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Graphical abstract

Streptococcus pneumoniae colonization associates with impaired adaptive immune responses against SARS-CoV-2

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Keywords: SARS-CoV-2, *S. pneumoniae*, immune responses, healthcare workers, hospital presented patients

Abstract

Background: Although recent epidemiological data suggest that pneumococci may contribute to the risk of SARS-CoV-2 disease, cases of co-infection with *Streptococcus pneumoniae* in COVID-19 patients during hospitalisation have been reported infrequently. This apparent contradiction may be explained by interactions of SARS-CoV-2 and pneumococcus in the upper airway, resulting in the escape of SARS-CoV-2 from protective host immune responses.

Methods: Here, we investigated the relationship of these two respiratory pathogens in two distinct cohorts of a) healthcare workers with asymptomatic or mildly symptomatic SARS-CoV-2 infection identified by systematic screening and b) patients with moderate to severe disease who presented to hospital. We assessed the effect of co-infection on host antibody, cellular and inflammatory responses to the virus.

Results: In both cohorts, pneumococcal colonisation was associated with diminished anti-viral immune responses, which affected primarily mucosal IgA levels among individuals with mild or asymptomatic infection and cellular memory responses in infected patients.

Conclusion. Our findings suggest that *S. pneumoniae* impair host immunity to SARS-CoV-2 and raises the question if pneumococcal carriage also enables immune escape of other respiratory viruses and facilitates reinfection occurrence.

Trials registration: ISRCTN89159899 for FASTER study and Clinicaltrials.gov identifier: NCT03502291 for LAIV study

INTRODUCTION

Despite the widespread global effects of the coronavirus disease 2019 (COVID-19) pandemic, few reports have assessed potential interactions between upper airway bacterial colonisation and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Consequently, the contribution of respiratory bacterial pathogens to SARS-CoV-2 infection and pathogenesis remains poorly understood (1, 2). Post-hoc analysis of two randomised clinical trials has found that individuals vaccinated with pneumococcal conjugate vaccines (PCVs) showed a reduction of 30-35% in hospitalisations for endemic human coronavirus (HCoV, OC43 and HKU1) associated pneumonia in adults (3, 4) and lower respiratory infection in children (5). A recent observational study reported that 13-valent pneumococcal conjugate vaccine (PCV13) in older adults was associated with a reduction of approximately 30% in COVID-19 disease, hospitalisation, and death (6). Also, a recent epidemiological study reported higher mortality observed in patients with SARS-CoV-2 co-infection or subsequent infection (although rare events) within 28 days after invasive pneumococcal disease (IPD) in the UK (7).

Traditionally, viral-pneumococcal interaction in the upper airway has been thought to increase the risk of secondary pneumococcal pneumonia, particularly during influenza and RSV seasonal outbreaks (8). However, a substantially low proportion of COVID-19 patients have documented pneumococcal pneumonia based on culture of blood or sputum samples collected during hospitalisation, with specimen collection often occurring after provision of antibiotics (1, 7, 9, 10) Bacterial and viral interaction in the upper airways could act synergistically to promote viral evasion by direct and indirect mechanisms (11, 12). Adaptive immune mechanisms play a critical role in protecting against viral infection, including against SARS-CoV-2 (13-15). *S. pneumoniae* (Spn) has been shown to have a modulatory effect on the anti-viral immune responses mounted by the host and the sequence of pathogen exposure in co-infection cases may also alter the disease outcome. Mice exposed to *S. pneumoniae* prior to influenza A exhibited reduced antiviral serum IgG a month after infection (16), whereas a randomised controlled human study of experimental pneumococcus/influenza co-infection reported diminished mucosal IgA responses to influenza antigens associated with pneumococcal carriage (17), resembling findings of the current study. Non-pneumococcal specific cleavage of mucosal IgA1 by pneumococcal IgA1 proteases (18) could be a potential mechanism which contributes to anti-viral IgA reductions identified in both studies.

To study interactions of pneumococcus and SARS-CoV-2 and the effect of pneumococcus on host anti-viral immune responses, we longitudinally sampled a cohort of healthcare workers (HCW) at high risk for SARS-CoV-2 infection and patients with suspected COVID-19 disease. In both cohorts, we studied prevalence of SARS-CoV-2 and pneumococcal colonisation, associations of co-infection and disease

severity and evaluated immune responses and inflammation levels in the context of SARS-CoV-2 mono-infection and co-infection with pneumococcus. Lastly, we sought to assess whether pneumococcal carriage associated reduction in mucosal IgA to respiratory viruses could be due the activity of pneumococcus IgA1 protease as well as whether the order of infection of virus and pneumococcus was important. Consequently, we evaluated samples from our previous studies of live attenuated influenza virus vaccine (LAIV) and pneumococcus co-infections (12, 17, 19) to assess that.

RESULTS

SARS-CoV-2 and *S. pneumoniae* prevalence in HCW and Patient Cohort

The impact of pneumococcal carriage on SARS-CoV-2 viral replication and clinical outcome was assessed in a cohort of frontline HCWs (n=85, median age: 35; IQR: 27.5- 46.5) and a cohort of patients presented to hospital with suspected COVID-19 disease (patients, n=400, median age: 61; IQR:48 -72). Participants were screened for both SARS-CoV-2 and *S. pneumoniae* presence in the naso/oropharynx (Figure 1A). Amongst HCWs, 34% (29/85) tested positive for SARS-CoV-2 at any time point during the 3-months follow-up period of the study on a combined nose and throat (NT) swab or on NT swab and saliva sample and all of these experienced asymptomatic or mildly symptomatic viral infection (20). In the patient cohort, 63.5% (255/400) were tested positive for SARS-CoV-2 at the point of recruitment to the study on NT swabs, and their symptoms ranged from moderate to severe (21).

In the HCW cohort, the overall pneumococcal colonisation rate was 20% (17/85). Increased pneumococcal prevalence was observed in SARS-CoV-2 positive compared to the SARS-CoV-2 negative participants [34.5% (10/29) vs 12.5% (7/56), respectively, p=0.023] (Figure 1B), with 7/10 participants acquiring SARS-CoV-2 while already being colonised with Spn and 3/10 having a concurrent infection (Supplemental figure 1). In the patient cohort, the overall Spn colonisation rate was 8.5% (35/400) and prevalence of Spn colonisation did not differ amongst SARS-CoV-2 positive and SARS-CoV-2 negative individuals [9.4% (24/255) vs 7.6% (11/145), respectively] (Figure 1C). In SARS-CoV-2 infected patients, pneumococcal colonisation did not associate with increased disease severity, as defined by NIH severity score (median: 4, IQR:3-4 in both groups) (Table 1) or reduced survival rate (Supplemental figure 2). Also, these two groups did not differ in days of sample collection post symptoms onset (median: 41, IQR:29-57 vs 47, IQR: 36-59) (Table 1). However, recruitment of patients who had already developed symptoms made the order of pathogen infection unknown. During the 9 months recruitment period of patient study, we observed fluctuations in pneumococcal carriage rate, with lower colonisation rates during periods of local and UK lockdowns (Figure 1D).

Although, the SARS-CoV-2 upper airway viral load did not differ significantly by Spn carriage status in either cohort, the HCW non-colonised group had a 4.4-fold higher median value (median: 2.01 $x10^2$ RNA copies/ml, IQR: 4.02 $x10^1$- 4.03 $x10^3$) compared to the Spn-colonised group (4.5 x 10^1 RNA copies/ml, IQR: 2.30 $x10^1$- 2.03 $x10^3$ in Spn+). Similarly, in patient cohort, the non-colonised group had a 6-fold higher viral load (median: 1.04 x 10^5 RNA copies.ml, IQR: 1.89 $x10^3$- 1.94 $x10^6$) compared to Spn-colonised group (1.74 x 10^4 RNA copies/ml, IQR: 6.12 $x10^1$- 8.14 $x10^6$ in Spn+). The patient cohort (both Spn colonised and non-colonised groups) had a higher viral load compared to the HCW cohort (Figure 1E).

Impaired mucosal antibody responses to SARS-CoV-2 in pneumococcal colonised individuals

IgA plays a crucial role in anti-viral immune defence of mucosal surfaces (14, 22, 23). Herein, levels of mucosal IgA to surface SARS-CoV-2 antigens, such as receptor binding domain (RBD), spike protein subunit-1(S1) and subunit-2 (S2) and the internal nucleocapsid protein (N) were measured in saliva samples in HCWs and nasal lining fluid in patient cohort (due to difficulties in acquiring saliva from patients) one month post SARS-CoV-2 infection.

In the HCW cohort, amongst SARS-CoV-2 positive subjects, non-colonised HCWs had greater salivary IgA levels compared to Spn-colonised for all SARS-CoV-2 antigens assessed, with statistically significant differences for S1 and S2 between the two HCW groups (median 4.1- and 6.4-fold change of IgA to S1 and S2, respectively) ($p=0.035$ and $p=0.028$, respectively) (Figure 2A).

Among SARS-CoV-2 positive patients, there was a trend of overall weakened IgA induction to SARS-CoV-2 antigens in Spn-colonised compared to non-colonised group, however there were no significant differences between the two groups (Figure 2B). Non-colonised subjects mounted robust nasal IgA to SARS-CoV-2 antigens (RBD, S2 and N), with the exception of S1, for which titres did not differ significantly from control. This group had the highest antibody fold rise against RBD and N (6.2-fold and 9.4- fold increase from control levels) ($p<0.0001$ in both)(Figure 2B). Spn-colonised subjects showed a lesser increase against RBD and N (2.2- and 3.2-fold increase from control levels) ($p=0.002$ and $p=0.014$, respectively) (Figure 2B) and a moderate induction of nasal IgA to S1 and S2, with titres that were not significantly higher from control group (Figure 2B).

