

Acquired immune responses to the seasonal trivalent influenza vaccination in COPD

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Key Words:	Human, Lung, Vaccination

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1 Acquired immune responses to the seasonal trivalent influenza vaccination in
2 COPD

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23 Keywords: COPD, influenza, vaccination

24 Abbreviations used: ASC: Antibody secreting cells; ATS: American Thoracic Society; COPD:
25 Chronic obstructive pulmonary disease; CPMP: Committee for Proprietary Medicinal
26 Products; FEV1: Forced expiratory volume in 1 sec; FVC: Forced vital capacity; GzmB:

- 1 Granzyme B; HAI: Haemagglutinin inhibition; IAV: Influenza A virus; PBMC: Peripheral blood
- 2 mononuclear cells; TIV: trivalent influenza vaccine

For Peer Review

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Summary

Epidemiological data suggests that influenza vaccination protects against all-cause mortality in COPD patients. However recent work has suggested there is a defect in the ability of some COPD patients to mount an adequate humoral response to influenza vaccination. The aim of our study was to investigate humoral and cell mediated vaccine responses to the seasonal trivalent influenza vaccination (TIV), in COPD subjects and healthy controls. 47 subjects were enrolled into the study; 23 COPD patients, 13 age-matched healthy control (HC - ≥ 50) and 11 young healthy control subjects (YC - ≤ 40). Serum and PBMC were isolated pre-TIV vaccination and at days 7, 28 and 6 months post vaccine for Haemagglutinin Inhibition (HAI) titre, antigen-specific T cell and antibody-secreting cell analysis. The kinetics of the vaccine response were similar between YC, HC and COPD patients and there was no significant difference in antibody titres between these groups 28d post-vaccine. As we observed no disease-dependent differences in either humoral or cellular responses, we investigated if there was any association of these measures with age. H1N1 ($r=-0.4253$, $p=0.0036$) and Influenza B ($r=-0.344$, $p=0.0192$) antibody titre at 28d negatively correlated with age as did H1N1-specific CD4+ T helper cells ($r=-0.4276$, $p=0.0034$). These results suggest that age is the primary determinant of response to trivalent vaccine and that COPD is not a driver of deficient responses per se. These data support the continued use of the yearly trivalent vaccine as an adjunct to COPD disease management.

1 Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by airway inflammation, resulting from the inhalation of tobacco smoke or other irritants (1). The course of the disease is one of progressive, irreversible airflow limitation, functional impairment and periods of acute symptom deterioration called exacerbations (2). Acute exacerbations are important events in the natural history of COPD associated with impaired quality of life and accelerated decline in lung function, placing a considerable burden on health-care resources (3-5). Respiratory infection is a key determinant with viral infections implicated in between 40% and 60% of COPD exacerbations (6, 7). Consequently, preventative strategies aimed at reducing the impact of respiratory viral infection, are key interventions in reducing the morbidity and health-care burden associated with COPD exacerbations.

Influenza A and B are single stranded RNA viruses of the *Orthomyxoviridae* family that circulate in the human population causing acute infection of both the upper and lower respiratory tract. In patients with COPD, influenza is associated with seasonal exacerbations, with the virus detected in up to 28% of exacerbating COPD patients (8, 9). The seasonal influenza vaccination is currently the best method for reducing the incidence of influenza infection, and the associated complications such as pneumonia, and is recommended for the elderly and in those with chronic conditions such as COPD(10). Therapies to treat influenza infection, once present in COPD, are currently very limited, both in terms of the window of opportunity for treatment and therapeutic efficacy. Thus, vaccination is a critical component in exacerbation prevention. In the UK, the most commonly used formulation is the trivalent influenza vaccine (TIV) which currently contains inactive viral haemagglutinin (HA) from H1N1, H3N2 and influenza B strains. A recognised correlate of vaccine protection is the production of sufficient concentrations of antibodies to HA, as assessed by haemagglutinin inhibition (HAI) assays (11). The response to TIV can be demonstrated by either seroprotection (HAI antibody titre $\geq 1:40$) or seroconversion (minimum 4-fold rise in HAI antibody titre) (12). Whilst the use and efficacy of TIV in COPD is supported by epidemiological studies (13, 14), recent

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laboratory-based work has begun to question the effectiveness of these vaccines in disease (15-17).

Whilst trials and epidemiological data point to a benefit of TIV in COPD patients (18), a small study from Australia, demonstrated that COPD patients had lower titres of H1N1-specific antibodies 28 days post-vaccination and lower serum IL-21 levels, which is thought to play a role in B-cell antibody synthesis (16). In addition, the same group reported a reduced proportion of COPD patients who seroconverted 28 days after vaccination, compared to healthy controls (15). More recently, Parpaleix et al demonstrated decreased geometric mean HAI antibody titres in COPD patients at 30 days post-vaccine compared to healthy controls, but no significant difference in the proportion of volunteers who experienced seroconversion between health and disease (17). Furthermore, this decrease in geometric mean HAI antibody titres was associated with a decrease in vaccine-specific CD4+ T cell responses prior to vaccination.

The aim of our study was to conduct a detailed assessment of the HAI antibody, T cell and B cell responses to TIV of COPD patients, compared to age-matched healthy and young, healthy controls enabling the identification of any differences in vaccine responses between the groups. .

Methods

Volunteer Recruitment

Twenty-three COPD patients, 13 age-matched (≥ 50 years old) healthy control subjects and 11 younger (< 40 years old) healthy control subjects were studied (Fig 1). Power calculations were based on the proportion of responders achieving at least a 4-fold increase in the H1N1 HAI antibody titre in each group. A minimum sample size of 22 subjects in the COPD group and 11 subjects in the control groups was determined as sufficient for a two-sided, type-1 error rate of 0.05 and a power of 80%. A diagnosis of COPD was confirmed by post-bronchodilator spirometry, with a forced expiratory volume in 1 second (FEV₁)/forced vital capacity (FVC) ratio of < 0.7 required for enrolment. Spirometry was conducted in accordance with ATS standards (19). COPD subjects had a smoking history of at least 10 pack years. All subjects had received the TIV in the year prior to the study (2014). The vaccine strains for the 2014/2015 Northern hemisphere vaccine were A/California/7/2009 (H1N1) pdm09-like virus, A/Texas/50/2012 (H3N2)-like virus and B/Massachusetts/2/2012-like virus. Exclusion criteria included a history of other pulmonary disease, long-term use of immunosuppressant medications (including oral corticosteroids) and an exacerbation within the month prior to recruitment. All subjects gave written informed consent and the study was approved by the National Research Ethics Service (NRES) South Central – Oxford C Committee (15/SC/0528).

Study Procedures

As this was a purely observational study, the intramuscular influenza vaccine was administered by the volunteer's usual health care provider as part of usual care between October 2015 and December 2015. All of the young healthy group were healthcare professionals. The vaccine strains for the 2015/2016 Northern hemisphere were A/California/7/2009 (H1N1) pdm09-like virus, A/Switzerland/9715293/2013 (H3N2)-like virus and B/Phuket/3073/2013-like virus. Phlebotomy was performed at a pre-vaccine visit and then at 7-10 days (Visit 1), 28 ± 3 days (Visit 2) and 180 ± 14 days (Visit 3) post-vaccination.

Serum HAI Antibody Titres

The HAI assays were performed by the Public Health England (PHE) laboratories, Porton Down, Salisbury, UK. Briefly, serial 2-fold dilutions for each set of sera was incubated with standardised concentrations (4 HA units) of influenza virus representing either the H1N1, H3N2 and influenza B 2015/16 viral strains. Chicken red blood cells were then added and allowed to settle. After 30 min, the strain-specific HAI antibody titres at each time point for each individual were calculated as the highest dilution of sera that inhibited hemagglutination. Seroconversion rates (i.e., numbers of individuals with HAI antibody titres ≥ 40) and seroprotection rates (i.e., numbers of individuals with HAI antibody titres ≥ 10 at D0 and HAI antibody titres ≥ 40 after vaccination or with HAI antibody titres ≥ 10 at D0 and ≥ 4 -fold increase in HAI antibody titres after vaccination) are also indicated.

PBMC isolation & storage

PBMCs from volunteers were isolated from heparinized blood by means of centrifugation on Ficoll-Paque (GE Healthcare). Purified PBMC were frozen in Heat-inactivated Foetal Bovine Serum containing 10% (v/v) DMSO (Sigma) and stored in liquid nitrogen until analysis.

Flow cytometry Analysis

Flow cytometry was performed as previously described (20). To analyse influenza-specific T cells, PBMCs were stimulated overnight with inactivated A/H3N2 Wisconsin/67/2005 and A/H1N1/California/04/2009 in the presence of monensin/brefeldin A mix (Sigma). Cells were first stained for viability and surface markers: Live dead (Molecular Probes), CD4 (OKT4 clone), CD3 (SK7 clone), CD8 (RPA-T8 clone), CD49d (9F10 clone) and CD49a (SR84 clone; BD Biosciences). Cells were then resuspended in Cytofix/Cytoperm (BD Biosciences) before staining for intracellular markers: IL-2 (N7.48 A clone; Miltenyi), IFN γ (B27 clone), IL-17a (N49-653 clone), Granzyme B (GB11 clone), TNF α (MAb11 clone; BD Biosciences). Flow

cytometric analysis was performed on a FACSFortessa using FACSDiva software v5.0.3 (BD Biosciences). At least 1×10^6 live events, according to forward- and side-scatter parameters, were accumulated and analysed for boolean combination gating with FlowJo software (Tree Star Inc.). The percentage of influenza A virus-specific CD4⁺ or CD8⁺ T cells expresses the sum of the 15 different cytokine boolean combinations (IL-2, IFN γ , IL-17a and/or TNF α). Background cytokine responses detected in negative controls were subtracted from those detected in stimulated samples for every specific combination.

Antibody-Secreting Cell (ASC) detection.

A/California/07/2009 (H1N1) influenza HA-specific IgG-secreting B cells were performed as previously described (21). Briefly, PBMC were stimulated with a mixture of Pokeweed mitogen (Sigma), *Staphylococcus aureus* Protein A (Sigma) and CpG ODNs (Invivogen) for 6 days before ELISpot assay. H1N1 HA protein (Protein Sciences) was used to coat the plate (Millipore), and IgG- or IgA-paired antibodies (Mabtech) were used to reveal. ELISpot readouts were expressed as the number of HA-specific IgG or IgA ASC/ 10^6 PBMC.

Statistics and Analysis

Statistical analyses were performed using either a Wilcoxon's matched-pairs signed-rank test, Mann-Whitney U test, Kruskal-Wallis or Friedman test with Dunn's multiple comparison testing as appropriate (GraphPad Prism v7.0, GraphPad Software Inc., San Diego, USA). For the paired analysis, if data was missing for a given visit, all data from that volunteer was excluded and there was no data imputation. Data are expressed as medians. Results were considered significant if $P < 0.05$. The radar charts were designed with R, a free software environment for statistical computing and graphics (<http://www.r-project.org/>).

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Results

Subject Demographics

47 subjects were successfully enrolled into the study, 23 COPD patients, 13 age-matched healthy controls and 11 young control subjects (Fig 1). The COPD and healthy control subjects were well matched for age and gender. As expected there were significant differences in the proportion of current smokers and FEV1 measures. To ensure that recent vaccination history was known, our inclusion criteria required subjects to have received the prior year's (2014/15) influenza vaccination. The descriptive characteristics are shown in Table 1.

Humoral responses to TIV

To ascertain the effectiveness of the vaccine to induce a humoral response, we first analysed the antibody titres to all three vaccine components via individual haemagglutinin inhibition (HAI) assays in the whole cohort (Fig 2A-C). The median (IQR) level of pre-vaccine HAI titres were 40 (10-40) for A/H1N1, 20 (10-40) for A/H3N2 and 17.5 (5-40) for B/Phuket strains. These data suggest that half the cohort were already seroprotected against A/H1N1 virus but not A/H3N2 or B/Phuket strain before vaccination. There was a significant increase in antibody titres 7 days (V1) and 28 days (V2) following vaccination for all vaccine components, although this was not sustained out to 6 months (V3) for B/Phuket strain. In addition, the HAI antibody increases in FluB were not as strong and remained consistently lower than the titres induced by the FluA components.

In line with other studies (17), we next compared the HAI titres to all three vaccine components between groups at 28d post-vaccine, when all groups demonstrated a significant increase in titres against the FluA components (Fig 3). Only the COPD group demonstrated a significant increase in HAI antibody titres to B/Phuket at 28d post-vaccination ($p=0.0127$, Fig 3C). However there was no significant difference in the HAI antibody titres between the groups for any of the vaccine components at day 28 (Fig 4A-C). Neither was there a significant difference in the fold change in HAI antibody titres between groups at V2 compared to the pre-vaccination visit, except for B/Phuket between COPD patients and Young controls (Fig 4D-F).

1 Whilst there appeared to be differences in the proportion of volunteers seroprotected (titre
2 ≥ 40) between groups for each vaccine component, the proportion was again only significantly
3 different for B/Phuket where 80% of the young controls were seroprotected compared to the
4 62.5% of Healthy controls and 53% of COPD patients ($p=0.0469$, Chi-squared).

5 Since it is also recommended that seroconversion be taken into account as well as
6 seroprotection (12) we analysed the number of patients who did not seroconvert at visit 2
7 (Figure 4D-F). The only significant difference in seroconversion we observed was a decrease
8 in the response of young controls to B/Phuket compared to COPD patients (Figure 4F). In
9 order to assess any differences in the response to vaccine using either definition, we also
10 assessed the proportions of volunteers who were not seroprotected at baseline and also did
11 not seroconvert by day 28 (Table 2). Whilst there was evidence of differential responses to
12 different vaccine components, there were no significant differences in the proportions of the
13 groups that were not protected against the individual vaccine components.

