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ORIGINAL RESEARCH ARTICLE

Immunogenicity and crossreactivity of antibodies to the nucleocapsid protein of SARS-CoV-2: utility and limitations in seroprevalence and immunity studies



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COVID-19 patients elicit strong responses to the nucleocapsid (N) protein of SARS-CoV-2 but binding antibodies are also detected in prepandemic individuals, indicating potential crossreactivity with common cold human coronaviruses (HCoV) and questioning its utility in seroprevalence studies. We investigated the immunogenicity of the full-length and shorter fragments of the SARS-CoV-2 N protein, and the crossreactivity of antibodies with HCoV. We identified a C-terminus region in SARS-CoV-2 N of minimal sequence homology with HCoV that was more specific for SARS-CoV-2 and highly immunogenic. IgGs to the full-length SARS-CoV-2 N also recognized N229E N, and IgGs to HKU1 N recognized SARS-CoV-2 N. Crossreactivity with SARS-CoV-2 was stronger for alpha- rather than beta-HCoV despite having less sequence identity, revealing the importance of conformational recognition. Higher preexisting IgG to OC43 N correlated with lower IgG to SARS-CoV-2 N in rRT-PCR negative individuals, reflecting less exposure and indicating a potential protective association. Antibodies to SARS-CoV-2 N were higher in patients with more severe and longer duration of symptoms and in females. IgGs remained stable for at least 3 months, while IgAs and

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Submitted for Publication December 22, 2020; received submitted February 7, 2021; accepted for publication February 9, 2021.

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1931-5244/\$ - see front matter

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<https://doi.org/10.1016/j.trsl.2021.02.006>

IgMs declined faster. In conclusion, N protein is a primary target of SARS-CoV-2-specific and HCoV crossreactive antibodies, both of which may affect the acquisition of immunity to COVID-19. (Translational Research 2021; 232:60–74)

Abbreviations: ADE = antibody-dependent disease enhancement; COVID-19 = coronavirus disease 2019; CT = C-terminus; FL = full-length; HCoV = common cold human coronavirus; LOESS = locally estimated scatterplot smoothing; M = month; MFI = median fluorescence intensity; N = nucleocapsid; NT = N-terminus; RBD = receptor-binding domain; rRT-PCR = real-time reverse-transcriptase polymerase chain reaction; S = spike; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

AT A GLANCE COMMENTARY

Dobaño C, et al.

Background

COVID-19 patients elicit antibodies to the nucleocapsid (N) protein of SARS-CoV-2 but responses are also detected in pre-pandemic subjects, suggesting crossreactivity with common cold human coronaviruses (HCoV) and questioning its utility in seroprevalence studies. Detailed information on the immunogenicity of SARS-CoV-2 N, the extent of crossreactivity of antibodies with HCoV and its impact on immunity, are lacking.

Translational Significance

The C-terminus of SARS-CoV-2 N of minimal sequence homology with HCoV was more specific for SARS-CoV-2 and highly immunogenic and thus suitable for diagnostics. Crossreactivity was stronger for alpha- than beta-HCoV, and a negative correlation between HCoV and SARS-CoV-2 IgGs indicates a potential protective role.

INTRODUCTION

The identification of the antigens and epitopes that induce antibody responses after exposure to SARS-CoV-2 infection is one of the requirements to estimate the seroprevalence in a population. In addition, it is essential to understand immunity to COVID-19 disease. Previous knowledge on other related coronaviruses and the prompt sequencing of the SARS-CoV-2 genome early in the pandemic allowed to identify the spike (S)¹ and the nucleocapsid (N)² structural proteins as major targets of antibodies. Consequently, both antigens constituted the basis for most immunoassays developed to study COVID-19 distribution and protective immune responses. The surface glycoprotein S, which contains the receptor-binding domain (RBD), has a better known function in immunity³⁻⁵ and is the

leading antigen candidate for vaccine development.^{6,7} N is smaller than S, lacks a glycosylation site, and is extensively used in leading serodiagnostics kits⁸⁻¹¹ due to its abundant expression during infection¹²⁻¹⁴ and early antibody response,^{15,16} but its immunological relevance is less established. The pattern of antibody responses to S compared to N may vary according to disease severity¹⁷ and age.¹⁸ N forms ribonucleoprotein complexes during the virion assembly process by binding to the viral RNA genome and packing it into long helical structures.¹⁹ Its main function is to regulate viral RNA transcription during replication, promoting the synthesis of its own proteins¹⁴ while interfering with the metabolism,^{19,20} protein translation,²¹ and proliferation²² of the infected host cell. During the process of infection, N dissociates itself from the genome and is exposed to the host immune system,²³ and its high immunogenicity has also prompted its exploration as vaccine target.^{13,24}

We recently developed a multiplex quantitative suspension array (qSAT) assay using the xMAP Luminex technology.²⁵ We included antigen fragments of S, N, and membrane (M) SARS-CoV-2 proteins to establish the utility of each construct for seroprevalence and correlates of immunity studies. Initial investigations using plasma or serum samples from individuals infected with SARS-CoV-2 and pre-pandemic samples (negative controls), showed different levels of immunogenicity and specificity across the antigens and fragments tested. Even though antibodies to full-length N (N FL) constructs were usually high in adult COVID-19 cases, moderate to high responses were frequently detected in samples collected before the pandemic,²⁵ as it had been reported before also for SARS-CoV-1 samples.²⁶ This questioned the use of N FL for seropositivity calculations that were consequently restricted to S-based antigens in our initial evaluations.²⁷ We hypothesized that such antibody signals in negative controls, more prominent for N than S antigens, reflected crossreactivity with human coronaviruses of the common cold (HCoV) due to highly conserved regions, rather than nonspecific or polyreactive responses. The HCoV include alphacoronaviruses (229E and NL63) and betacoronaviruses (HKU1 and OC43). In fact, increasing number of reports in the literature are consistent with some antibodies to HCoV being

crossreactive with the betacoronaviruses SARS-CoV-2 and SARS-CoV-1.²⁸⁻³² Interestingly, significant protein similarity between SARS-CoV-1, SARS-CoV-2, and other HCoV has been reported for N, including a highly conserved motif in the N-terminal (NT) half of the protein (FY YLGTGP)³³ and relevant immunodominant epitope regions.^{24,34} Likewise, preexisting SARS-CoV-2-specific T cells have also been reported in ~40%–60% of unexposed individuals and attributed to crossreactivity with HCoV previously encountered.³⁵⁻³⁸

A key question is the relevance of those preexisting antibodies on acquisition of COVID-19 immunity. Is this crossreactivity sufficient to protect against disease?³⁹ If this is the case, it could be one of the reasons why children may be more protected than older adults.⁴⁰⁻⁴² Alternatively, these preexisting crossreactive antibodies could interfere with the development and/or maintenance of effective levels of anti-SARS-CoV-2 antibodies^{43,44} and, even worse, they could have a negative impact by mediating antibody-dependent disease enhancement (ADE),^{45,46} which could be associated with severe prognosis.^{47,48}

Our study aimed to better characterize the immunogenicity, specificity and crossreactivity of anti-N antibodies from SARS-CoV-2 and 229E, HKU1, NL63, and OC43 HCoV, measured simultaneously. To this end, we tested several antigenic fragments in multiplex to measure different immunoglobulin (Ig) isotypes, and compared their relative immunogenicity in pre-pandemic and pandemic samples, including SARS-CoV-2 positive cases. In addition, we aimed to better understand the demographic, clinical, and epidemiological variables affecting the levels of antibodies to N SARS-CoV-2 in exposed people. Our study helps addressing the extent and characteristics of this crossreactivity in the immune response to COVID-19 and the utility of various N-based antigens in serodiagnostics and seroprevalence studies.

