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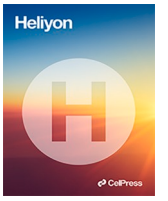

Research article

SARS-CoV-2 natural infection, but not vaccine-induced immunity, elicits cross-reactive immunity to OC43

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ABSTRACT

Background: The recent SARS-CoV-2 pandemic renewed interest toward other non-severe acute respiratory syndrome human coronaviruses. Among these, OC43 is a seasonal human coronavirus widely diffused in the population (90 % seroprevalence in adults) which is responsible for mild respiratory symptoms. As OC43 protective immunity is short lasting, we investigated whether humoral immunity to SARS-CoV-2, induced by vaccination or spontaneous infection, protects against OC43 re-infection at either systemic or mucosal level.

Methods: A neutralization assay was conducted against "wild type" SARS-CoV-2 lineage B.1 (EU) and OC43 in VeroE6 cell lines using plasma and saliva samples from 49 subjects who were never infected and received three BNT162b2 RNA vaccine doses (SARS-CoV-2-vaccinated: SV) and from 25 SARS-CoV-2-infected and vaccinated subjects (SIV). The assays were performed right before (T0), fifteen days (T1) and three months (T2) after the third dose administration (SV) or post-infection (SIV).

Results: After the third vaccination dose was administered, SARS-CoV-2-specific neutralizing activity (NA) significantly augmented in SV saliva ($p < 0.05$) and plasma ($p < 0.0001$); yet, this NA was not protective against OC43. Conversely, in SIV, at T1, natural infection significantly increased NA against both SARS-CoV-2 ($p < 0.01$) and OC43 ($p < 0.05$) at systemic as well as mucosal level; still, this cross-reactivity vanished at T2. Of note, NA against SARS-CoV-2 and OC43 was shown to be higher in SIV compared to SV in plasma and saliva, as well; though, statistically significant differences were evident only in the oral mucosa at T1 ($p < 0.05$).

Conclusions: Our findings show that SARS-CoV-2 spontaneous infection triggers a more comprehensive and cross-reactive immunity than vaccine-induced immunity, protecting against OC43 at the systemic and mucosal levels. These results support the development of a pan-coronavirus vaccine able to prompt cross-reactive immunity even against seasonal coronaviruses, which could have enormous economic and health benefits globally.

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1. Introduction

Coronaviruses (CoV) are classified into alpha, beta, gamma and delta genera. The first Coronavirus able to infect humans (HCoV) was discover in 1965. To date seven strains have been identified. Of these, two belong to the alpha genus (HCoV-229E; HCoV-NL63), while five belong to the beta genus (SARS-CoV, MERS-CoV, SARS-CoV-2, HCoV-OC43, HCoV-HKU1) [1]. The respiratory and gastrointestinal tract's epithelial cells are the main targets of HCoVs [2]; however, many patients also experience neurological and neuromuscular problems during and after the acute phase of the illness, reminiscent of viral neuro-tropism [3–5].

HCoV-229E; HCoV-NL63, HCoV-HKU1 and HCoV-OC43 are seasonal viruses widely spread in the global human population, indeed almost 90 % of individuals are seropositive to these viruses [6]. Although severe and sometimes fatal lower respiratory tract infections can occasionally strike newborns, the elderly, or individuals with impaired immune systems, these viruses typically cause moderate upper-respiratory tract sickness and account for 15%–30 % of adult instances of the common cold [7]. Conversely, SARS-CoV, MERS-CoV and the early variants of SARS-CoV-2 infect the lower respiratory tract, possibly causing acute respiratory distress syndrome (ARDS), septic shock and multi-organ failure, with high case -fatality ratio [8].

Oral and nasal mucosa represent the main gate of HCoV entrance, and this is why virus-specific neutralizing immunity at this level is likely to be highly protective in controlling viral infection and replication [9–11].

It has been demonstrated that natural infection triggers a stronger response than vaccine administration leading to a higher protection rate to re-infection at both systemic and mucosal level [12]. This is particularly relevant considering that re-infections represent a common feature for all of the above mentioned HCoVs with a median time to re-infection ranging from 30 to 36 months, but early re-infections are also found between 6 and 12 months after the previous one [13]. These observations suggest that HCoV-acquired immunity wanes over time and, once exhausted, subjects are no-longer protected from infection as widely documented during the recent SARS-CoV-2 pandemic [14].

All seven HCoV genomes contain open reading frame (ORF) encoding 4 structural proteins, 15 non-structural proteins (nsp), and 1 or more accessory proteins [15,16]. The four structural proteins include the Spike (S), the Nucleocapsid (N), the Membrane (M) and the Envelope (E) proteins [1] and their alignment shows a high sequence identity ranging from 50 to 95 % [17]. Of these, S protein is the most mutable and immunogenic subunit, thus representing the main target for SARS-CoV-2 vaccine set-up. M and N also trigger a significant immune response and are more conserved than the S subunit [1,18].