15 *Cellular responses to TIV*

16 In addition to antibodies, T cell responses are also key parameters of influenza protection (20,
17 22-26). We therefore analysed the polyfunctional CD4+ and CD8+ T cell responses to
18 inactivated whole virus (A/H1N1 and A/H3N2) in PBMC from our volunteer cohort at each time
19 point (Figure S1). We did not observe any significant difference in the quality of polyfunctional
20 CD4+ and CD8+ T cell responses between groups (Figure S2). We did observe a significant
21 increase ($p=0.0035$) in the proportion of virus- specific CD4+ T cells 28 days post vaccination
22 (Fig 5A) compared to prevaccination in the whole cohort. In contrast, there was no increase
23 in the proportion of virus-specific CD8+ T cells (Fig 5B). However, there was a significant
24 decrease in the proportion of virus-specific GzmB+ CD8+ T cells at all time-points following
25 vaccination (Fig 5C). We also compared the proportions of virus-specific T cells between
26 groups at 28d post-vaccine (Fig 5D-F). Similarly to the HAI data, we again observed no
27 significant differences in CD4+ or CD8+ T cell proportions between the groups.

At the same time we also analysed the proportions of both A/H1N1 HA-specific IgG-secreting B cells in these samples using ELISpot (Fig 6A-D). However, we observed no significant change in the proportion of A/H1N1 HA-specific IgG-secreting B cells either in response to vaccination or between the groups at 28d post-vaccine. A/H1N1 HA-specific IgA-secreting B cells are very low (data not shown).

In order to present a complete picture of our work, we have also analysed all of the variables reported above at 7 days and 6 months post vaccination and present these data as radar plots (Fig 7B&D). Furthermore we evaluated the capacity of CD4 and CD8 T cells to acquire the cell migration capacity to mucosal with the expression of integrin CD49a and CD49d. There were no statistically significant differences in any of the measurements between groups at any time-point post-vaccination. Thus, overall there appear to be no differences in vaccine responses across the groups. This is despite a statistically significant difference in B/Phuket HAI antibody titres between groups at baseline, which appears more due to age, rather than COPD (Fig 7A). Furthermore, there are also differences in the proportions of influenza-specific cytokine-secreting CD4⁺ T cells at baseline which, surprisingly are significantly lower in healthy age-matched controls compared to both young controls.

Age correlates with antibody titres

To further investigate the effects of age on responses to vaccination, we correlated the age of the volunteers with the antibody titres of the separate vaccine components at 28d (Figure 8A-C). There were weak, but significant negative correlations between age and A/H1N1 ($r = -0.4253$, $p = 0.0036$) and B/Phuket ($r = -0.344$, $p = 0.0192$) but not A/H3N2 titres.

As we have previously shown that pre-existing CD4⁺ T helper cells are essential for adequate responses to influenza (24), we next analysed the correlation between age and pre-existing virus-specific CD4⁺ T cells (Figure 8D). Similarly to the HAI data, we observed a weak, but significant negative correlation between age and the proportion of virus-specific CD4⁺ cells at the pre-vaccination visit ($r = -0.4276$, $p = 0.0034$).

Discussion

In this study we have shown that in the majority of volunteers across disease and age groups, who were vaccinated with TIV, there was adequate seroprotection (titre ≥ 40) to at least one component of the vaccine at 28d post-vaccination. Importantly, there were minimal differences in the vaccine responses of COPD patients, compared to age-matched healthy controls and young controls at any time-point. Although there were no significant age-related differences between groups in many of the immune measures analysed prior to vaccine administration there was evidence of a relationship between age and vaccine responses. Further analysis demonstrated a negative correlation between age and HAI antibody titres for two out of three vaccine components. Taken together these data suggest that age-related immune senescence may have a stronger dampening effect on vaccine responses than COPD per se.

In the Western world, COPD is a disease associated with life-long smoking and due to the pernicious nature of symptom onset, is not usually diagnosed until the late forties/early fifties. Thus, COPD itself is a disease already associated with ageing and therefore it is essential that the age of any control group is well matched with the COPD group. In the previous studies from the Australian group demonstrating a disease effect on vaccine responses, there were significant differences between the ages of the control group and the COPD group (15, 16). Indeed, Burel et al demonstrated that age was associated with post-vaccination A/H1N1 antibody titres by univariate, although not multivariate analysis (16).

The use of HAI antibody titres as a correlate of protection is further complicated by the use of different methods to assess vaccine responsiveness. The Committee for Proprietary Medicinal Product (CPMP) criteria state that post-vaccination serum is considered seroprotected if the HAI antibody titre is ≥ 40 (12). However, the CPMP have two definitions of seroconversion, with the seroprotected level of ≥ 40 only being considered a seroconversion if the pre-vaccine levels were negative. If the serum sample already has a positive HAI titre, then a minimum four-fold increase in HAI antibody titre is required to be considered a seroconversion (12). However, it is unclear what the relevance of the definition of

seroconversion is if the baseline titre is already 40 or greater and the volunteer is thus already considered to be seroprotected. For example, Nath et al demonstrate an average 120-fold increase in the HAI antibody titre in controls and only an average 2-fold increase in COPD patients (15). Nevertheless the majority of COPD patients were already seroprotected with a median pre-vaccine titre of 320 which was significantly higher than the pre-vaccine titre of controls (median titre of 60)(15). Parpaleix et al present geometric mean titres, but when we analyse the geometric means of our own HAI data (not shown), there are no impacts on the conclusions we reached using the mean titres. But, whilst the titres are reported as being lower in COPD compared to controls, there were no significant differences in the proportions of these groups that underwent seroconversion to any of the vaccine components (17).

The data presented here and by Parpaleix et al highlight that there are differential responses to the different components of the TIV (17). We demonstrate that the magnitude of the response to B/Phuket in the whole cohort is approximately 2-fold lower than the two FluA components and is reflected in both the seroprotection and seroconversion rates. This observation may be explained by the decreased sensitivity of the HAI assay for influenza B whole viral antigens (27). Whilst the high-level of pre-existing seroprotection to A/H1N1 in all groups is likely a result of the inclusion of the same components in both the 2014 and 2015 TIV, the other differences between the groups are harder to reconcile. For example, the young controls appeared to respond less well to the A/H3N2 components than either the COPD or age-matched controls. The reason for this is unclear, but may result from the original antigenic sin hypothesis where exposure to different circulating viral strains that prime the immune system during childhood which may then go on to determine which response is able to be boosted by the vaccine (28). Thus, the A/H3N2 may have been more dominant during the maturation of the immune response in the older volunteers, whereas A/H1N1 may have been more dominant at the time of immune maturation in the young volunteers. This is supposition and further work will be required to confirm or refute this notion. This difference in response to A/H3N2 strain certainly impacts on the correlation with age, reversing the direction of the

1 association compared to the other components even though the association is not statistically
2 significant.

3 The impact of ageing on the antibody responses to the influenza vaccination has long
4 been recognised, with the clinical vaccine efficacy being reduced from 70-90% in the young
5 to 17-53% in those subjects who were ≥ 65 (29). When this older age group were further
6 categorised into those above or below 75 it was the over 75s who had significantly lower
7 vaccine responses suggesting that it is only those over 75 that have a diminished HAI antibody
8 response (29). The mean age of our COPD and age-matched healthy volunteers was 69 and
9 65 respectively and only 5 COPD patients were over 75 at the time of sampling, which may
10 explain the weak negative correlation with age in our study. It has been proposed that the
11 manifestation of COPD is a result of accelerated ageing, but current evidence suggests that
12 this phenomenon is confined to the lung rather than a manifestation of systemic disease as
13 would be required for an impact on vaccine response (30). In order to overcome the effects of
14 ageing on the vaccine response, recent studies have demonstrated that increasing the dose
15 of the vaccine components leads to better vaccine responses in the elderly (31, 32). If the
16 administration of high-dose vaccine is taken into practice, our data would suggest that COPD
17 patients may be just as likely to respond as other elderly patients. However, clinical trials of
18 the high dose vaccine in COPD would be need to confirm this.

19 A limitation of our study, that we share with others, is the differences in the number of
20 current and ex-smokers between the COPD groups and the controls (15-17). This is an
21 important issue as smoking is associated with increased susceptibility to influenza infection
22 (33). However, given the toxic effects of smoking there is some debate about whether there is
23 such a thing as healthy smoker. Despite this, Burel et al demonstrate that smoking was not
24 statistically associated with vaccine responsiveness in a univariate analysis (16). A further
25 limitation of our study was that there was no age continuum in either our healthy or COPD
26 cohort, but rather we recruited to discrete age groups. This was deliberate on our part to try
27 to ensure that if there was an effect of age it would be clearly visible. Furthermore, our ability

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1 to only demonstrate associations with age may be a function of the study being powered to
2 detect differences due to disease rather than age differences. The biggest limitation to this
3 and all other studies using HAI antibody titre as an outcome is that this is not a functional
4 measure of protection from influenza vaccination but is only a surrogate marker of protection.
5 Thus, larger scale randomised control trials with clinical endpoints and confirmed influenza
6 diagnosis are required to fully investigate the impact of both age and COPD on vaccine
7 responses.

8 In summary, our data suggest that there is no primary defect in the responsiveness of
9 COPD patients to the TIV. However, there was substantial heterogeneity in the responses to
10 the three vaccine components amongst the different age groups, suggesting that age is the
11 primary driver to reduced vaccine responsiveness for at least two of the vaccine components.
12 These data would support continuing the yearly influenza vaccine schedule as an adjunct to
13 COPD disease management in an effort to reduce the burden of influenza in this patient group.

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Conflict of Interest

KJS and TMAW have received grants from GlaxoSmithKline and AstraZeneca outside of the current work. The rest of the authors have no relevant conflicts of interest.

For Peer Review

References

1. Decramer M, Janssens W, Miravittles M. Chronic obstructive pulmonary disease. *Lancet*. 2012;379(9823):1341-51.
2. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American journal of respiratory and critical care medicine*. 2007;176(6):532-55.
3. Miravittles M, Ferrer M, Pont A, Zalacain R, Alvarez-Sala JL, Masa F, et al. Effect of exacerbations on quality of life in patients with chronic obstructive pulmonary disease: a 2 year follow up study. *Thorax*. 2004;59(5):387-95.
4. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax*. 2002;57(10):847-52.
5. Anzueto A. Impact of exacerbations on COPD. *Eur Respir Rev*. 2010;19(116):113-8.
6. Wilkinson TMA, Aris E, Bourne S, Clarke SC, Peeters M, Pascal TG, et al. A prospective, observational cohort study of the seasonal dynamics of airway pathogens in the aetiology of exacerbations in COPD. *Thorax*. 2017.
7. Rohde G, Wiethage A, Borg I, Kauth M, Bauer TT, Gillissen A, et al. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax*. 2003;58(1):37-42.
8. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *American journal of respiratory and critical care medicine*. 2006;173(10):1114-21.
9. Sapey E, Stockley RA. COPD exacerbations . 2: aetiology. *Thorax*. 2006;61(3):250-8.
10. Excellence NifHaC. Immunizations - seasonal influenza Clinical Knowledge Summary <https://cks.nice.org.uk/immunizations-seasonal-influenza#!scenario>: National Institutes for Health and Care Excellence; 2018 [updated May 2018].
11. Sanei F, Wilkinson T. Influenza vaccination for patients with chronic obstructive pulmonary disease: understanding immunogenicity, efficacy and effectiveness. *Ther Adv Respir Dis*. 2016;10(4):349-67.
12. Trombetta CM, Perini D, Mather S, Temperton N, Montomoli E. Overview of Serological Techniques for Influenza Vaccine Evaluation: Past, Present and Future. *Vaccines (Basel)*. 2014;2(4):707-34.
13. Poole PJ, Chacko E, Wood-Baker RW, Cates CJ. Influenza vaccine for patients with chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2006(1):CD002733.
14. Schembri S, Morant S, Winter JH, MacDonald TM. Influenza but not pneumococcal vaccination protects against all-cause mortality in patients with COPD. *Thorax*. 2009;64(7):567-72.
15. Nath KD, Burel JG, Shankar V, Pritchard AL, Towers M, Looke D, et al. Clinical factors associated with the humoral immune response to influenza vaccination in chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease*. 2014;9:51-6.
16. Burel JGN, K.; Pritchard, A.L.; White, O.J.; Davies, J.M.; Towers, M.;Looke, D.;Upham, J.W.;. Evaluation of Immune Responses to Influenza Vaccination in Chronic Obstructive Pulmonary Disease. *J Vaccines Vaccin*. 2012(S4):001.
17. Parpaleix A, Boyer L, Wiedemann A, Lacabaratz C, Margarit L, Enouf V, et al. Impaired humoral and cellular immune responses to influenza vaccination in chronic obstructive pulmonary disease patients. *The Journal of allergy and clinical immunology*. 2017;140(6):1754-7 e6.
18. Bekkat-Berkani R, Wilkinson T, Buchy P, Dos Santos G, Stefanidis D, Devaster JM, et al. Seasonal influenza vaccination in patients with COPD: a systematic literature review. *BMC Pulm Med*. 2017;17(1):79.
19. Culver BH, Graham BL, Coates AL, Wanger J, Berry CE, Clarke PK, et al. Recommendations for a Standardized Pulmonary Function Report. An Official American Thoracic Society Technical Statement. *American journal of respiratory and critical care medicine*. 2017;196(11):1463-72.