Anti-viral IgG responses were measured in convalescent sera in both cohorts. In the HCW cohort, there was a trend of overall lower levels of IgG induction observed for RBD and S2 antigens in the Spn colonised participants compared to non-colonised (Figure 2C). Non-colonised participants showed a moderate rise in IgG titres against all SARS-CoV-2 proteins, except S1, whereas the Spn colonised counterparts mounted lower IgG responses against those viral antigens, with only anti-RBD IgG levels

differing significantly from those observed in healthy controls. (Figure 2C). In the patient cohort, IgG levels against N protein were greater in the non-colonised patients when compared with those mounted by the Spn colonised counterparts (median 12-fold difference, p=0.014) and 2-fold higher against RBD in non-colonised vs Spn colonised group (p=0.10) (Figure 2C). The two patient groups raised similar IgG levels to the spike subunits. In agreement with findings that disease severity correlates with increased levels of systemic IgG to SARS-CoV-2 (24), we observed that IgG titres to viral antigens were consistently higher in patients than HCWs (Figure 2C).

Experimentally induced pneumococcal colonisation impairs nasal IgA against influenza antigens but only when colonisation precedes viral infection.

We have previously observed reduced mucosal IgA, but not IgG and IgM, responses to influenza antigens in Spn colonised subjects when colonised with serotype 6B three days before administration of LAIV (17). Pneumococcal IgA1 protease is a cell-associated enzyme which cleaves human IgA1, but not IgA2 (18). To test its involvement in the reduction of anti-viral mucosal IgA, we evaluated the association of pneumococcal carriage with virus specific IgA1 vs IgA2 levels in nasal mucosa samples previously collected in two LAIV-pneumococcal co-infection studies with known onset of viral and Spn infection (12, 19).

Influenza-specific IgA, IgA1 and IgA2 levels were measured in nasal wash samples at baseline and day 24 after LAIV administration for a subset of participants (15 Spn+ and 15 Spn-) that received LAIV 3 days after pneumococcus. While no to little induction of total IgA and IgA subclasses to influenza antigens were observed from baseline levels in Spn colonised participants, non-colonised group exhibited a median 3-, 1.9- and 1.7-fold rise of influenza specific IgA, IgA1 and IgA2 titres, respectively (Supplemental figure 3A). When the order of infection was inverted (LAIV infection occurred 3 days before pneumococcal challenge)(19) levels of influenza-specific IgA did not differ between Spn colonised and non-colonised participants (IgA median 1.5- and 1.6-fold increase, respectively) (p=0.28) (Supplemental figure 3B). Due to insufficient nasal material from the current study cohorts, we could not assess IgA subclass in those samples.

***S. pneumoniae* colonisation is associated with decreased levels of memory B cells to SARS-CoV-2**

Memory B cells are of great importance for long term humoral immunity. To identify SARS-CoV-2 specific memory B cells, fluorescently labelled S1 and S2 antigens were used in PBMCs from HCWs and recovered patients (Supplemental figure 4). Overall, Spn colonised participants showed a trend of reduced frequency of memory B cells to SARS-CoV-2 antigens compared to their non-colonised counterparts, which was observed in both HCW and patient cohorts (Figure 3). In HCW cohort, the

proportion of S1-specific memory B cells was significantly higher from healthy controls in non-colonised (0.12% vs 0.01%, p=0.003) and Spn colonised group (and 0.06% vs 0.01%, p=0.031) but less pronounced in the latter group (Figure 3A). In the patient group, non-colonised individuals had the highest frequencies of S1- and S2-specific memory B cells when compared to Spn colonised participants and any other group. Specifically, non-colonised patients had greater proportion of S1-specific memory B cells (0.18% vs 0.08%, p=0.027) and a strong trend of higher S2-specific memory B cell proportion compared to Spn-colonised counterparts (0.35% vs 0.14%, p=0.09) (Figure 3B).

Lack of SARS-CoV-2 specific T cell responses in patients colonised with *S. pneumoniae.*

To assess $CD4^+$ and $CD8^+$ T cell (Supplemental figure 5) mediated recall responses in HCWs and patients, as well as healthy uninfected controls, PBMCs were stimulated *ex vivo* with N, S and S1 defined peptide pools from SARS-CoV-2. In the HCW cohort, overall $CD4^+$ T cells responses did not differ significantly from healthy controls (Figure 4A), either between Spn colonised and non-colonised HCWs. In the patient cohort, the magnitudes of T cell responses to N, S1 and S were greater in the non-colonised compared to Spn colonised group and one of the highest in both study cohorts. Median percentages of specific $CD4^+$ T cells for N, S1 and S were 1.64% (IQR: 0.53-2.77), 0.22 (IQR: 0.08-0.54) and 0.57 (IQR: 0.41-1.09), respectively in the non-colonised group vs 0.14% (IQR: 0.07-0.22), 0.08% (IQR: 0.018-0.38) and 0.12% (IQR: 0.02-0.60), respectively in the Spn-colonised group. IL-2 was the most abundantly produced cytokine, and similar secretion patterns were observed for TNF-α and IFN-γ, as described above. The cytokine specific (IFN-γ, TNF-α and IL-2) $CD4^+$ T cells responses to each peptide per group are shown in Supplemental figure 6.

SARS-CoV-2 N, S and S1 specific $CD8^+$ T cell responses were also assessed in the same samples. Similar to $CD4^+$ T cell responses, both the number of responders and magnitudes of $CD8^+$ responses to N and S1 were the highest and most robust, respectively, in the non-colonised patients. In this group, median $CD8^+$ T cell responses for N and S1 were 2.03% (IQR: 0.35%-2.85%) and 0.36% (IQR: 0.11%-0.84%), respectively (Figure 4B). $CD8^+$ T cell responses to S peptide pool were close to the lower limit of detection (LOD) in all groups. $CD8^+$ responses to N and S1 were impaired in the Spn colonised patients and differed significantly from their non-colonised counterparts (median: 0.08% vs 2.03%, p=0.019 and median: 0.025% vs 0.36%, p=0.009, respectively) (Figure 4B).

Distinct nasal inflammation profile between HCWs and patients with COVID-19 infection

SARS-CoV-2 induced nasal and systemic inflammatory responses were assessed by measuring levels of 30 cytokines in the nasal fluid and serum during the early phase of the viral infection in HCW and patient cohorts. In the nose, HCW groups showed a lack of upregulation in cytokines which

functionally promote T and B cell maturation and differentiation (Figure 5A). In blood, non-colonised patients exhibited an increased inflammatory profile (16/30 cytokines were upregulated) compared to the Spn colonised counterparts (upregulation of 9/30 cytokines) (Figure 5A). It is important to note, IL-2 and IL-12, which are key cytokines for T cell proliferation and activation, did not differ from control in both HCW and patient Spn colonised groups (Figure 5A). Levels of nasal and serum cytokines per group were also plotted based on significance and fold-change difference from the control group (Figure 5B).

We also used an unsupervised analysis to assign profiles to each group. Principal-component analysis (PCA) was applied on all analytes in nasal lining fluid (Figure 6A) and serum (Figure 6B) for all groups and the control. In the nose, patient groups were segregated together and away from the healthy control group in the second principal component (Figure 6A), with the non-colonised group having a more distinctive profile. HCW groups exhibited a similar inflammatory profile, which clustered between control and patient groups (Figure 6A). In serum, patient groups also segregated together and showed a very similar profile, which differed from the control group in the second principal component. The HCW groups clustered again between control and patient groups, with the Spn colonised HCWs appearing as a heterogeneous group (Figure 6B).

DISCUSSION

Here we report the first immunological analysis to our knowledge of SARS-CoV-2 infection in the context of co-infection with pneumococcus among two distinct cohorts - asymptomatic and mildly symptomatic HCWs and patients who experienced moderate to severe symptoms during SARS-CoV-2 infection. More importantly this is the first comprehensive analysis showing the potential role of *S. pneumoniae* carriage in modulating the host immune responses against SARS-CoV-2. Our findings have potential implications for other respiratory viruses.

Humoral and cellular antiviral immune responses varied substantially by pneumococcal carriage status both in HCW and patient cohort, suggesting that colonisation of upper airways by pneumococcus affects host immunity to SARS-CoV-2. This effect was more apparent in the nasal mucosa of HCWs, where diminished salivary anti-S1 and S2 IgA levels were observed in the pneumococcal colonised individuals. In the patient cohort pneumococcal carriage was associated with reduced SARS-CoV-2 specific memory B cells and weakened T cell responses, particularly $CD4^+$ T cell responses.

SARS-CoV-2 infection and virus replication starts in the naso/oropharynx- the primary site of infection (25). Mucosal antibodies, particularly secretory IgA, play an important role in defence against respiratory viruses (26, 27). For SARS-CoV-2 infection, in vitro studies with monoclonal anti-spike IgA

demonstrated the superiority of IgA to block binding to the ACE2 receptor compared with the IgG isotype (28) and SARS-CoV-2 challenge studies in mice highlighted that musosal anti-spike IgA production is critical for sterilizing immunity in the upper respiratory tract (27). We found that Spn colonised HCWs had dimished IgA responses against SARS-CoV-2 S1 and S2 proteins compared to non-colonised counterparts, and that these responses did not differ between Spn colonised and non-colonised groups in the patient cohort. This may indicate that such a immunesuppressive effect is more important at the early phases of SARS-CoV-2 infection and less relevant once infection has progressed to symptomatic and severe disease.

