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ABSTRACT

Background: The systemic antibody responses to SARS-CoV-2 in COVID-19 patients has been extensively studied. However, less is known about the mucosal responses in the upper airways, the site of initial SARS-CoV-2 replication.

Methods: The IgG and IgA antibody responses were analysed in plasma and nasopharyngeal swabs from the first four confirmed COVID-19 patients in France. Two were pauci-symptomatic while two developed severe disease. We characterized their antibody profiles by using an in-house ELISA to detect antibodies directed against SARS-CoV-2 Nucleoprotein and Spike.

Results: Anti-N IgG and IgA antibodies were detected in the NPS of severe patients only. The levels of antibodies in the plasma markedly differed amongst the patients. The most distinctive features are a strong anti-N IgG response in the severe patient who recovered, and a high anti-N IgA response specifically detected in the fatal case of COVID-19.

Conclusions: Anti-N IgG and IgA antibodies are detected in NPS only for severe patients, with levels related to serological antibodies. The severe patients showed different antibody profiles in the plasma, notably regarding the IgA and IgG response to the N antigen, that may reflect different disease outcome. By contrast, pauci-symptomatic patients did not exhibit any mucosal antibodies in NSP, which is associated with a low or absent serological response against both N and S.

1. Background

The coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a highly transmissible and pathogenic coronavirus that emerged in 2019 and spread around the world in 2020 [1]. Considerable efforts are being made to understand SARS-CoV-2 epidemiology and pathogenesis, which is crucial for antiviral drug development and vaccine design [2,3].

A detailed characterization of the immune response to SARS-CoV-2 infection is mandatory, to understand its contribution to virus clearance and establishment of protection.

SARS-CoV-2 infects a host cell via mucosa of upper respiratory tract [4]. Hence, it would be interesting to characterize and compare immune

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Table 1
Summary of sampling dates along with patient's illness history. The data are from (14).

	day PSO[a]	illness history[b]	SARS CoV2 RT-qPCR[c]	NPS[d]	plasma[e]
Patient 1	0				
	1	influenza like symptom			
	6	hospital admission, diagnosed COVID-19	PCR +		
	10	ICU admission, symptoms worthening	PCR -		
	13	ICU discharge, patient improvement	PCR -		X
	14	PCR neg	PCR -	X	
	19	asymptomatic		X	X
	21	asymptomatic		X	X
	23	asymptomatic		X	X
	25	hospital discharge			X
	32				X
Patient 3	0				
	1	fever and diarrhoea			
	4	hospital admission- Not diagnosed as COVID19			
	5	ICU admission- acute respiratory failure			
	7	ICU-diagnosed COVID19	PCR +		
	14			x	
	15	ICU		x	x
	16	ICU		x	x
	17	ICU			x
	18	ICU		x	x
	19	ICU		x	x
	20	ICU	PCR +	x	x
	21	ICU		x	x
	22	ICU	PCR -		x
	24	death	PCR -	x	x
Patient 4	0				
	1	moderate influenza-like symptoms			
	2	hospital admission-diagnosed COVI19	PCR +		
	3	mild symptoms	PCR +		
	8	mild symptoms	PCR +		
	9	mild symptoms	PCR -		
	11	asymptomatic			
	12	asymptomatic	PCR -		
	14	asymptomatic		x	x
	16	asymptomatic		x	x
	18	asymptomatic			x
	19	asymptomatic		x	
	20	asymptomatic			x
	21	hospital discharge			
	28	asymptomatic			x
Patient 5	0				
	1	hospital admission-mild syptoms- dry cough			
	2	PCR +-high viral load–COVID diagnosed	PCR +		
	3	mild symptoms	PCR +		
	8	asymptomatic	PCR +		
	10	asymptomatic	PCR -	x	x
	12	asymptomatic		x	x
	14	asymptomatic	PCR -	x	x
	16	asymptomatic	PCR -		x
	21	hospital discharge			
	23	asymptomatic			x

[a] days post symptoms onset
[b] mains steps in clinical evolution
[c] Detection of SARS CoV2 viral RNA RT-qPCR in NPS
[d] NPS samples used in this study,
[e] plasma samples used in this study

responses in mucosal and plasma specimens of COVID-19 patients. IgA have important roles in the immune response of mucosal surfaces [5]. The antibody pool in the mucosa contains a higher proportion of IgA than in the plasma, and IgA may be important contributors to protection against viruses that target the mucosal surfaces [6].