The structural homology among HCoV genes suggests that cross-reactive immune responses could be induced by HCoV infection as recently documented by studies assessing SARS-CoV-2 susceptibility and COVID-19 severity in subjects previously infected by HCoVs [6,19–22]. In particular, Gou et al. reported a positive correlation between HCoV-OC43 S-IgG titres and disease severity in COVID-19 patients [7]. This cross-immunity includes T-cell and B-cell compartments [19], and may result in beneficial or detrimental effects. Indeed, on the one hand, cross-reactive T-cell response control disease severity, reduce viral transmission, and boost secondary immune response; on the other hand, it can cause tissue damage by increasing cytokine secretion. Likewise, cross-reactive B-cell response can modulate viral replication and contribute to foster viral clearance, but the presence of low-avidity cross-reactive memory B-cells may decrease the functionality of antibody response, reducing the magnitude of high-affinity, protective de novo antibodies [6].

The impact of cross-reactivity induced by previous HCoV infections on SARS-CoV-2 has been extensively reviewed [7,19,21]; the effects of cross-reactive immune responses induced by SARS-CoV-2 infection and/or vaccination on common seasonal HCoVs has nevertheless not yet been investigated. This issue will be addressed in this study with a special focus on HCoV-OC43, which overall displays a 69 % sequence homology with SARS-CoV-2 [6].

2. Materials and methods

2.1. Study design

A longitudinal, retrospective study was set up to assess how cross-reactive humoral immunity developed in two distinct subject groups: (i) Subjects who were immunized against SARS-CoV-2 and received three doses of the vaccine (SV); (ii) and subjects who were vaccinated against SARS-CoV-2 and became infected after receiving two doses of the vaccine (SIV). Blood and saliva samples were taken at T0 (nearly 15 days before booster dose administration), at T1 (15 days after booster dose administration), and at T2 (3 months after booster dose administration). SV received the third dose (booster dose) of the BNT162b2 RNA vaccine approximately six months after the second one. Subjects in the SIV group were enrolled because they were scheduled the third dose of vaccine, but they were infected shortly before administration. Thus, the T0 of the SIV corresponds to the T0 of the SV. Specimens from SIV were taken 15 days (T1) or 3 months (T2) after recovery.

The main goal of the investigation was to verify whether SARS-CoV-2 neutralizing activity (NA) induced by either vaccination or infection may have a cross-reactive protective impact against OC43. The secondary objectives were to: (i) assess the protection against OC43 provided by natural infection and/or the SARS-CoV-2 vaccine at various time points; and (ii) determine if the pattern of cross-reactive NA in the oral cavity mirrors NA at the systemic level. The Graphical Abstract provides a summary of the study design.

2.2. Viral strains and cell lines

Positive nasopharyngeal swabs (NPS) were used to isolate SARS-CoV-2, lineage B.1 (EU) (accession number: EPI_ISL_412973), which was thought to be the comparator virus. Whole genome sequencing was used to identify the strain, and the sequences were submitted to GISAID. HCoV-OC43 strain was purchased by Bei resources (BEI Resources NR-52725). The viruses were propagated in

VeroE6 cells purchased and certified by ATCC® (ATCC® VERO C1008, CRL-1586™; RRID: CVCL_0574). The presence of mycoplasma was excluded by Mycoplasma Detection Kit (MycoStrip™, InvivoGen). The Median Tissue Culture Infectious Dose ($TCID_{50}$) endpoint dilution assay was used to determine the virus titres. In summary, 96-well plates were plated with successive 10-fold dilutions of the viral suspension ranging from 10^6 to 10^{-4} $TCDI_{50}$/mL (50 μL). The plates were then incubated at 37 °C in 5 % CO_2, and the virus-induced cytopathic effect (CPE) was monitored every day using an optical microscope (ZOETM Fluorescent Cell Imager, Bio-Rad, Hercules, CA, USA). Viral titre was ascertained 72 h post-infection (hpi) using the crystal violet dying procedure, as previously reported [23]. Every experiment involving the handling of HCoVs was carried out in a BSL3 facility.

2.3. Study population and sample Collection

Plasma and saliva specimens were voluntarily collected from students and healthy volunteers at the University of Milan's Medical School in Italy.

The immune-biology laboratory at the University of Milan (Italy) enlisted individuals: 49 were SARS-CoV-2-Vaccinated (SV) [mean age (years) ± DS: 32.4 ± 13.9; range: 20–78; female: 67 %] while 25 were SARS-CoV-2 infected after receiving two vaccination doses (SIV) [mean age (years) ± SD: 36.4 ± 16.9; range: 18–78; female: 48 %]. A molecular test for SARS-CoV-2 and OC43 was employed to assess ongoing HCoV on saliva samples. The anti-SARS-CoV-2 vaccine, BNT162b2 (Comirnaty), was given to each and every participant. According to the prescribed immunization regimens, all enrolled participants had received two doses at T0 (BNT162b2: dose II administered 21 days after dosage I). Smokers, people with inflammatory disorders, and people receiving immunosuppressive treatments were excluded from the study.