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3 1 20. Bonduelle O, Carrat F, Luyt CE, Leport C, Mosnier A, Benhabiles N, et al. Characterization of
4 2 pandemic influenza immune memory signature after vaccination or infection. *The Journal of clinical*
5 3 *investigation*. 2014;124(7):3129-36.
6 4 21. Bonduelle O, Yahia N, Siberil S, Benhabiles N, Carrat F, Krivine A, et al. Longitudinal and
7 5 integrative biomodeling of effector and memory immune compartments after inactivated influenza
8 6 vaccination. *Journal of immunology*. 2013;191(2):623-31.
9 7 22. Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T
10 8 lymphocytes: a correlate of protection and a basis for vaccine development. *Curr Opin Biotechnol*.
11 9 2007;18(6):529-36.
12 10 23. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *The New*
13 11 *England journal of medicine*. 1983;309(1):13-7.
14 12 24. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, et al. Preexisting influenza-
15 13 specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nature*
16 14 *medicine*. 2012;18(2):274-80.
17 15 25. Huang KY, Li CK, Clutterbuck E, Chui C, Wilkinson T, Gilbert A, et al. Virus-specific antibody
18 16 secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model.
19 17 *The Journal of infectious diseases*. 2014;209(9):1354-61.
20 18 26. Pleguezuelos O, Robinson S, Fernandez A, Stoloff GA, Mann A, Gilbert A, et al. A Synthetic
21 19 Influenza Virus Vaccine Induces a Cellular Immune Response That Correlates with Reduction in
22 20 Symptomatology and Virus Shedding in a Randomized Phase Ib Live-Virus Challenge in Humans. *Clin*
23 21 *Vaccine Immunol*. 2015;22(7):828-35.
24 22 27. Kendal AP, Cate TR. Increased sensitivity and reduced specificity of hemagglutination
25 23 inhibition tests with ether-treated influenza B/Singapore/222/79. *J Clin Microbiol*. 1983;18(4):930-4.
26 24 28. Monto AS, Malosh RE, Petrie JG, Martin ET. The Doctrine of Original Antigenic Sin: Separating
27 25 Good From Evil. *The Journal of infectious diseases*. 2017;215(12):1782-8.
28 26 29. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly:
29 27 a quantitative review. *Vaccine*. 2006;24(8):1159-69.
30 28 30. Mercado N, Ito K, Barnes PJ. Accelerated ageing of the lung in COPD: new concepts. *Thorax*.
31 29 2015;70(5):482-9.
32 30 31. DiazGranados CA, Dunning AJ, Kimmel M, Kirby D, Treanor J, Collins A, et al. Efficacy of high-
33 31 dose versus standard-dose influenza vaccine in older adults. *The New England journal of medicine*.
34 32 2014;371(7):635-45.
35 33 32. Izurieta HS, Thadani N, Shay DK, Lu Y, Maurer A, Foppa IM, et al. Comparative effectiveness of
36 34 high-dose versus standard-dose influenza vaccines in US residents aged 65 years and older from 2012
37 35 to 2013 using Medicare data: a retrospective cohort analysis. *Lancet Infect Dis*. 2015;15(3):293-300.
38 36 33. Cruijff M, Thijs C, Govaert T, Aretz K, Dinant GJ, Knottnerus A. The effect of smoking on
39 37 influenza, influenza vaccination efficacy and on the antibody response to influenza vaccination.
40 38 *Vaccine*. 1999;17(5):426-32.
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Figure legends

Figure 1: CONSORT flow diagram detailing subject enrolment and subgroupings in the study. Where samples are missing this is due to a visit being missed and a sample not collected.

Figure 2: Cohort HAI antibody responses to TIV. HAI antibody titres of trivalent influenza vaccine constituents (**A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket) in serum from the whole cohort of volunteers at each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, y-axes are presented as log2 scale and median values are indicated by line whilst + indicates mean. Dotted red line indicates seroprotection (titre of ≥ 40). Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3: HAI antibody responses of the cohort subgroups to different vaccine components. HAI antibody titre to **A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD patients, age-matched healthy controls and young controls at each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4: HAI antibody responses of the cohort subgroups to the different vaccine components 28 days post vaccination. HAI antibody titre to **A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination). Fold change in HAI antibody titre to **D**) A/H1N1 **E**) A/H3N2 and **F**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD

volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination) compared to the pre vaccination visit. For all graphs, median values are shown. Dotted red line indicates (**A-C**) seroprotection (titre of ≥ 40) or (**D-F**) seroconversion (4-fold increase in titre). Statistical significance was determined by Kruskal-Wallis ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$

Figure 5: T cell responses to influenza virus pre and post vaccination. H3N2 and H1N1 inactivated virus-specific CD4 and CD8 T cells were measured by presence of intracellular cytokines (boolean gates for IFN γ , IL-2, IL-17 and/or TNF α) or GzmB in each subject. (**A-C**) Proportions of T cells responding to whole H1N1 and H3N2 viral stimulation from the whole cohort of volunteers after each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. (**D-F**) Proportion of T cells responding to whole virus from COPD volunteers ($n=23$; filled circles), age-matched healthy controls ($n=13$; filled squares) and young healthy controls ($n=10$; filled triangles) at V2 (Day 28 post vaccination). For all graphs, median values are shown. Statistical significance was determined by Kruskal-Wallis ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, ** $P < 0.01$.

Figure 6: B cell responses to H1N1 HA pre and post-vaccination. **(A)** Number of H1N1-specific B cells releasing IgG per million PBMC from the whole cohort of volunteers after each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). Median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. **(B)** Number of B cells releasing IgG from COPD volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination). Median values are shown and statistical significance was determined by Kruskal-Wallis

1 ANOVA with a Dunn's post-hoc test.

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3 **Figure 7: Radar plot of responses in each volunteer group pre and post-vaccination.**

4 Radar charts comparing flu virus-specific immune compartments of COPD (black), Healthy
5 control (blue) and Young Healthy control (red) groups **A)** prevaccination, and fold change (Visit
6 / prevaccination) of influenza specific-immune response at V1 (**B**), V2 (**C**), and V3 (**D**). Radar
7 charts show A/H3N2, A/H1N1 and B/Phuket strain HAI antibody titres, A/H1N1 HA-specific
8 IgG and IgA secreting B cells, virus-specific CD4 and CD8 T cells, GzmB+ CD8 T cells and
9 CD49a+CD49d+ homing markers evaluated on IFN γ + CD4 and CD8 T cells. The values on
10 the axis represent the mean of each parameter derived from the upper and lower 95%
11 confidence intervals of the mean of each assay for all tested subjects. Statistical significance
12 was determined by Kruskal-Wallis ANOVA with a Bonferroni correction ** P<0.01.

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14 **Figure 8: Correlations of viral titres and pre-existing CD4+ T cells with age.** Associations
15 between (**A-C**) viral titres at V2 (28d post-vaccine) to individual vaccine components or (**D**)
16 pre-vaccine virus-specific CD4+ T cells and age were analysed by using the Spearman rank
17 correlation test.

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Table 1

	COPD n=23	Healthy Control n=13	P value	Young Control n=11
Age *	69.0 (55-83)	65.1 (56-72)	0.112	33.2 (22-40)
Gender, Male n, %	13 (56.5)	5 (38.5)	0.489	5 (45.5)
Current smoker n, %	8 (34.8)	1 (7.7)	0.001	1 (9.1)
Pack years	38 (22)	4 (20)	<0.001	0 (2)
FEV1 (L)	1.46 (1.0)	2.95 (1.3)	<0.001	3.53 (0.85)
FEV1%	55 (25)	109 (18)	<0.001	98 (20)

Table 1. Demographics and clinical characteristics. Data represented as median (IQR) or frequency (%), * represented as mean (range). Statistical significance was determined using a Mann Whitney test

Table 2

	COPD	Healthy Control	Young Control
H1N1	26.1%	30.8%	9.1%
H3N2	17.4%	23.1%	36.4%
FluB	47.8%	53.8%	36.4%

Table 2: Proportions of volunteers who did not seroconvert (<4-fold increase in HAI titre) by V2 and were not seroprotected (HAI titre <1:40) at baseline to each of the vaccine components.

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Supplemental Figure Legends

Supplemental Figure 1: Representative gating strategy for identification of IFN- γ , IL-2, IL-17, TNF- α and Granzyme B CD4 and CD8 T cells. Gate represents percentage of mother gate.

Supplemental Figure 2: No difference of polyfunctional influenza-specific T-cell responses between COPD, Healthy controls and Younger controls at V2 (28d post-vaccine). Detail analyses of quadruple (blue), triple (red), double (green) and single (yellow) virus-specific CD4 T-cell response (A) and CD8 T-cell response (B) are shown on the x-axis for COPD (left graph), Healthy controls (middle graph) and Younger controls (right graph).

Acquired immune responses to the seasonal trivalent influenza vaccination in COPD

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Keywords: COPD, influenza, vaccination

Abbreviations used: ASC: Antibody secreting cells; ATS: American Thoracic Society; COPD: Chronic obstructive pulmonary disease; CPMP: Committee for Proprietary Medicinal Products; FEV1: Forced expiratory volume in 1 sec; FVC: Forced vital capacity; GzmB:

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- 1 Granzyme B; HAI: Haemagglutinin inhibition; IAV: Influenza A virus; PBMC: Peripheral blood
- 2 mononuclear cells; TIV: trivalent influenza vaccine

For Peer Review

Summary

Epidemiological data suggests that influenza vaccination protects against all-cause mortality in COPD patients. However recent work has suggested there is a defect in the ability of some COPD patients to mount an adequate humoral response to influenza vaccination. The aim of our study was to investigate humoral and cell mediated vaccine responses to the seasonal trivalent influenza vaccination (TIV), in COPD subjects and healthy controls. 47 subjects were enrolled into the study; 23 COPD patients, 13 age-matched healthy control (HC - ≥ 50) and 11 young healthy control subjects (YC - ≤ 40). Serum and PBMC were isolated pre-TIV vaccination and at days 7, 28 and 6 months post vaccine for Haemagglutinin Inhibition (HAI) titre, antigen-specific T cell and antibody-secreting cell analysis. The kinetics of the vaccine response were similar between YC, HC and COPD patients and there was no significant difference in antibody titres between these groups 28d post-vaccine. As we observed no disease-dependent differences in either humoral or cellular responses, we investigated if there was any association of these measures with age. H1N1 ($r=-0.4253$, $p=0.0036$) and Influenza B ($r=-0.344$, $p=0.0192$) antibody titre at 28d negatively correlated with age as did H1N1-specific CD4⁺ T helper cells ($r=-0.4276$, $p=0.0034$). These results suggest that age is the primary determinant of response to trivalent vaccine and that COPD is not a driver of deficient responses per se. These data support the continued use of the yearly trivalent vaccine as an adjunct to COPD disease management.

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by airway inflammation, resulting from the inhalation of tobacco smoke or other irritants (1). The course of the disease is one of progressive, irreversible airflow limitation, functional impairment and periods of acute symptom deterioration called exacerbations (2). Acute exacerbations are important events in the natural history of COPD associated with impaired quality of life and accelerated decline in lung function, placing a considerable burden on health-care resources (3-5). Respiratory infection is a key determinant with viral infections implicated in between 40% and 60% of COPD exacerbations (6, 7). Consequently, preventative strategies aimed at reducing the impact of respiratory viral infection, are key interventions in reducing the morbidity and health-care burden associated with COPD exacerbations.

Influenza A and B are single stranded RNA viruses of the *Orthomyxoviridae* family that circulate in the human population causing acute infection of both the upper and lower respiratory tract. In patients with COPD, influenza is associated with seasonal exacerbations, with the virus detected in up to 28% of exacerbating COPD patients (8, 9). The seasonal influenza vaccination is currently the best method for reducing the incidence of influenza infection, and the associated complications such as pneumonia, and is recommended for the elderly and in those with chronic conditions such as COPD(10). Therapies to treat influenza infection, once present in COPD, are currently very limited, both in terms of the window of opportunity for treatment and therapeutic efficacy. Thus, vaccination is a critical component in exacerbation prevention. In the UK, the most commonly used formulation is the trivalent influenza vaccine (TIV) which currently contains inactive viral haemagglutinin (HA) from H1N1, H3N2 and influenza B strains. A recognised correlate of vaccine protection is the production of sufficient concentrations of antibodies to HA, as assessed by haemagglutinin inhibition (HAI) assays (11). The response to TIV can be demonstrated by either seroprotection (HAI antibody titre $\geq 1:40$) or seroconversion (minimum 4-fold rise in HAI antibody titre) (12). Whilst the use and efficacy of TIV in COPD is supported by epidemiological studies (13, 14), recent

laboratory-based work has begun to question the effectiveness of these vaccines in disease (15-17).

Whilst trials and epidemiological data point to a benefit of TIV in COPD patients (18), a small study from Australia, demonstrated that COPD patients had lower titres of H1N1-specific antibodies 28 days post-vaccination and lower serum IL-21 levels, which is thought to play a role in B-cell antibody synthesis (16). In addition, the same group reported a reduced proportion of COPD patients who seroconverted 28 days after vaccination, compared to healthy controls (15). More recently, Parpaleix et al demonstrated decreased geometric mean HAI antibody titres in COPD patients at 30 days post-vaccine compared to healthy controls, but no significant difference in the proportion of volunteers who experienced seroconversion between health and disease (17). Furthermore, this decrease in geometric mean HAI antibody titres was associated with a decrease in vaccine-specific CD4+ T cell responses prior to vaccination.

The aim of our study was to conduct a detailed assessment of the HAI antibody, T cell and B cell responses to TIV of COPD patients, compared to age-matched healthy and young, healthy controls enabling the identification of any differences in vaccine responses between the groups. .

