APPENDIX

Increased risk of non-influenza respiratory virus infections associated with receipt of inactivated influenza vaccine

Follow-up for illnesses in subjects and their household members

We recruited and randomized 119 subjects to receive 2008-09 seasonal trivalent influenza inactivated vaccine (TIV) (0.5ml Vaxigrip, Sanofi Pasteur) or saline placebo [1]. Two randomized subjects dropped out of the study prior to administration of TIV/placebo, and two subjects dropped out immediately after receipt of TIV/placebo. The present report focuses on the 115 subjects who were successfully followed up.

All subjects and their household contacts were instructed to record the presence of any systemic and respiratory symptoms in a symptom diary daily throughout the study. Symptom diaries were completed by proxy for younger children, and verified by study nurses during home visits. Telephone calls were made biweekly to monitor for any acute respiratory illnesses, and to remind households to report any acute upper respiratory tract infections (URTIs) to the study hotline as soon as possible after illness onset. Home visits were triggered by the presence of at least two symptoms or signs of fever (body temperature ≥37.8°C), chills, headache, sore throat, cough, presence of phlegm, coryza or myalgia in any household member. During home visits, nasal and throat swabs (NTS) were collected from all household members regardless of illness and pooled for laboratory analysis [1].

We defined episodes of acute respiratory illness (ARI) and febrile acute respiratory illness (FARI) as periods of 1 or more day when participants met the criteria for ARI or FARI, respectively, according to data from symptom diaries or telephone follow-up, and episodes occurring with less than 7 days separation were merged together. If two ARI episodes had occurred with less than 7 days separation and different respiratory viruses detected in each episode, they would not have been merged together, but this situation did not occur in our study. Laboratory results from multiple NTS collected from the same ARI episode were consistent, i.e. in episodes from which two or more specimens were collected, the test results were both positive for the same virus, or some were positive for the same virus and others were negative for all viruses, or all specimens were negative for all viruses. In the analyses reported we considered virus detections on a per-episode basis rather than a per-detection basis.

Determination of person-years of follow-up

Serum specimens were drawn from subjects immediately before vaccination in November–December 2008, one month after vaccination, in mid-study around April 2009, and at the end of the study in August–October 2009. We defined the follow-up period for each subject from 14 days after receipt of TIV/placebo until collection of mid-study sera as the "winter" season and from collection of mid-study sera until collection of the final sera as the "summer" season. Person-years of follow-up were calculated accordingly.

Technical details of laboratory methods

We tested NTS collected from subjects for 19 respiratory viruses by the ResPlex II Plus multiplex array using Templex technology [2] followed by product detection and identification using a Luminex suspension microarray according to the manufacturers instructions [3,4]. The 19 virus targets included influenza types A and B (including 2009-H1N1), RSV types A and B, parainfluenza types 1-4, metapneumovirus, rhinovirus, coxsackievirus/echovirus, adenovirus types B and E, bocavirus, coronavirus types NL63, HKU1, 229E and OC43.

Total nucleic acids were prepared from samples by using the NucliSens easyMAG instrument (bioMerieux, SA, France) and RT-PCR was performed using the OneStep RTPCR Kit (Qiagen, Hilden, Germany). Briefly, the 50μL reaction mixture included 10μL of eluted nucleic acids, 10μL of 5x Qiagen OneStep RT-PCR buffer, 2μL of dNTP mix, 6μL of ResPlex II primer mix, 2μL of Qiagen OneStep RT-PCR enzyme mix, 0.75μL of amplification enhancer and 19.25μL of RNase-free water. RT-PCR was carried out with an initial RT step at 50°C for 35 minutes, followed by 15 minutes of PCR activation at 95°C, 15 cycles of enrichment cycling (94°C for 30 seconds, 52°C for 1 minute, 72°C for 1 minute), six cycles of two-step cycling (94°C for 15 seconds, 70°C for 1.5 minute), and 30 cycles of three-step cycling (94°C for 15 seconds, 52°C for 15 seconds, 72°C for 15 seconds). A final extension of 3 minutes at 72°C concluded the amplification. For detection, 5μL of RT-PCR product, 10μL of ResPlex II Bead Mix, and 35μL of detection buffer were incubated at 52°C for 10 minutes. Then 10μL of streptavidin–phycoerythrin conjugate mixed with detection buffer were added. Mixtures were maintained at 52°C for 5 minutes, and then 120μL of stopping

buffer was added. Samples were analyzed on a Luminex 100 system (Luminex, Austin, TX) running QIAplex MDD Software 1.2. Raw mean fluorescence intensity data were exported to Microsoft Excel for storage and analysis. One internal and one sample control were processed in parallel for each analysis to check for the viral RNA isolation procedure, possible PCR inhibition and sample integrity.