The sampling timing and main characteristics of the subject enrolled are summarized in Table1A and 1B.

Whole blood was centrifuged at 1500 g for 10 min to isolate plasma, which was then stored at −20 °C until needed. Since the assay targets both nucleocapsid and spike proteins, plasma samples were incubated at 56 °C for 30 min before being analyzed using iFlash SARS-CoV-2 IgG and IgM (C86095G–C86095M–Shenzhen YHLO Biotech Co, Shenzhen, China). This was done to rule out the possibility of a prior asymptomatic infection. SARS-CoV-2 N plus S antigens were only detected in positive participants in the SIV group (data not shown).

Saliva specimens were obtained according to a previously validated protocol [9]. The University of Milan Ethics Committee granted ethical clearance (number 14/22). Participants signed a written consent form, all of which were anonymized.

2.4. SARS-CoV-2 and OC43 Virus Neutralization assay (vNTA)

Virus Neutralization assay (vNTA) in plasma and saliva samples from every enrolled subject was performed as exhaustively described in Ref. [9]. To assess the level of CPE in relation to the viral control, wells were scored. The wells' blue staining revealed the presence of NA. A neutralizing titre is the highest dilution at which 90 % of the CPE is reduced. If the results and the information were greater than or equivalent to 1:10 for serum titre or 1:1 for saliva specimens, they were deemed positive. Neutralization activity (NA) was assessed against SARS-CoV-2 B.1 (EU) strain and HCoV-OC43 strain.

2.5. Statistical analyses

When appropriate, the Kruskall-Wallis test and the unpaired Student's T-test were used. Variance analysis (ANOVA) was applied to compare variables between analyzed groups. A significant p-value <0.05 was established. Analysis were performed by GraphPad Prism 9. The GLP guidelines that our laboratories have adopted were followed during every procedure.

3. Results

3.1. SARS-CoV-2 and OC43 neutralization activity (NA) in plasma and saliva samples from SV and SIV

Assessment of NA in plasma samples of SV showed that following third dose vaccine administration NA titer against SARS-CoV-2 significantly increased ($p < 0.0001$) (Fig. 1A). NA levels observed at three months (T2), decreased compared to T1 ($p < 0.05$), but were still higher compared to T0 ($p < 0.0001$) (Fig. 1A). Conversely, NA against OC43 was not modified in response to a third SARS-CoV-2 vaccine dose administration (Fig. 1B). The same trend was observed by analyzing saliva samples; thus, a significant increase in NA titer against SARS-CoV-2 was observed at T1 compared to T0 ($p < 0.05$) and was maintained at T2 ($p < 0.01$) (Fig. 1C); however, such NA was not protective against OC43 (Fig. 1D).

Different results were obtained by performing NA analyses in biological samples of SIV. Thus, both SARS-CoV-2 ($p < 0.05$) (Fig. 2A) and OC43 ($p < 0.05$) (Fig. 2B) specific NA were significantly increased in SIV plasma samples (Fig. 2A). However, at T2 such protective NA was maintained only against SARS-CoV-2 ($p < 0.0001$) (Fig. 2A). Likewise, the hybrid immunity induced by natural infection plus vaccination in SIV resulted in a significantly increased NA in saliva samples against both SARS-CoV-2 ($p < 0.01$) (Fig. 2C) and OC43 ($p < 0.05$) (Fig. 2D). However, unlike plasma specimens, in saliva samples, NA levels dropped at T2, and as for OC43 they were significantly reduced compared with T1 ($p < 0.05$)

All NA results are summarized in table 1.

Tab.1A
SV cohort demographical features and NA results in plasma and saliva at different time-points