METHODS

Antigens. SARS-CoV-2. Heterologously expressed N FL protein was either purchased from a commercial source (GenScript, Z03480), or purified in house after HEK cell⁴⁹ or *Escherichia coli* expression.²⁵ Furthermore, 2 *E. coli* expressed His-tagged N protein constructs comprising the NT domain (residues 43–180) and C-terminal (CT) domain (residues 250–360) were purified by affinity chromatography, as described.²⁵ Previous works carried out with SARS-CoV-1 N protein, sharing >90% identity to SARS-CoV-2 N, revealed the presence of 4 main immunodominant regions (EP1–4) in different domains of the N protein (Fig S1A).^{24,28} We focused on the B cell immunodominant domain EP4 (residues 348–416) showing a lower percentage of aminoacid identity with other HCoVs,

which corresponds to the CT region of SARS-CoV-2 N protein (Fig S1B). Thus, a shorter fragment of the CT region (CT-short) was also heterologously expressed in *E. coli* cells and His-tag purified, as described.²⁵ Finally, an even shorter peptide, based on a less conserved region of the CT end (QRQKKQQT VLLPAADLDDFSKQ, residues 384–406, referred as CT-peptide) was synthesized by BCNPeptides (Barcelona, Spain) (Fig S1).⁵⁰ Additional information on the N constructs is provided in the Supplementary material.

HCoV. N FL recombinant proteins from OC43, HKU1, NL63, and 229E HCoVs were codon-optimized for *E. coli* heterologous expression and His-tag purified as in Dobaño *et al.*²⁵

Study volunteers and samples. We analyzed 3 set of samples (Table S1), (i) pre-pandemic plasmas from healthy adults collected before the COVID-19 outbreak (negative controls, $n = 128$), (ii) pandemic plasmas from health care personnel working at Hospital Clínic in Barcelona (Spain) collected as part of a study on SARS-CoV-2 seroprevalence at the March–April 2020 outbreak ($n = 578$) of which SARS-CoV-2 infected cases were asymptomatic or had mild symptoms,^{27,51} and (iii) pandemic plasmas from 49 COVID-19 patients recruited at the Clínica Universidad de Navarra in Pamplona (Spain), of which 47 had severe symptoms and were hospitalized and 2 had mild symptoms.²⁵

For the characterization of the immunogenicity of the various SARS-CoV-2 N protein constructs, we used all pre-pandemic and 104 pandemic samples. For the analysis of HCoV N FL crossreactivity, we selected 30 pre-pandemic samples with the highest levels of IgG to SARS-CoV-2 N FL and 30 pre-pandemic samples with the lowest levels, among our set of negative controls tested previously (Fig S2).²⁵ Pandemic samples included: (i) 60 plasmas from individuals with a SARS-CoV-2 infection confirmed by real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) and seropositive for RBD tested in our prior studies^{27,51} (29 plasmas with the highest and 31 plasmas with the lowest levels of IgG to SARS-CoV-2 N FL), (ii) 30 plasmas from individuals with a negative rRT-PCR and RBD serology but with high IgG to N FL, and (iii) 7 negative individuals at baseline (month [M]0) who later got infected and seroconverted for RBD at M1.

For the analysis of factors associated with antibody levels to SARS-CoV-2 N FL protein and their kinetics, we tested all the plasma samples available from the cohort of health care workers in Barcelona at baseline (M0 = 578) and 1 and 3 months later (M1 = 565, M3 = 70).^{27,51}

The research was carried out according to the principles of the Declaration of Helsinki and informed consent was obtained. Samples analyzed in this study received ethical clearance for immunological

evaluation and/or inclusion as controls in immunoassays, and the protocols and informed consent forms were approved by the Institutional Review Board at Hospital Clínic in Barcelona (Refs. CEIC-7455 and HCB/2020/0336) or Universidad de Navarra (Ref. UN/2020/067) prior to study implementation.

Measurement of IgM, IgG, and IgA antibodies. qSAT assays to measure plasma IgG, IgA, and IgM against SARS-CoV-2 N protein constructs were performed as reported²⁷ and analyzed in a Luminex 100/200 instrument. For the assessment of IgG crossreactivity between HCoV and SARS-CoV-2 N proteins, we applied an optimized protocol, as described,²⁵ and samples were analyzed with a FlexMap 3D instead of a Luminex 100/200 instrument, to increase the dynamic quantification range of antibody values in high responders (Fig S3). Briefly, N constructs coupled to magnetic microspheres (Luminex Corporation, Austin) were incubated with plasma samples (1/500 and/or 1/3500 dilutions) or blank controls in 96-well plates. For the CT-peptide, avidin-beads were used that bound the biotin conjugated to a PEG12 biopolymer linked to the peptide, and the coupling performed following manufacturer's instructions. Before multiplexing N protein constructs coupled to MagPlex beads, we tested for potential interference among N constructs comparing multiplex to singleplex assays (Fig S3). After sample incubation with beads, plates were washed and a labeled secondary antibody (anti-human IgG, IgM, or IgA) was added. Following the last incubation, plates were washed and read in a Luminex xMAP analyzer. Crude median fluorescent intensities (MFI) and background fluorescence from blank wells were exported using the xPONENT software.

Statistical data analysis. Boxplots and Wilcoxon Rank Sum tests were used to compare antibody levels to each N construct between study groups. To assess for crossreactivity, we performed correlations (Spearman) and heatmaps of antibody responses to SARS-CoV-2 and HCoV N antigens in prepandemic and pandemic samples separately, stratifying by rRT-PCR positivity and by high vs low anti-N FL IgG responders, as appropriate. Seropositivity was defined by a cutoff calculated with prepandemic samples as 10 to the mean plus 3 standard deviations of \log_{10} -transformed MFI. A variable called magnitude of responses to HCoV N FL was created by adding up the levels of IgG to the 4 HCoV N FL. To evaluate the factors associated with levels of antibodies to SARS-CoV-2 N FL protein, we used Wilcoxon Rank Sum test. The LOESS (locally estimated scatterplot smoothing) method was used to fit a curve to depict kinetics of antibody levels over time. The analysis was carried out using the statistical software R studio version R-4.0.2⁵² (packages used: tidyverse,⁵³ pheatmap,⁵⁴ and corplot⁵⁵).