We further investigated a potential mechanism responsible for the association between bacterial colonisation and IgA immunosuppressed responses against viral infections using an influenza virus/*S. pneumoniae* co-infection model. We observed that preceding pneumococcal colonisation impaired the induction of both influenza-specific IgA1 and IgA2 nearly a month after influenza infection- an effect that was not seen in the absence of pneumococcal colonisation. This suggests that cleavage of non-pneumococcal specific IgA1 by pneumococcal IgA1 protease most likely is not a mechanism, through which pneumococcus contributes to the reduction in mucosal IgA to other pathogens. When influenza infection preceded experimentally induced pneumococcal colonisation, both Spn colonised and non-colonised, LAIV recipients mounted similar influenza-specific IgA levels. These findings imply that the order of exposure to respiratory pathogens during co-infection can affect some of the defence mechanisms. Differential nasal inflammatory responses during the early stages of infection, driven by either the virus or *S. pneumoniae,* depending on the order of infection, may have a differential effect on downstream immune responses (12, 16, 17), altering the dynamics between the pathogens (19).

It has been shown that *S. pneumoniae* stimulates IFN-I production and upregulates the expression of IFN-stimulated genes in both mice and human studies (17, 29). Therefore, it is possible that pneumococcal colonisation interferes with the replication cycle of the virus (30, 31) and contributes to host antiviral defences by governing the production of IFNs (32, 33). Here, despite a trend of higher viral load in the non-colonised groups, we did not observe significant viral load difference between pneumococcal colonised and non-colonised individuals. However, as SARS-CoV-2 viral load changes rapidly from day to day, the nature of the study prohibited the assessment of such a time-course dependent variable (34, 35).

Consistent with published studies of COVID-19 infection (36, 37), we observed inflammatory responses, including IL-6, IP-10, IL-1b and IL-8, in nasal lining fluid and serum in both cohorts, with increased cytokine induction in the patient groups, particularly the non-colonised individuals. The induction of cytokines that influence T cell activation (IL-2, IL-12, IFN-γ, IL-15, IL-17A), which subsequently assists B cell maturation (38), was distinctive in the nasal mucosa of patient cohort.

Impairment of inflammatory response in the nasal mucosa could also affect the influx of effector immune cells and influence downstream immune responses (12, 17). Also, pneumococcal colonised individuals in both cohorts exhibited a lack of IL-2 and IL-12 induction in serum, which could partially explain the weakened T cell responses observed in those groups.

Consistent with previous studies, convalescent patients mounted higher serum IgG levels compared to asymptomatic and mildly symptomatic HCWs (39, 40), showed increased frequency of memory B cells, broader and stronger T cell responses in the convalescent phase (41) and had elevated acute proinflammatory responses both in the nose and blood (42). Coordinated immunity by all three branches of adaptive immunity is more likely to protect against SARS-CoV-2 reinfection, as it is seen in protection against other infectious diseases (40), whereas suboptimal immunity against SARS-CoV-2 could allow reinfection to occur.

Our study has limitations. Diagnosis of co-infection was complex amongst patients, as pneumococcus might be carried by the patient before the viral infection or might be picked up later. High use of antibiotics (potentially prescribed at an outpatient visit) and reduced social mixing may have affected - prevalence and dynamics of transmission of other respiratory pathogens, such as RSV(1, 43), and most likely pneumococcus. Hence, we observed decreased prevalence of pneumococcus amongst patients- particularly during periods of national lockdown, which subsequently limited the number of SARS-CoV-2/ pneumococcus co-infected individuals studied here. Thus, despite inclusion of nearly 500 subjects in our study, we were able to evaluate pneumococcal mediated immunomodulatory effects only for a relatively small number of individuals, limiting the ability to do further stratifications. In addition, differences in age and underlying disease between the HCW and patient cohorts are some factors that have potentially affected the course and outcome of the disease. Further studies, ideally in the setting of controlled human co-infection model, are needed to explore *S. pneumoniae*/respiratory virus interactions and the biological mechanisms through which pneumococcus assists viruses to subvert immune responses at the primary site of infection.

Despite the observational design, our study has identified pneumococcal colonisation as a variable which can modulate host immune responses to SARS-CoV-2 infection; an effect that was observed in both cohorts despite the aforementioned differences. An impaired adaptive immunity against SARS-CoV-2 natural infection could potentially increase susceptibility to subsequent SARS-CoV-2 infection. The increased evidence on PCV-induced protection against lower respiratory infections associated with viral infection and the broader ability of pneumococcus to interact with respiratory viruses in a way that increases pneumococcal virulence, viral pathogenicity or impairs anti-viral immune responses highlights the importance of PCVs in both paediatric and older adults as an additional public health tool for those who are at increased risk of pneumococcal and viral lower respiratory infections.

METHODS

Study design

This study combined participants recruited into two prospective cohort studies of a) frontline HCWs (n=85) and b) patients (n=400) presented to the hospital. HCWs in a variety of roles were enrolled onto SARS-CoV-2 Acquisition in Frontline Healthcare Workers - Evaluation to inform Response (SAFER) study between 30th March and 9th April 2020 at Royal Liverpool University Hospital (RLUH) in Liverpool, UK. Eligible HCWs (aged ≥ 18 years) were asymptomatic at the time of enrolment to the study. Screening against SARS-CoV-2 and *S. pneumoniae* was performed on the nose and throat (NT) swabs and saliva samples (Figure 1A). Symptom reporting was via a questionnaire completed twice weekly, accompanying each sampling episode (20).

For the patient cohort, adults (aged ≥ 18 years) with signs and symptoms of suspected COVID-19 infection attending RLUH, Aintree University and Whiston Hospital in Merseyside between April 2020 to January 2021 were recruited into Facilitating A SARS Cov-2 Test for Rapid Triage (FASTER), regardless of disease severity, race, ethnicity, gender, pregnancy or nursing status, or the presence of other medical conditions (Table 2). Screening against SARS-CoV-2 and *S. pneumoniae* was performed on throat swabs, NT swabs and saliva samples (Figure 1A).

Bacterial DNA extraction and *S. pneumoniae* qPCR

Bacterial genomic DNA was extracted from both raw and culture-enriched (CE) material from throat swabs, NT swabs and saliva samples for the patient cohort, and NT and saliva samples for the HCW cohort, as previously described (44). Briefly, bacterial DNA was extracted using the Agowa Mag mini-DNA extraction kit (LGC Genomics, Berlin, Germany). Pneumococcal presence was determined by sequential singleplex qPCR targeting the *lyt*A (45) and the *pia*B genes (46), using the QuantStudio 5 system (ThermoFisher, UK), as previously described (46). Briefly, 20µL PCR mix consisted of 12.5µL 1 × TaqMan Universal PCR Master Mix (Life Technologies Ltd, Paisley, UK), 0.225µL or 0.2µL *100µ*M each *lytA* or *piaB* primer respectively, 0.125µLor 0.175µL 100µM *lytA* or *piaB* probe respectively, molecular graded water (Fisher Scientific, Loughborough, UK) and 2.5µL of the extracted DNA. Thermal cycling conditions were: 10min at 95°C and 40 cycles of 15secs at 95°C and 1min at 60°C. A negative DNA extraction control (parallel extraction from sample buffer only), a qPCR negative control (master mix only), a qPCR positive control (pneumococcal Spn15B strain) and duplicates of each sample were amplified. A standard curve of a ten-fold dilution series of genomic DNA extracted from TIGR4 was used. Pneumococcal positive samples considered those that both genes were present. All samples were assessed by a *lytA* qPCR and those positive underwent a *piaB* qPCR. Samples were considered

296 *lytA*-positive if one or two yielded a C_T < 40 cycles. Threshold between plates was normalised according
297 to positive control C_T values.
298
299 **SARS-CoV-2 RNA extraction and RT-qPCR**
300 SARS-CoV-2 RNA was extracted from NT swabs in amies solution or saliva, as previously described (47).
301 Briefly, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany), and 8µL of
302 extracted RNA was tested using the genesig Real-Time Coronavirus COVID-19 PCR (genesig, UK). Virus
303 copies/ml were quantified using the manufacturer's positive control (1.67×10^5 copies/µl) as a
304 reference.
305
306 **Immunological analyses of SARS-CoV-2 positive participants**

307 SARS-CoV-2 positive individuals from both cohorts (all Spn colonised individuals and a subset of non-
308 colonised HCW and patients) were stratified by pneumococcal colonisation status. Pre-pandemic
309 samples from healthy unexposed individuals were also included, resulting in 5 groups used for the
310 immunological analysis: i) HCW_nCOV+ Spn-, ii) HCW_nCOV+/Spn+, iii) Patient_nCOV+/Spn-, iv)
311 Patient_nCOV+/Spn+ and v) Healthy controls. Demographic and clinical characteristics of these 5
312 groups are shown in Table 1. The selection of HCW (n=27) and COVID-19 patients (n=89) that were
313 used in the analysis of immunological parameters was mainly based on a) the availability of
314 convalescent sample and b) the pneumococcal carriage status. To assess and compare immune
315 responses to SARS-CoV-2, we analysed convalescent blood and upper respiratory samples (saliva and
316 nasal lining fluid) in HCW and patient groups. Access to convalescent samples was restricted in the
317 patient cohorts, as only 39% (100/255) of SARS-CoV-2 positive individuals donated samples at the
318 convalescent phase of COVID-19 infection. In both cohorts, we also assessed nasal and systemic
319 inflammation during the acute phase of COVID-19 infection. Samples from healthy adults collected
320 prior to June 2019 were used as healthy controls.

321 **Enzyme-linked immunosorbent assay for SARS-CoV-2 and influenza virus antigens**
322 ELISA was used to quantify levels of IgG and IgA to SARS-CoV-2 antigens in serum and saliva or nasal
323 lining fluid samples, respectively, whereas IgA, IgA1 and IgA2 were measured in nasal wash samples
324 of live attenuated influenza vaccine (LAIV) recipients (Demographics are shown in Supplemental
325 Tables 1 & 2). LAIV was administered intranasally Recombinant SARS-CoV-2 RBD and N protein were
326 produced at Jenner institute, Oxford, UK, as reported elsewhere (48). Recombinant S1 and S2 subunits
327 (full length proteins) were commercially available (Biosciences, UK). Seasonal TIV (either 2015/2016
328 or 2016/2017 formulation) was used as the source of influenza antigens for measuring mucosal IgA
329 and subclasses in nasal washes of study participants.

