Spike-based COVID-19 immunization increases antibodies to nucleocapsid antigen

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Antibodies to the nucleocapsid (N) antigen are suggested to be used to monitor infections after COVID-19 vaccination, as first generation subunit vaccines are based on the spike (S) protein. We used multiplex immunoassays to simultaneously measure antibody responses to different fragments of the SARS-CoV-2 S and N antigens for evaluating the immunogenicity of the mRNA-1273 (Spykevax) and the BNT162b2 (Comirnaty) vaccines in 445 health care workers. We report a >4-fold increase post-vaccination of IgG levels to the full length (N FL) and C-terminus of N (N CT) in 5.2% and 18.0% of individuals, respectively, and of IgA in 3.6% (N FL) and 9.0% (N CT) of them. The increase in IgG levels and avidity was more pronounced after Spykevax than Comirnaty vaccination (36.2% vs 13.1% for N CT, and 10.6% vs 3.7% for N FL). Data suggest the induction of cross-reactive antibodies against the N CT region after administering these S-based vaccines, and this should be taken into account when using N seropositivity to detect breakthroughs. (Translational Research 2021; 000:1−7)

Abbreviations: spike = S; nucleocapsid = N; C-terminal = CT; full length = FL; receptor-binding domain = RBD; median fluorescence intensity = MFI; standard deviations = SD; confidence intervals = CI; interquartile range = IQR; health care workers = HCW; fold change = FC; N-terminal = NT

INTRODUCTION

With the successful completion of phase 3 clinical trials[1] and subsequent global rollout of COVID-19 vaccines since December 2020, there is a need to monitor the occurrence of breakthroughs and the duration of protection, for the evaluation of the effectiveness of immunization. This is becoming of paramount importance with the appearance of new more transmissible variants that may potentially escape immune responses elicited by COVID-19 vaccines. Indeed, effectiveness of COVID-19 vaccines may be lower to the Delta variant.[2] As first generation subunit vaccines are based on the spike (S) protein from SARS-CoV-2,[3] infections in vaccinated individuals using serological testing cannot be detected with antibodies to this target. Therefore, current studies suggest using antibodies to the

nucleocapsid (N) antigen to diagnose incident infections after vaccination.[4,5]

As part of a longitudinal evaluation of the immunogenicity of the mRNA-1273 (Spykevax)[1] and the BNT162b2 (Comirnaty) vaccines in a cohort of health care workers followed up since the onset of the COVID-19 pandemic in 2020, we set out to monitor IgM, IgA and IgG responses to several SARS-CoV-2 antigens including S and N proteins after vaccination started in early 2021.

METHODS

Study population. We followed up a total of 445 health care workers (HCW, n = 353 from hospital[6] and n = 92 from primary care[7]) in Barcelona, Spain, with different histories of prior COVID-19 exposure. Venous or finger prick blood was collected at different time points before and after immunization with the mRNA-1273 (Spykevax)[1] or the BNT162b2 (Comirnaty),[8] products from Moderna Biotech and Pfizer-BioNTech, respectively. Plasma was isolated and cryopreserved at -80°C.

Antibody determinations. We used a multiplex immunoassay able to simultaneously measure levels of antibodies to different fragments of the S and N proteins. The N antigens included a C-terminal (CT) fragment (residues 340-416) and the full length (FL) of N, both cloned in pET22b expression vectors, fusing a C-terminal 6xHis-tag, transformed in *E. coli* BL21 DE3, induced with IPTG, and purified by affinity chromatography using HisTrap columns. The integrity of the proteins was checked by Coomassie stained SDS-PAGE, showing >90% purity.[9, 11] The S antigens included a full length protein, as well as its subregion S2, and the receptor-binding domain (RBD), as previously reported.[10, 11] Antigen-coupled microspheres were added to 384 well plates in multiplex and incubated with test plasma samples, positive and negative controls (1:500 final dilution) for 1 hour at room temperature, protected from light. After washing, goat anti-human IgG (1:400) or IgA (1:200) phycoerythrin conjugates (Moss Bio) were added to each well and incubated for 30 minutes. After washing, plates were acquired on a Flexmap 3D reader, at least 50 microspheres per analyte per well, and median fluorescence intensity (MFI) reported for each analyte. Isotype and antigen specific seropositivity cutoffs were calculated as 10 to the mean plus 3 standard deviations (SD) of $\log_{10}$-transformed MFI of 128 pre-pandemic negative control samples.