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Methods

Volunteer Recruitment

Twenty-three COPD patients, 13 age-matched (≥ 50 years old) healthy control subjects and 11 younger (< 40 years old) healthy control subjects were studied (Fig 1). Power calculations were based on the proportion of responders achieving at least a 4-fold increase in the H1N1 HAI antibody titre in each group. A minimum sample size of 22 subjects in the COPD group and 11 subjects in the control groups was determined as sufficient for a two-sided, type-1 error rate of 0.05 and a power of 80%. A diagnosis of COPD was confirmed by post-bronchodilator spirometry, with a forced expiratory volume in 1 second (FEV1)/forced vital capacity (FVC) ratio of < 0.7 required for enrolment. Spirometry was conducted in accordance with ATS standards (19). COPD subjects had a smoking history of at least 10 pack years. All subjects had received the TIV in the year prior to the study (2014). The vaccine strains for the 2014/2015 Northern hemisphere vaccine were A/California/7/2009 (H1N1) pdm09-like virus, A/Texas/50/2012 (H3N2)-like virus and B/Massachusetts/2/2012-like virus. Exclusion criteria included a history of other pulmonary disease, long-term use of immunosuppressant medications (including oral corticosteroids) and an exacerbation within the month prior to recruitment. All subjects gave written informed consent and the study was approved by the National Research Ethics Service (NRES) South Central – Oxford C Committee (15/SC/0528).

Study Procedures

As this was a purely observational study, the intramuscular influenza vaccine was administered by the volunteer's usual health care provider as part of usual care between October 2015 and December 2015. All of the young healthy group were healthcare professionals. The vaccine strains for the 2015/2016 Northern hemisphere were A/California/7/2009 (H1N1) pdm09-like virus, A/Switzerland/9715293/2013 (H3N2)-like virus and B/Phuket/3073/2013-like virus. Phlebotomy was performed at a pre-vaccine visit and then at 7-10 days (Visit 1), 28 ± 3 days (Visit 2) and 180 ± 14 days (Visit 3) post-vaccination.

Serum HAI Antibody Titres

The HAI assays were performed by the Public Health England (PHE) laboratories, Porton Down, Salisbury, UK. Briefly, serial 2-fold dilutions for each set of sera was incubated with standardised concentrations (4 HA units) of influenza virus representing either the H1N1, H3N2 and influenza B 2015/16 viral strains. Chicken red blood cells were then added and allowed to settle. After 30 min, the strain-specific HAI antibody titres at each time point for each individual were calculated as the highest dilution of sera that inhibited hemagglutination. Seroconversion rates (i.e., numbers of individuals with HAI antibody titres ≥ 40) and seroprotection rates (i.e., numbers of individuals with HAI antibody titres ≥ 10 at D0 and HAI antibody titres ≥ 40 after vaccination or with HAI antibody titres ≥ 10 at D0 and ≥ 4 -fold increase in HAI antibody titres after vaccination) are also indicated.

PBMC isolation & storage

PBMCs from volunteers were isolated from heparinized blood by means of centrifugation on Ficoll-Paque (GE Healthcare). Purified PBMC were frozen in Heat-inactivated Foetal Bovine Serum containing 10% (v/v) DMSO (Sigma) and stored in liquid nitrogen until analysis.

Flow cytometry Analysis

Flow cytometry was performed as previously described (20). To analyse influenza-specific T cells, PBMCs were stimulated overnight with inactivated A/H3N2 Wisconsin/67/2005 and A/H1N1/California/04/2009 in the presence of monensin/brefeldin A mix (Sigma). Cells were first stained for viability and surface markers: Live dead (Molecular Probes), CD4 (OKT4 clone), CD3 (SK7 clone), CD8 (RPA-T8 clone), CD49d (9F10 clone) and CD49a (SR84 clone; BD Biosciences). Cells were then resuspended in Cytofix/Cytoperm (BD Biosciences) before staining for intracellular markers: IL-2 (N7.48 A clone; Miltenyi), IFN γ (B27 clone), IL-17a (N49-653 clone), Granzyme B (GB11 clone), TNF α (MAb11 clone; BD Biosciences). Flow

cytometric analysis was performed on a FACSFortessa using FACSDiva software v5.0.3 (BD Biosciences). At least 1×10^6 live events, according to forward- and side-scatter parameters, were accumulated and analysed for boolean combination gating with FlowJo software (Tree Star Inc.). The percentage of influenza A virus-specific CD4⁺ or CD8⁺ T cells expresses the sum of the 15 different cytokine boolean combinations (IL-2, IFN γ , IL-17a and/or TNF α). Background cytokine responses detected in negative controls were subtracted from those detected in stimulated samples for every specific combination.

Antibody-Secreting Cell (ASC) detection.

A/California/07/2009 (H1N1) influenza HA-specific IgG-secreting B cells were performed as previously described (21). Briefly, PBMC were stimulated with a mixture of Pokeweed mitogen (Sigma), *Staphylococcus aureus* Protein A (Sigma) and CpG ODNs (Invivogen) for 6 days before ELISpot assay. H1N1 HA protein (Protein Sciences) was used to coat the plate (Millipore), and IgG- or IgA-paired antibodies (Mabtech) were used to reveal. ELISpot readouts were expressed as the number of HA-specific IgG or IgA ASC/ 10^6 PBMC.

Statistics and Analysis

Statistical analyses were performed using either a Wilcoxon's matched-pairs signed-rank test, Mann-Whitney U test, Kruskal-Wallis or Friedman test with Dunn's multiple comparison testing as appropriate (GraphPad Prism v7.0, GraphPad Software Inc., San Diego, USA). For the paired analysis, if data was missing for a given visit, all data from that volunteer was excluded and there was no data imputation. Data are expressed as medians. Results were considered significant if $P < 0.05$. The radar charts were designed with R, a free software environment for statistical computing and graphics (<http://www.r-project.org/>).

Results

Subject Demographics

47 subjects were successfully enrolled into the study, 23 COPD patients, 13 age-matched healthy controls and 11 young control subjects (Fig 1). The COPD and healthy control subjects were well matched for age and gender. As expected there were significant differences in the proportion of current smokers and FEV1 measures. To ensure that recent vaccination history was known, our inclusion criteria required subjects to have received the prior year's (2014/15) influenza vaccination. The descriptive characteristics are shown in Table 1.

Humoral responses to TIV

To ascertain the effectiveness of the vaccine to induce a humoral response, we first analysed the antibody titres to all three vaccine components via individual haemagglutinin inhibition (HAI) assays in the whole cohort (Fig 2A-C). The median (IQR) level of pre-vaccine HAI titres were 40 (10-40) for A/H1N1, 20 (10-40) for A/H3N2 and 17.5 (5-40) for B/Phuket strains. These data suggest that half the cohort were already seroprotected against A/H1N1 virus but not A/H3N2 or B/Phuket strain before vaccination. There was a significant increase in antibody titres 7 days (V1) and 28 days (V2) following vaccination for all vaccine components, although this was not sustained out to 6 months (V3) for B/Phuket strain. In addition, the HAI antibody increases in FluB were not as strong and remained consistently lower than the titres induced by the FluA components.

In line with other studies (17), we next compared the HAI titres to all three vaccine components between groups at 28d post-vaccine, when all groups demonstrated a significant increase in titres against the FluA components (Fig 3). Only the COPD group demonstrated a significant increase in HAI antibody titres to B/Phuket at 28d post-vaccination ($p=0.0127$, Fig 3C). However there was no significant difference in the HAI antibody titres between the groups for any of the vaccine components at day 28 (Fig 4A-C). Neither was there a significant difference in the fold change in HAI antibody titres between groups at V2 compared to the pre-vaccination visit, except for B/Phuket between COPD patients and Young controls (Fig 4D-F).

1 Whilst there appeared to be differences in the proportion of volunteers seroprotected (titre
2 ≥ 40) between groups for each vaccine component, the proportion was again only significantly
3 different for B/Phuket where 80% of the young controls were seroprotected compared to the
4 62.5% of Healthy controls and 53% of COPD patients ($p=0.0469$, Chi-squared).

5 Since it is also recommended that seroconversion be taken into account as well as
6 seroprotection (12) we analysed the number of patients who did not seroconvert at visit 2
7 (Figure 4D-F). The only significant difference in seroconversion we observed was a decrease
8 in the response of young controls to B/Phuket compared to COPD patients (Figure 4F). In
9 order to assess any differences in the response to vaccine using either definition, we also
10 assessed the proportions of volunteers who were not seroprotected at baseline and also did
11 not seroconvert by day 28 (Table 2). Whilst there was evidence of differential responses to
12 different vaccine components, there were no significant differences in the proportions of the
13 groups that were not protected against the individual vaccine components.

15 *Cellular responses to TIV*

16 In addition to antibodies, T cell responses are also key parameters of influenza protection (20,
17 22-26). We therefore analysed the polyfunctional CD4⁺ and CD8⁺ T cell responses to
18 inactivated whole virus (A/H1N1 and A/H3N2) in PBMC from our volunteer cohort at each time
19 point (Figure S1). We did not observe any significant difference in the quality of polyfunctional
20 CD4⁺ and CD8⁺ T cell responses between groups (Figure S2). We did observe a significant
21 increase ($p=0.0035$) in the proportion of virus-specific CD4⁺ T cells 28 days post vaccination
22 (Fig 5A) compared to prevaccination in the whole cohort. In contrast, there was no increase
23 in the proportion of virus-specific CD8⁺ T cells (Fig 5B). However, there was a significant
24 decrease in the proportion of virus-specific GzmB⁺ CD8⁺ T cells at all time-points following
25 vaccination (Fig 5C). We also compared the proportions of virus-specific T cells between
26 groups at 28d post-vaccine (Fig 5D-F). Similarly to the HAI data, we again observed no
27 significant differences in CD4⁺ or CD8⁺ T cell proportions between the groups.

At the same time we also analysed the proportions of both A/H1N1 HA-specific IgG-secreting B cells in these samples using ELISpot (Fig 6A-D). However, we observed no significant change in the proportion of A/H1N1 HA-specific IgG-secreting B cells either in response to vaccination or between the groups at 28d post-vaccine. A/H1N1 HA-specific IgA-secreting B cells are very low (data not shown).

In order to present a complete picture of our work, we have also analysed all of the variables reported above at 7 days and 6 months post vaccination and present these data as radar plots (Fig 7B&D). Furthermore we evaluated the capacity of CD4 and CD8 T cells to acquire the cell migration capacity to mucosal with the expression of integrin CD49a and CD49d. There were no statistically significant differences in any of the measurements between groups at any time-point post-vaccination. Thus, overall there appear to be no differences in vaccine responses across the groups. This is despite a statistically significant difference in B/Phuket HAI antibody titres between groups at baseline, which appears more due to age, rather than COPD (Fig 7A). Furthermore, there are also differences in the proportions of influenza-specific cytokine-secreting CD4⁺ T cells at baseline which, surprisingly are significantly lower in healthy age-matched controls compared to both young controls.

Age correlates with antibody titres

To further investigate the effects of age on responses to vaccination, we correlated the age of the volunteers with the antibody titres of the separate vaccine components at 28d (Figure 8A-C). There were weak, but significant negative correlations between age and A/H1N1 ($r = -0.4253$, $p = 0.0036$) and B/Phuket ($r = -0.344$, $p = 0.0192$) but not A/H3N2 titres.

As we have previously shown that pre-existing CD4⁺ T helper cells are essential for adequate responses to influenza (24), we next analysed the correlation between age and pre-existing virus-specific CD4⁺ T cells (Figure 8D). Similarly to the HAI data, we observed a weak, but significant negative correlation between age and the proportion of virus-specific CD4⁺ cells at the pre-vaccination visit ($r = -0.4276$, $p = 0.0034$).

Discussion

In this study we have shown that in the majority of volunteers across disease and age groups, who were vaccinated with TIV, there was adequate seroprotection (titre ≥ 40) to at least one component of the vaccine at 28d post-vaccination. Importantly, there were minimal differences in the vaccine responses of COPD patients, compared to age-matched healthy controls and young controls at any time-point. Although there were no significant age-related differences between groups in many of the immune measures analysed prior to vaccine administration there was evidence of a relationship between age and vaccine responses. Further analysis demonstrated a negative correlation between age and HAI antibody titres for two out of three vaccine components. Taken together these data suggest that age-related immune senescence may have a stronger dampening effect on vaccine responses than COPD per se.

In the Western world, COPD is a disease associated with life-long smoking and due to the pernicious nature of symptom onset, is not usually diagnosed until the late forties/early fifties. Thus, COPD itself is a disease already associated with ageing and therefore it is essential that the age of any control group is well matched with the COPD group. In the previous studies from the Australian group demonstrating a disease effect on vaccine responses, there were significant differences between the ages of the control group and the COPD group (15, 16). Indeed, Burel et al demonstrated that age was associated with post-vaccination A/H1N1 antibody titres by univariate, although not multivariate analysis (16).

The use of HAI antibody titres as a correlate of protection is further complicated by the use of different methods to assess vaccine responsiveness. The Committee for Proprietary Medicinal Product (CPMP) criteria state that post-vaccination serum is considered seroprotected if the HAI antibody titre is ≥ 40 (12). However, the CPMP have two definitions of seroconversion, with the seroprotected level of ≥ 40 only being considered a seroconversion if the pre-vaccine levels were negative. If the serum sample already has a positive HAI titre, then a minimum four-fold increase in HAI antibody titre is required to be considered a seroconversion (12). However, it is unclear what the relevance of the definition of

seroconversion is if the baseline titre is already 40 or greater and the volunteer is thus already considered to be seroprotected. For example, Nath et al demonstrate an average 120-fold increase in the HAI antibody titre in controls and only an average 2-fold increase in COPD patients (15). Nevertheless the majority of COPD patients were already seroprotected with a median pre-vaccine titre of 320 which was significantly higher than the pre-vaccine titre of controls (median titre of 60)(15). Parpaleix et al present geometric mean titres, but when we analyse the geometric means of our own HAI data (not shown), there are no impacts on the conclusions we reached using the mean titres. But, whilst the titres are reported as being lower in COPD compared to controls, there were no significant differences in the proportions of these groups that underwent seroconversion to any of the vaccine components (17).

