the Fisher exact test for categorical covariates. Regression models for interval-censored data were used to estimate geometric mean titers (GMT) for HI assays [23] and to compare GMT between groups. Evolution of GMT over time was evaluated using these models (accounting for repeated data). The association between (log 2-transformed) HI estimates (GMT of the upper and lower bounds of the interval censored HI measures) at inclusion and protection against infection was modeled using logistic regression among all ILI subjects and adjusted for age at inclusion using three age groups (subjects <30 years, 30–50 years and >50 years old). Keeping with previously published work [24,25], we define protection in this analysis as a negative result by PCR for influenza infection.

Statistical analyses were performed with the R statistical program (v2.15.2).
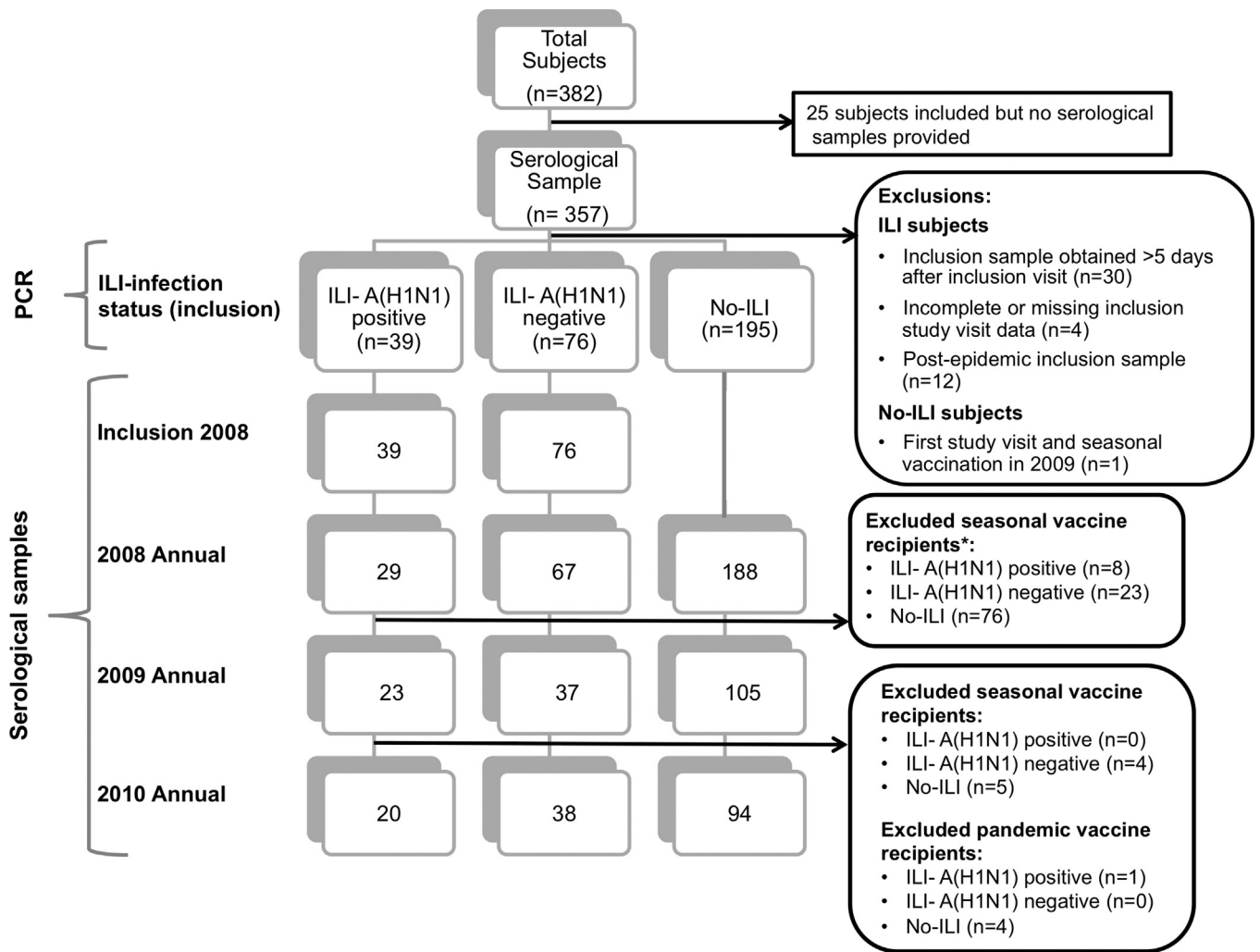
Fig. 1. Study blood sample flowchart. Total number of subjects per group, according to ILI and seasonal 2007 A(H1N1) infection status (PCR for ILI subjects only) at inclusion, and number of serological samples obtained at inclusion and annual study visits. * Includes missing vaccine status: ILI-A(H1N1) positive ($n = 2$); ILI-A(H1N1) negative ($n = 3$); no-ILI ($n = 2$).