Pooled NTS were also tested for influenza A and B by reverse-transcription polymerase-chain-reaction (RT-PCR) [5,6]. We refer to infections determined by virologic assays as 'confirmed' infections.

Serum specimens were drawn from subjects at baseline, one month after vaccination, in mid-study around April 2009, and at the end of the study. These serum specimens were tested for antibody responses to the vaccine strains A/Brisbane/ 59/2007(H1N1), A/Brisbane/10/2007(H3N2), and B/Florida/ 4/2006-like (Yamagata-lineage) by hemagglutination inhibition and for antibody responses to A/California/04/ 2009(H1N1) by viral microneutralization using standard methods as previously described [1,6]. Post-season antibody titers were compared with mid-study titers, which were in turn compared with post-vaccination antibody titers to determine serologic evidence of infection during the summer and winter influenza seasons, respectively, as 4-fold or greater rises in antibody titers.

Additional details of statistical analyses

Because duration of follow-up varied by subject, we estimated the incidence rates of ARI and FARI episodes overall and in the winter and summer seasons,

and estimated the relative risk of ARI and FARI and confirmed respiratory virus infections for subjects who received TIV versus placebo via the incidence rate ratio. We fitted negative binominal regression models but did not find evidence of over-dispersion in incidence rates of ARI and FARI and therefore in the final analyses we used Poisson regression models with duration of follow-up for each individual as an offset term to estimate the relative risks with 95% confidence intervals (CIs).

Subjects with more than one confirmed infection

No subject experienced a confirmed influenza infection plus a confirmed non-influenza respiratory virus infection. One subject had a confirmed infection with rhinovirus in both winter and summer 2009 (156 days apart), and another subject had confirmed infections with coxsackie/echovirus twice in the winter (58 days apart).

Additional analysis including influenza serology

Because we were not able to obtain NTS from some subjects during illness episodes and therefore confirmed infections underestimated all infections, we estimated the rates of influenza virus infection based on serological evidence as a 4-fold or greater rise in antibody titer during the winter or summer influenza seasons. Six individuals had 4-fold or greater rises to more than one virus in the winter or summer season, and we classified one infecting strain per season correcting for cross-reactions as previously described [7]. We then explored the association between the risk of confirmed non-influenza respiratory virus infection versus serological evidence of influenza infection, stratified by the

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Comment [s1]: How can they do that for the vaccinated arm. They are expected to have a 4 fold rise so that does not give you their infection rate necessarily. Perhaps because they checked it after the vaccine was given and then waited for it to rise. Still what about those who were vaccinated and had an acute rise in antibody?

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winter and summer seasons. We used Fisher's exact tests and estimated odds ratios with 95% CIs using Fisher's conditional maximum likelihood approach [8].

Appendix Table 1 below shows the incidence rates of influenza infection determined by serology or RT-PCR, and the incidence rates of respiratory virus infection as determined by the multiplex assay. Only approximately 15% of the infections indicated by serology were confirmed by RT-PCR, for various reasons [1]. Of the placebo recipients, 24% and 13% had serologic evidence of seasonal influenza infection in the winter and summer seasons, respectively. TIV recipients appeared to be protected against seasonal influenza (p=0.04). However, it is important to be cautious when interpreting serologic data on influenza infections from individuals that have received influenza vaccination, because antibody titers rise substantially in the weeks following vaccination and then tend to fall over the following months [9]. High post-vaccination titers can mask further rises associated with infection, due to a ceiling effect. More rapidly falling titers post-vaccination can also mask smaller rises associated with infection. Finally, some infections and particularly those infections associated with milder subclinical illness may not lead to 4-fold or greater rises in antibody titers.

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In the winter 2009 season, 97/115 subjects had paired sera available, and 1/17 (6%) subjects with any influenza infection indicated by serology had a confirmed infection with a non-influenza respiratory virus (on 23 January), while 15/80 (19%) subjects without serologic evidence of influenza infection had a confirmed infection with a non-influenza respiratory virus (odds ratio, OR: 0.27; 95% CI:

0.01, 2.05). In the summer season, 106/115 subjects had paired sera available, and 2/36 (6%) subjects with any influenza infection indicated by serology had a confirmed infection with a non-influenza respiratory virus, while 5/70 (7%) subjects without serologic evidence of influenza infection had a confirmed infection with a non-influenza respiratory virus (OR: 0.77; 95% CI: 0.07, 4.99).