Antibody levels to SARS-CoV-2 and influenza antigens were quantified, as previously described (17, 49) with minor modifications. Briefly, Nunc 96-well plates were coated with 1µg/ml SARS-CoV-2 antigen or 0.2 µg/ml TIV and stored at 4°C overnight for at least 16h. After coating, plates were washed 3 times with PBS/0.05%Tween and blocked with 2% BSA in PBS for 1h at room temperature. Thawed serum, saliva, nasal fluid and nasal wash samples diluted in 0.1% BSA-PBS were plated in duplicate and incubated for 2h at room temperature alongside an internal positive control (dilution of a convalescent serum) to measure plate to plate variation. For the standard curve, a pooled sera of SARS-CoV-2 infected participants was used in a two-fold serial dilution to produce either eight or nine standard points (depending on the antigen) that were assigned as arbitrary units. Goat anti-human IgG (γ-chain specific, A9544, Sigma-Aldrich) or IgA (α-chain specific, A9669, Sigma-Aldrich) or mouse anti-human IgA1 (Fc-specific, ab99794, Abcam) or IgA2 (Fc-specific, ab99800, Abcam) conjugated to alkaline phosphatase was used as secondary antibody and plates were developed by adding 4-nitrophenyl phosphate in diethanolamine substrate buffer. Optical densities were measured using an Omega microplate reader at 405nm. Blank corrected samples and standard values were plotted using the 4-Parameter logistic model (Gen5 v3.09, BioTek).

Flow Cytometry assays

Cryopreserved PBMCs were used, and all samples were acquired on an Aurora cytometer (Cytek Biosciences) and analysed using Flowjo software v.10 (Treestar).

Direct *ex vivo* immune B and T cell phenotyping

B cell phenotyping: PBMCs seeded in 96-well plates were washed (440g for 5 min), stained with Live/dead e506 viability dye for 15 minutes at 4°C (Thermofisher), following an extracellular cocktail of monoclonal antibodies, including SARS-CoV-2 S1 and S2 protein conjugated with Biotin-Streptavidin, for 20 minutes at 4°C protected from light (Supplemental Table 3). S1 and S2 proteins were conjugated with Biotin (EZ Link conjugation kit, Thermofisher) and labelled with Streptavidin-BV785 and PE (Biolegend), respectively.

T cell phenotyping: Following stimulation in 96-well U plates, PBMCs were washed (440g for 5 min), stained with viability dye for 15min at 4°C (Thermofisher), following an extracellular cocktail of monoclonal antibodies for 20 minutes at 4°C protected from light (Supplemental Table 4). Cells were washed again (440g for 5 min), then fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences) for 15min at 4°C. After the incubation, cells were stained with an intracellular cocktail of monoclonal antibodies (Supplemental Table 4).

T cell stimulation and Intracellular cytokine staining assay

Cells were cultured for 18h at 37°C in the presence of SARS-CoV-2 specific peptides (2µg/ml) in 96-well U bottom plates at $1x10^6$ PBMCs per well. Overlapping peptides spanning the immunogenic domains of the SARS-CoV-2 Spike (Prot_S), nucleocapsid (Prot_N) and S1 subunit (Prot_S1) proteins were purchased from Miltenyi Biotec. Golgi-Plug containing brefeldin A and golgi-stop containing monensin (BD Biosciences, San Diego, CA) were added 2h after the peptide addition. A stimulation with an equimolar amount of DMSO was performed as a negative control and Staphylococcal enterotoxin B (SEB, 2 µg/mL) was included as a positive control. The following day cells were harvested from plates, washed and stained for surface markers (Supplemental Table 4).

Luminex analysis of nasal lining fluid or serum.

The Cytokine Human Magnetic 30-plex panel was used to quantitate human nasal and serum cytokines, as previously described (12). Triton-treated nasal fluid and serum were acquired on an LX200 using a 30-plex magnetic human Luminex cytokine kit (Thermo Fisher Scientific) and analysed with xPonent3.1 software following the manufacturer's instructions. Samples were run in duplicate, and standards run on all plates. Calibration and verification beads were run prior to all runs.

Statistical Analysis

Statistical analyses were performed using R software (version 4.0.4) or GraphPad Prism (version 9.0). Two-tailed statistical tests were used throughout the study. Categorical variables were compared using Fisher's exact or Chi-squared test. Continuous variables were tested for normality and appropriate statistical tests applied. Non-normally distributed measurements were expressed as the median and Mann-Whitney (two group comparison) or Kruskal-Wallis (three to five group comparison) tests were used. Differences were considered significant at $p < 0.05$, and multiple testing correction was employed where appropriate. False discover rate (FDR) corrections were performed using the Benjamini-Hochberg test at an $FDR < 0.05$ significance threshold.

Study approval

The two study protocols were reviewed and approved by the NHS Health Service Research Ethics Committees (REF: 20/SC/0147 for SAFER and 20/SC/0169 for FASTER). LAIV clinical trial (LAIV/pneumococcus challenge studies) had been previously approved by NHS REC (14/NW/1460). All participants provided written informed consent and were free to withdraw from the studies at any point.

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Author contributions

EM: conception and design of the study, protocol development, assay development, data analysis and interpretation and manuscript writing. JR and BCU: assay development, conduction of experiments and data analysis. CS, EN, SP, AH, LH, ELG, KSC, RLB, CTW, ACA contributed to sample processing, conducting, and analysing experiments. AHW, SG, MF, KL, HH, AMC, TF and ERA contributed to recruitments, samples collection, protocol development and design of the study. NFW: conception and design of SAFER, SAFER protocol development and implementation, data analysis, review and comment on the manuscript. SJD, DP produced and provided reagents, reviewed and commented on the manuscript. RB: study oversight, protocol development, review and comment on the manuscript. CT: data interpretation, review and comment on the manuscript. EB: study oversight, protocol development, data interpretation, review and comment on the manuscript. LJ, BDG and DMF: conception and design of the study, protocol development, data interpretation and manuscript writing. All authors have read and approved the manuscript.

Competing Financial Interests: RB**,** CT, EB, LJ and BDG are employees of Pfizer, and may own Pfizer stock.

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609

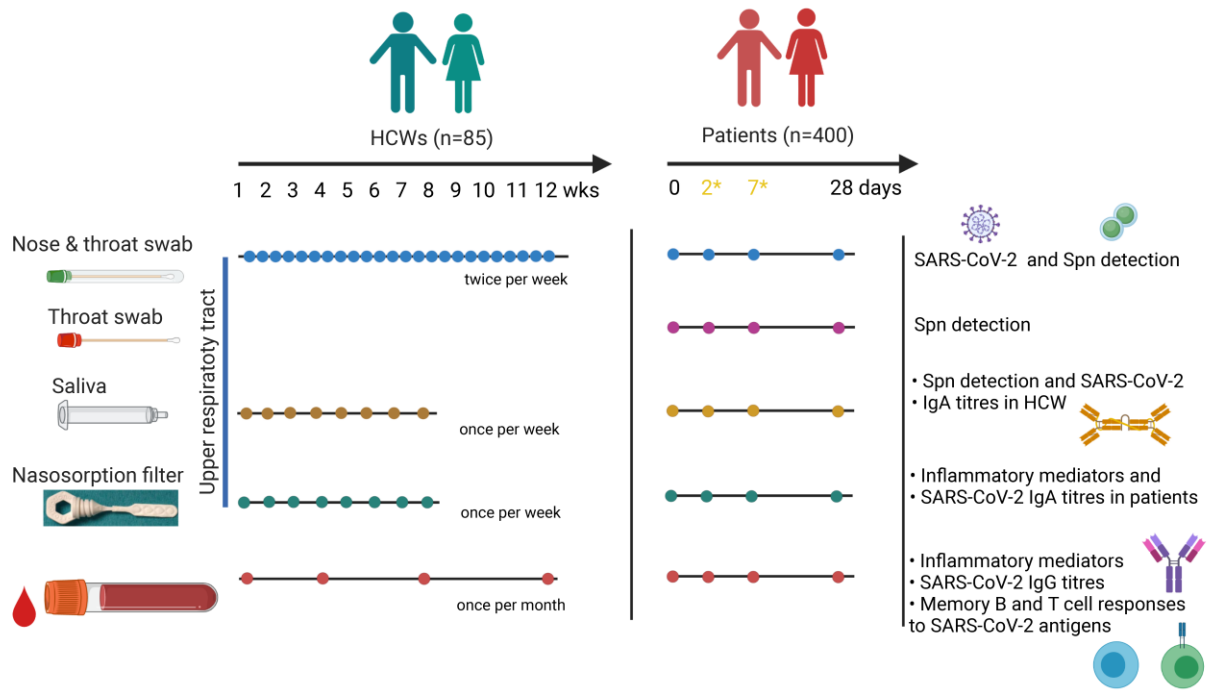
	Units	HCW nCOV+ Spn- (n=17)	HCW nCOV+ Spn+ (n=10)	Patient nCOV+ spn- (n=70)	Patient nCOV+ spn+ (n=19)	Patient groups P value	Overall p value	Healthy Control
Age	median (IQR)	36 (28 -49)	34.5 (29-38)	60 (51-69)	61 (50-73)		<0.0001[α]	28 (19-42)
Female	n (%)	14 (73.3%)	7 (77.8%)	31 (44.3%)	7 (36.9%)		0.007[b]	14 (82.4%)
Smoking	n (%)	0 (0%)	2 (20%)	12 (17.1%)	1 (5.3%)		0.167	0 (0%)
NIH Clinical Score	median (IQR)	0 (0-1)	0 (0-1)	4 (3-4)	4 (3-4)		<0.0001[a]	0 (0-0)
ISARIC 4C Clinical Score	median (IQR)	0 (0-1)	0 (0-1)	7 (4-11)	7 (3-9)	0.71[d]	<0.0001[a]	0 (0-0)
Oxygen required at admission to hospital	n (%)	N/A	N/A	10 (14.3%)*	6 (31.6%)*	0.098[c]		N/A
Die in hospital	n (%)	N/A	N/A	(5.7%)*	(10.5%)*	0.61[c]		N/A
Hospital length of stay (survivors only)	median (IQR)	N/A	N/A	5 (1-11)	3 (1-8)	0.24[d]		N/A
Day from symptoms onset to hospital admission	median (IQR)	N/A	N/A	7 (3-10)	5 (2-7)	0.26[d]		N/A