2.6. Ethics

This study received French ethics committee (Comité de Protection des Personnes Ile-de-France V (no. 07715)) and Data Protection Authority approval (no. 1261460) and all study participants provided written informed consent.

3. Results

Out of a total of 382 subjects included in the study in 2008, 357 provided at least one serological sample (Fig. 1). ILI subjects with incomplete inclusion visit data ($n = 4$), whose inclusion sample was obtained >5 days after inclusion visit ($n = 30$) and after the seasonal 2007 epidemic ($n = 12$) were excluded. One additional subject was not included due to seasonal vaccination during first study visit in 2009. Seasonal vaccination was reported (or missing) during the 2009 ($n = 107$) and 2010 study visits ($n = 9$). Five other subjects reported pandemic vaccination. This analysis is therefore based on 716 samples from 310 subjects. Sixty-nine percent of subjects ($n = 213$) provided at least two blood samples, 48% ($n = 148$) provided at least three blood samples and 15% ($n = 45$) provided four blood samples. Regarding the timing of the annual study visits, 99%, 86%, and 100% were conducted before the start of the seasonal epidemic period for the 2008, 2009 and 2010 annual study visits, respectively (Fig. 2).

Study subjects were 18-85 years old at inclusion; No-ILI subjects were older than ILI-subjects (Median: 53 (IQR: 36; 66) vs Median: 44 (IQR: 35; 57), $p = 0.0206$) and were more likely to have had seasonal 2007 vaccination (No-ILI: 76 (39%) vs 24 (21%); $p = 0.0010$). ILI and no-ILI subjects also differed at baseline with regard to household size (No-ILI: Median 2 (IQR: 2; 4) vs ILI subjects: Median 1 (IQR: 1; 3); $p < 0.0001$), history of hypercholesteremia (No-ILI: 35 (18%) vs ILI subjects: 8 (7%); $p = 0.0064$), and cardiovascular illness (No-ILI: 62 (32%) vs ILI subjects: 24 (21%); $p = 0.0485$) (Table 1).

Of the 115 ILI subjects included in the protection analysis, 39 were found to have laboratory confirmed A(H1N1) infection. Proportion of infection decreased with increasing age group, with the highest proportion (52%) of infection among the subjects <30 years old ($p < 0.0001$). ILI–A(H1N1) positive subjects had lower GMT at inclusion compared to ILI–A(H1N1) negative subjects (Fig. 3) (33.8 [95CI: 29.8; 38.4] vs 43.5 [95CI: 39.0; 48.6], $p = 0.0056$).

Over study follow-up, 3 subjects were diagnosed with seasonal A(H3N2) infection (2008–2009 season) and 1 subject with A(H1N1)pdm09 infection (2009–2010); all infected subjects were enrolled in the no-ILI group at inclusion.

3.1. HI protection curves

Serological titer against seasonal 2007 A(H1N1) at inclusion among ILI subjects (ILI-A(H1N1) positive, $n = 39$; ILI-A(H1N1)
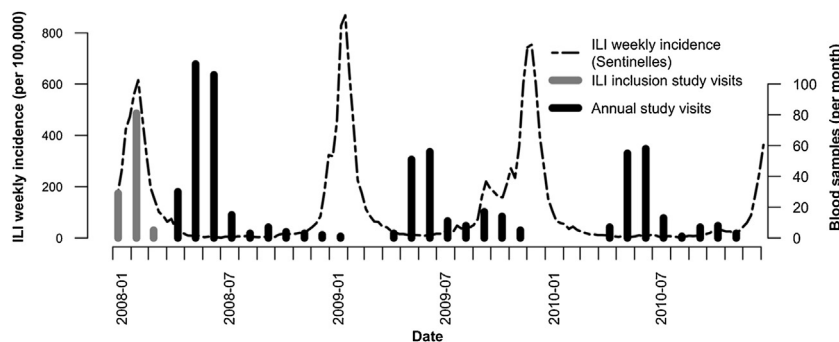
Fig. 2. Weekly incidence of influenza-like illnesses (ILIs) in France according to French Sentinel network and monthly study blood collection from January 2008 to November 2010. Dotted line (left scale) ILI weekly incidence per 100,000; monthly study blood collection (right scale), gray bars represent ILI inclusion visit samples and black bars represent annual study visit samples.