Additional comments on detection of respiratory viruses

We were only able to detect a respiratory virus in 49% of NTS collected during ARI episodes, and the etiology of the remaining ARI episodes remains uncertain. It is likely that the fraction of ARI episodes associated with respiratory viruses was underestimated in our study due to imperfect sensitivity of the multiplex assay, potentially low levels of viral shedding in some infections, and the lack of timely identification of some illnesses. In addition, some of the ARI episodes without confirmed viral etiology may be associated with bacterial infections, which may not be affected by prior virus-induced innate immunity in the same way as viral infections [10]. This is consistent with our observation of no significant or substantial difference between TIV and placebo recipients in incidence of ARI episodes without a virus detected (Table 3).

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Additional comments on the implications for test-negative studies

Viral interference may bias estimates of influenza vaccine effectiveness in observational studies. One approach to estimating vaccine effectiveness involves identifying medically-attended acute URTIs and attributing them to influenza or other respiratory viruses using laboratory tests [11-20]. Influenza vaccine coverage is then compared between the influenza-positive patients (cases) and

the influenza-negative patients (controls), adjusting for potential confounders. An assumption required for this 'test-negative' case-control study design to be valid is that the risk of illness associated with non-influenza infections must be independent of receipt of influenza vaccine [21]. The existence of temporary non-specific immunity would invalidate this assumption. If unvaccinated individuals faced an increased risk of influenza infection, and other respiratory viruses co-circulated during the influenza season, one might expect those unvaccinated individuals to face a lower risk of non-influenza respiratory virus infections due to temporary non-specific immunity. In this case, test-negative studies would tend to overestimate vaccine effectiveness.

One test-negative study used a multiplex assay to examine non-influenza etiologies of outpatients with influenza-like illness [16]. Among the control group of outpatients with influenza-like illness that tested negative for influenza virus, influenza vaccination coverage was significantly higher in the patients who had a confirmed non-influenza respiratory virus compared to those with no viral etiology. While the authors commented that this observation did not seem biologically plausible [16], the existence of temporary non-specific immunity could explain the results. The implication of this observation is that in test-negative studies it might be preferable to include as controls only those patients who test negative for co-circulating respiratory viruses rather than all patients who test negative for influenza virus.

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Appendix Table 1. Incidence rates of influenza virus infections by serology or RT-PCR, and respiratory virus infections detected by the multiplex assay in specimens collected from 115 subjects aged 6-15 years who received trivalent influenza vaccine (TIV) or placebo.

	TIV (n=69)			Placebo (n=46)			p-value
	n	**Rate***	**(95% CI)**	**n**	**Rate***	**(95% CI)**	
Any seasonal influenza	13	252	(147, 435)	18	530	(334, 841)	0.04
Seasonal influenza A(H1N1)	6	116	(52, 259)	8	235	(118, 471)	0.19
Seasonal influenza A(H3N2)	3	58	(19, 181)	6	177	(79, 393)	0.10
Seasonal influenza B	4	78	(29, 207)	4	118	(44, 314)	0.56
Pandemic influenza A(H1N1)	20	388	(250, 602)	6	176	(79, 393)	0.07
Any non-influenza virus†	21	408	(266, 625)	4	118	(44, 314)	0.01
Rhinovirus	12	233	(132, 410)	2	59	(15, 235)	0.04
Coxsackie/echovirus	8	155	(78, 311)	1	29	(4, 209)	0.06

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Other respiratory virus‡	6	116	(52, 259)	1	29	(4, 209)	0.14

* Incidence rates estimated as the number of infections per 1,000 person-years of follow-up. Due to the possibility of cross-reactive antibody, a maximum of one influenza infection per season (winter or summer) was inferred from the serologic data as described previously [7].

† In TIV recipients there were 4 detections with both rhinovirus and coxsackie/echovirus, and 1 detection with both coxsackie/echovirus and coronavirus NL63.

‡ including positive detections of coronavirus, human metapneumovirus, parainfluenza, RSV. The ResPlex II multiplex array tested for 19 virus targets including influenza types A and B (including 2009-H1N1), RSV types A and B, parainfluenza types 1-4, metapneumovirus, rhinovirus, coxsackievirus/echovirus, adenovirus types B and E, bocavirus, coronavirus types NL63, HKU1, 229E and OC43.

Abbreviations: TIV = trivalent inactivated influenza vaccine; CI = confidence interval.