Table 1: Demographic and clinical Characteristics of the study groups, used in the immunological analysis. Health care workers with RT-qPCR confirmed COVID-19 infection, colonised or non-colonised with pneumococcus. Patients presented to the hospital with RT-qPCR confirmed COVID-19 infection, colonised or non-colonised with pneumococcus. Healthy controls were samples collected from human studies before 2019. * Positivity proportion was calculated using the denominator for individual variables. NIH; National Institutes of Health clinical score for assessment of clinical spectrum of SARS-CoV-2 infection. a: Kruskal-Wallis test, b: Chi-square test, c: Fisher's exact test, d: Mann-Whitney test.

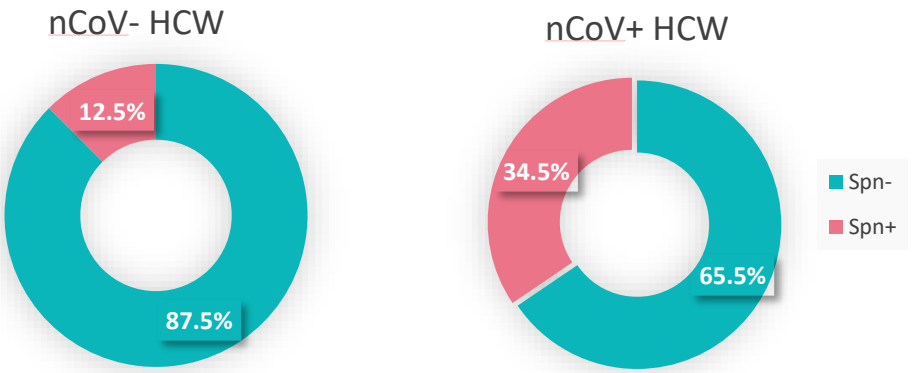
	Units	Patient_nCoV+/Spn- (n=70)	Patient_nCoV+/Spn- (n=19)	p-value
COPD	n (%)	12 (17.1%)	1 (5.3%)	0.22
Asthma	n (%)	13 (18.6%)	2 (10.5%)	0.75
Cancer	n (%)	4 (5.7%)	0 (0.0%)	0.57
Treatment with immunosuppressants	n (%)	13 (18.6%)	2 (10.5%)	0.51
Corticosteroids during admission	n (%)	29 (41.4%)	12 (63.2%)	0.12
- **Dexamethasone, 6mg**	n (%)	16 (22.9%)	7 (36.8%)	0.85

Table 2: Comorbidities and treatments used in patient subset included in the immunological analysis. Patients presented to the hospital with RT-qPCR confirmed COVID-19 infection, colonised or non-colonised with pneumococcus. Percentages between the two groups were compared using Fisher's exact test.

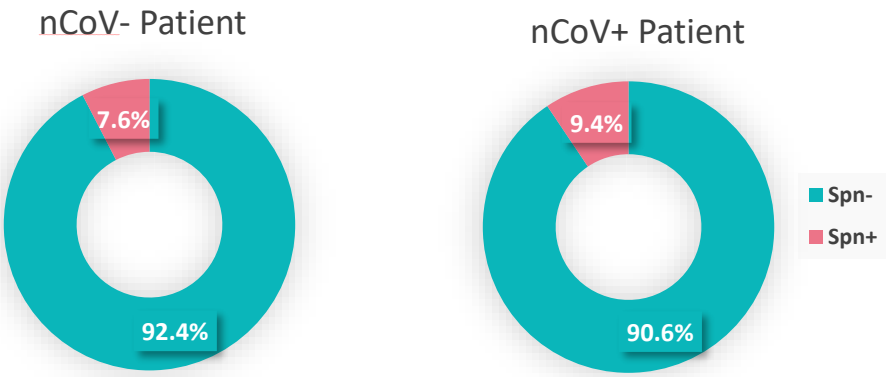
A



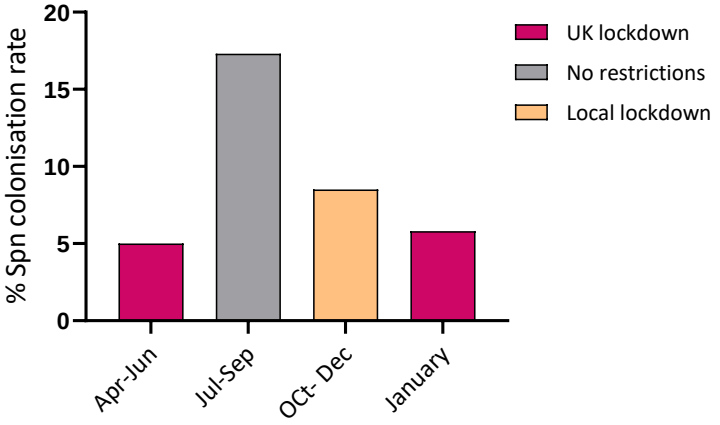
B


nCoV- HCW
12.5%
87.5%
nCoV+ HCW
34.5%
65.5%
Spn-
Spn+

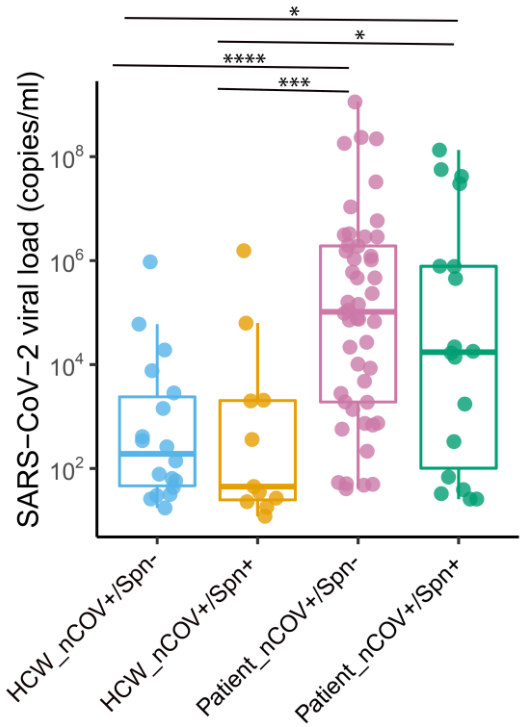
C


nCoV- Patient
7.6%
92.4%
nCoV+ Patient
9.4%
90.6%
Spn-
Spn+

D


% Spn colonisation rate
20
15
10
5
0
Apr-Jun
Jul-Sep
OCt- Dec
January
Circulation restrictions
UK lockdown
No restrictions
Local lockdown

E


SARS−CoV−2 viral load (copies/ml)
10^8
10^6
10^4
10^2
*
*
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HCW_nCOV+/Spn-
HCW_nCOV+/Spn+
Patient_nCOV+/Spn-
Patient_nCOV+/Spn+

Figure 1. Prevalence of pneumococcal colonisation amongst SARS-CoV-2 positive and negative HCWs and patients. A) Experimental design of the study with sample type, sample collection schedule and measurable per sample type depicted for both HCW and patient cohorts. In patient cohort, day2 and day7 samples were collected only for individuals who were hospitalised. **B-C**) Doughnut charts showing the percentage of pneumococcal prevalence in **B)** HCWs (n=85) and **C)** patients (n=400), infected and non-infected with SARS-CoV-2. Fisher's exact test was used to compare percentages. **D)** Percentage of pneumococcal colonisation rate detected in patient cohort during calendar periods of different circulation restrictions rules applied. 5% (6/119) from April- June, 17.3% (13/75) from July to September, 8.5% (13/154) from October to December and 5.8% (3/52) in January. **E)** Levels of viral load, expressed as RNA copies/ml as detected by Genesig RT-qPCR in HCW non-colonised (n=19, light blue) and Spn-colonised (n=10, yellow) and patient non-colonised (n=73, lilac) and Spn-colonised (n=19, green). *p <0.05, ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test for comparisons between groups.