In this study, we used in-house ELISA [7] to detect IgG and IgA directed against the SARS-CoV-2 nucleoprotein (N) and spike (S) in the NPS and in the plasma of four of the first recognized COVID-19 patients in France [8]. Serological and mucosal antibody profiles were specific to each patient, with higher antibody levels in the plasma of the severe patients as previously reported [9,10] that were also reflected in the NPS. Our study suggests a diversified humoral response to SARS-CoV-2 infection, with IgA to IgG ratios both in blood and nasopharyngeal swabs NPS potentially underlying different disease outcome.

2. Methods

2.1. Study design

This study analyses the levels of anti-N and anti-S SARS-CoV-2 IgA and IgG antibodies in NPS and plasma of four confirmed COVID-19 cases with different clinical histories. We used in-house ELISA tests, the performance of which was evaluated using NPS and serum from pre-epidemic individuals.

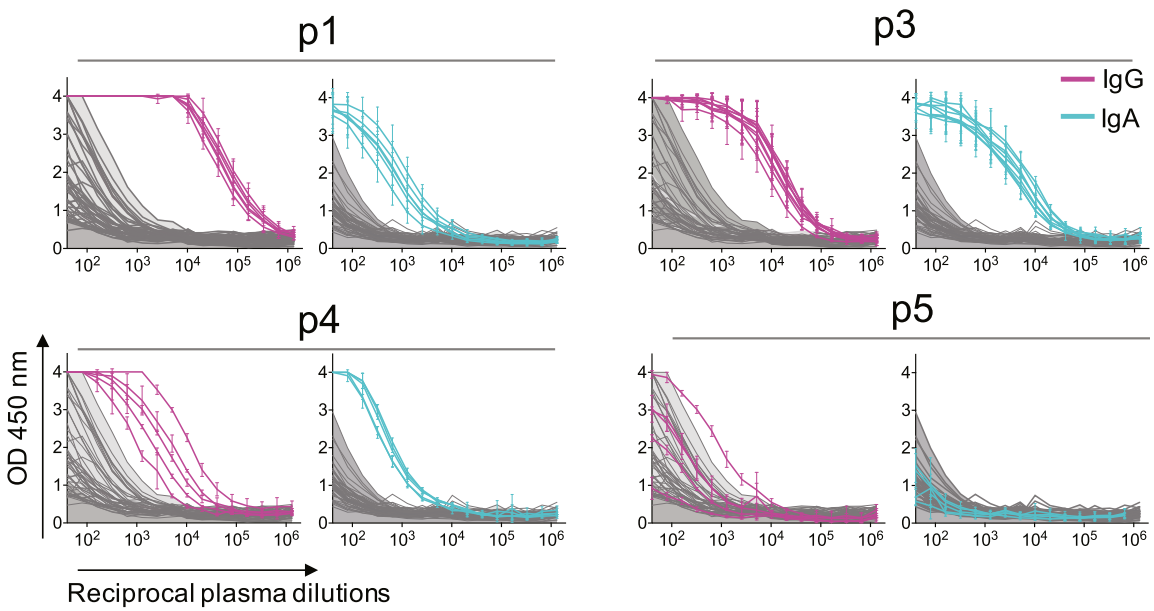

Fig. 1. Detection of IgG and IgA anti-N antibodies in patient's plasma. ELISA measuring plasma IgG and IgA reactivity to SARS CoV2-N protein for each patient. Graphs show the optical density units at 450 nm (*Y* axis) and reciprocal plasma dilutions (*X* axis) of each measured sample, taken at different times PSO, as described in Table. Dilutions of pre-epidemics control serum are in grey, the filled light grey area shows ELISA signals generated by negative serum samples.

2.2. Cohorts

Pre-epidemic NPS originated from the National Reference Center for respiratory viruses were confirmed to be SARS-CoV-2 negative by RT-PCR (data not shown). Pre-epidemic sera were provided before November 2019 from Institut Pasteur by the ICAReB platform (Clinical Investigation and Access to Research Bioresources) or the Center for translational Science. Samples from donors were collected in accordance with local ethical guidelines by Institut Pasteur.