The data presented here and by Parpaleix et al highlight that there are differential responses to the different components of the TIV (17). We demonstrate that the magnitude of the response to B/Phuket in the whole cohort is approximately 2-fold lower than the two FluA components and is reflected in both the seroprotection and seroconversion rates. This observation may be explained by the decreased sensitivity of the HAI assay for influenza B whole viral antigens (27). Whilst the high-level of pre-existing seroprotection to A/H1N1 in all groups is likely a result of the inclusion of the same components in both the 2014 and 2015 TIV, the other differences between the groups are harder to reconcile. For example, the young controls appeared to respond less well to the A/H3N2 components than either the COPD or age-matched controls. The reason for this is unclear, but may result from the original antigenic sin hypothesis where exposure to different circulating viral strains that prime the immune system during childhood which may then go on to determine which response is able to be boosted by the vaccine (28). Thus, the A/H3N2 may have been more dominant during the maturation of the immune response in the older volunteers, whereas A/H1N1 may have been more dominant at the time of immune maturation in the young volunteers. This is supposition and further work will be required to confirm or refute this notion. This difference in response to A/H3N2 strain certainly impacts on the correlation with age, reversing the direction of the

1 association compared to the other components even though the association is not statistically
2 significant.

3 The impact of ageing on the antibody responses to the influenza vaccination has long
4 been recognised, with the clinical vaccine efficacy being reduced from 70-90% in the young
5 to 17-53% in those subjects who were ≥ 65 (29). When this older age group were further
6 categorised into those above or below 75 it was the over 75s who had significantly lower
7 vaccine responses suggesting that it is only those over 75 that have a diminished HAI antibody
8 response (29). The mean age of our COPD and age-matched healthy volunteers was 69 and
9 65 respectively and only 5 COPD patients were over 75 at the time of sampling, which may
10 explain the weak negative correlation with age in our study. It has been proposed that the
11 manifestation of COPD is a result of accelerated ageing, but current evidence suggests that
12 this phenomenon is confined to the lung rather than a manifestation of systemic disease as
13 would be required for an impact on vaccine response (30). In order to overcome the effects of
14 ageing on the vaccine response, recent studies have demonstrated that increasing the dose
15 of the vaccine components leads to better vaccine responses in the elderly (31, 32). If the
16 administration of high-dose vaccine is taken into practice, our data would suggest that COPD
17 patients may be just as likely to respond as other elderly patients. However, clinical trials of
18 the high dose vaccine in COPD would be need to confirm this.

19 A limitation of our study, that we share with others, is the differences in the number of
20 current and ex-smokers between the COPD groups and the controls (15-17). This is an
21 important issue as smoking is associated with increased susceptibility to influenza infection
22 (33). However, given the toxic effects of smoking there is some debate about whether there is
23 such a thing as healthy smoker. Despite this, Burel et al demonstrate that smoking was not
24 statistically associated with vaccine responsiveness in a univariate analysis (16). A further
25 limitation of our study was that there was no age continuum in either our healthy or COPD
26 cohort, but rather we recruited to discrete age groups. This was deliberate on our part to try
27 to ensure that if there was an effect of age it would be clearly visible. Furthermore, our ability

1 to only demonstrate associations with age may be a function of the study being powered to
2 detect differences due to disease rather than age differences. The biggest limitation to this
3 and all other studies using HAI antibody titre as an outcome is that this is not a functional
4 measure of protection from influenza vaccination but is only a surrogate marker of protection.
5 Thus, larger scale randomised control trials with clinical endpoints and confirmed influenza
6 diagnosis are required to fully investigate the impact of both age and COPD on vaccine
7 responses.

8 In summary, our data suggest that there is no primary defect in the responsiveness of
9 COPD patients to the TIV. However, there was substantial heterogeneity in the responses to
10 the three vaccine components amongst the different age groups, suggesting that age is the
11 primary driver to reduced vaccine responsiveness for at least two of the vaccine components.
12 These data would support continuing the yearly influenza vaccine schedule as an adjunct to
13 COPD disease management in an effort to reduce the burden of influenza in this patient group.

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Author contributions: Conception & design – KJS, NPW, TMAW Data acquisition, analysis and interpretation – KJS, NPW, OB, AJH, DC, ACM, BC; Drafting of manuscript for important intellectual content – KJS, NPW, OB, BC, TMAW

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Conflict of Interest

KJS and TMAW have received grants from GlaxoSmithKline and AstraZeneca outside of the current work. The rest of the authors have no relevant conflicts of interest.

For Peer Review

References

1. Decramer M, Janssens W, Miravittles M. Chronic obstructive pulmonary disease. *Lancet*. 2012;379(9823):1341-51.

2. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *American journal of respiratory and critical care medicine*. 2007;176(6):532-55.

3. Miravittles M, Ferrer M, Pont A, Zalacain R, Alvarez-Sala JL, Masa F, et al. Effect of exacerbations on quality of life in patients with chronic obstructive pulmonary disease: a 2 year follow up study. *Thorax*. 2004;59(5):387-95.

4. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax*. 2002;57(10):847-52.

5. Anzueto A. Impact of exacerbations on COPD. *Eur Respir Rev*. 2010;19(116):113-8.

6. Wilkinson TMA, Aris E, Bourne S, Clarke SC, Peeters M, Pascal TG, et al. A prospective, observational cohort study of the seasonal dynamics of airway pathogens in the aetiology of exacerbations in COPD. *Thorax*. 2017.

7. Rohde G, Wiethage A, Borg I, Kauth M, Bauer TT, Gillissen A, et al. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax*. 2003;58(1):37-42.

8. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *American journal of respiratory and critical care medicine*. 2006;173(10):1114-21.

9. Sapey E, Stockley RA. COPD exacerbations . 2: aetiology. *Thorax*. 2006;61(3):250-8.

10. Excellence NifHaC. Immunizations - seasonal influenza Clinical Knowledge Summary <https://cks.nice.org.uk/immunizations-seasonal-influenza#!scenario>: National Insititutes for Health and Care Excellence; 2018 [updated May 2018].

11. Sanei F, Wilkinson T. Influenza vaccination for patients with chronic obstructive pulmonary disease: understanding immunogenicity, efficacy and effectiveness. *Ther Adv Respir Dis*. 2016;10(4):349-67.

12. Trombetta CM, Perini D, Mather S, Temperton N, Montomoli E. Overview of Serological Techniques for Influenza Vaccine Evaluation: Past, Present and Future. *Vaccines (Basel)*. 2014;2(4):707-34.

13. Poole PJ, Chacko E, Wood-Baker RW, Cates CJ. Influenza vaccine for patients with chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2006(1):CD002733.

14. Schembri S, Morant S, Winter JH, MacDonald TM. Influenza but not pneumococcal vaccination protects against all-cause mortality in patients with COPD. *Thorax*. 2009;64(7):567-72.

15. Nath KD, Burel JG, Shankar V, Pritchard AL, Towers M, Looke D, et al. Clinical factors associated with the humoral immune response to influenza vaccination in chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease*. 2014;9:51-6.

16. Burel JGN, K.; Pritchard, A.L.; White, O.J.; Davies, J.M.; Towers, M.;Looke, D.;Upham, J.W.;. Evaluation of Immune Responses to Influenza Vaccination in Chronic Obstructive Pulmonary Disease. *J Vaccines Vaccin*. 2012(S4):001.

17. Parpaleix A, Boyer L, Wiedemann A, Lacabaratz C, Margarit L, Enouf V, et al. Impaired humoral and cellular immune responses to influenza vaccination in chronic obstructive pulmonary disease patients. *The Journal of allergy and clinical immunology*. 2017;140(6):1754-7 e6.

18. Bekkat-Berkani R, Wilkinson T, Buchy P, Dos Santos G, Stefanidis D, Devaster JM, et al. Seasonal influenza vaccination in patients with COPD: a systematic literature review. *BMC Pulm Med*. 2017;17(1):79.

19. Culver BH, Graham BL, Coates AL, Wanger J, Berry CE, Clarke PK, et al. Recommendations for a Standardized Pulmonary Function Report. An Official American Thoracic Society Technical Statement. *American journal of respiratory and critical care medicine*. 2017;196(11):1463-72.

- 1 20. Bonduelle O, Carrat F, Luyt CE, Leport C, Mosnier A, Benhabiles N, et al. Characterization of
2 pandemic influenza immune memory signature after vaccination or infection. *The Journal of clinical*
3 *investigation*. 2014;124(7):3129-36.
- 4 21. Bonduelle O, Yahia N, Siberil S, Benhabiles N, Carrat F, Krivine A, et al. Longitudinal and
5 integrative biomodeling of effector and memory immune compartments after inactivated influenza
6 vaccination. *Journal of immunology*. 2013;191(2):623-31.
- 7 22. Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T
8 lymphocytes: a correlate of protection and a basis for vaccine development. *Curr Opin Biotechnol*.
9 2007;18(6):529-36.
- 10 23. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *The New*
11 *England journal of medicine*. 1983;309(1):13-7.
- 12 24. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, et al. Preexisting influenza-
13 specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nature*
14 *medicine*. 2012;18(2):274-80.
- 15 25. Huang KY, Li CK, Clutterbuck E, Chui C, Wilkinson T, Gilbert A, et al. Virus-specific antibody
16 secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model.
17 *The Journal of infectious diseases*. 2014;209(9):1354-61.
- 18 26. Pleguezuelos O, Robinson S, Fernandez A, Stoloff GA, Mann A, Gilbert A, et al. A Synthetic
19 Influenza Virus Vaccine Induces a Cellular Immune Response That Correlates with Reduction in
20 Symptomatology and Virus Shedding in a Randomized Phase Ib Live-Virus Challenge in Humans. *Clin*
21 *Vaccine Immunol*. 2015;22(7):828-35.
- 22 27. Kendal AP, Cate TR. Increased sensitivity and reduced specificity of hemagglutination
23 inhibition tests with ether-treated influenza B/Singapore/222/79. *J Clin Microbiol*. 1983;18(4):930-4.
- 24 28. Monto AS, Malosh RE, Petrie JG, Martin ET. The Doctrine of Original Antigenic Sin: Separating
25 Good From Evil. *The Journal of infectious diseases*. 2017;215(12):1782-8.
- 26 29. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly:
27 a quantitative review. *Vaccine*. 2006;24(8):1159-69.
- 28 30. Mercado N, Ito K, Barnes PJ. Accelerated ageing of the lung in COPD: new concepts. *Thorax*.
29 2015;70(5):482-9.
- 30 31. DiazGranados CA, Dunning AJ, Kimmel M, Kirby D, Treanor J, Collins A, et al. Efficacy of high-
31 dose versus standard-dose influenza vaccine in older adults. *The New England journal of medicine*.
32 2014;371(7):635-45.
- 33 32. Izurieta HS, Thadani N, Shay DK, Lu Y, Maurer A, Foppa IM, et al. Comparative effectiveness of
34 high-dose versus standard-dose influenza vaccines in US residents aged 65 years and older from 2012
35 to 2013 using Medicare data: a retrospective cohort analysis. *Lancet Infect Dis*. 2015;15(3):293-300.
- 36 33. Cruijff M, Thijs C, Govaert T, Aretz K, Dinant GJ, Knottnerus A. The effect of smoking on
37 influenza, influenza vaccination efficacy and on the antibody response to influenza vaccination.
38 *Vaccine*. 1999;17(5):426-32.

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Figure legends

Figure 1: CONSORT flow diagram detailing subject enrolment and subgroupings in the study. Where samples are missing this is due to a visit being missed and a sample not collected.

Figure 2: Cohort HAI antibody responses to TIV. HAI antibody titres of trivalent influenza vaccine constituents (**A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket) in serum from the whole cohort of volunteers at each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, y-axes are presented as log2 scale and median values are indicated by line whilst + indicates mean. Dotted red line indicates seroprotection (titre of ≥ 40). Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3: HAI antibody responses of the cohort subgroups to different vaccine components. HAI antibody titre to **A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD patients, age-matched healthy controls and young controls at each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4: HAI antibody responses of the cohort subgroups to the different vaccine components 28 days post vaccination. HAI antibody titre to **A**) A/H1N1 **B**) A/H3N2 and **C**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination). Fold change in HAI antibody titre to **D**) A/H1N1 **E**) A/H3N2 and **F**) B/Phuket constituents of the trivalent influenza vaccine in serum from COPD

volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination) compared to the pre vaccination visit. For all graphs, median values are shown. Dotted red line indicates (**A-C**) seroprotection (titre of ≥ 40) or (**D-F**) seroconversion (4-fold increase in titre). Statistical significance was determined by Kruskal-Wallis ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$

Figure 5: T cell responses to influenza virus pre and post vaccination. H3N2 and H1N1 inactivated virus-specific CD4 and CD8 T cells were measured by presence of intracellular cytokines (boolean gates for IFN γ , IL-2, IL-17 and/or TNF α) or GzmB in each subject. (**A-C**) Proportions of T cells responding to whole H1N1 and H3N2 viral stimulation from the whole cohort of volunteers after each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). For all graphs, median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. (**D-F**) Proportion of T cells responding to whole virus from COPD volunteers (n=23; filled circles), age-matched healthy controls (n=13; filled squares) and young healthy controls (n=10; filled triangles) at V2 (Day 28 post vaccination). For all graphs, median values are shown. Statistical significance was determined by Kruskal-Wallis ANOVA with a Dunn's post-hoc test. P values from Dunn's test are shown * $P < 0.05$, ** $P < 0.01$.