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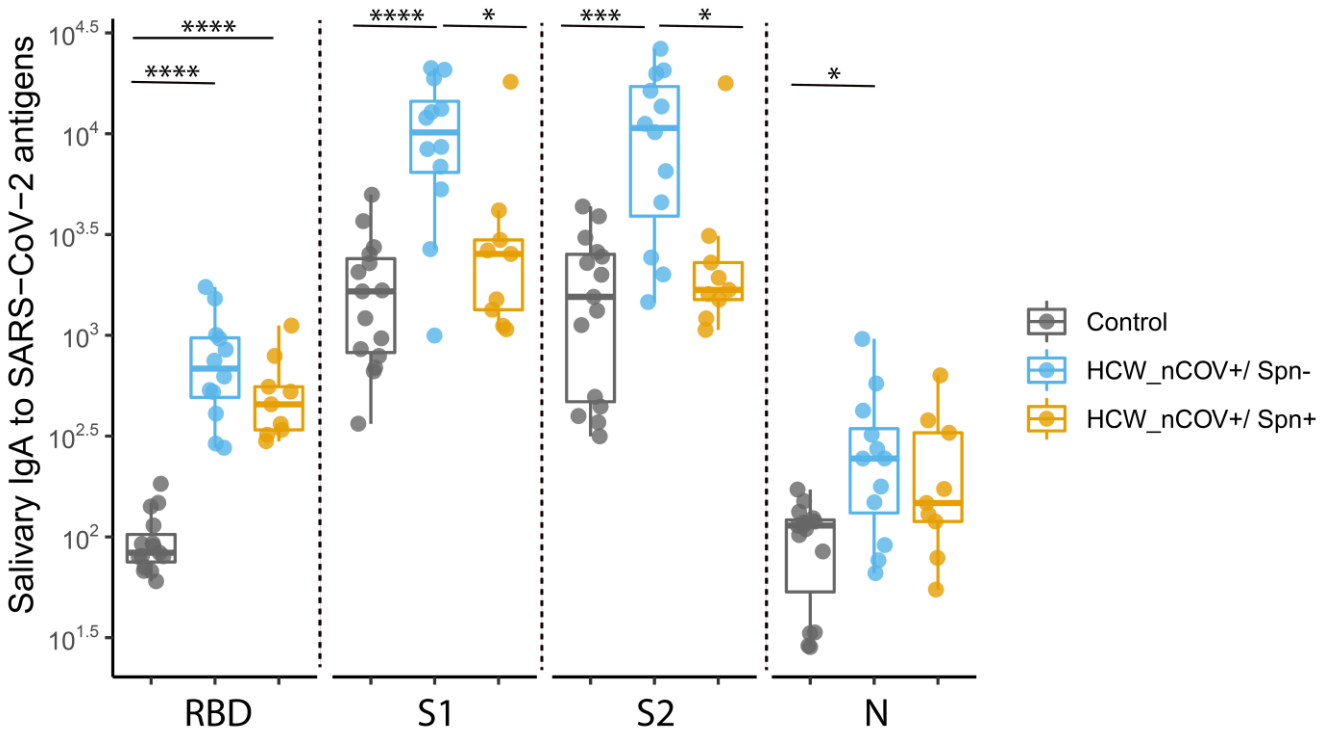

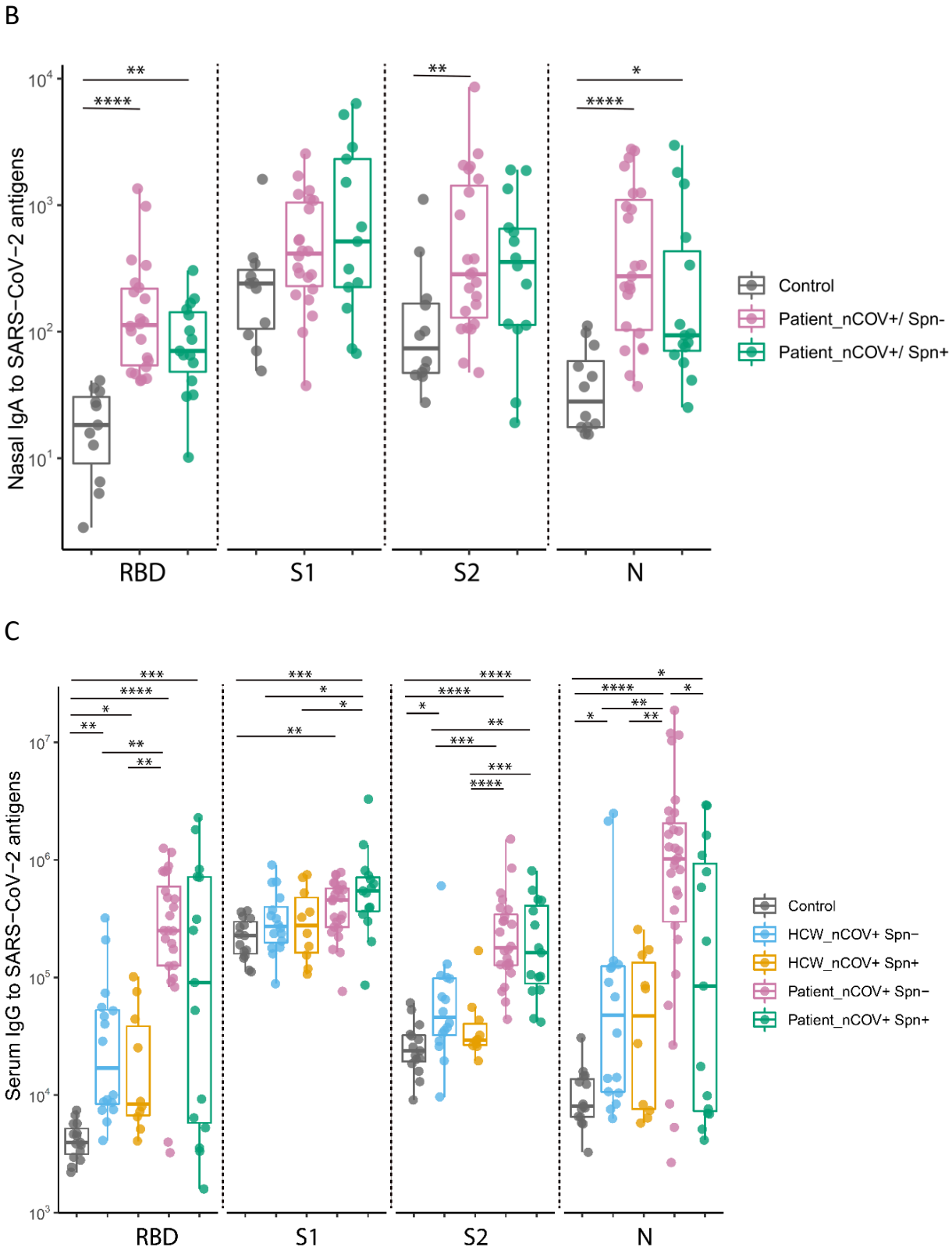

Figure 2. Mucosal and systemic antibody responses to SARS-CoV-2 in HCWs and patients. **A)** Salivary IgA titres to SARS-CoV-2 RBD, S1, S2 and N protein in HCWs, divided into non-colonised (n=12) and Spn-colonised (n=9), and unexposed healthy controls (n=15). **B)** Nasal IgA titres to SARS-CoV-2 RBD, S1, S2 and N protein in patients, divided into non-colonised (n=23) and Spn-colonised (n=15) and unexposed healthy control collected before 2019 (n=12). **C)** Serum IgG titres in HCW (non-colonised; n=16 and Spn-colonised; n=10), patients (non-colonised, n=24 and Spn-colonised, n=14) and

unexposed healthy controls (n=15). Both mucosal and serum antibody titres from SARS-CoV-2 positive participants were measured during the convalescent phase of the viral infection. Antibody levels are expressed as arbitrary units. Medians with IQRs are depicted for anti-viral responses. *p <0.05, **p<0.01, ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test for comparisons between groups.