Table 1
Comparison of baseline characteristics between ILI ($n = 115$) and no-ILI subjects ($n = 195$) at inclusion.

	No-ILI ($n = 195$)	ILI ($n = 115$)	p
Covariate			
Age (yrs)—median (IQR)	53 (36; 66)	44 (35; 57)	0.0206
Number of household members—median (IQR)	2 (2; 3.5)	1 (1; 3)	<0.0001
Sex (Female)—N (%)	111 (57)	68 (59)	0.7225
Asthma—N (%)	8 (4)	6 (5)	0.7782
ILI season 2006–2007[*]—N (%)	27 (14)	13 (12)	0.7246
Smoker[†]—N (%)	41 (21)	29 (27)	0.2595
Chronic Illness—N (%)	100 (51)	47 (41)	0.0790
Hypercholesterolemia—N (%)	35 (18)	8 (7)	0.0064
Cardiovascular—N (%)	62 (32)	24 (21)	0.0485
Respiratory—N (%)	11 (6)	10 (9)	0.3515
Other—N (%)	19 (10)	5 (4)	0.1222
2007–2008 seasonal vaccination—N (%)	76 (39)	24 (21)	0.0010

[*] Indicate unspecified values (ILI season 2006–2007, $n = 9$).
[†] Indicate covariate missing values (Smoker, $n = 8$).

negative, $n = 76$) was positively associated with probability of protection against A(H1N1) infection. We found significant differences between older (>50 years old) and younger subjects (<30 years old) ($p = 0.0091$); no significant differences were found between subjects 30–50 years old and subjects <30 years old ($p = 0.4271$). GMT associated with 50% probability of protection decreased with increasing age: we predicted a GMT of 40.8 at inclusion was associated with 50% [95CI: 29.3%; 70.7%] probability of protection among subjects <30 years old; GMT 26.8 for 50% [95CI: 34.4%; 65.6%] among subjects 30–50 years old; GMT 8.9 for 50% [95CI: 15.3%;

84.7%] among subjects >50 years old (Fig. 4). Overall, increasing serological titer was associated with a steady increase in probability of protection, however, the increase in protection was much smaller for HI titers >80.

3.2. Evolution of antibody titer

Among ILI–A(H1N1) positive subjects highest GMT (54.0 [95CI: 45.3; 64.4]) was observed at the first annual study visit, conducted 3–12 months post-infection (April 2008 and January 2009) (Fig. 5).
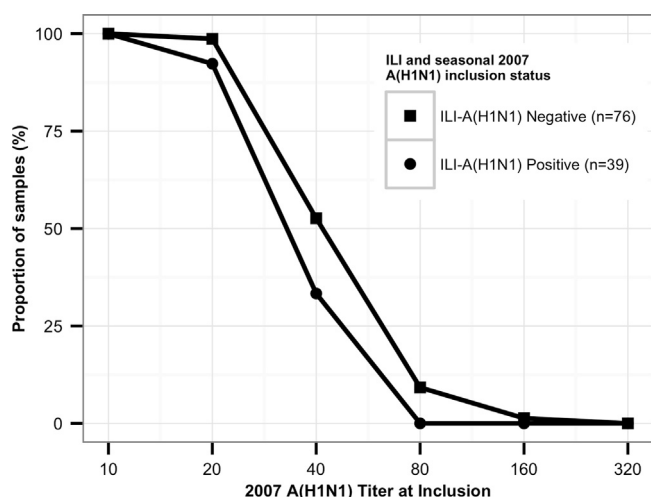
Fig. 3. Cumulative distribution curves for 2007H1N1 titers at inclusion among ILI subjects ($n = 115$) according to seasonal 2007 A(H1N1) infection status by PCR.

Fig. 4. HI protection curve by age group, based on predictions from the logistic model among ILI subjects at inclusion (ILI-A(H1N1) negative: subjects <30 years old ($n = 10$), 30–50 years old ($n = 26$), >50 years old ($n = 40$); ILI-A(H1N1) positive: subjects <30 years old ($n = 11$), 30–50 years old ($n = 20$), >50 years old ($n = 8$)).