symbol	T0	T1	T2	subject	Gender	age	T0 SARS-CoV-2 NA titer		T0 OC43 NA titer		T1 SARS-CoV-2 NA titer		T1 OC43 NA titer		T2 SARS-CoV-2 NA titer		T2 OC43 NA titer	
							Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva
							N°49	N°15	N°49	N°15	N°49	N°15	N°49	N°15	N°29	N°15	N°29	N°15
SV	Before 3 dt dose administration	15 days post booster dose	3 months after booster dose	1	F	25	120		800		3200		800		1600	1	100	1
Uninfecte SARS-CoV-2 vaccinated with 3 doses of vacine				2	F	49	1		800		640		100		80		100	
				3	M	58	20	1	100	4	800	4	1	8	640		1	
				4	M	26	80		800		400		800		200		200	
				5	F	25	320		800		1600		800		800		1600	
				6	M	25	160		400		3200		400		400		200	
				7	M	26	80		200		800		800		800	4	1	2
				8	M	26	80		200		1600		400					
				9	F	26	80	1	400	1	800	2	100	1	100		200	
				10	F	26	20		1	4	1600		100	4		2		1
				11	F	26	320	2	100		1600	2	100			1		2
				12	F	26	80	1	100	2	800	1	1	4	400		800	
				13	F	20	80	1	400	4	200	1	400	4	6400		200	
				14	M	22	400		400	1	1600		1600	1	160		100	
				15	F	23	160		400		400		200			4		1
				16	F	25	20		100		800		100					
				17	M	28	10	1	400	1	1600	2	400	1	320		400	
				18	M	23	80		800		3200		800					
				19	F	24	80		400		800		200					
				20	F	25	40		200		400		200					
				21	F	51	20		800		400		800		160		2000	
				22	F	40	20		2000		400		2000		320		400	
				23	F	68	20		400		1600		400		3200		400	
				24	M	23	40		800		800		800			2		1
				25	F	35	40		100		1600		800			4		2
				26	M	40	80	2	800		800	1	400			1		1
				27	M	30	1	1	200	4	400	1	800	4		2		4
				28	F	33	10	1	1600	4	1600	2	1600	4				
				29	F	36	10	1	800	1	3200	8	800	2				
				30	F	20	80		400		1600		400			1		2
				31	F	23	80		200		800		200					
				32	F	24	20	1	400	1	400	1	800	1	200		400	
				33	M	20	80		1		1600		200		100		200	
				34	F	23	80		800		12800		800		80			
				35	F	47	10		400		400		400		800			
				36	M	22	80		100		400		100		200			
				37	F	57	80		200		1600		800		640	2	400	1
				38	M	40	10		800		800		1600				1600	
				39	F	23	20	1	1600	4	400	4	1600	8	800	2		2
				40	F	26	1		100		800		800		320			1
				41	F	43	40	1	400	4	1600	2	3200	1	160			
				42	F	37	40		200		1600		200		400			
				43	F	78	1		400		200		400		1600	2		
				44	M	47	1		800		3200		1600					1
				45	F	20	160		200		1600		400			4		1
				46	F	35	20	1	100	2	6400	1	100	1	320	1		
				47	F	63	1	1	800	16	800	8	3200	16	80			
				48	M	31	80		200		400		200					
				49	F	23	80		400		800		1600		640		800	

Tab.1B
SIV cohort demographical features and NA results in plasma and saliva at different time-points

symbol	T0	T1	T2	subject	Gender	age	T0 SARS-CoV-2 NA titer		T0 OC43 NA titer		T1 SARS-CoV-2 NA titer		T1 OC43 NA titer		T2 SARS-CoV-2 NA titer		T2 OC43 NA titer	
							Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva	Plasma	Saliva
							N°24	**N°25**	**N°19**	**N°18**	**N°24**	**N°25**	**N°19**	**N°18**	**N°24**	**N°10**	**N°24**	**N°10**
SIV	Before	15 days	3 months	1	M	58	120	1	800	4	800	8	400	4	800	8	400	2
SARS-CoV-2 vaccinated	Infection	post	after	2	F	26	1	1	800	1	1600	2	400	1	800	2	400	2
subjects with 2 doses of		booster	booster	3	F	26	20	2	100	4	3200	2	200	1	3200		100	
vaccine and infected before		dose	dose	4	M	28	80	1	800	2	800	4	800	1	400		400	
the Third booster dose				5	M	40	320	1	800	4	1600	2	200	2	1600	2	200	1
				6	M	30	160	1	400	1	200	1	200	2	200	1	200	1
				7	F	33	80	8	200	1	800	4	800	8	200		800	
				8	F	36	80	1	200	4	3200	2	800	1	1600	4	800	8
				9	F	23	80	2	400	4	1600	4	800	8	1600		400	
				10	F	25	20	1	1	1	1600	2	6400	32	800	1	3200	4
				11	F	22	320	1	100	1	16000	1	400	32	3200		400	
				12	F	34	80	1			3200	2			3200	1		1
				13	F	58	80	1	400	4	1600	2	6400	32	800		6400	
				14	M	21	400	1	400	4	1600	1	400	2	1600	2	400	2
				15	M	36	160	1			800	2			400			
				16	F	34		1				8						
				17	M	74	10	1	400	2	3200	32	6400	32	800	4	3200	8
				18	M	78	80	1	800	16	32000	16	800	32	800	1	800	2
				19	M	54	80	1	400	4	1600	4	6400	32	800		3200	
				20	F	43	40	2			1600	16			1600			
				21	M	18	20	1			1600	4						
				22	M	36	20	1	2000	1	320	4	400	1	1600		400	
				23	F	22	40	1	400	2	800	2	1600	8	100		800	
				24	M	29	40	1	800		640	2	100		800		100	
				25	M	23	80	2			1600	4			200			