Comment [s2]: Ok so they claim that about half the rate of infections for flu happened in the vaccinated but 5 times the rate of non flu also happened in the vaccinated.

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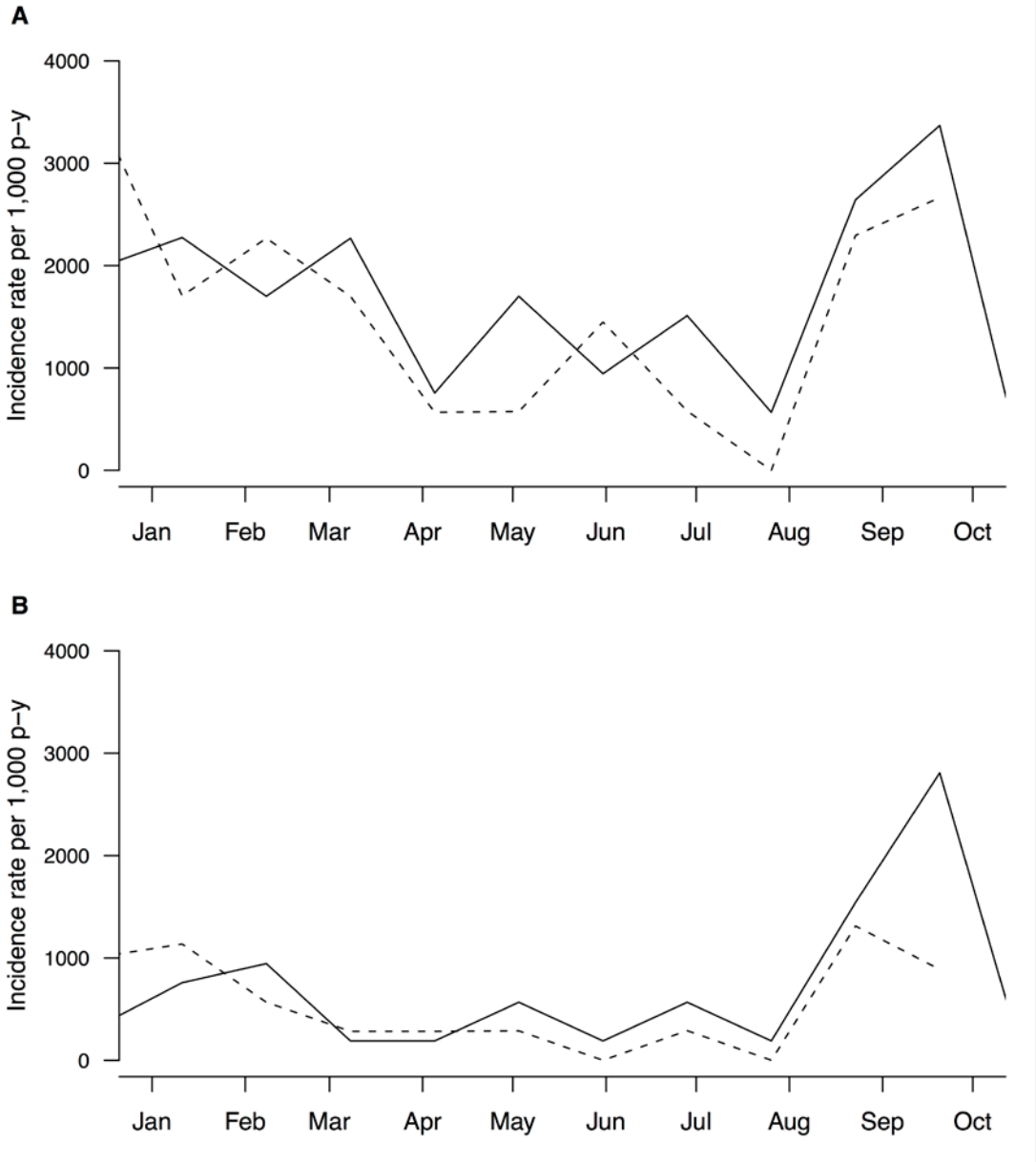
Appendix Figure 1. Incidence rates of acute upper respiratory tract infections in subjects who received trivalent inactivated influenza vaccine (TIV) or placebo. Panel (A) compares incidence rates of acute respiratory illness defined as at least two of body temperature ≥37.8°C, cough, sore throat, headache, runny nose, phlegm and myalgia; panel (B) compares incidence rates of febrile acute respiratory illness defined as body temperature ≥37.8°C plus cough or sore throat.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