Samples from a subset of 82 individuals (41 vaccinated with Comirnaty [5 pre-exposed] and 41 with Spykevax vaccine [14 pre-exposed]), were selected for avidity assays. Antibody avidity was determined as the percentage of IgG and IgA levels against S, N FL and N CT antigens measured incubating plasma samples (diluted 1:500) with a chaotropic agent (urea 4M, 30 min at room temperature) over the IgG or IgA levels measured in the same samples without chaotropic agent.

Statistical analysis. MFIs were $\log_{10}$-transformed. Groups were compared with the Wilcoxon Sum Rank test for continuous non-parametric variables and with the Wilcoxon Signed-Sum Rank Test for paired non-parametric continuous data. Correlations between continuous variables were analyzed using linear regression models and Spearman's rank test. A *P*-value of < 0.05 was considered statistically significant and 95% confidence intervals (CI) were calculated for all estimates. We performed the statistical analysis using GraphPad Prism V9.

Ethics. The study protocol was approved by the *Comitè Ètic d'Investigació Clínica IDIAP Jordi Gol* and the CEIm of Hospital Clínic, and written informed consent was obtained from all participants.

RESULTS

While responses to S proteins were generally induced at high levels pre- to post-vaccination, as expected, we observed an unanticipated statistically significant increase in antibody responses to N proteins, particularly against the N CT fragment (Fig 1A). The rise in antibody levels for each antigen was consistent with an increase in percent seropositivity (Table I). As predictable, most naïve subjects were seronegative for anti-N antibodies prior to vaccination.

Specifically, levels of IgG and IgA, to the N CT fragment rose >4-fold in 18.06% and 9.0% of vaccinated individuals, respectively (Table S1). This increase was not observed for IgM (data not shown). Remarkably, the 4-fold increase in IgG was observed in more individuals for the Spykevax (36.2%) than the Comirnaty (13.1%) vaccination (Tables 1, S1) and the increase in IgG reached more statistical significance for the Moderna product (*P* < 0.001) (Fig 1A).

Examining the antibody responses to N FL, commonly used in serological evaluations, 5.2% and 3.6% of individuals had a >4-fold increase in IgG and IgA levels, respectively (Table S1), but the patterns depended on the vaccine. Following COVID-19 Spykevax vaccination, levels of IgG and IgA to N FL increased but to a lesser extent than to N CT (Fig 1A), with 10.6% of individuals showing a >4-fold change in IgG levels (Table S1). In contrast, N FL IgG levels
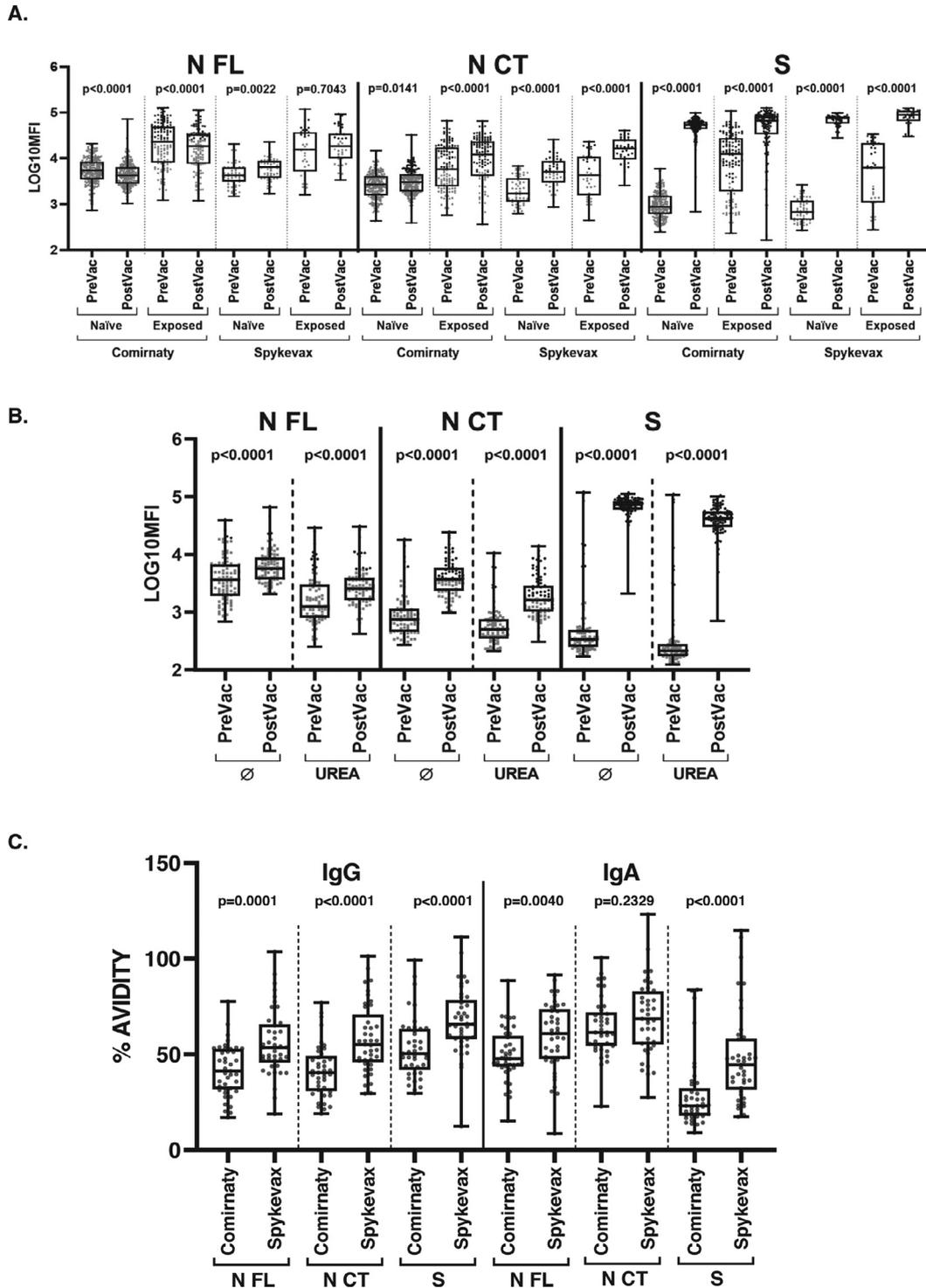
Fig 1. Comparison of IgG levels to N FL, N CT and S, before and after COVID-19 mRNA vaccination, and IgG and IgA avidity post-vaccination. IgG levels ($\log_{10}$ MFI) against the N FL, N CT and S proteins of SARS-CoV-2 before and after COVID-19 vaccination stratifying by vaccine and prior SARS-CoV-2 exposure status (A); IgG levels comparing with and without urea incubation (B); and IgG and IgA % of avidity post-vaccination stratifying by vaccine (C). The center line of boxes depicts the median of MFIs; the lower and upper hinges correspond to the first and third quartiles; the distance between the first and third quartiles corresponds to the interquartile range (IQR); whiskers extend from the hinge to the highest or lowest value within $1.5 \times$ IQR of the respective hinge. Black dots correspond to seropositive samples and grey dots correspond to seronegative samples. Wilcoxon rank test was used to assess statistically significant differences in antibody levels between paired samples.