The NPS and plasma samples for the four patients are part of patients included in one of the participating center (i.e., Hôpital Bichat, APHP, Paris, France) of the French COVID cohort assessing hospitalized patients with a virologically-confirmed COVID-19 (NCT04262921) [11]. The NPS were used for viral genome detection by qRT-PCR, then heated 30 minutes at 56 °C for virus inactivation. This study was conducted with the understanding and the consent of each participant or his/her surrogate. Ethics approval was obtained from the French Ethic Committee CPP-Ile-de-France VI (ID-RCB: 2020-A00256-33). NPS and plasma were heat-inactivated 30 min at 56 °C

2.3. ELISA

The anti-N and anti-S ELISA tests were performed as described in Grzelak et al [7]. Briefly, 96-well ELISA plates were coated overnight with 50 ng of purified N protein (expressed in bacterial cells) in PBS/ of SARS-CoV-2 or with 125 ng of purified S protein produced in HEK 293T cells. After blocking, diluted plasma or NPS were added and incubated at 37 °C. Plates were then incubated with peroxidase-conjugated goat anti-human IgG or IgA (#2040-05 and #2050-05 -Southern Biotech respectively). Revelation was performed by adding HRP chromogenic substrate (TMB, Eurobio Scientific). Optical densities were measured at 450 nm (OD 450). All tests were performed in triplicate.

3. Results

3.1. Nasopharyngeal swabs and blood samples

Nasopharyngeal swabs and serum samples collected before November 2019 were used as negative samples. To investigate the kinetics of anti-SARS-CoV-2 mucosal antibody responses in comparison with systemic responses, we took advantage of the longitudinal sampling performed for four of the first COVID-19 cases detected in France.

The patients were admitted at Hospital Bichat, Paris, and confirmed for SARS-CoV-2 infection by RT-PCR in NPS at the time of admission. They experienced different clinical evolutions: two exhibited severe disease, one being fatal, and two had mild disease [8]. Three to eight NPS samples collected for detection of viruses between days 10 and 24 post symptoms onset (PSO), and five to nine blood samples collected between days 10 and 32 PSO, were used to explore the IgG and IgA anti-N response of each of the patient. Clinical history and sampling periods are summarized in Table 1.

3.2. Detection of anti-N and anti-S antibodies by ELISA

We previously developed anti-N and anti-S ELISA tests to assess antibody level in plasma or sera [7]. We adapted the ELISAs to the detection of antibodies in the NPS, and used it to compare the production of antibodies in the plasma and NPS. Serial 2-fold dilutions of the samples were assessed for anti-N and anti-S IgG and IgA antibodies, using anti-IgG and anti IgA secondary antibodies in otherwise strictly identical experimental settings. Anti-S IgG and IgA antibodies could be measured in only 7 NPS samples". The rest of the samples had insuffisient volumes. Sera or NPS samples of pre-epidemic patients were run in parallel to patient samples, to give the background in each fluid type.

Signals of both IgG and IgA anti-N and anti-S antibodies (OD450) in the plasma specimens were clearly above that observed for pre-pandemic samples for all patients except for patient 5 anti- N antibodies (Figs. 1,2).

Anti-N signals in the NPS were distinct from the pre-pandemic NPS for the two severe patients, indicating that anti-N IgG and IgA antibodies can be specifically detected in NPS samples from COVID-19 patients (Fig. 3). Similar levels of anti-S IgG and IgA were detected in the NPS where they have been measured (Fig. 3).

Antibody titers were derived from the dilution curves, and expressed as RD50 (*i.e* the reciprocal serum dilution necessary to obtain 50% of maximum ELISA OD450 values). For both IgG and IgA measurements, matched cut off values were calculated for the plasma and NPS by calcu-