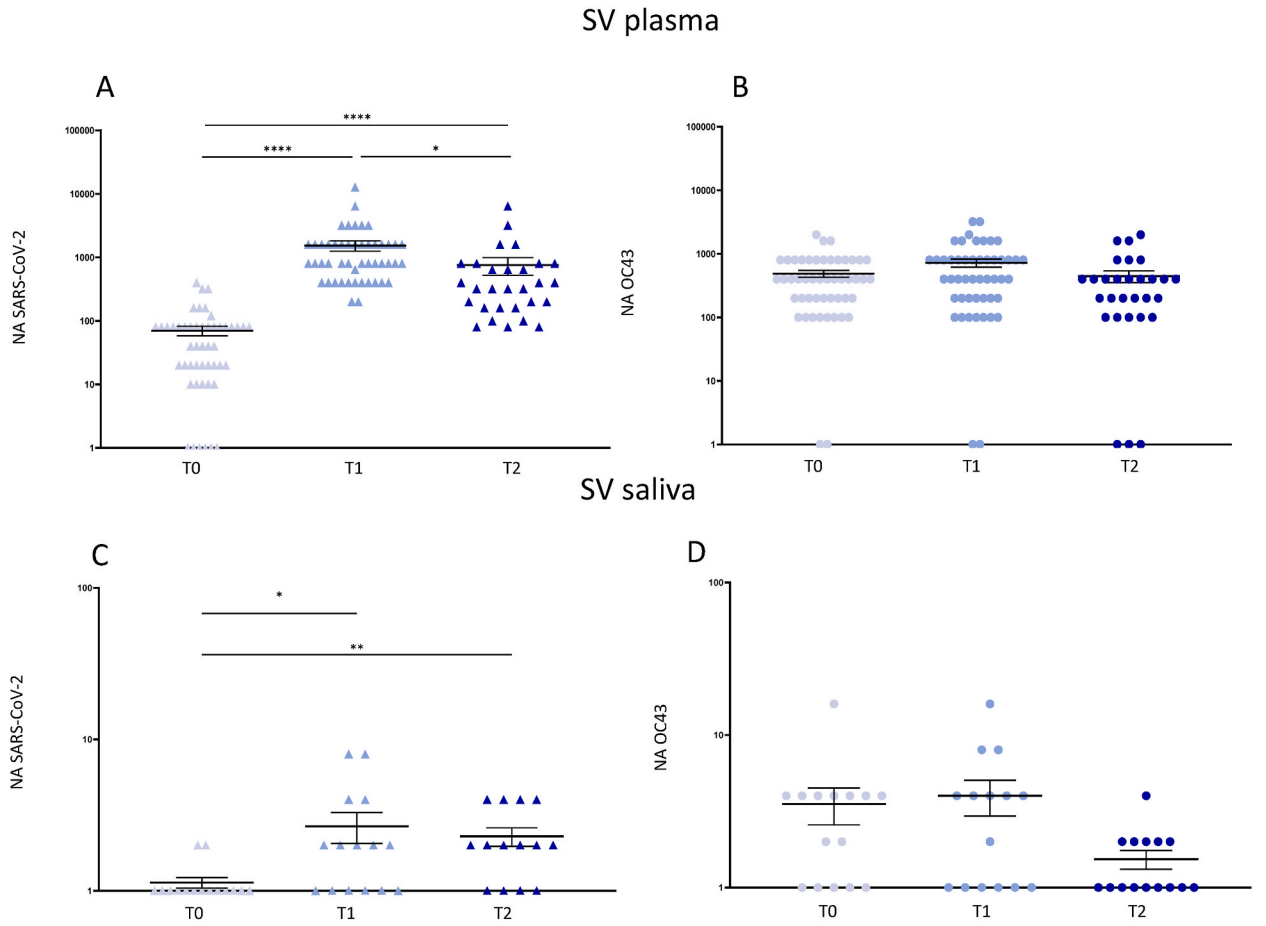

Fig. 1. Plasma and saliva neutralizing activity (NA) in SARS-CoV-2-vaccinated subjects (SV). NA against SARS-CoV-2 and OC43 in plasma samples of SV group are reported in panel A and B respectively, while NA against SARS-CoV-2 in saliva samples are shown in panel C and OC43 in panel D. 49 plasma samples and 15 saliva samples of SV were tested against SARS-CoV-2 and OC43. Mean values ± standard errors are reported. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$,$****p < 0.0001$ To compare three time points in SV NA response one way ANOVA Kruskall-Wallis test was performed.

3.2. Comparison of SARS-CoV-2 and OC43 NA in plasma and saliva samples from SV and SIV

By comparing NA in SV and SIV at T0, T1 and T2, following third dose vaccine administration (SV) or infection (SIV), we observed a more robust response at mucosal level induced by hybrid immunity compared to vaccine alone. In fact, as shown in Fig. 4A and B, NA in saliva from SIV, is visibly more robust at T1 compared to that observed in SV samples, against both SARS-CoV-2 and OC43 ($p < 0.05$ for both viruses). Although this trend is maintained at T2, after three months such differences are no longer significant. Conversely, these variations were not statistically significant in plasma samples against both SARS-CoV-2 (Fig. 3A) and OC43 (Fig. 3B), but we can observe a trend at T1 with higher mean response in SIV compared to SV, against both viruses.