Table I. Antibody seropositivity in health care personnel before and after COVID-19 mRNA vaccination, and percentage of subjects with a >4-fold increase in antibody levels, stratifying by antibody isotype, antigen, prior SARS-CoV-2 exposure status and vaccine.

		Naïve										Exposed*												
		Comirnaty						Spykevax						Comirnaty						Spykevax					
		PreVac		PostVac		FC >4		PreVac		PostVac		FC >4		PreVac		PostVac		FC >4		PreVac		PostVac		FC >4	
IgG	N CT	0.0%	(0)	21.3%	(48)	9.8%	(22)	0.0%	(0)	50.9%	(28)	29.1%	(16)	42.9%	(54)	67.5%	(85)	19.0%	(24)	38.5%	(15)	94.9%	(37)	46.2%	(18)
	N FL	0.0%	(0)	0.9%	(2)	4.0%	(9)	0.0%	(0)	0.0%	(0)	3.6%	(2)	46.0%	(58)	38.9%	(49)	3.2%	(4)	41.0%	(16)	41.0%	(16)	20.5%	(8)
	RBD	0.0%	(0)	99.6%	(224)	99.6%	(224)	1.8%	(1)	100.0%	(55)	100.0%	(55)	69.0%	(87)	94.4%	(119)	62.7%	(79)	59.0%	(23)	100.0%	(39)	92.3%	(36)
	S	0.0%	(0)	99.6%	(224)	99.6%	(224)	1.8%	(1)	100.0%	(55)	100.0%	(55)	70.6%	(89)	92.9%	(117)	51.6%	(65)	56.4%	(22)	100.0%	(39)	76.9%	(30)
	S2	0.9%	(2)	97.3%	(219)	93.3%	(210)	0.0%	(0)	100.0%	(55)	100.0%	(55)	68.3%	(86)	85.7%	(108)	23.8%	(30)	56.4%	(22)	100.0%	(39)	46.2%	(18)
IgA	N CT	1.3%	(3)	2.7%	(6)	0.4%	(1)	0.0%	(0)	14.5%	(8)	7.3%	(4)	22.2%	(28)	69.0%	(87)	23.8%	(30)	5.1%	(2)	46.2%	(18)	12.8%	(5)
	N FL	1.8%	(4)	0.4%	(1)	0.4%	(1)	1.8%	(1)	1.8%	(1)	1.8%	(1)	16.7%	(21)	34.9%	(44)	11.1%	(14)	12.8%	(5)	7.7%	(3)	0.0%	(0)
	RBD	0.0%	(0)	96.0%	(216)	79.6%	(179)	0.0%	(0)	98.2%	(54)	94.5%	(52)	47.6%	(60)	95.2%	(120)	77.0%	(97)	35.9%	(14)	100.0%	(39)	84.6%	(33)
	S	0.4%	(1)	98.2%	(221)	94.2%	(212)	0.0%	(0)	98.2%	(54)	98.2%	(54)	57.1%	(72)	96.0%	(121)	84.9%	(107)	46.2%	(18)	100.0%	(39)	92.3%	(36)
	S2	1.3%	(3)	84.4%	(190)	85.8%	(193)	0.0%	(0)	87.3%	(48)	94.5%	(52)	52.4%	(66)	92.9%	(117)	65.1%	(82)	38.5%	(15)	94.9%	(37)	76.9%	(30)
Number subjects	225							55						126						39					