RESULTS

Immunogenicity, specificity, and seropositivity of SARS-CoV-2 N antibodies. Plasmas from individuals diagnosed with COVID-19 had statistically significantly higher levels of IgG, IgA, and IgM to the N FL protein as well as the NT and CT domains of SARS-CoV-2 N protein than prepandemic plasmas (Fig 1). Levels were statistically significantly higher in patients who were hospitalized compared to nonhospitalized (asymptomatic and mild cases) (Fig 1A). However, prepandemic plasmas also contained IgG, IgM and IgA antibodies recognizing N antigens. In the case of IgG, the responses against N FL protein were the highest, followed by the N NT and N CT domains. This high reactivity of negative control plasmas resulted in a higher seropositivity cutoff and lower seroprevalence estimates for IgG to N FL protein in our cohort of health care workers, with 10.1% at M1 (Fig S4A) compared with 11.3% for RBD.²⁷ Higher plasma dilutions (1/3500 vs 1/500) reduced antibody levels proportionally more in prepandemic than pandemic samples, increasing signal-to-noise ratio, sensitivity and overall seropositivity (Fig S4B). Nevertheless, seroreactivity in prepandemic samples was still patent, which could be attributed to the presence in our antigens of stretches of immunogenic aminoacid sequences similar to other HCoV.^{24,28} Searching for SARS-CoV-2 specific responses, we tested the N CT-short protein fragment and the N CT-peptide that had a lower percentage of identity with HCoVs (Fig S1). The shorter CT constructs were immunogenic in pandemic positive samples and less seroreactive in prepandemic samples than the N FL protein, with higher signal-to-noise ratios obtained (Fig 1B). Additional information on the serological characterization of the rest of N FL constructs is provided in the Supplementary material, Figs S5 and S6.

Crossreactivity of IgG antibodies to SARS-CoV-2 and HCoV N FL proteins. To test whether the antibodies recognizing SARS-CoV-2 N antigens in negative samples could be due to crossreactivity with HKU1, OC43, NL63 or 229E HCoV N antigens, we compared the patterns of IgG responses to N FL proteins from the 5 coronaviruses in prepandemic and COVID-19 samples at 2 timepoints, also including the SARS-CoV-2 N CT-short fragment. Overall, anti-N IgG levels to SARS-CoV-2 were significantly higher in COVID-19 cases vs prepandemic samples compared to anti-N IgG levels to HCoV, which were similar or slightly higher in pandemic positive vs prepandemic samples, like for IgG to N from NL63 (Fig 2A). This pattern indicates both high immunogenicity of SARS-CoV-2 N antigens in COVID-19 samples, but also some level of crossreactivity against some HCoV N antigens. Furthermore, data support the higher SARS-CoV-2-specificity of the

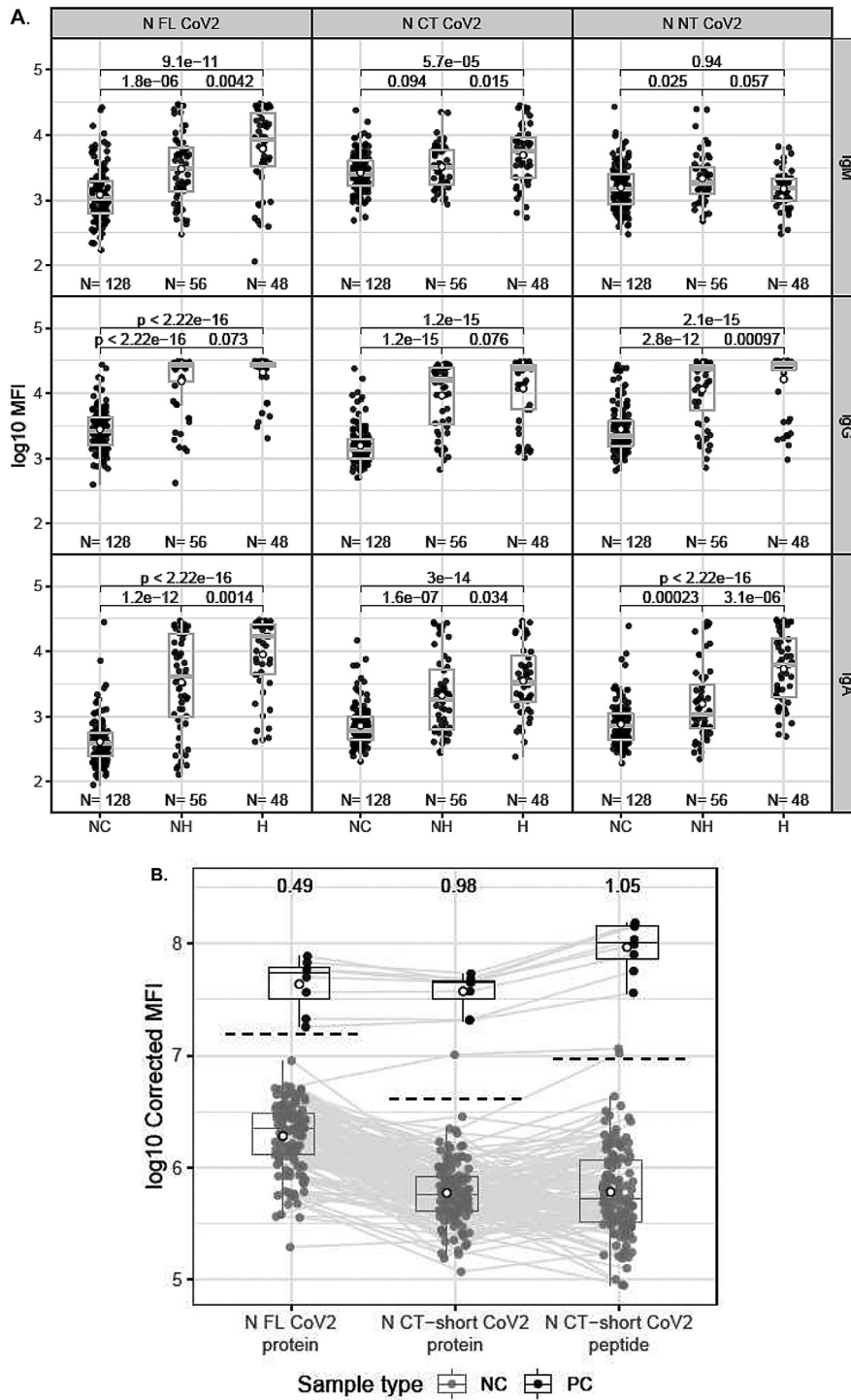


Fig 1. Levels (log₁₀ median fluorescence intensity, MFI) of antibodies to nucleocapsid (N) SARS-CoV-2 antigens in pre-pandemic (NC) and pandemic samples. The lower and upper hinges of the boxplots correspond to the 25th and 75th percentiles (IQR) and extend 1.5 * IQR from the hinge; open circles are means. (A) N FL: full-length produced at ISGlobal; N C-terminus [CT]; and N N-terminus [NT] domains produced at CRG. NH, non-hospitalized; H, hospitalized. *P* values were calculated by the Wilcoxon test. (B) N FL and N CT-short from ISGlobal and N CT-peptide. Numerical values on the top of the boxplots are the ratios of the means of positive controls (PC) to the seropositivity cutoff values indicated by black dashed lines, calculated as 10 to the mean plus 3 standard deviations of the log₁₀-transformed MFI of the NC. The gray lines link the samples from the same individuals. PC are the different points of the titration curve corrected to have the same dilution factor.