Figure 6: B cell responses to H1N1 HA pre and post-vaccination. **(A)** Number of H1N1-specific B cells releasing IgG per million PBMC from the whole cohort of volunteers after each individual visit (prevaccine, 7 days (V1), 28 days (V2) and 6 months (V3) following vaccination). Median values are indicated by line whilst + indicates mean. Statistical significance was determined by Friedman ANOVA with a Dunn's post-hoc test. **(B)** Number of B cells releasing IgG from COPD volunteers (filled circles), age-matched healthy controls (filled squares) and young healthy controls (filled triangles) at V2 (Day 28 post vaccination). Median values are shown and statistical significance was determined by Kruskal-Wallis

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ANOVA with a Dunn's post-hoc test.

Figure 7: Radar plot of responses in each volunteer group pre and post-vaccination.

Radar charts comparing flu virus-specific immune compartments of COPD (black), Healthy control (blue) and Young Healthy control (red) groups **(A)** prevaccination, and fold change (Visit / prevaccination) of influenza specific-immune response at V1 **(B)**, V2 **(C)**, and V3 **(D)**. Radar charts show A/H3N2, A/H1N1 and B/Phuket strain HAI antibody titres, A/H1N1 HA-specific IgG and IgA secreting B cells, virus-specific CD4 and CD8 T cells, GzmB+ CD8 T cells and CD49a+CD49d+ homing markers evaluated on IFN γ + CD4 and CD8 T cells. The values on the axis represent the mean of each parameter derived from the upper and lower 95% confidence intervals of the mean of each assay for all tested subjects. Statistical significance was determined by Kruskal-Wallis ANOVA with a Bonferroni correction ** P<0.01.

Figure 8: Correlations of viral titres and pre-existing CD4+ T cells with age. Associations between **(A-C)** viral titres at V2 (28d post-vaccine) to individual vaccine components or **(D)** pre-vaccine virus-specific CD4+ T cells and age were analysed by using the Spearman rank correlation test.

Table 1

	COPD	Healthy Control	P value	Young Control
	n=23	n=13		n=11
Age *	69.0 (55-83)	65.1 (56-72)	0.112	33.2 (22-40)
Gender, Male n, %	13 (56.5)	5 (38.5)	0.489	5 (45.5)
Current smoker n, %	8 (34.8)	1 (7.7)	0.001	1 (9.1)
Pack years	38 (22)	4 (20)	<0.001	0 (2)
FEV1 (L)	1.46 (1.0)	2.95 (1.3)	<0.001	3.53 (0.85)
FEV1%	55 (25)	109 (18)	<0.001	98 (20)

Table 1. Demographics and clinical characteristics. Data represented as median (IQR) or frequency (%), * represented as mean (range). Statistical significance was determined using a Mann Whitney test

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Table 2

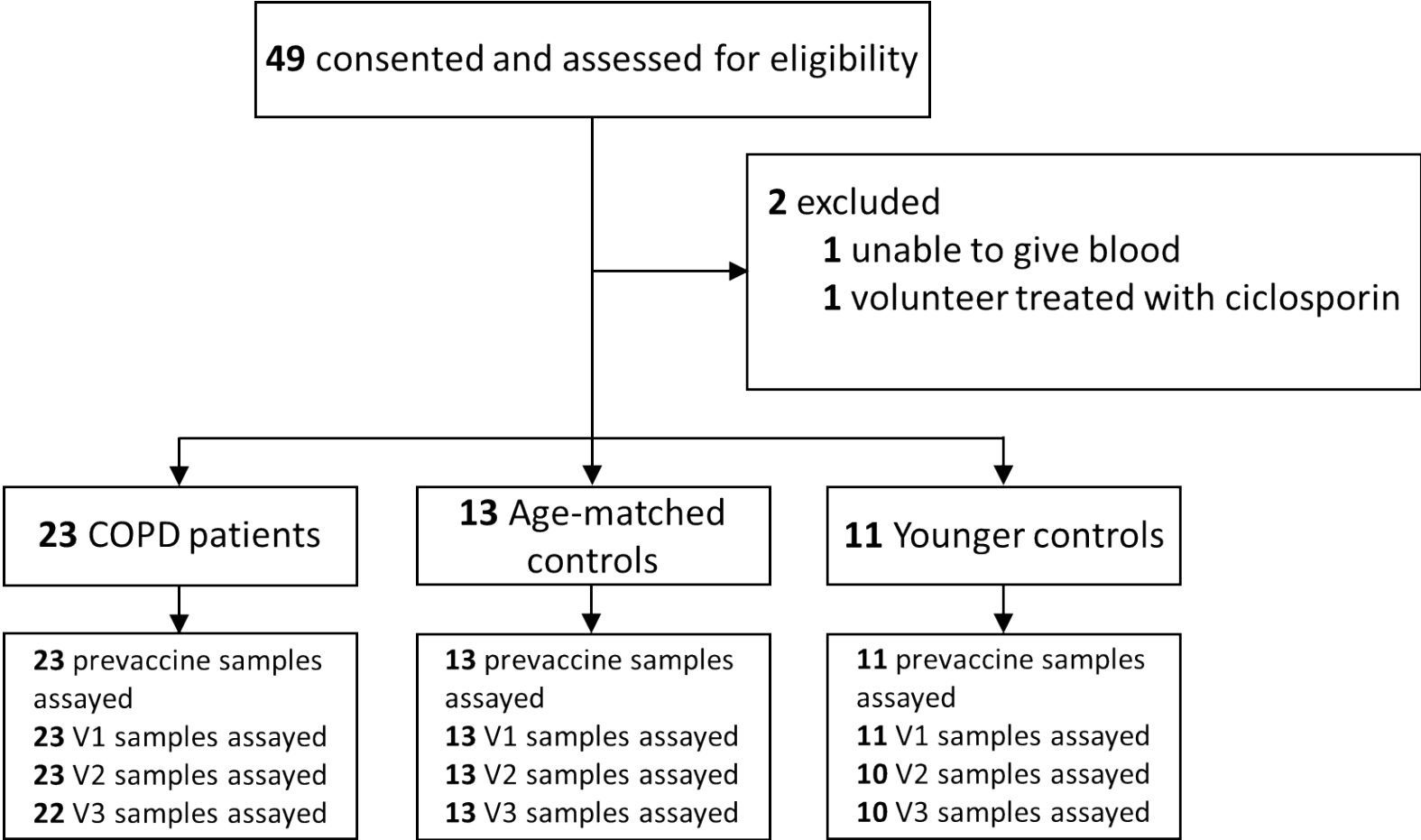
	COPD	Healthy Control	Young Control
H1N1	26.1%	30.8%	9.1%
H3N2	17.4%	23.1%	36.4%
FluB	47.8%	53.8%	36.4%

Table 2: Proportions of volunteers who did not seroconvert (<4-fold increase in HAI titre) by V2 and were not seroprotected (HAI titre <1:40) at baseline to each of the vaccine components.

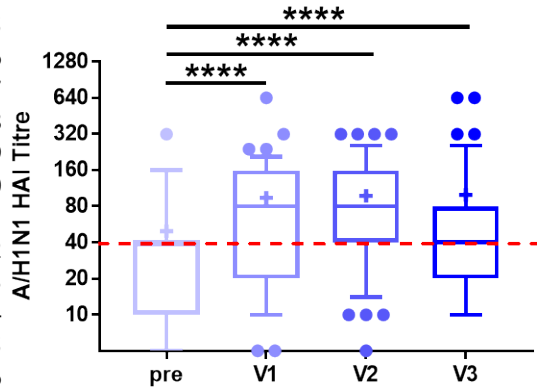
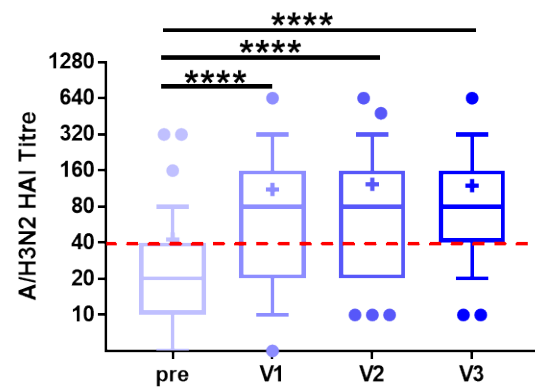
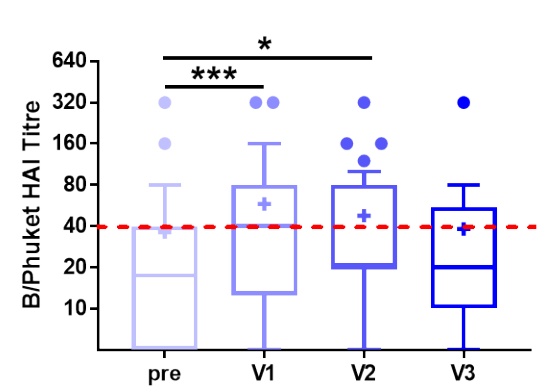
Supplemental Figure Legends

Supplemental Figure 1: Representative gating strategy for identification of IFN- γ , IL-2, IL-17, TNF- α and Granzyme B CD4 and CD8 T cells. Gate represents percentage of mother gate.

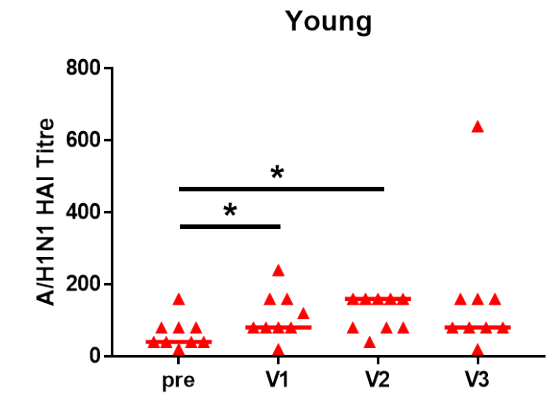
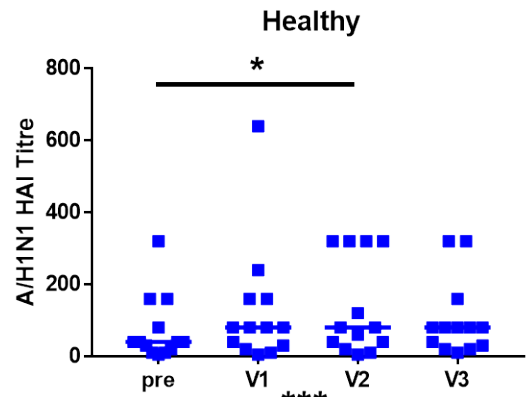
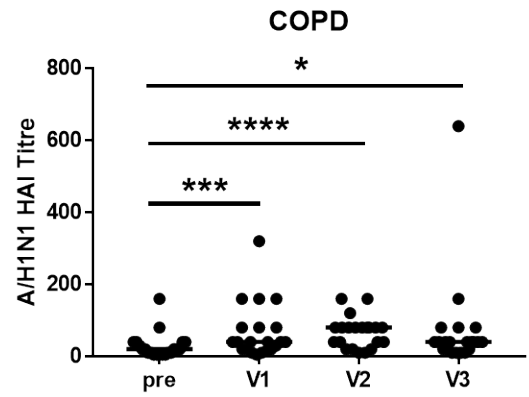
Supplemental Figure 2: No difference of polyfunctional influenza-specific T-cell responses between COPD, Healthy controls and Younger controls at V2 (28d post-vaccine). Detail analyses of quadruple (blue), triple (red), double (green) and single (yellow) virus-specific CD4 T-cell response (**A**) and CD8 T-cell response (**B**) are shown on the x-axis for COPD (left graph), Healthy controls (middle graph) and Younger controls (right graph).



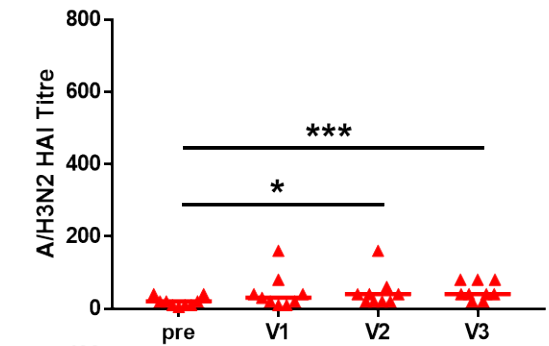
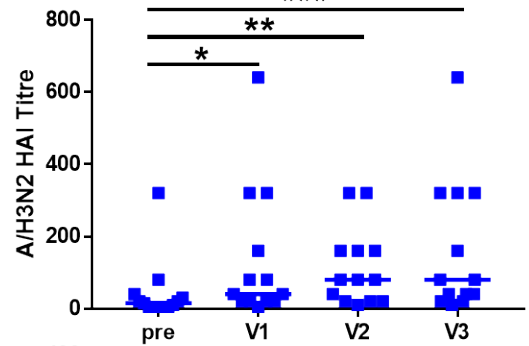
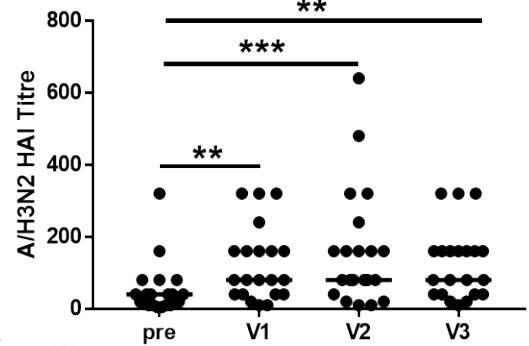
Staples et al
Figure 1