Abbreviations: FC, fold change; N, nucleocapsid; RBD, receptor binding domain; S, spike.
*There were 39 (8.7%) and 126 (28.3%) SARS-CoV-2 pre-exposed participants among those vaccinated with Spykevax (Moderna BioTech) and with Comirnaty (Pfizer-BioNTech), respectively, defined by positive SARS-CoV-2 rRT-PCR and/or serology diagnosis before vaccination in the prospective cohorts of health care workers (HCW). Antibody levels were measured in the prior visit of the longitudinal follow up that was available pre-immunization (PreVac), depending on the study cohort (ranging from 1 day to 6 months), and post-immunization (PostVac) at 5 to 34 days after the 2nd dose (except for 77 HCW samples measured 3 to 21 days post 1st dose). Percentages of seropositivity are shown (%), and in parenthesis the number of individuals, over the total of paired samples shown in the bottom row.

decreased pre- to post-vaccination with Comirnaty in most of the participants (Fig 1A).

Correlations between levels of antibodies to N CT and S proteins (full length S, S2, RBD) post-vaccination were significant (Spearman rho, 0.70-0.83 for IgG, and 0.68-0.88 for IgA, $P < 0.0001$), and a similar but weaker correlation was observed between levels of antibodies to N FL and S proteins post-vaccination (Spearman rho, 0.50-0.76 for IgG, and 0.53-0.85 for IgA, $P < 0.0001$). Correlations were also significant but less strong when analyzing pre-vaccination antibody levels: N CT, rho 0.51-0.72 for IgG, and 0.44-0.75 for IgA, $P < 0.0001$; N FL, rho 0.31-0.53 for IgG, and 0.36-0.67 for IgA, $P < 0.0001$.

We performed additional experiments to understand the observed raise in antibodies to an antigen (N) that is not included in the vaccine. We first discarded non-specific effects of the mRNA COVID-19 vaccines, or bystander activity, since antibody responses to other vaccine antigens (hepatitis B virus, diphtheria, tetanus, pertussis) or to N from common cold human coronaviruses did not significantly increase; in fact, some of them decreased after vaccination like N FL (data not shown). We also discarded that this antibody increase was due to multispecific or polyreactive memory T helper or B cells in SARS-CoV-2 pre-exposed individuals, whereby a booster response against S through vaccination would also recall responses against non-vaccine antigens in the virus (N), since this phenomenon was also observed in naïve volunteers. The finding that pre-exposed individuals had a stronger increase in N CT IgG levels after vaccination mirrors the booster effect that is seen with S (Fig 1A).

Since correlation analyses suggested that antibodies induced by both mRNA vaccines could be due to cross-reactivity between N and S epitopes, we aligned the primary amino acid sequences of N CT (residues 340-416)[9] and S but found a negligible identity (1.5%) in the overall sequence. A short C-terminal stretch in S1 (upstream of RBD), between amino acids 179 and 256, showed a 13% of identity with N CT, similar to that observed between specific immunodominant regions of common cold human coronaviruses with minimal cross-reactivity with the same N CT fragment.[9] Although these alignments did not provide strong evidence for cross-reactivity, at least at the linear epitope level, we also tested other fragments of the N protein to try mapping the target epitopes. Different fragments of N based on the N-terminal domain (NT, residues 43-180), C-terminal domain (residues 250-360) or shorter peptides within the C-terminal domain (residues 384-406), were not recognized by antibodies elicited upon vaccination in this cohort (Figure S2 and data not shown).