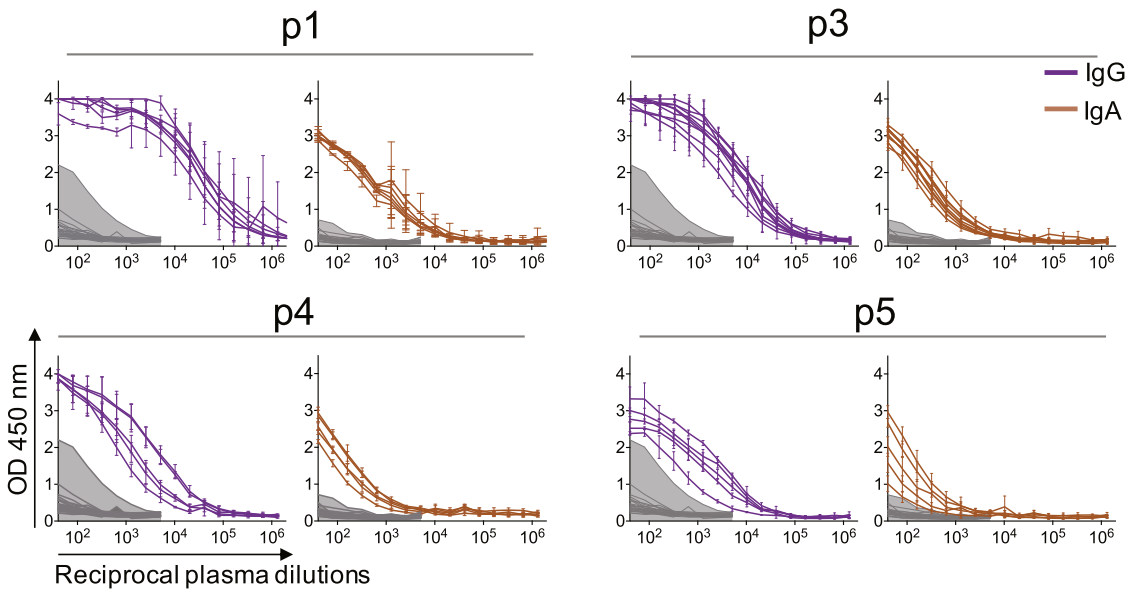

Fig. 2. Detection of IgG and IgA anti-S antibodies in patient's plasma. ELISA measuring plasma IgG and IgA reactivity to SARS CoV2-S protein for each patient. Graphs show the optical density units at 450 nm (*Y* axis) and reciprocal plasma dilutions (*X* axis) of each measured sample, taken at different times PSO, as described in Table. Dilutions of pre-epidemics control serum are in grey, the filled light grey area shows ELISA signals generated by negative serum samples.

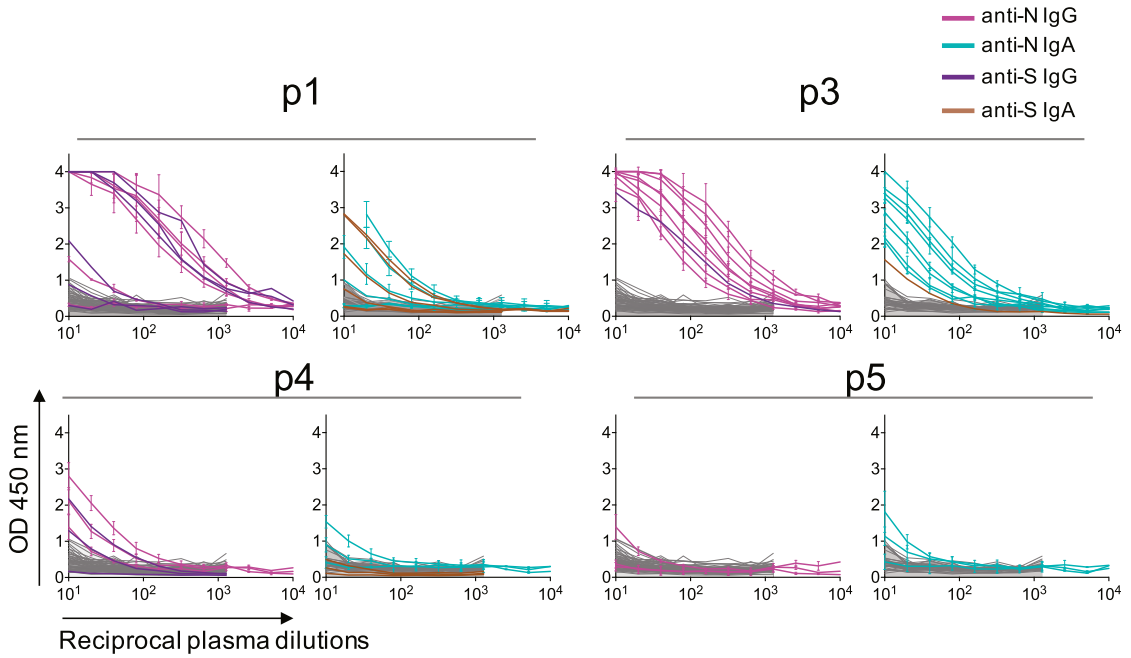

Fig. 3. Detection of IgG and IgA antibodies in patient's NPS. ELISA measuring the IgG and IgA anti-N reactivity and anti-S antibodies for NPS samples with enough volume. Graphs show the optical density units at 450 nm (*Y* axis) and reciprocal plasma dilutions (*X* axis) of each measured sample, taken at different times PSO, as described in Table 1. Dilutions of pre-epidemics control NPS are in grey, the filled light grey area shows ELISA signals generated by negative NPS samples.

lating the mean RD_{50} titer of negative samples + 3 Standard deviations (sera: *n*=46; NPS: *n*=48 for IgG; *n*= 27 for IgA).