A**B****C**

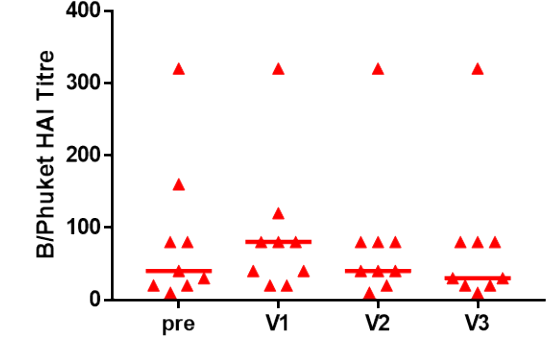
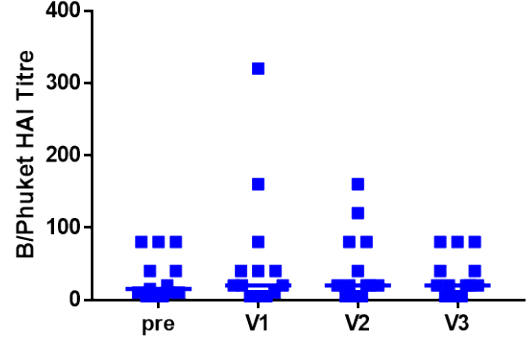
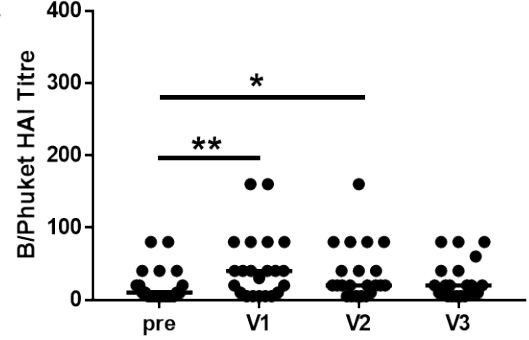
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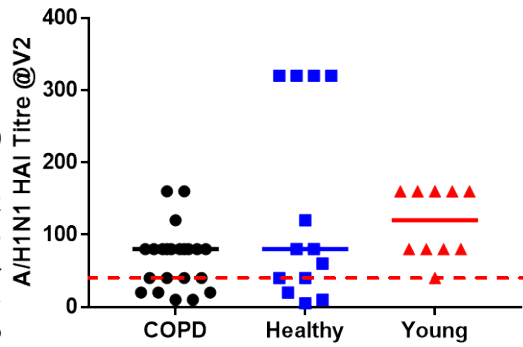
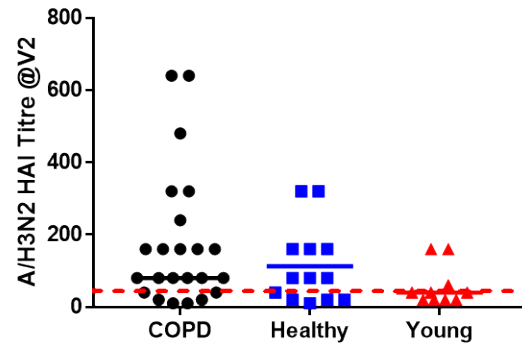
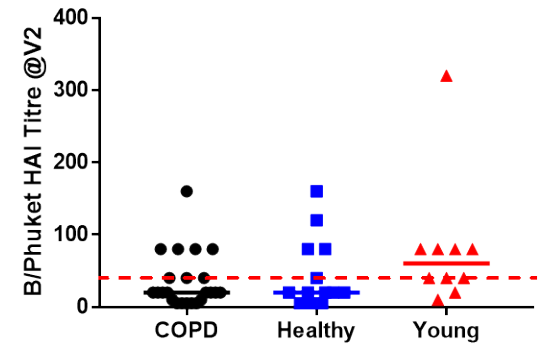
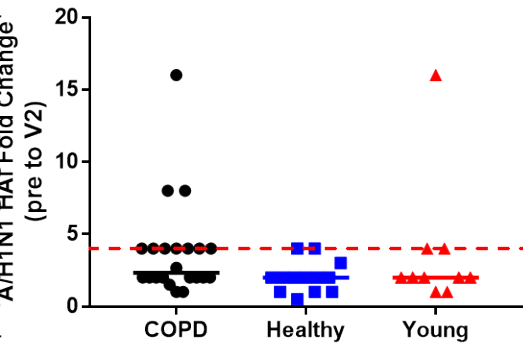
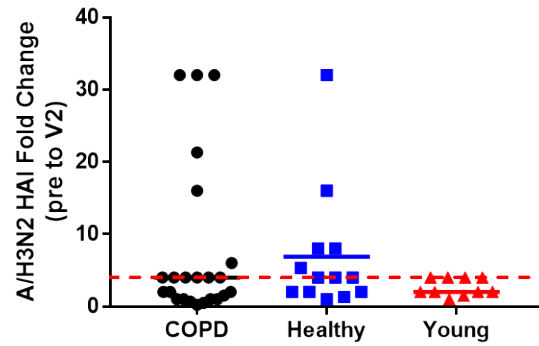
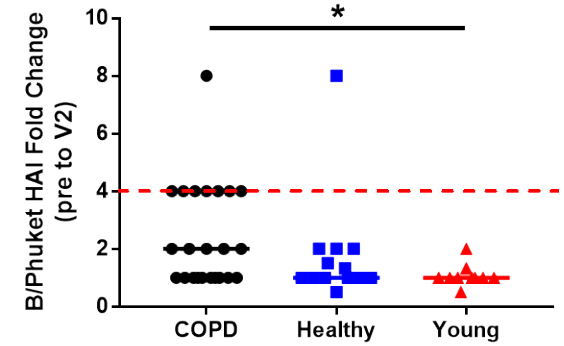


B



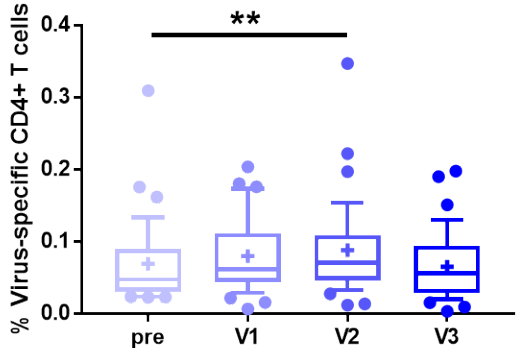
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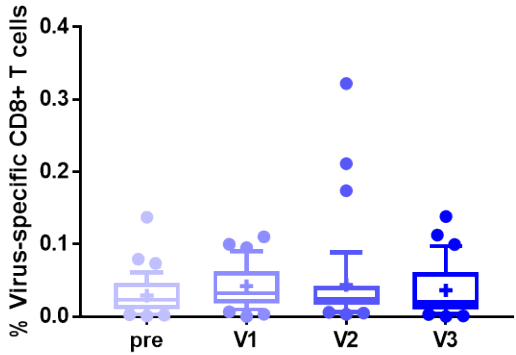
A**B****C****D****E****F**

Staples et al Figure 4

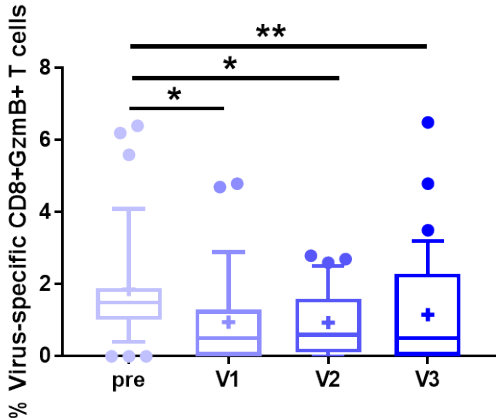
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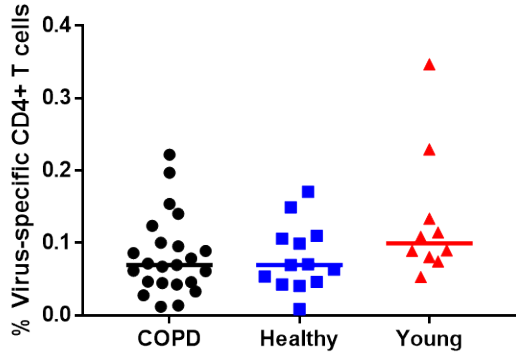
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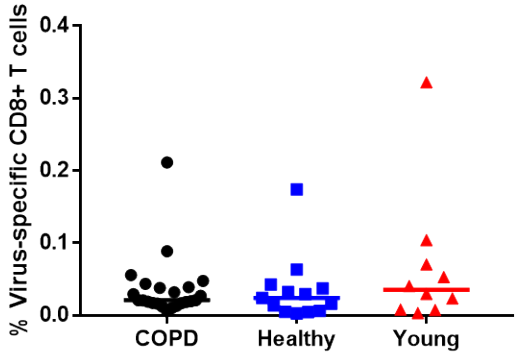
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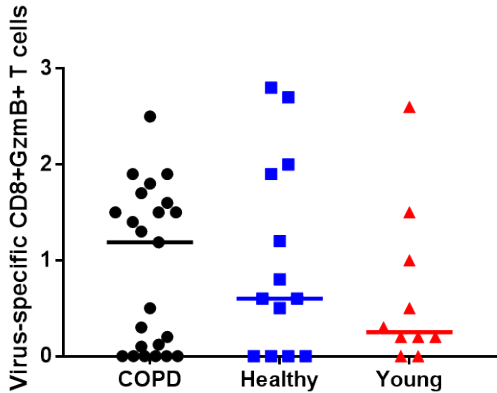
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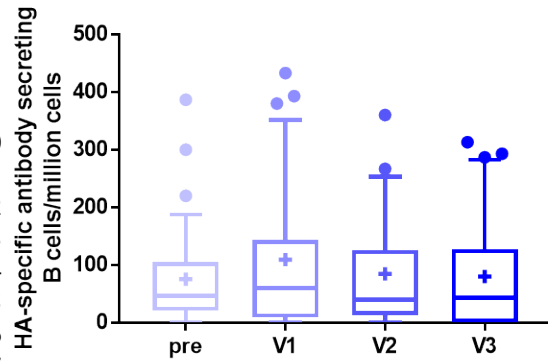
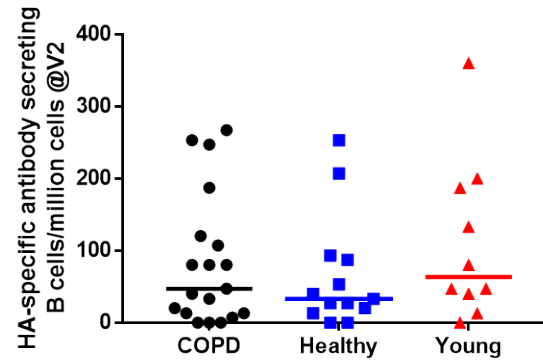
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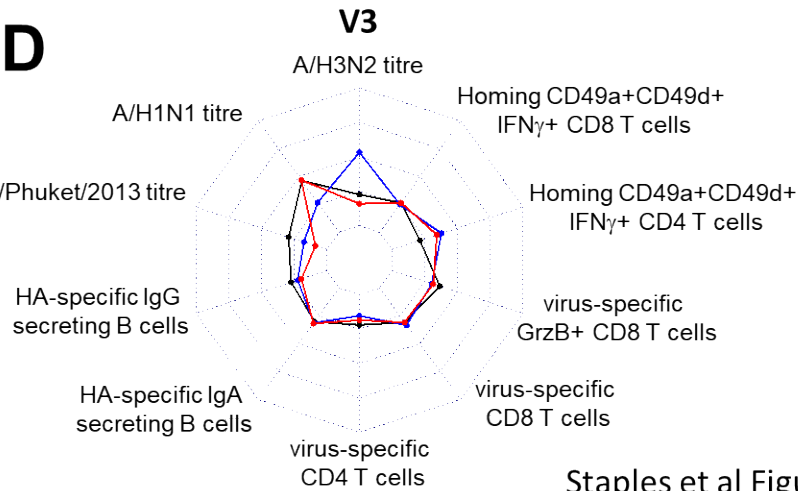
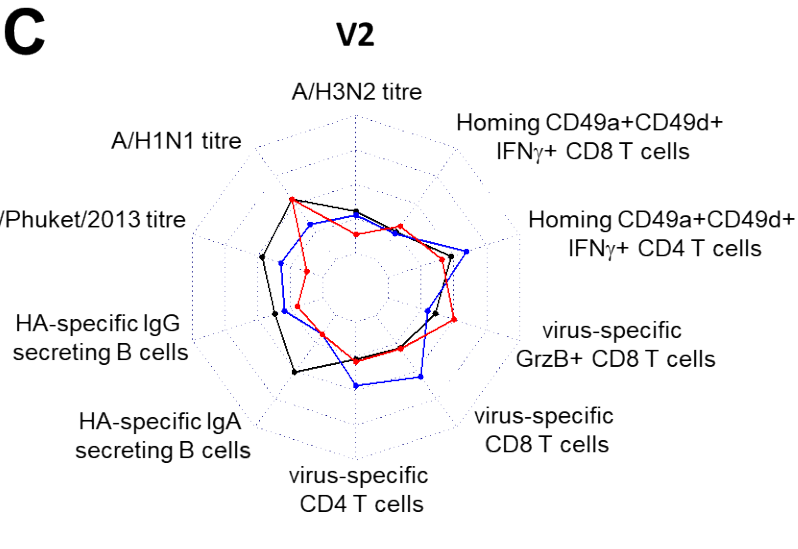
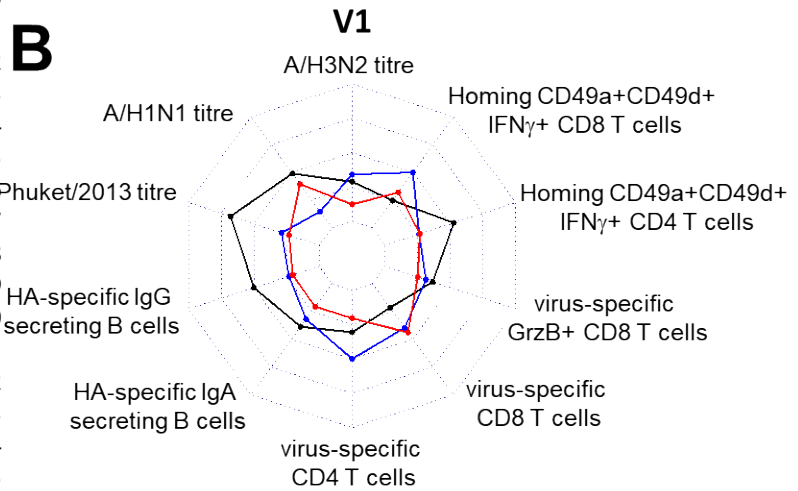
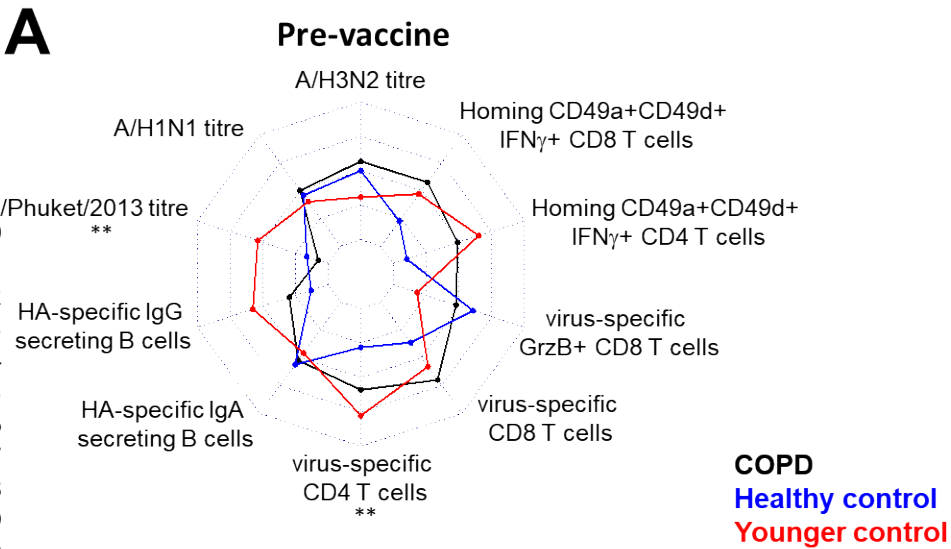