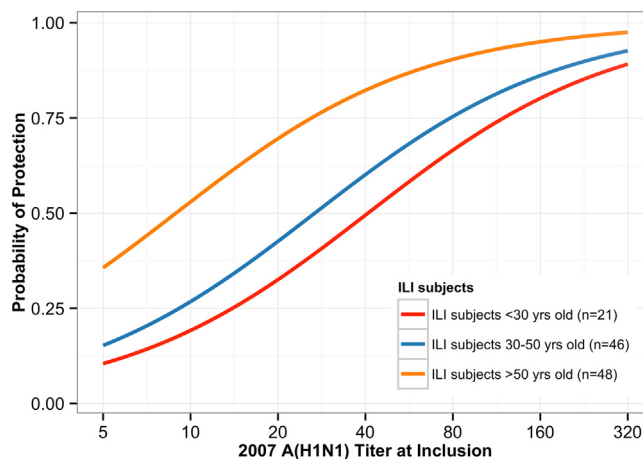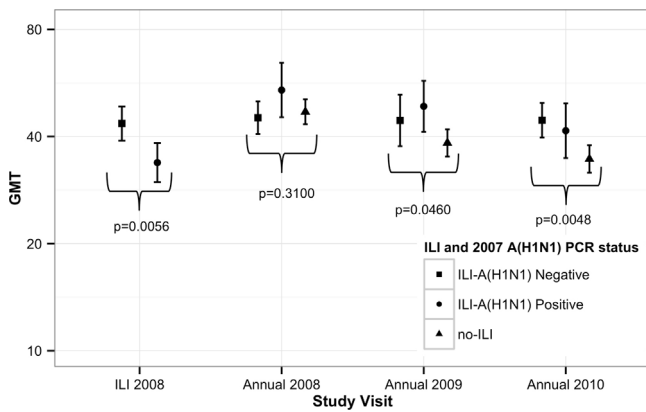**Fig. 5.** Evolution of GMT according to ILI and A(H1N1) infection status at inclusion; error bars represent 95% CI.

HI titers declined thereafter to GMT 41.5 [95CI: 34.8; 49.5] at the time of the 2010 study visit but remained significantly higher compared to GMT at inclusion ($p = 0.0157$). Whereas ILI-A(H1N1) negative subjects maintained a stable GMT throughout the study period ($p = 0.7502$), GMT among no-ILI subjects decreased steadily ($p < 0.0001$). GMT was significantly different between the three ILI groups at the 2009 ($p = 0.0460$) and 2010 ($p = 0.0048$) annual study visits. GMT among no-ILI subjects differed from ILI-A(H1N1) positive subjects at the 2009 study visit ($p = 0.0230$) and from ILI-A(H1N1) negative subjects at the 2010 study visit ($p = 0.0118$).

4. Discussion

We studied the relationship between HI titer and probability of protection against seasonal 2007 influenza A(H1N1) infection in three age groups and explored how HI titer evolves over three years (2008 to 2010) according to ILI and infection status at inclusion. Our analysis supports, among naturally infected subjects, previous findings with regard to the HI protection curve and identifies differences according to age. We found evidence to suggest elevated HI titers associated with seasonal 2007 A(H1N1) infection decrease post-infection, but persist into subsequent influenza seasons.

Previous studies demonstrating a positive association between HI titer and clinical protection have relied primarily on vaccine study data [11,24]. Hobson et al. [11] cautioned that the estimated HI titer (18–36) associated with a 50% protection may not be generalizable to naturally infected subjects, however this remains a prominent outcome or endpoint in serological and vaccine efficacy studies regardless of type of influenza exposure. We are the first, to our knowledge, to identify differences in the HI protection curve according to age; age-related differences in susceptibility to influenza infection are well established [3,5,26,27]. Furthermore, our results indicate that low antibody titer, particularly among older subjects, may confer a significant level of protection in the context of natural infection. Our estimates of GMT at inclusion associated with 50% probability of protection, ranging from 8.9 to 40.8 according to age group, are comparable to a recent estimate by Coudeville et al. (HI titer 17 (Credible Interval: 10; 29)) [24] as well as other published work [11,28,29]. However, one household study found that 50% protection was associated with HI titer 255 (CI: 1:62 to 1:917); the higher estimate attributed to the intensity of exposures in the confined setting of the home [30].

Our model of the relationship between HI titer and probability of protection did not support the designation of a single threshold level [11] and is therefore depicted by a curve [24]. We found that probability of protection increased steadily with increasing HI titer for HI titers ≤ 80; higher HI titers were associated with smaller increases in probability of protection. This phenomenon has been observed previously for HI titers ≥ 150 [24,28].