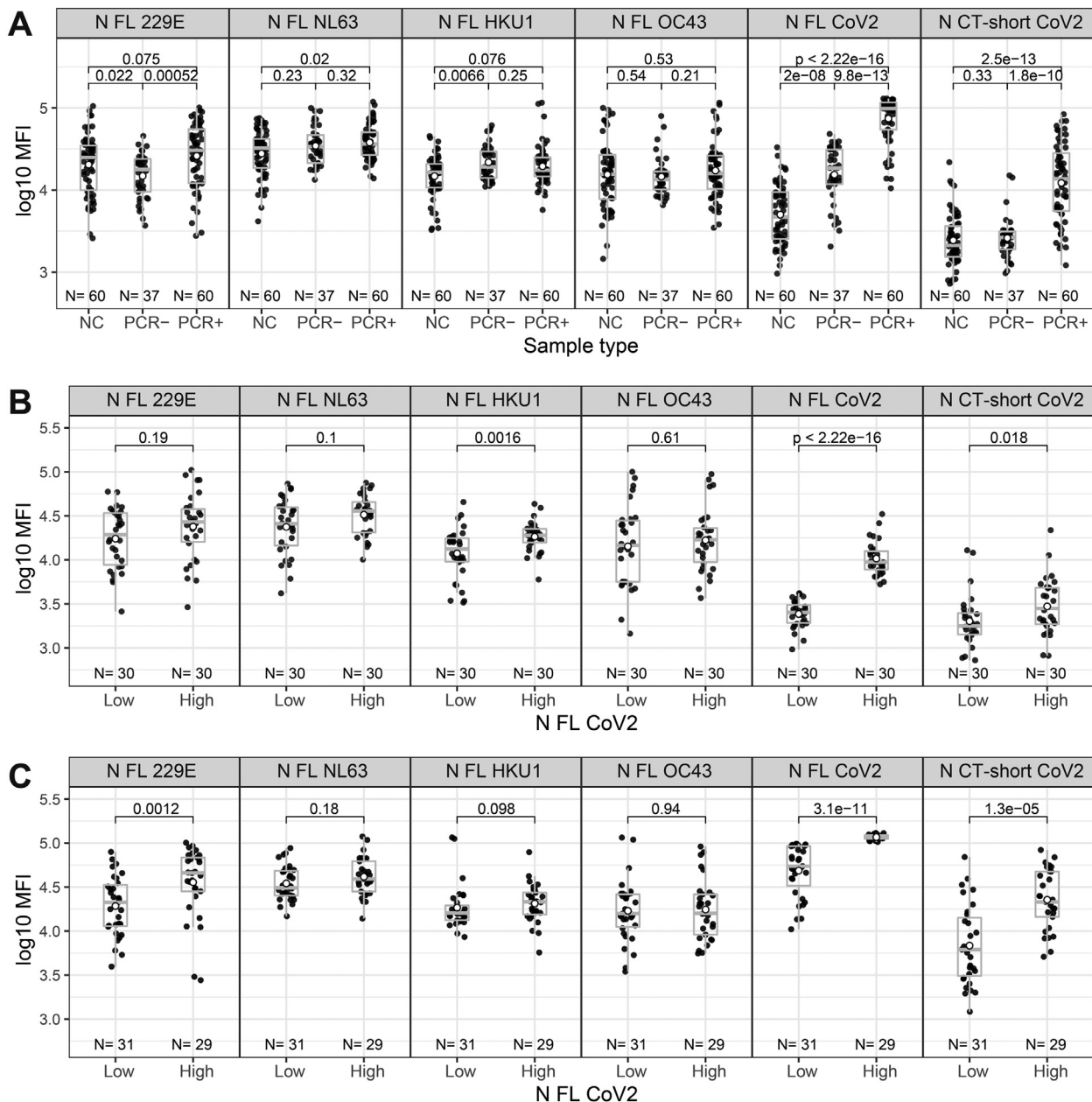


Fig 2. Levels (\log_{10} median fluorescence intensity, MFI) of IgG antibodies to N antigens of human coronaviruses. (A) Plasmas from pre-pandemic (NC) and pandemic (rRT-PCR positive and negative) individuals. Plasmas stratified according to low vs high IgG responses to SARS-CoV-2 N FL in (B) pre-pandemic, and (C) pandemic rRT-PCR positive. Boxplots indicate median and IQR, open circles are means, and *P* values were calculated by Wilcoxon test.

IgG response to the N CT-short fragment because IgG levels in pre-pandemic individuals were the lowest and similar to levels in pandemic rRT-PCR negative subjects (Fig 2A). Pandemic samples from rRT-PCR negative health care workers had significantly higher levels of IgG to N FL protein, but not N CT-short fragment, than pre-pandemic samples and significantly lower levels than rRT-PCR positive individuals.

In pre-pandemic samples, IgG levels to HKU1 N FL protein were statistically significantly higher in samples with higher IgG levels to SARS-CoV-2 N FL protein (Fig 2B). In addition, there was a significant correlation ($\rho = 0.35$, $P = 0.0065$) between IgG to N FL from SARS-CoV-2 and HKU1, and to a lesser extent with N FL from NL63 (Fig 3A), suggestive of crossreactivity. In samples from pandemic rRT-PCR positive