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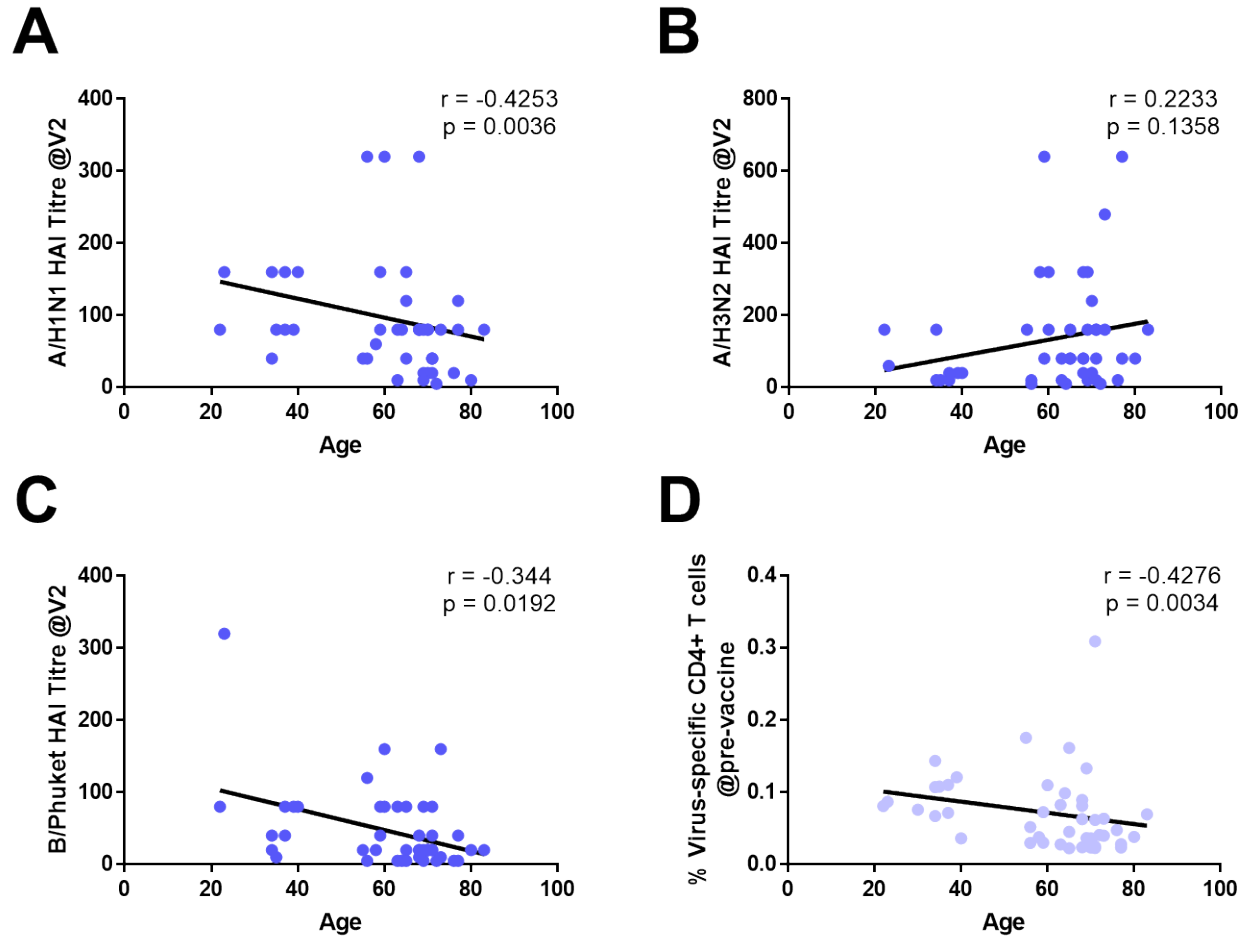


Staples et al Figure 5

A**B**

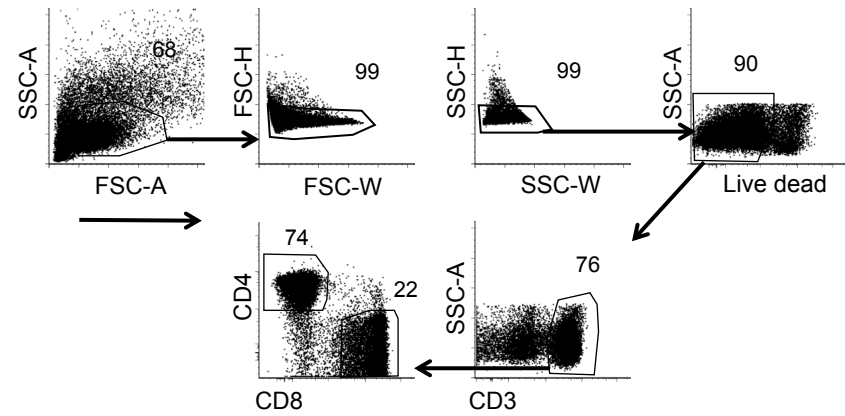


Staples et al Figure 7

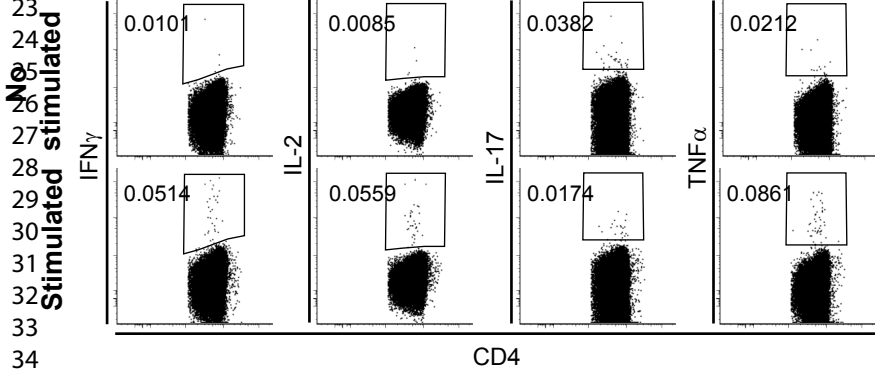


Staples et al Figure 8

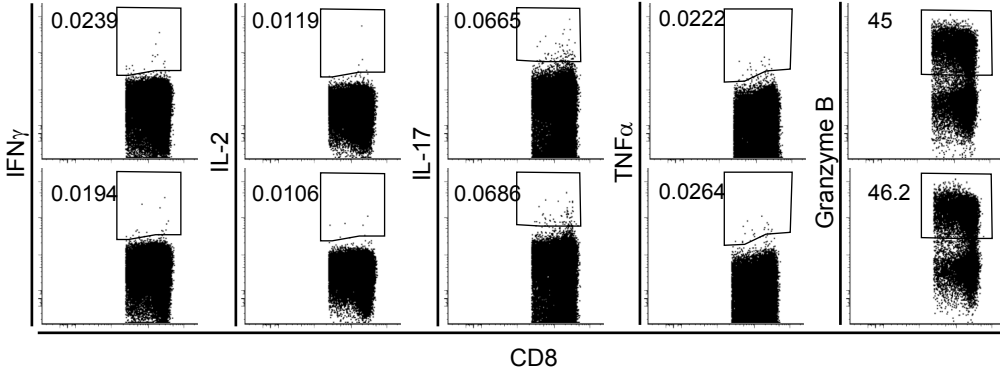
Gating strategy for cytokine-T cell analysis



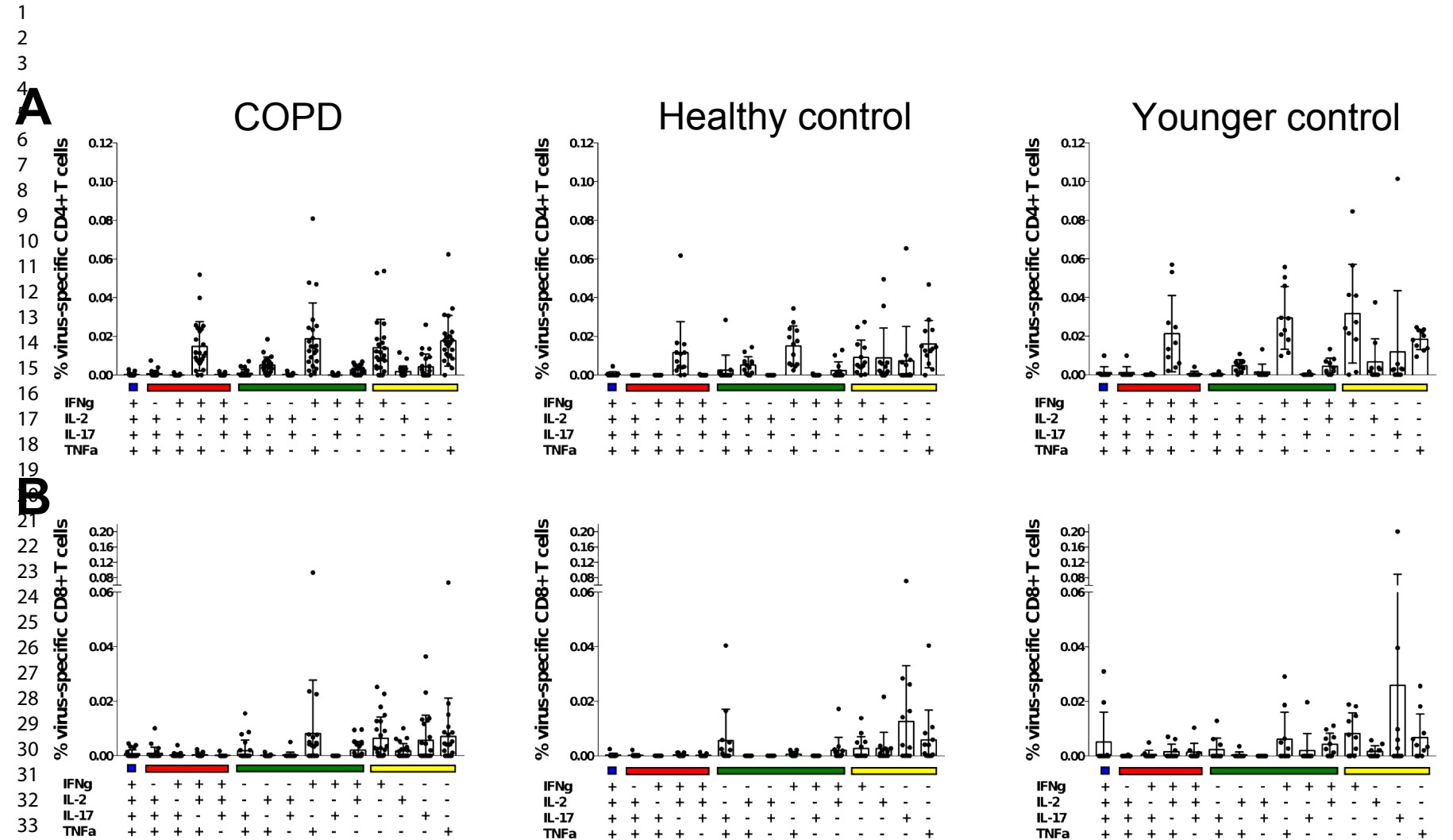
On CD4 T cells



On CD8 T cells



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Supplemental Figure 1



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Supplemental Figure 2