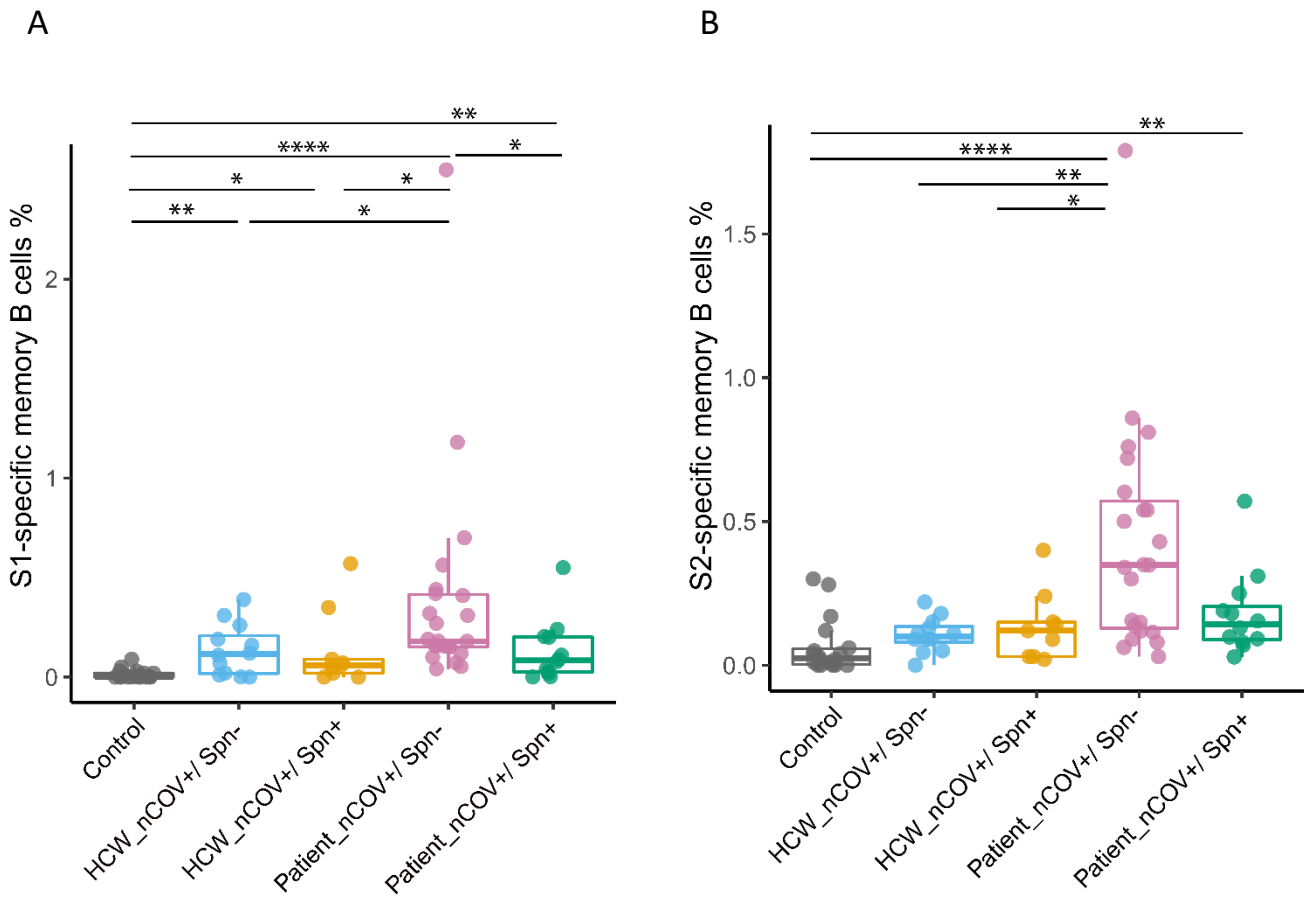

Figure 3. SARS-CoV-2 specific memory B cells in HCW and patients. Percentage of **A)** S1 and **B)** S2 specific memory B cells, within CD19+CD27+ memory B cells in HCW (non-colonised; n=12 and Spn-colonised group; n=9), recovered patients (non-colonised; n=23 and Spn-colonised; n=12) and healthy controls (n=18). Medians with IQRs are depicted and each spot represents an individual. *p <0.05, **p<0.01, ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test.

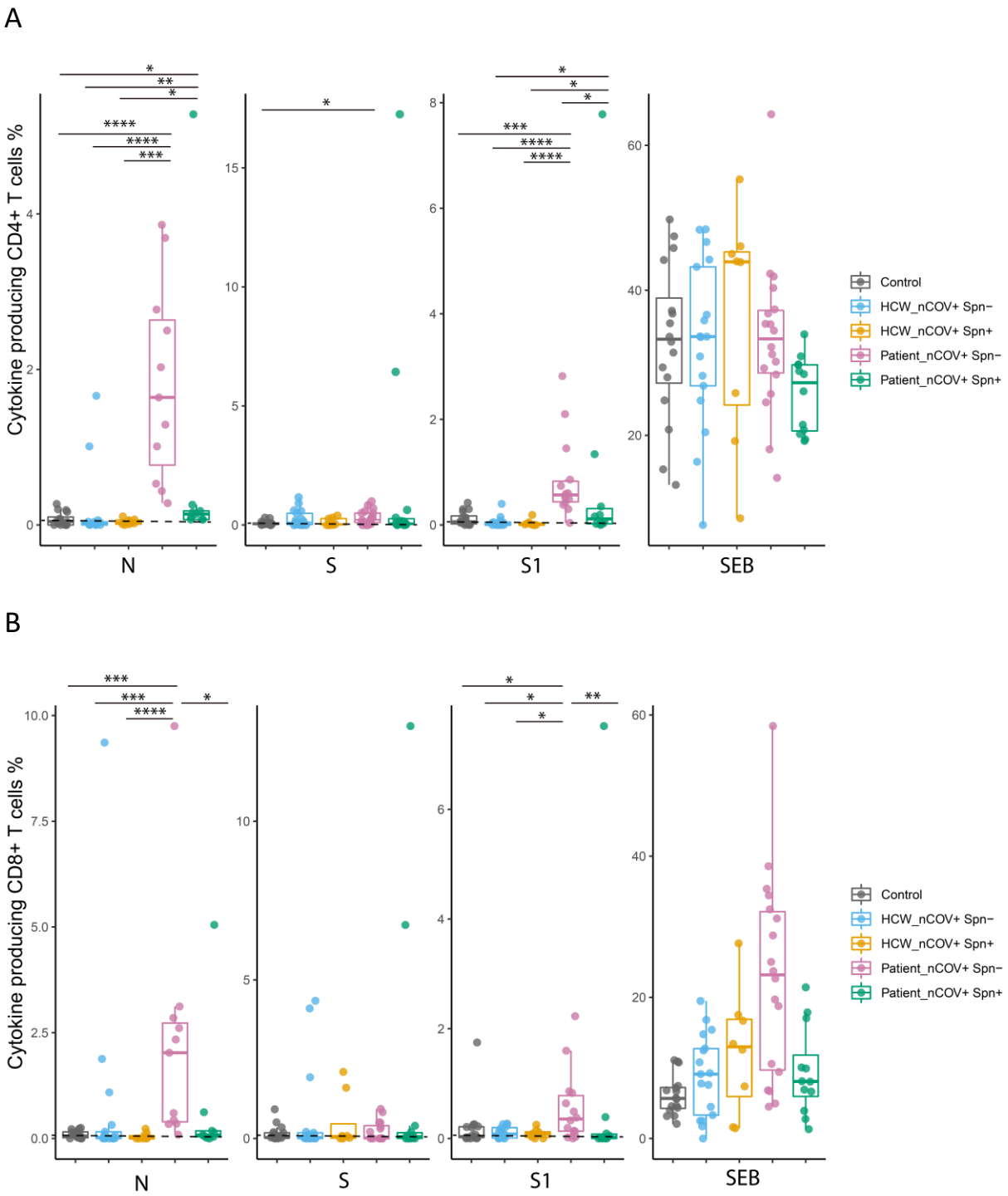

Figure 4. SARS-CoV-2 specific T cell responses in HCW and patients. Percentage of **A)** cytokine producing (IFN-γ, TNF-α, IL-2) $CD4^+$ and **B)** $CD8^+$ T cells after *ex vivo* PBMC stimulation with N, S1 and S peptides pools in SARS-CoV-2 positive HCWs (non-colonised; n=17 and Spn-colonised; n=8), recovered patients (non-colonised; n=17 and Spn-colonised; n=14) and healthy control (n=16). One peptide pool was used per condition. SEB was used as a positive control and DMSO as the negative control (non-stimulated cell condition-mock). Background (mock) was subtracted from peptide-stimulated conditions to remove non-specific signal. Data indicate positivity for any of the three measured cytokines. Medians with IQRs are depicted and each spot represents an individual. *p <0.05, **p<0.01, ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test.

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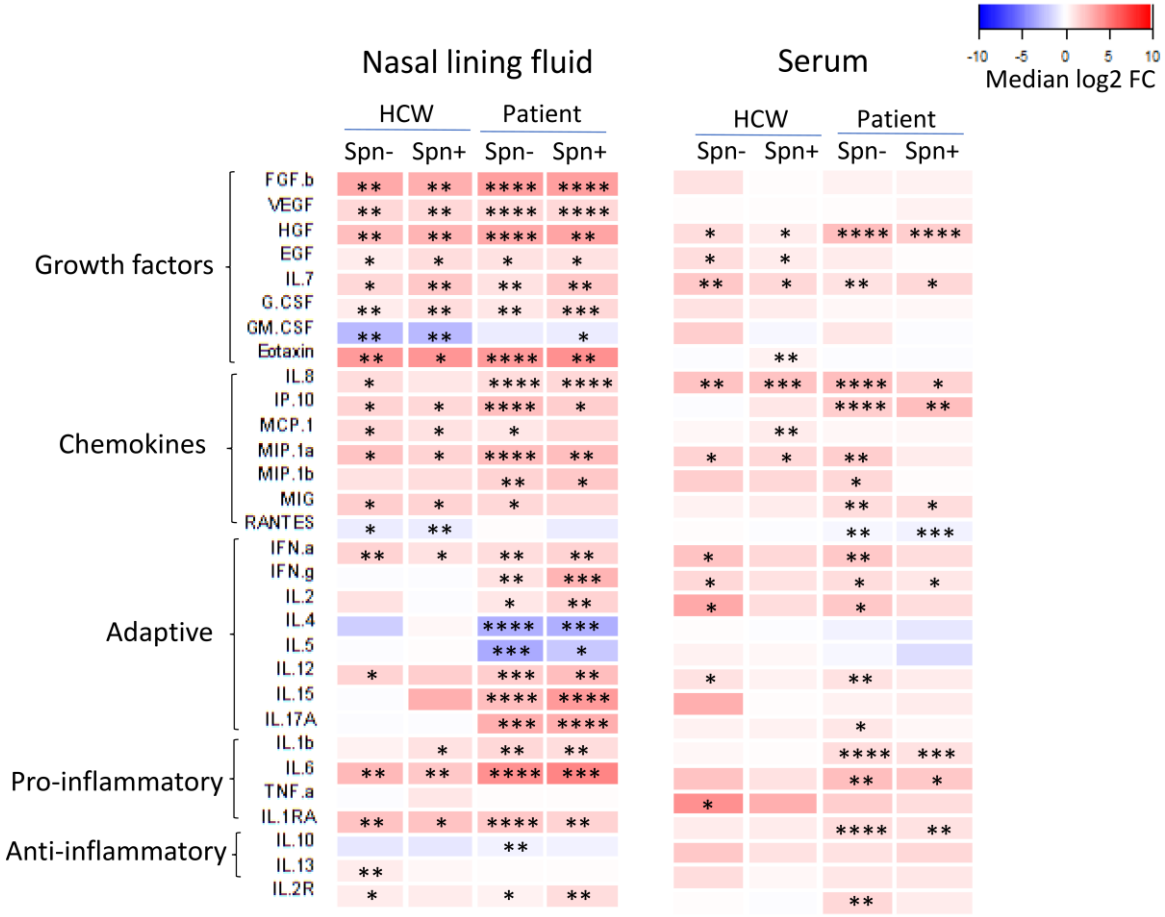
Nasal lining fluid
Serum
-10 -5 0 5 10
Median log2 FC
HCW
Patient
Spn- Spn+ Spn- Spn+
Growth factors
FGF.b
VEGF
HGF
EGF
IL.7
G.CSF
GM.CSF
Eotaxin
Chemokines
IL.8
IP.10
MCP.1
MIP.1a
MIP.1b
MIG
RANTES
Adaptive
IFN.a
IFN.g
IL.2
IL.4
IL.5
IL.12
IL.15
IL.17A
Pro-inflammatory
IL.1b
IL.6
TNF.a
IL.1RA
Anti-inflammatory
IL.10
IL.13
IL.2R