3.3. The serological response of the patients

Patient 1, who developed secondary severe symptoms, showed a robust serological response all along the sampling period, from day 12 until day 32 PSO after the patient's full recovery and discharge from hospital (Table 1). For both N and S antigens, the IgG levels were constantly high, and the IgA levels low (Fig. 4). Patient 1 therefore demonstrated markedly different levels of IgG and IgA in the plasma, with IgG titers 70- to 130-fold higher than IgA titers. The humoral response of this patient appears prominently driven by IgG, and persists over time.

Patient 3, with a rapidly progressing severe disease, had lower IgG levels against both N and S antigens compared to Patient 1 (Fig. 4). His levels of IgA differed according to the targeted antigen, anti-N IgA levels are around 20 fold higher than anti-S IgA. IgG levels were only 2.5- to 4.5-fold higher than IgA for the N antigen, while for the S antigen IgG levels were 17.5 to 44 fold higher than IgA. Antibody levels did not vary until a decline on day 24 PSO, when patient died.

Patient 4 (contact case of patient 1) had IgG levels progressively increasing over time in the plasma, while the levels of IgA were constant and low. Anti-S and anti-N serologies followed the same progression,

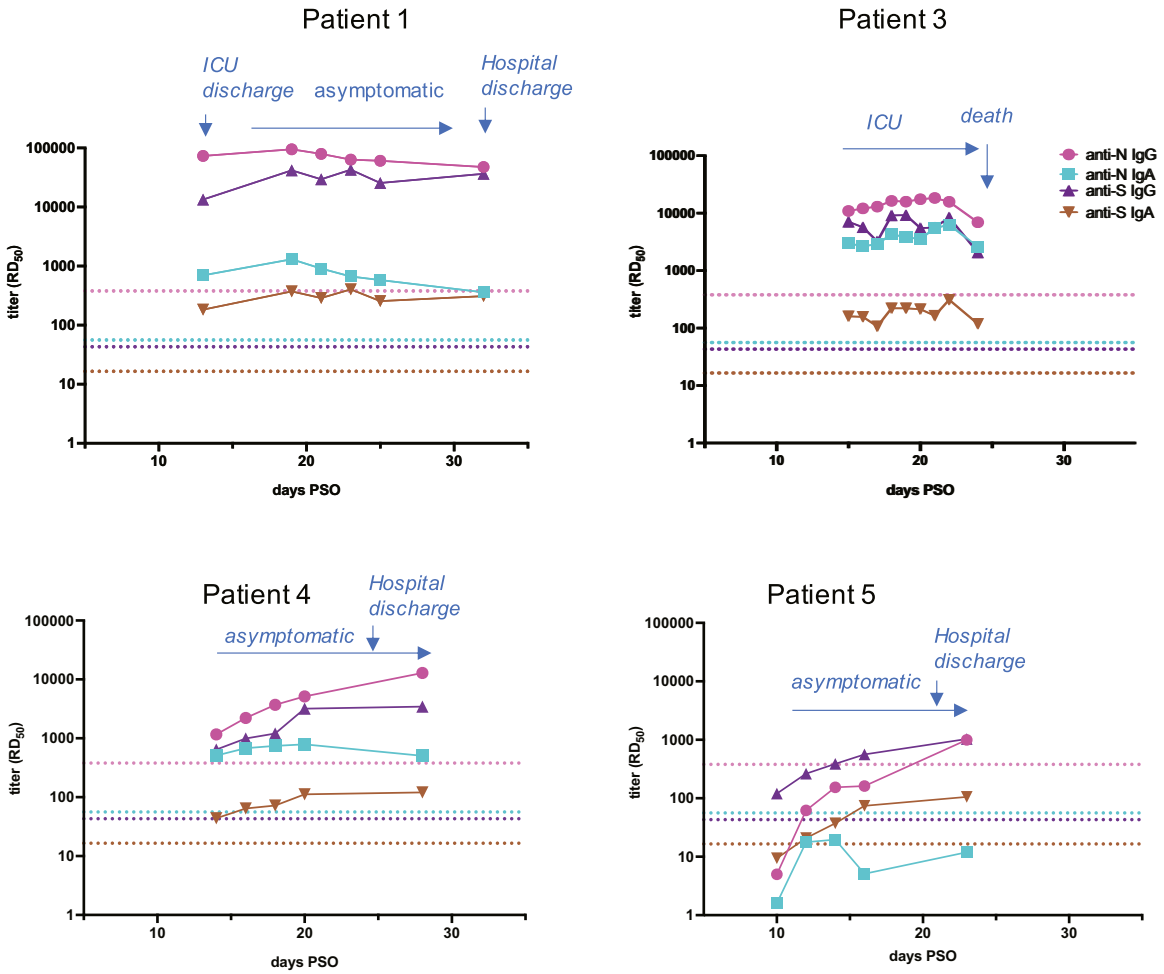

Fig. 4. Anti-N and S titers in patient's plasma. Antibody titers calculated as RD_{50} (*Y* axis) are plotted according to the day PSO (*X* axis). Main steps of the patient's clinical history are shown by arrows. The positive thresholds of IgG and IgA detection for anti N and anti S, deduced from the mean IC_{50} of pre-epidemic samples + 3SD, are shown as pink (anti-N IgG), blue (anti-N IgA), purple (anti-S IgG) and brown (anti-S IgA) dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the IgA response being mainly directed against the N (Fig. 4). The IgG/IgA ratio increased for both antigens from days 14 to 28 PSO. This reflects a developing serological IgG response after patient's recovery, while the IgA stabilized after 20 days PSO.

Patient 5 (contact case of patient 3) did not show any significant seroconversion to SARS-CoV-2 N protein. Mild and increasing levels of anti-S antibodies were detected, with higher levels of IgG antibodies (Fig. 4).

3.4. Detection of anti N antibodies in nasopharyngeal swab