4. Discussion

Overall, our findings emphasize the capacity of SARS-CoV-2 to stimulate the production of neutralizing immunity that exhibit cross-reactivity against other human coronaviruses (HCoVs), providing protection against OC43 infection at least 15 days following infection. Notably, this protective response appears to be elicited mainly by natural infection, as individuals who received three SARS-CoV-2 vaccine doses but were never infected did not show such cross-reactivity. Notwithstanding, it is worthwhile to underline that such cross-reactivity seems to be lost overtime as three months after infection even though the trend was maintained at both plasma and mucosal level, differences were no longer statistically significant. Additional investigates are required to verify if this result is just a consequence to the limited number of samples analyzed at the follow-up compared to T0 and T1 or if such cross-reactivity is lost due to the decrease in the overall NA.

These results confirm and expand prior studies [7,19,24] suggesting that hybrid immunity drives a more robust and comprehensive immune response. Indeed, unlike vaccine-induced immune response, which specifically targets the Spike protein alone, both humoral

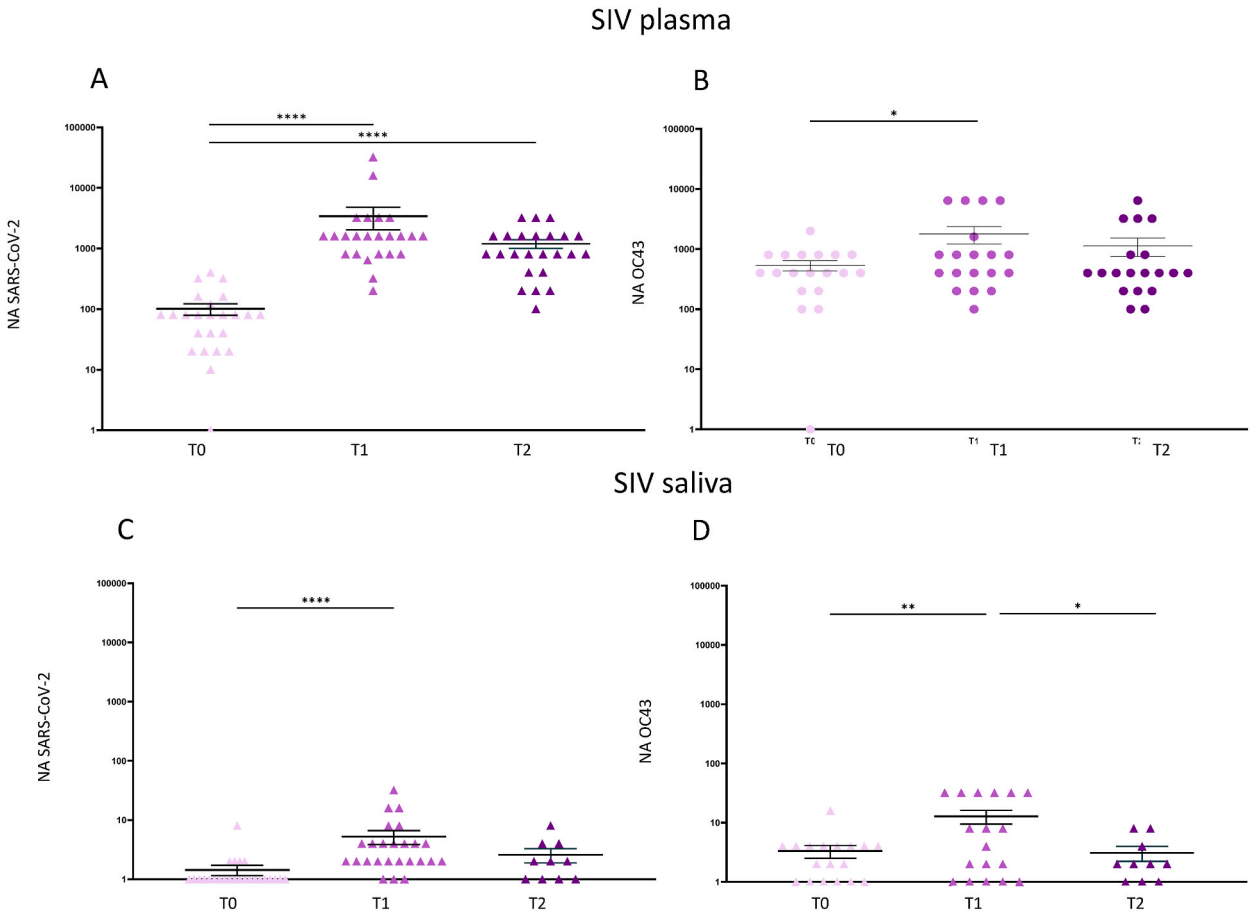

Fig. 2. Plasma and saliva neutralizing activity (NA) in SARS-CoV-2-infected subjects (SIV). NA against SARS-CoV-2 and OC43 in plasma samples of SIV group are reported in panel A and B respectively, while NA against SARS-CoV-2 in saliva samples are shown in panel C and OC43 in panel D. 24 plasma have been tested against SARS-CoV-2 and 19 against OC43. 25 saliva samples were tested against SARS-CoV-2 and 18 aganist OC43. Mean values ± standard errors are reported. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,****$p < 0.0001$ To compare three time points in SIV NA response one way ANOVA Kruskall-Wallis test was performed.