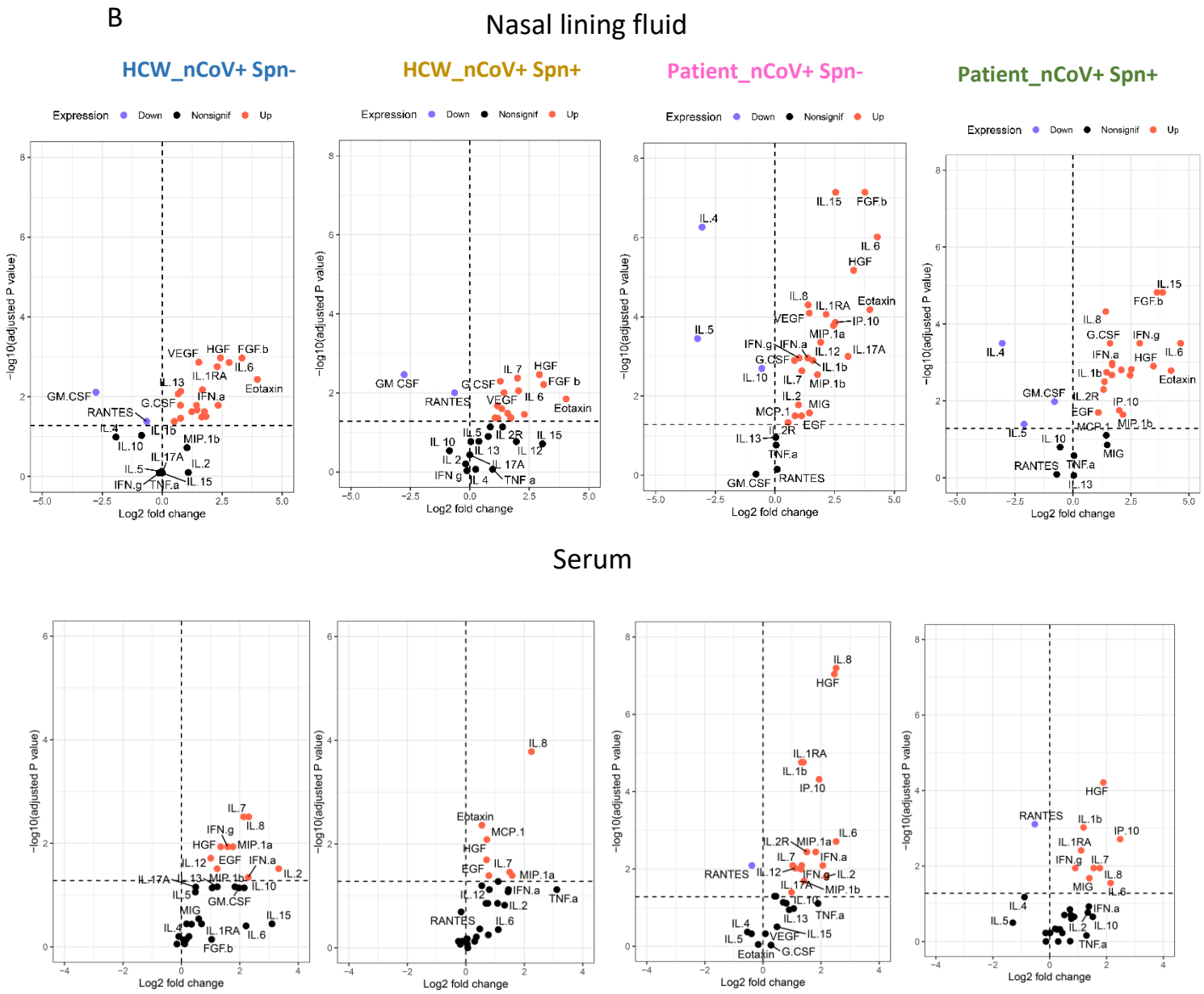

Figure 5. Cytokine concentrations in nasal lining fluid and serum. A) Heatmaps showing median log_2FC of 30-cytokine levels from unexposed healthy control group in nasal lining fluid and serum of non-colonised and Spn-colonised HCWs and patients during the acute phase of SARS-CoV-2 infection. Upregulation (red) and downregulation (blue) in cytokines' levels from control group. Cytokines were clustered in active cytokine families**. B)** Volcano plots showing median log2 fold-change from healthy control (n=17) per cytokine in nasal lining fluid and serum of non-colonised HCW(n=17) and Spn-colonised HCW (n=9), non-colonised patient (n=70) and Spn-colonised patient group (n=19). The horizontal dotted line represents the cut-off of significance (p adjusted=0.05, after correction of p value for false discovery rate), while the vertical dotted line represents a cut-off point for determining whether the levels of cytokines were higher (right, red) or lower (left, blue) compared to healthy control group. Statistical comparisons were applied between each study group and the healthy control group using Mann-Whitney test, following Benjamini-Hochberg correction for multiple testing.

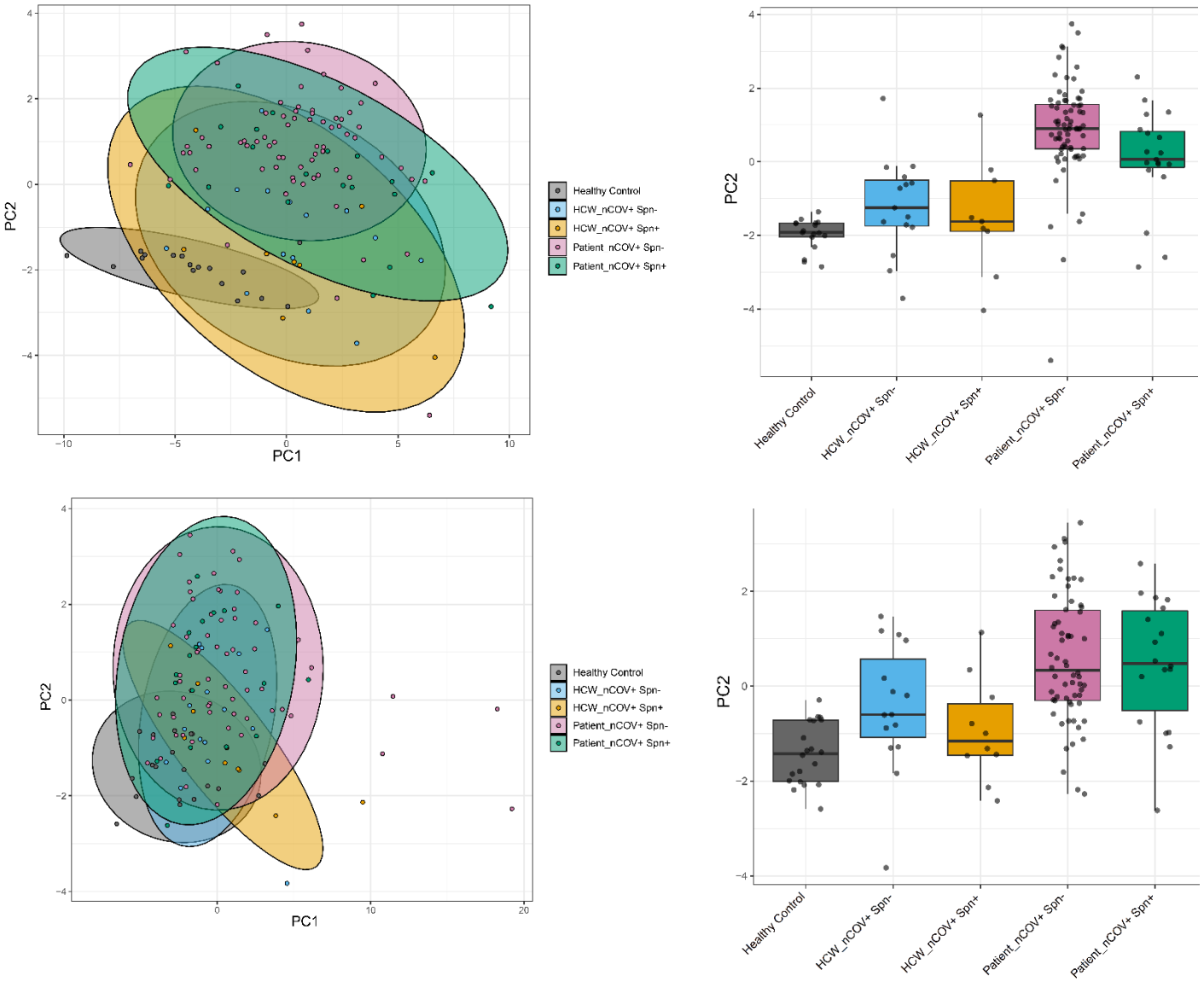

Figure 6. Nasal and serum inflammatory profiles of SARS-CoV-2 infection and co-infection with *S. pneumoniae* in HCWs and patients. Principal component analysis (PCA) of 30 cytokines in **A)** nasal lining fluid and **B)** serum of healthy control (grey), non-colonised HCW (light blue), Spn-colonised HCW (yellow), non-colonised patients (lilac) and Spn-colonised patient group (green). PC1: principal component 1; PC2, principal component 2.

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