IgG and IgA anti-N ELISA signals above the cut-off were detected in NPS of **patients 1 and 3,** indicating the occurrence of a humoral response in the nasopharyngeal mucosa of these patients (Fig. 5). However, the IgG and IgA profiles differed over time, the IgG levels constantly being over the background while the IgA exhibited more variable levels. When anti-S antibodies have been measured, NPS samples exhibited constant IgG and IgA levels (see patient 1 in supplemental Fig. 1)

For **patient 1,** the anti-N IgG to IgA ratio in NPS decreased from 80 at day 14 PSO to 9 and 14 at days 19 and 21 PSO, respectively. Then a sharp increase was observed at day 23, reflecting the collapse of IgA levels while IgG antibodies remained at high levels (Fig. 5). Such IgA drop in the NPS occurred one week after the patient became free of symptoms (Table 1), and did not occur for the anti-S IgA antibodies. **Patient 3** exhibited an anti-N IgG/IgA ratio in NPS oscillating between 4 and 8 over time. No evolution of this ratio was observed along with illness severity, which led to death on day 24. At 14 days PSO, anti-S IgG titers were about as high as anti-N IgG, while IgA against S could hardly be detected (supplementary Fig. 1). **Patient 4** showed only a weak anti-N and anti-S IgG responses in NPS samples at days 16 and 19 PSO, indicating a moderate but significant activation of humoral response in the nasal mucosa. No significant IgA response could be detected against neither of the antigens. **Patient 5** did not demonstrate any anti-N IgG response in NPS. As both patients 4 and 5 are devoid of anti N IgA in NPS, the IgG/IgA ratio is irrelevant.

4. Discussion

We report here the matched detection of SARS-CoV-2 anti-N and anti-S IgG and IgA antibodies, in the plasma and NSP of four COVID-19 patients, two severe patients, and two pauci-symptomatic patients who remained under survey at the hospital despite their mild symptoms given that the pandemic was in its early stage. This small set of patients is valuable owing to (i) the different and well-characterized disease progression between patients; (ii) the sera and NPS longitudinal sampling over the COVID-19 illness period. This provides an opportunity to compare the systemic and mucosal antibody responses in severe patients during the course of illness, and in pauci-symptomatic individuals after their full recovery