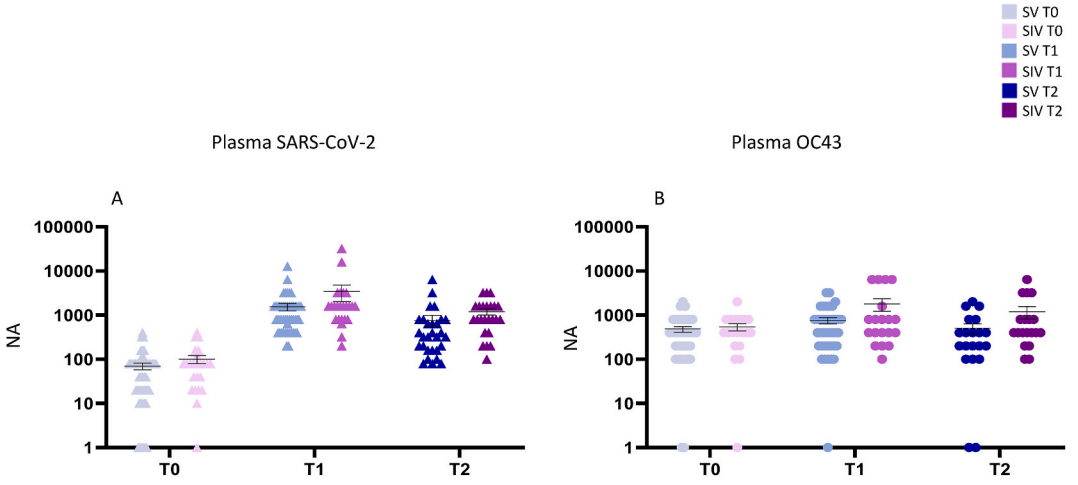

Fig. 3. Neutralizing activity (NA) in plasma specimens from SARS-CoV-2-infected (SIV) and vaccinated (SV) individuals. Comparison of neutralizing activity (NA) in plasma against SARS-CoV-2 (A) and OC43 (B) from SIV and SV at T0, T1, and T2. Mean values ± standard errors are reported. *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001. To compare SV and SIV groups unpaired T-test was used.

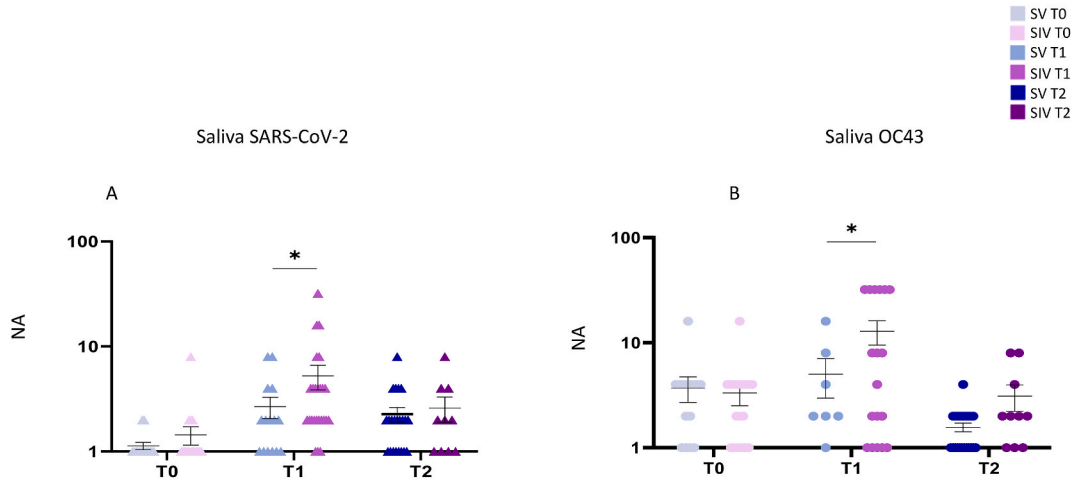

Fig. 4. Comparison of neutralizing activity (NA) in SARS-CoV-2-vaccinated (SV) and SARS-CoV-2-infected (SIV) saliva specimens. Panel A shows comparison between SV and SIV in saliva specimens against SARS-CoV-2, and panel B shows the results against OC43. Triangles identify NA to SARS-CoV-2 while circles identify NA to OC43. Mean values ± standard errors are reported. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,****$p < 0.0001$. To compare SV and SIV groups unpaired T-test was used.

and cell-mediated immune responses elicited by SARS-CoV-2 infection recognize all viral epitopes. This broader recognition presumably results into an enhanced cross-reactivity against multiple OC43 antigens, highlighting the superior immunogenicity of natural infection. As already demonstrated by several studies and in this same cohort [12] a hybrid immunity is also more protective against SARS-CoV-2 variants such as delta and omicron one. Indeed, subjects who were both infected/vaccinated display a stronger neutralization activity against WT virus and variants compared than subjects only vaccinated [23,25,26].