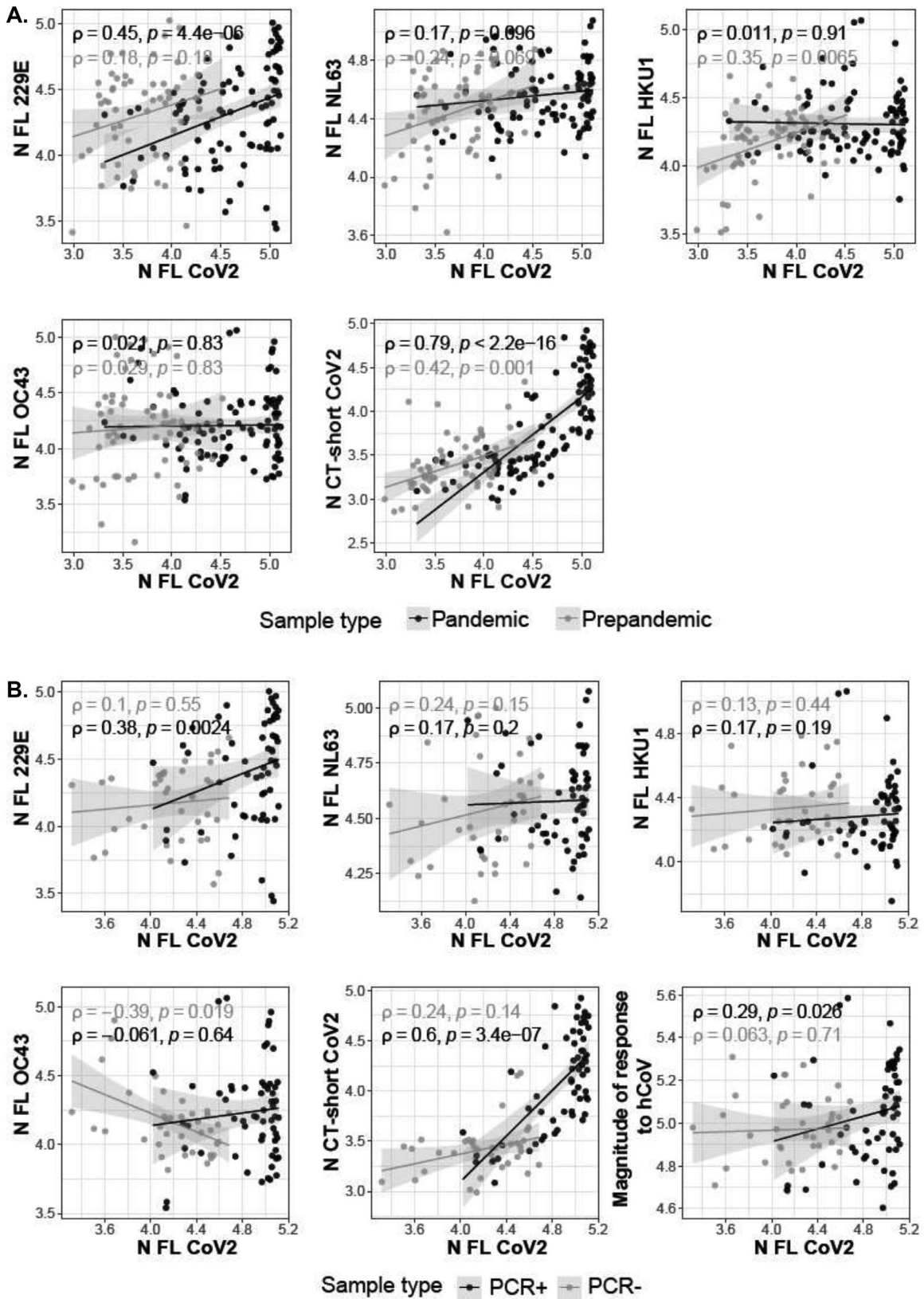


Fig 3. Correlations of plasma IgG levels (\log_{10} MFI) to SARS-CoV-2 and HCoV N antigens. (A) Pandemic and prepandemic samples. (B) Pandemic rRT-PCR positive and negative. (C) Magnitude of response to HCoV in pandemic and prepandemic samples. ρ (rho) and P values were calculated by Spearman, shaded areas represent 0.95 confidence intervals.

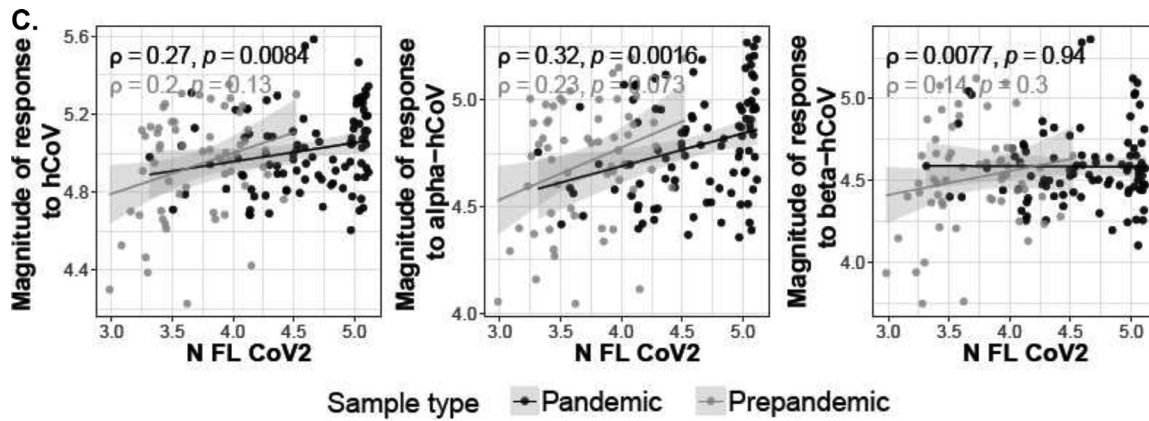


Fig 3 Continued.

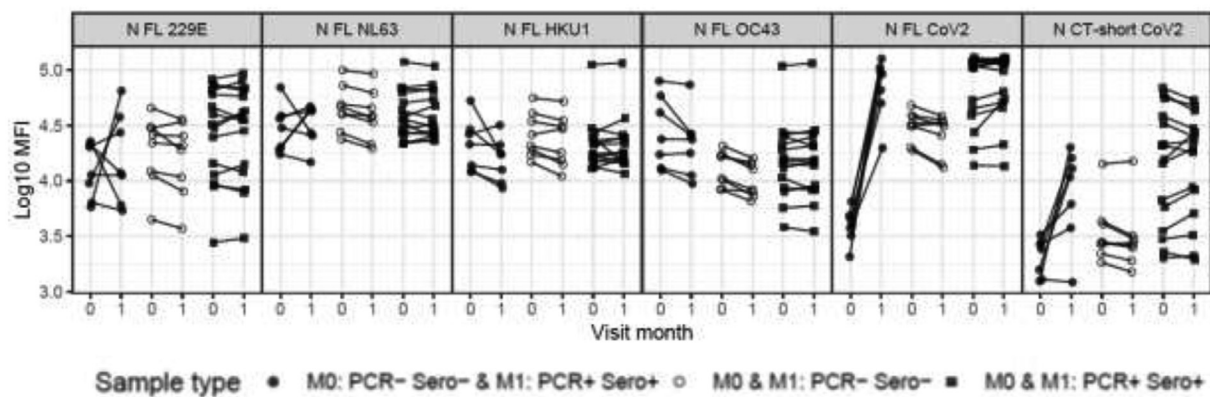


Fig 4. Change in antibody levels to human coronavirus nucleocapsid (N) proteins from baseline (M0) to 1 month later (M1) depending on the rRT-PCR SARS-CoV-2 infection and seropositivity to RBD status (positive [+] or negative [-]).

individuals, IgG levels to 229E N FL protein were statistically significantly higher in samples with higher IgG levels to SARS-CoV-2 N FL protein (Fig 2C), and there was a significant correlation of N FL IgG responses between SARS-CoV-2 and 229E ($\rho = 0.38-0.45, P < 0.0024$) (Fig 3A and B). Correlation between N FL protein and N CT-short fragment was higher and more significant in samples from pandemic ($\rho = 0.79, P < 0.001$) and rRT-PCR positive individuals ($\rho = 0.6, P < 0.001$), compared to samples from prepandemic and rRT-PCR negative subjects, respectively (Fig 3A and B). In contrast, there was a significant inverse correlation between OC43 and SARS-CoV-2 IgG levels in samples from rRT-PCR negative pandemic individuals ($\rho = -0.39, P = 0.019$) (Fig 3B). Integrating the magnitude of antibody responses to the four HCoV N FL proteins, the correlation with SARS-CoV-2 N FL was statistically significant in samples from pandemic ($\rho = 0.29, P = 0.026$, Fig 3B) and rRT-PCR positive subjects ($\rho = 0.27, P = 0.0084$, Fig 3C), and it was higher and only significant for alpha- (229E and NL63) than for

beta-HCoV (HKU1 and OC43) (Fig 3C). The correlation in prepandemic samples improved when IgG to N from OC43 beta-HCoV was excluded from the HCoV antibody summation ($\rho = 0.25, P = 0.053$ vs $\rho = 0.2, P = 0.13$), although IgG levels to OC43 N protein strongly correlated with those of IgG to N from alpha-coronaviruses ($\rho = 0.522$ for 229E, $\rho = 0.503$ for NL63; Fig S7A). Antibody levels to SARS-CoV-2 N FL correlated more significantly with antibody levels to N NT fragment in prepandemic ($\rho = 0.526$), and with antibody levels to N CT fragment in pandemic ($\rho = 0.624$) samples. The breadth of antibody responses to human coronaviruses was heterogeneous among individuals, but responses of antibodies to alpha- and beta-HCoV tended to cluster together (Fig S7B).