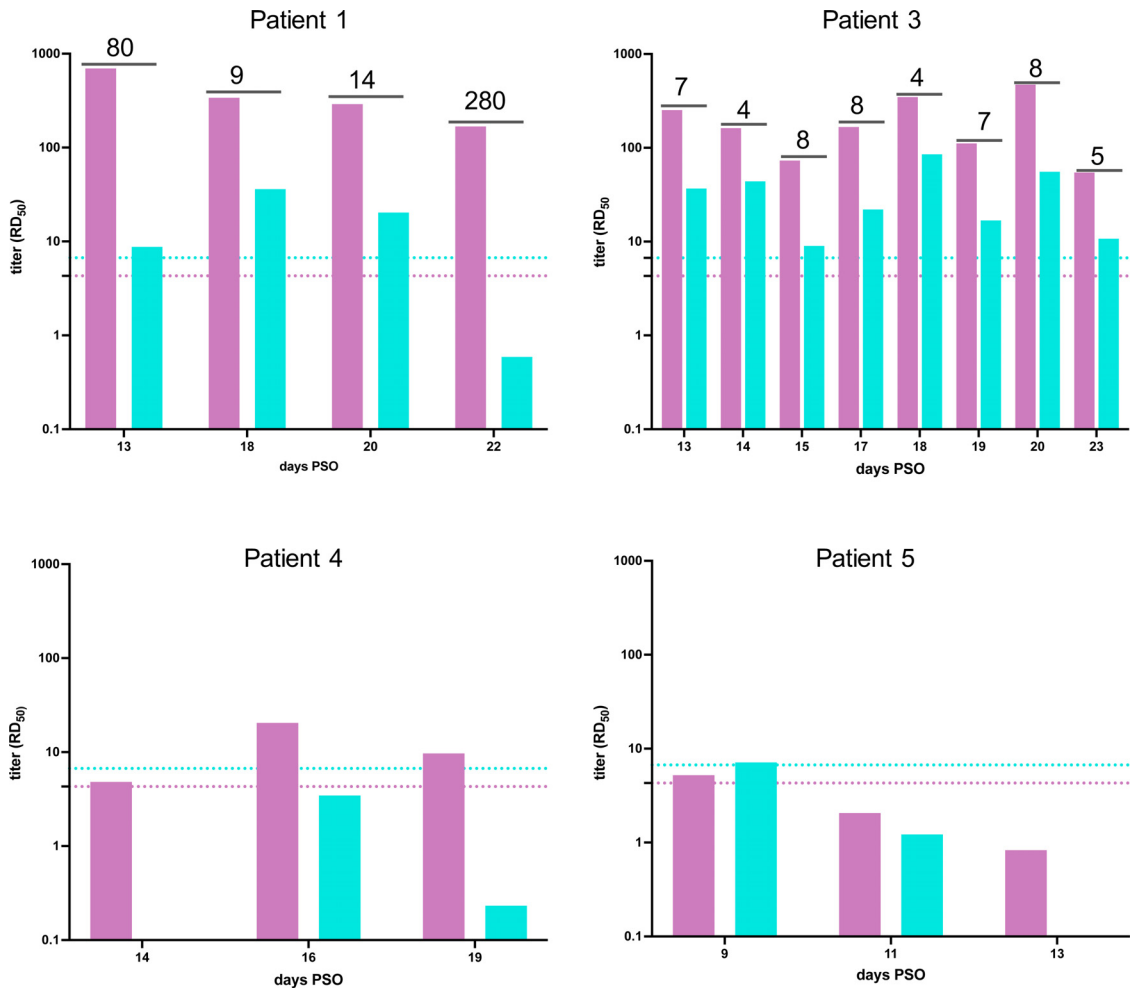

Fig. 5. IgG ang IgA anti-N titers in patient's NPS. Antibody titers calculated as RD_{50} (*Y* axis) are plotted according to the day PSO (*X* axis). The positive thresholds of IgG and IgA detection, deduced from the mean IC_{50} of pre-epidemic NPS samples + 3SD, are shown as pink (IgG) and blue (IgA) dotted lines respectively. The ratio of IgG to IgA titers is indicated when appropriate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The anti-N antibodies are used to sensitively detect ongoing or past infection, regardless of the protective immune activity. In contrast, the anti-S immunity provides to some extend protection against SARS-CoV2 re-infection.

The patients 1 and 3 with severe disease showed stable IgG and IgA responses in the plasma, indicating that the maximal serological response had been reached, which is in line with the time post symptoms sampled [13]. Their serological response was nevertheless clearly distinct. The humoral response in the plasma of patient 1 was dominated by a strong IgG response against both N and S viral antigens, the IgG/IgA ratio being constantly around 100. Patient 3 had a less robust IgG response against both N and S, and a stronger anti-N IgA response than patient 1, with an IgG /IgA ratio between 2.5 and 4.5 for the N antigen. We could also detect anti-N IgG and IgA in the NPS of these severe patients, indicating the occurrence of a humoral response at the initial site of infection. For some of these NPS samples, anti-S IgG ang IgA have been measured and detected as well, confirming previous results where anti-S antibodies have been detected in the NPS of hospitalized patients [12].