NA generated following SARS-CoV-2 natural infection and specifically targeting OC43 is cross-reactive not only in the systemic circulation, as previously reported by other studies [27], but also at mucosal level in the oral cavity. Indeed, neutralizing activity against both SARS-CoV-2 and OC43 turned out to be higher in SIV compared to SV in both anatomical districts, although statistically significant variances were detected exclusively in the oral mucosa. This observation could be clinically important considering that HCoVs typically initiate infection in the upper respiratory tract through epithelial cells of the oral mucosa, serving as both an early viral entry point and as a frontline defense [28]. Saliva, in particular, enriched with antibodies, cytokines, and chemokines, carries the ability to recognize and neutralize viruses while attracting innate and acquired immune cells to the infection site [29,30]. In particular, the local activation of antigen-specific B and T lymphocyte responses epitomizes the immune system's capability to recognize and target viral infection and/or replication. This orchestrated response generates a protective milieu, potentially providing a barrier against future infections and preventing the further dissemination of virus into the respiratory system. In this frame, the complex cross-reactive immunity prompted by SARS-CoV-2 infection at mucosal level holds critical importance in efficiently thwarting not only SARS-CoV-2 but also other HCoVs, thereby preventing their spreading [31].

Several studies agree on the importance of cross-reactivity, as this significantly improves immune responses to other pathogens displaying high sequence homology, mainly within HCoV family. For instance, Guo et al. [7] recently reported that cross-reactivity of antibodies directed against seasonal coronaviruses appears to be protective against SARS-CoV-2 infection, as it correlates with disease severity. T cell-mediated cross-reactivity against multiple SARS-CoV-2 antigens has been documented even in healthy individuals not once exposed to SARS-CoV-2 [32]; presumably because of previous exposure to other seasonal coronaviruses, which in virtue of their sequence homology are able to confer long-term coverage [33].

It was recently reported that [34] the greater the sequence homology among different microbes, the greater the antibody cross-reactivity will be. In fact, evaluation of cross-reactivity of anti-Spike specific IgG in the sera of SARS-CoV-2 infected convalescents showed that higher protection is achieved against SARS-CoV, OC43 and MERS, sharing more than 30 % sequence homology in their Spike proteins, compared to 229E and NL63 which display less than 30 % sequence homology.

This phenomenon is even more interesting if cross-reactivity can confer protection against more aggressive viruses. Hicks et al., for example, found that SARS-CoV-2-specific spike IgG could cross-react against several seasonal coronaviruses, including SARS-CoV, OC43, HKU1, but also against MERS, which among all HCoVs is the most lethal [35]. Therefore, these data support the development and administration of a pan-coronavirus vaccine which beyond preventing seasonal coronavirus infections, could also neutralize MERS-CoV that still endures in dromedary camels in Middle Eastern countries, leading to sporadic infections in human beings [36]. as well as new emerging coronaviruses. In a scenario of socioeconomic globalization, this type of vaccine would be a huge economic and health benefit globally.

This study presents some limitations. First, we could not assess SARS-CoV-2 cross-reactive immunity against HCoVs other than OC43, because of the paucity of biological samples (mainly saliva) collected during the pandemic period. Second, as PBMCs were used for other studies we could not assess cellular cross-reactive immunity, which would have allowed to get an exhaustive overview on Coronavirus cross-reactive immune response. Despite these limits, to our knowledge this is the first report demonstrating the

development of HCoV cross-reactive mucosal immunity following SARS-CoV-2 infection. These findings are likely to be important considering that: 1) the oral mucosa represents the main gate of viral entrance; 2) immunity to seasonal HCoVs rapidly wanes overtime [13]; and 3) although such infections mostly develops as a common cold in healthy adults, severe outcomes have also been observed in subjects whose immune system is still evolving or it is not properly working [37,38].

Further studies are warranted to confirm these results and to verify if such cross-reactive protection in the oral mucosa may be extended even to more life-threatening viruses.

Data availability statement

Data will be made available on request.

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CRediT authorship contribution statement

Micaela Garziano: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mario Cano Fiestas:** Methodology. **Claudia Vanetti:** Methodology. **Sergio Strizzi:** Methodology. **Maria Luisa Murno:** Methodology. **Mario Clerici:** Writing – review & editing, Funding acquisition. **Mara Biasin:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Non-standard abbreviations

SV	SARS-CoV-2 vaccinated with 3 vaccine doses
SIV	SARS-CoV-2 infected after two vaccine doses
vNTA	Virus Neutralization assay
NA	Neutralizing activity

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