In individuals who became infected with SARS-CoV-2 from M0 to M1, IgG levels increased significantly for SARS-CoV-2 N FL protein ($P = 0.015$) and SARS-CoV-2 N CT-short fragment ($P = 0.031$), while antibody profiles against HCoV N proteins were variable (Fig 4). IgG to N antigen from beta-

HCoV tended to decrease or were unchanged, whereas IgG to N from alpha-HCoV (NL63 and 299E) increased in about half of individuals but overall not significantly. There did not seem to be a relationship between IgG levels at baseline and the change in levels at M1. In uninfected individuals, IgG levels declined from M0 to M1, significantly for 4 of 6 N antigens (Fig 4).

Kinetics and determinants of antibody levels to SARS-CoV-2 N FL. Having shown that SARS-CoV-2 infections induce a specific antibody response to the N antigen, despite having in some cases preexisting crossreactive antibodies due to prior exposure to HCoV, we investigated the kinetics and demographic, clinical and epidemiological variables associated with the levels of immunoglobulins to N FL protein. We analyzed samples from the cohort of health care workers followed up for 3 months in whom we had previously characterized RBD antibodies.^{27,51} We performed 2 analyses, including (i) all individuals and (ii) only those who were seropositive for SARS-CoV-2 RBD antibodies and, therefore, with evidence of exposure to the virus. IgA and IgM to SARS-CoV-2 N FL protein declined more markedly than IgG, which maintained high levels over the 3 months of study (Fig 5).

Having symptoms had a statistically significant association with higher IgA and IgG levels to SARS-CoV-2 N FL protein, and having a longer duration of symptoms had a statistically significant association with higher IgA and IgM levels to SARS-CoV-2 N FL protein (Fig 6A). IgA levels to SARS-CoV-2 N FL protein tended to be higher in older individuals, and IgM responses were statistically significantly higher in females than males (Fig 6B). No other clear associations were found for occupation or number of COVID-19 contacts.

DISCUSSION

Our study shows that SARS-CoV-2 infection induces specific IgM, IgA and IgG antibody responses to epitopes located at both the NT and CT domains of the N protein, consistent with immunogenicity data reported in the literature.^{15,20,56-58} All antibodies positively correlated with disease severity, and longer duration of symptoms was associated with higher IgA and IgM levels. Importantly, anti-N IgG antibodies remained quite stable over a period of at least 3 months following mild or asymptomatic infection, while IgAs and IgMs declined faster, similar to anti-SARS-CoV-2 RBD responses.⁵¹

We also found that significant levels of IgG, IgM, and IgA recognizing SARS-CoV-2 N protein were present in so-called pre-pandemic negative controls. This reactivity

could limit the utility of the N antigen in seroprevalence studies, as the cutoff values for IgG seropositivity are higher and hence the percentage of seropositive individuals may be underestimated, compared to calculations based on S antigens.²⁷ However, point prevalence estimates during outbreaks could still be assessed with IgA and IgM to N protein, as pre-pandemic samples have lower levels of such preexisting isotypes probably due to their shorter half-life, which is consistent with our kinetics data. The seroreactivity of N protein in negative controls also leads to lower specificity of SARS-CoV-2 diagnostic tests based on this antigen. To circumvent this, assays with truncated versions of N protein based on immunogenic regions have been developed (Roche, Abbot),^{8,10} as we have done here with N CT-short fragment and N CT-peptide.

Among the 3 regions of N protein (NT RNA-binding domain, central linker, and CT dimerization domain), the NT and CT are the major structural and functional domains.¹⁹ The N CT-short fragment and the N CT-peptide presented epitopes that were more specific and still immunogenic, thus applicable for seroprevalence and immunity studies. Correlation between N FL protein and N CT-short fragment was higher and more significant in samples from pandemic and rRT-PCR positive individuals compared to pre-pandemic and rRT-PCR negative individuals, respectively, indicative of a specific response to SARS-CoV-2 N protein upon infection.

Interestingly, pandemic rRT-PCR negative individuals had higher responses to N FL protein than pre-pandemic samples and significantly lower than rRT-PCR positive individuals. A possible explanation is that some of the rRT-PCR negative individuals might in fact have been exposed to SARS-CoV-2 despite being seronegative for RBD antibodies. This could indicate a higher sensitivity of N protein to detect low-responders or early infections.²⁸ Alternatively, the responses in rRT-PCR negatives could be due to crossreactivity with HCoV in this group of health care workers that due to their occupation might be at higher risk of recent exposure to common colds at a period when they were still prevalent (February-March). In contrast, the pre-pandemic samples had been collected at various times over the years but a large proportion in June 2008, some months after the cold season peak and the responses to HCoV are known to be short-lived.⁵⁹ In line with this, levels of IgG to HCoV N FL decreased from M0 to M1 in seronegative and rRT-PCR negative individuals. Therefore, the season of sample collection is an important variable to take into account. Other studies have reported a lack of N protein crossreactivity for antibody responses, with specificities >94%, which may be due to the protein regions included in the tests and/or the characteristics of the pre-pandemic controls included.^{60,61} In the case of SARS-CoV-1, it has been reported that