After an initial peak following infection or vaccination, antibody titers decline before reaching a stabilized level maintained over several years [31,32]. GMT among ILI-A(H1N1) positive subjects peaked at the time of the first annual study visit. However, due to the timing of this study visit (conducted 3–12 months post-infection) GMT at this time point likely captures the decline of HI titers rather than the peak GMT estimated to occur one month post-infection [33–35]. Other studies have also documented rapid GMT decline after reaching peak levels post-infection [33,35,36], however, our results also support recent findings showing persistence of elevated HI titers beyond 12 months post-infection [8]. Several studies [37–39], including one based on a subset of this cohort [15], suggested that previous seasonal A(H1N1) infection may have been protective against A(H1N1)pdm09 infection during the 2009 H1N1 pandemic via a cross-reactive antibody response. It is unlikely, however, that antibody persistence among ILI-A(H1N1) infected subjects is due to boosting during study follow-up. There is no known interaction between A(H3N2) (circulating during the 2008–2009 season) and A(H1N1) viruses. Regarding the pandemic season, none of the subjects with laboratory confirmed ($n = 1$) or suspected infection (seroconversion, $n = 6$) were in the ILI-A(H1N1) positive group.

GMT decline among no-ILI subjects was not surprising; antibody decline over time has been demonstrated [7,8,35] and may explain recurrent seasonal influenza epidemics. In contrast, GMT among ILI-A(H1N1) negative subjects remained stable throughout the study. Twenty-seven of the 76 ILI-A(H1N1) negative subjects were found to have laboratory-confirmed influenza B at inclusion, although, cross-reactive responses between influenza A and B viruses have not been demonstrated [40]. Stable GMT may suggest effects from undetected respiratory viruses or asymptomatic infections prior to study enrolment.

4.1. Strengths/limitations

This study is among few longitudinal studies investigating durability of the immune response [8,14]. Recent studies have primarily focused on the 2009 A(H1N1) pandemic and/or duration of vaccine-induced antibody response [33–35]; those investigating seasonal influenza infection were conducted almost 30 years ago [2–4,41]. Finally, while infected subjects may have also been identified by seroconversion, this study relied on a virological definition of infection, thus avoiding the sensitivity issues associated with a serological definition [42].

Our results may not be generalizable to children as this study is based on adult subjects. However our estimate of HI titer associated with 50% clinical protection against influenza among subjects <30 years old is comparable to recent findings from a randomized control trial investigating seasonal vaccination among children [29]. Another limitation of our study is the timing of the serological samples. Inclusion samples among ILI subjects were restricted to those collected within 5 days of the inclusion visit, limiting the delay between start of symptoms and blood collection. Nonetheless, we cannot assure that blood collection was performed before the start of HI titer increase for all ILI subjects.

The HI protection curves are based on ILI subjects seeking medical care, however, it is possible that their baseline risk for infection may be different from the general population. Furthermore, our study captures symptomatic infections among ILI subjects only; infected subjects with mild symptoms [3] that did not meet our definition of symptomatic illness and/or those with asymptomatic infections [43] were not identified. We are therefore unable to infer how protection and antibody persistence changes over time among asymptomatic subjects.

Although determination of HI titer by the HI assay is the gold standard, its variability is well known and this is another limitation for this study [42,44]. HI titer is the primary correlate of protection against influenza infection, however the association between HI titer and protection does not establish a direct mechanistic link. Recent research has explored the potential protective roles of local responses in the respiratory tract [8,45,46]. Furthermore, the low titers that we, and other studies [11,14,24], have found to correlate with protection may indicate that antibody responses may work in conjunction with other immune responses [30,47,48] to protect against infection. This may partly explain the difference in HI protection curves between older and younger subjects. Temporal data regarding cellular responses may further understanding of clinical protection against influenza infection, however this was outside the scope of this seroepidemiological study.

5. Conclusion

We found a positive association between HI titer and probability of protection among naturally infected subjects and identified differences according to age. This analysis provides a rare look into the persistence of the antibody response following natural seasonal A(H1N1) infection. Future studies should incorporate longitudinal data regarding other potential correlates of immunity, such as cellular immune responses, to better understand the durability of protection following seasonal influenza infection.

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Author contributions

Participated in the acquisition of data: M. Lemaitre and F. Carrat. Participated in the laboratory analyses: X. de Lamballerie, N. Salez, M. Leruez-Ville. Study concept and design: R. Delabre and F. Carrat. Analysis and interpretation of data: R. Delabre, M. Lemaitre, X. de Lamballerie, N. Salez, M. Leruez-Ville, and F. Carrat. R. Delabre and F. Carrat drafted the manuscript and all authors revised it critically. All authors gave final approval of this version of the manuscript.

Conflict of interest statement

F. Carrat received honoraria from Astra Zeneca, Boiron and GSK for advising on influenza epidemiology. Other authors have no conflict of interest to declare.

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