The IgA levels in NPS were consistently lower than IgG just as in plasma, suggesting that the mucosal response was dominated by an IgG response at late times of infection. This differs from the mucosal response that has been detected in COVID-19 patient's saliva earlier post infection, where higher proportions of IgA were detected [14].

The mucosal IgA response of patient 1 developed after symptoms improvement following the severe phase of his illness. The IgG/IgA ratio in the NPS followed a v-shaped progression, dropping from 80 to about 10 due to a transient rise in the IgA levels, then back up to 100 owing to anti-N IgA collapse. Interestingly, such drop did not occur in the levels of anti-S IgA. The transient rise in mucosal anti-N IgA antibodies between 19 and 21 days PSO indicates the development of an IgA-based anti-N mucosal response, that is delayed compared to the serological response, since at the same days PSO the IgA levels in the plasma progressively decreased (Figs. 3 and 4). Patient 3's anti-N IgG/IgA ratio is constant over time, and in the order of 5 in both plasma and NPS. Beyond patient's age, the antibody rate in the plasma, far higher for patient 1 than 3, could underlie different disease outcome. Patient 1 is characterized by a clear predominant IgG response in both plasma and NPS, detected upon disease resolution after the period of symptoms worsening. In contrast, patient 3's IgG response remained moderate, and was close to the anti-N IgA levels both in the plasma and in the nasal mucosa.

For the two pauci-symptomatic patients, we also observed divergent anti-N humoral responses. Patient 4 exhibited in the plasma an IgG response against N and S still increasing at 28 days PSO, which reflects a delayed IgG response compared to severe patients, in agreement with previous reports [9]. A sustained stable or increasing IgG response over time in symptomatic individuals with mild disease has recently been linked to a shorter time to disease resolution [15], as was the case of

patient 4. We did not detect any IgA in the NPS of patient 4, making the IgG/IgA ratio irrelevant. A weak and transient anti-N and anti-S IgG response could be detected in the NPS of this patient during the asymptomatic period, concomitant with the increase of the IgG response in the plasma.

Patient 5 did not develop any significant antibody response against N, neither in the plasma nor in NPS. In contrast, patient 5 seroconverted against the S antigen, mainly driven by an IgG response. Such different humoral responses could not be due to a different sampling time, since they covered a similar period post symptom for both pauci-symptomatic patients 4 and 5 (days 14-28 and 10-23 respectively). The case of patient 5 further demonstrates that a RT-PCR confirmed COVID-19 infected individual may show a delayed seroconversion at least against the N antigen, as IgG were only detected at day 23 PSO, and a progressing but low response against the S antigen. However, analysis of later time points would have been required to document a further increase in IgG titers, as shown for individuals with low initial antibody titers [16]. We extend here these observations to the IgA serological response, and show herein that patient 5 did not either develop any detectable mucosal humoral response. The fact that some pauci-symptomatic patients may not develop any humoral response neither in the nasal mucosa nor in serum, should be taken into account in seroprevalence surveys. It also suggests that non-humoral, innate or cell-mediated immunity may be sufficient to resolve infection as previously reported [17] .

In conclusion, exploring four Covid-19 patients with different disease profiles, we identified different antibodies responses according to (i) the severity of the symptoms (symptomatic vs pauci-symptomatic) (ii) disease progression (severe leading to death vs severe resolved), (iii) variations among pauci-symptomatic patients. These conclusions are nevertheless to be substantiated with other studies of both severe, pauci-symptomatic or asymptomatic individuals.

Author contributions

BCC handled laboratory logistics and generated data. BCC and CD conceived the project, analysed and summarized the data, and drafted the manuscript. SB and VE shared samples and data for the analysis. JG, ST, LB provided the specimens and sera for the analysis. SP and NE provided the purified N and S, critical for the study. SvdW provided input on interpretation of the results and critically reviewed the manuscript. All authors reviewed and the article.

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Declaration of Competing Interest

SvdW, BCC and NE have a patent “Use of proteins and peptides coded by the genome of a novel strain of sars associated coronavirus” issued, and SW a patent “Severe acute respiratory syndrome (sars) - associated coronavirus diagnostics” pending. SP, NE, SW and CD applied for a patent which includes claims describing N-based serological diagnosis of COVID. Professor Ghosn reports personal fees from ViiV Healthcare, Gilead Science, Janssen Cilag, and research grants from Gilead Sciences, MSD and ViiV Healthcare, outside the submitted work.

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