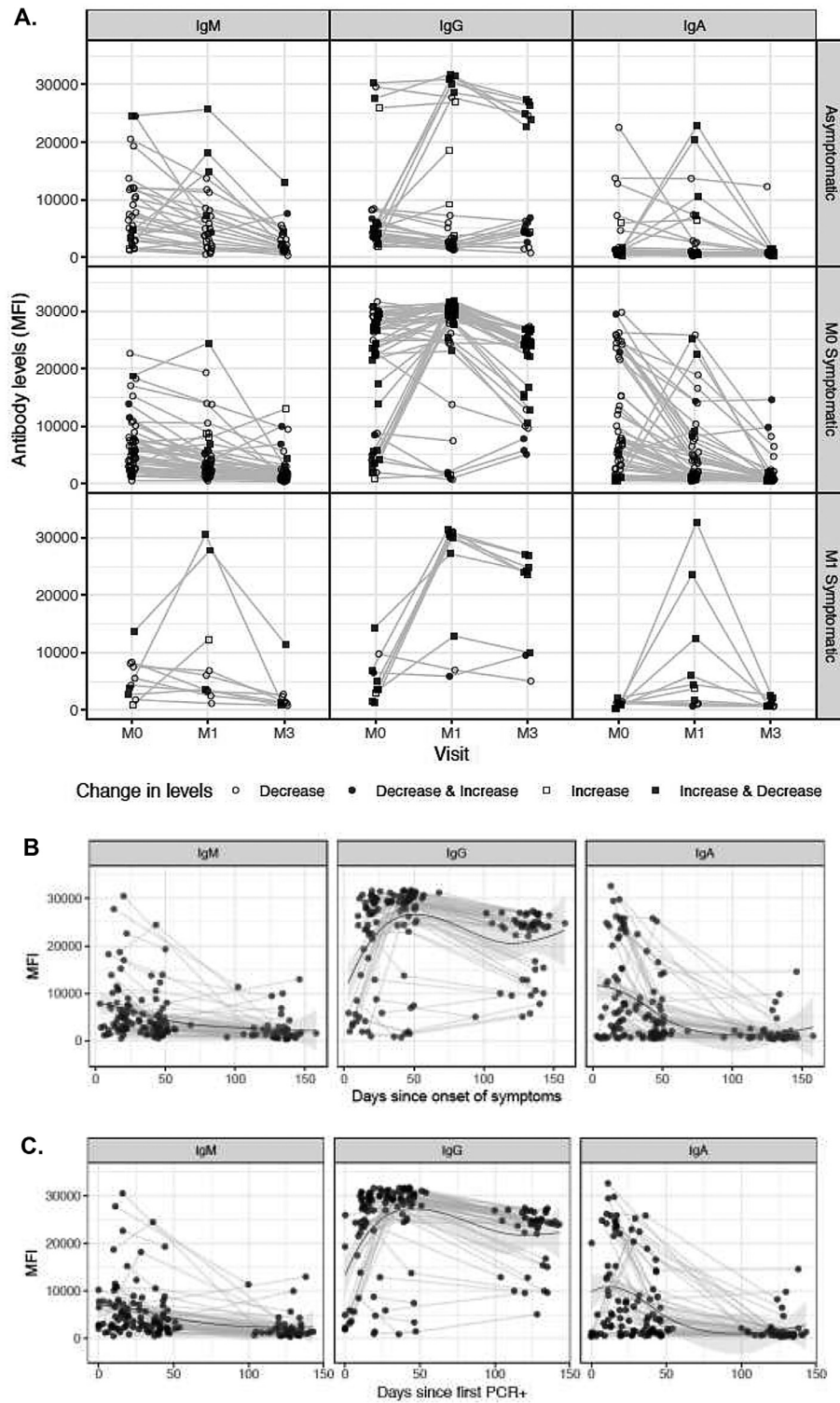


Fig 5. Kinetics of antibody responses to SARS-CoV-2 nucleocapsid (N) full-length protein in a cohort of health care workers with any evidence of infection (rRT-PCR positive or RBD antibody positive for any isotype) at any visit (month [M]0), M1, and M3). Grey lines connect the responses of the same subject across visits (A) or over time (B and C). (A) Antibody levels by visit and stratified by symptoms. (B) Antibody kinetics since onset of symptoms. (C) Antibody kinetics since first rRT-PCR positive. The curves represent the kinetics of the antibodies over time and were calculated by the LOESS (locally estimated scatterplot smoothing) method. The shaded area represents the 0.95 confidence intervals.

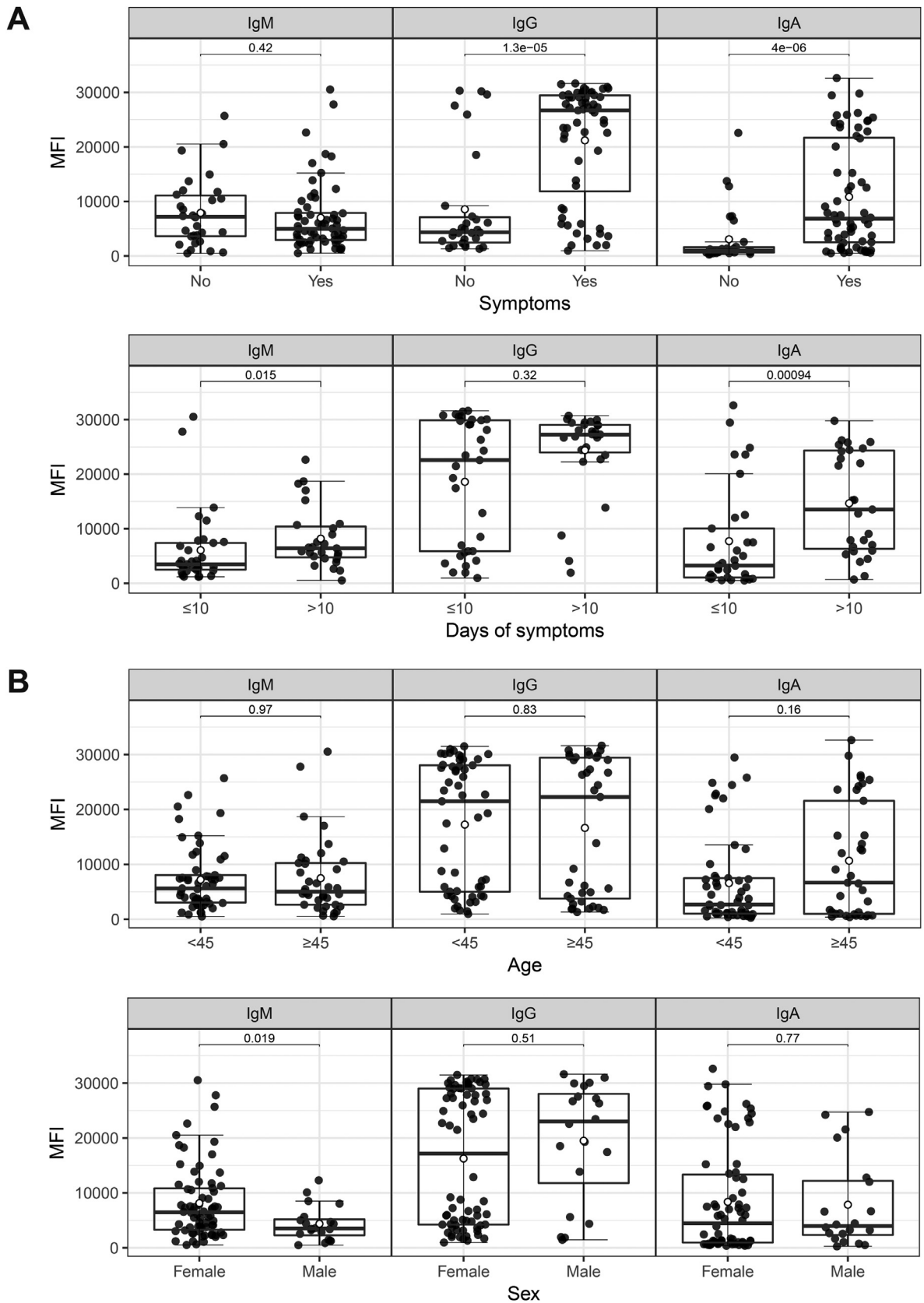


Fig 6. Factors associated to the levels of antibodies to SARS-CoV-2 nucleocapsid (N) full-length antigen. Box-plots indicate median and IQR, open circles are means, and *P* values were calculated by Wilcoxon test. Graphs