Finally, testing the avidity of antibodies following vaccination, we found that cross-reactive antibodies did not bind weakly to N (Fig 1B). Thus, the increase in anti-N antibody levels pre- to post-vaccination remained statistically significant when incubating samples with a chaotropic agent. The strength of IgG antibodies binding N CT was only slightly lower than that of IgG binding S, while IgA to N CT had higher avidity than IgA to S (Fig 1C). Spykevax vaccination induced statistically significantly higher avidity S and N CT antibodies than Comirnaty vaccination.

DISCUSSION

We report an increase in IgG and IgA to the N protein, specifically to the N CT, early after COVID-19 vaccination, with a more pronounced IgG rise for the Spykevax than the Comirnaty vaccine. These results suggest the presence of cross-reactive antibodies against the C-terminal region of the SARS-CoV-2 N protein after vaccination with S-based mRNA vaccines. Avidity assays indicate that the binding of vaccine-induced cross-reactive antibodies to N CT epitopes is not mediated by weak interactions that are characteristic of non-specific or polyreactive antibodies. We hypothesize that the amino acid residues recognized by these antibodies are either constituted by conformational epitopes,[13] or very short amino acid stretches not captured by sequence alignment, possibly located between amino acids 340-416 of N, considering that B cell epitopes can be as short as 4-5 amino acids in length.

A lack of antibody response against N FL after vaccination with mRNA vaccines has been previously reported,[5,12] and the decay we observe is consistent with a gradual waning of N antibodies with time. In kinetics studies, antibodies to N FL tend to decrease more markedly and rapidly with time post-infection compared to S antibodies. Therefore, the pattern found here could reflect the dynamics of the IgG response to N FL after infection with SARS-CoV-2 (in exposed) and/or to common cold human coronaviruses (in naive), which keep decreasing from pre to post vaccination. Besides the natural antibody decay, the decrease in IgG to N FL upon vaccination could be related to a redirection of antibody production to vaccine specific immunogens in detriment of other targets.

Finally, it is highly unlikely that the increase in IgG and IgA levels against N post-vaccination is due to asymptomatic infections given the high proportion of antibody increasers. Moreover, upon recent infection, the magnitude of the increase in N FL antibodies would

be much bigger particularly in previously exposed individuals, thus this option was also discarded.

Our results have significant public health relevance because the N protein is being suggested to detect breakthroughs in vaccinated people.[4] Our findings included HCW from separate cohorts, with serology assays performed in independent experiments, with antibodies measured at different time-points pre- and post-vaccination, and showing consistent results. Therefore, if this considerable number of individuals has cross-reactive antibodies between S and N CT, by using seropositivity to N as a marker of infection, they would falsely be classified as infected, and this would affect the accuracy of vaccine effectiveness estimates. The rise in antibodies is less marked for N FL, as the polyclonal response is the summation of different epitope specificities, including N CT but also N NT and central regions that were not affected by vaccination. Thus, the cross-reactive antibodies reaching the N CT contained in the N FL are proportionally less compared to the N CT epitopes alone, giving lower levels of cross-reactivity. The impact of false positive SARS-CoV-2 infections appears to occur more frequently with the COVID-19 Moderna vaccine, with which millions of people are being vaccinated. The higher increase in anti-N CT IgG in the Moderna vaccine recipients vs the Pfizer could be the result from higher levels of anti-S antibodies being induced by the Moderna vaccine.[14] In fact, the proportion of vaccine breakthrough infections in the USA[15] appears lower than the proportion of vaccinated individuals showing an increase in anti-N antibodies in our study.

In conclusion, in light of these findings, increase in antibody levels to N in vaccinated individuals may not be the best strategy to detect viral breakthroughs, particularly if using N CT fragments, although N FL seropositivity appears to be adequate. The World Health Organization indicates that careful consideration should be made as to which antibody target is selected, and alludes to the faster waning of anti-N protein antibodies after infection.[16] Therefore, combinations of multiple antigens including other less studied nonstructural proteins that have been shown to be immunogenic[17] have greater potential to accurately discriminate signatures of vaccine failure, for a more robust evaluation of vaccine effectiveness.

All authors have read the journal's policy on disclosure of potential conflicts of interest and the journal's authorship statement. The manuscript has been reviewed and approved by all authors.

The authors declare that no conflict of interests exist.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.10.004.

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