IgGs crossreact with the N antigen of endemic coronavirus, but only rarely the other way round.⁴¹

To assess if antibodies to SARS-CoV-2 and HCoV N proteins were crossreactive,^{28,41,62} we tested the longer-lasting IgG responses to N FL protein from 229E, NL63, HKU1 and OC43. These HCoV are widely distributed, with 229E and OC43 causing 15%–29% of all common colds.⁶³ 229E, OC43 and NL63 tend to be transmitted predominantly during the winter season in temperate-climate countries, while NL63 (accounting for an estimated 4.7% of common respiratory diseases) have a Spring-Summer peak. Data on SARS-CoV-1 reported false-positive results obtained from a recombinant SARS-CoV-1 N antigen ELISA due to the cross-reactivity with antibodies to OC43 and 229E.^{26,33} Our data suggest that IgGs to SARS-CoV-2 N protein also recognize 229E N, and that IgGs to HKU1 N protein recognize SARS-CoV-2 N, but not strongly. IgG levels to NL63 N protein were higher in pandemic positive vs pre-pandemic samples but this did not happen for the other HCoVs. Correlations of SARS-CoV-2 N CT-short fragment with HCoV N FL protein were similar to those seen for N FL protein. IgG responses to OC43 N protein correlated the least to IgG to SARS-CoV-2 N protein in the rest of analyses, although it had a highly significant correlation with IgG to N from the alpha-HCoV 229E and NL63, in contrast to other reports.⁶⁴ Interestingly, in samples from rRT-PCR negative pandemic individuals, there was a significant inverse correlation of IgG to N from SARS-CoV-2 with IgG to N from OC43 HCoV that is the beta-HCoV with the highest sequence homology with SARS-CoV-2 N protein, suggesting a potential role on immunity. If preexisting IgG to OC43 N protein bind to SARS-CoV-2 N protein, high antibody levels could neutralize SARS-CoV-2 infection upon exposure, resulting in sterile protection or low viral loads below the rRT-PCR detection threshold (rRT-PCR negative) that translates into low/no exposure and consequently less antibodies to SARS-CoV-2. If rRT-PCR negative individuals had not been exposed at all to SARS-CoV-2 (RBD seronegatives), then what would be measured as SARS-CoV-2 IgG might be IgG from crossreactive HCoVs. Nevertheless, a limitation of this study is that the design and sample size did not allow unraveling the potential effect of baseline HCoV antibodies on the acquisition of COVID-19 immunity,⁶⁴ which will be addressed in future longitudinal follow up analysis of our cohorts. This effect could be neutral, positive

(boost of responses) or negative, whereby having antibodies to certain viruses could adversely determine the profile of responses upon SARS-CoV-2 infection (“original antigenic sin”)^{43,44,47} or interfere by antigen masking. Findings from a recent study argue against a protective role for crossreactive HCoV T cells in SARS-CoV-2 infection.³⁷ In our study, we speculate that preexisting OC43 HCoV IgGs to N protein could be protective and, as a result, incoming SARS-CoV-2 infections would be more controlled and thus fewer antibodies induced against them.

Unexpectedly, higher serological correlations were consistently obtained for SARS-CoV-2 and alpha- rather than beta-HCoVs despite having less homology at the primary amino acid sequence level. The reactivity of the alpha- and beta-HCoVs clustered together within families, also by PCA analysis (data not shown). Therefore, it appears that crossreactivity is also and mostly driven by homologies at the conformational rather than the linear level.

Our study had some limitations. We did not have access to samples from HCoV infected (rRT-PCR positive) individuals and thus the crossreactivity among the N antigens could not be fully assessed.⁶⁵ In addition, we did not test IgG responses to other domains of N protein (like the NT that has significant sequence homologies except for a small amino acid region) or other structural proteins.⁵⁰ Our preliminary results show that crossreactivity may also exist for M and S2 proteins,⁶² due to antibody recognition in some pre-pandemic samples, as it had been similarly described for SARS-CoV infections.^{66,67} In fact, different antigens could have different levels of crossreactivity, as seen in a recent study of HCoV S antibody responses that reported an increase in IgG to OC43 (but not 229 or NL63) S proteins with SARS-CoV-2 infection.⁶⁸ This observation contrasts with our data that suggested boost of N IgG antibodies from M0 to M1 for 229 and NL63 but not for OC43. Other respiratory pathogens that are common coinfections may also have crossreactivity with SARS-CoV-2 and partially explain our results.⁶⁹

In conclusion, the N protein of SARS-CoV-2 is immunogenic but it is also recognized by partially crossreactive HCoV antibodies. More specific epitopes are located in the N CT-short region and could be used for seroprevalence and diagnostics purposes. Future larger prospective studies should determine whether the N antigen is a target of protective immune responses to COVID-19 and therefore a promising vaccine candidate together with S, and

show cumulative data from month 0 (M0) and month 1 (M1) individuals with any evidence of infection (seropositivity to receptor-binding domain (RBD) and/or rRT-PCR positive): M0 antibody levels from individuals with any evidence of infection at M0 plus M1 antibody levels from individuals who had a new evidence of infection from M0 to M1.

the role of preexisting HCoV antibodies in acquired immunity.

ACKNOWLEDGEMENTS

The assays development and sample collection were performed with internal funds from the investigators groups and institutions. G.M. had the support of the Department of Health, Catalan Government (SLT006/17/00109). J.C. and L.I. are supported by SAF2016-76080-R grant from the Spanish Ministry of Economy (AEI/FEDER, UE) and by PID2019-110810RB-I00 grant from the Spanish Ministry of Science & Innovation to L.I. A.T. and L.F.B. received support from Departament General de Recerca i Innovació en Salut (GENCAT-DGRIS-COVID19), CB 06/06/0028/CIBER de enfermedades respiratorias- Ciberes, ISCIII (FI19/00090 awarded to R.L.A.) and IDIBAPS group 2.603. ISGlobal receives support from the Spanish Ministry of Science and Innovation through the “Centro de Excelencia Severo Ochoa 2019-2023” Program (CEX2018-000806-S), and support from the Generalitat de Catalunya through the CERCA Program.

All authors have read the journal’s policy on disclosure of potential conflicts of interest. Authors have no financial or personal relationship with organizations that could potentially be perceived as influencing the described research. All authors have read the journal’s authorship agreement and the manuscript has been reviewed by and approved by all named authors.

We thank the volunteers who donated blood for COVID-19 studies and the clinical and laboratory staff who participated in the sample collection and processing. Special thanks to ISGlobal lab colleagues D. Barrios, P. Cisteró, L. Puyol, R.A. Mitchell, C. Jairoce, S. Alonso, J. Moreno, and those involved in data management and/or recruitment of volunteers and patients at the Pamplona and Barcelona hospitals, J.L. del Pozo, M. Fernández-Alonso, S. Sanz, S. Méndez, A. Llupià, E. Chóliz, A. Cruz, A. Figueroa-Romero, S. Folchs, M. Lamoglia, N. Ortega, N. Pey, M. Ribes, N. Rosell, P. Sotomayor, S. Torres, S. Williams, S. Barroso, A. Trilla and P. Varela.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.02.006.

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