

Anti-SARS-CoV-2 antibodies persistence after natural infection: a repeated serosurvey in Northern Italy

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Abstract

Introduction. To evaluate the decline of antibodies induced by SARS-CoV-2 infection, the individuals resident in 5 municipalities of the Autonomous Province of Trento, Northern Italy, who resulted IgG positive for anti-SARS-CoV-2 nucleocapsid (NC) in May 2020, were tested four months later.

Methods. Anti-SARS-CoV-2 NC antibodies were detected using the Abbott SARS-CoV-2 IgG assay. Samples that gave a negative result were re-tested using the Liaison SARS-CoV-2 IgG assay to assess anti-spike (S) S1/S2 antibodies. The fifty-percent tissue culture infective dose (TCID₅₀) neutralizing assay was performed on a subgroup of formerly positive sera. Statistical analysis was performed by STATA version 16.1 (STATA Corp., College Station, Texas, USA).

Results. Overall, 480 out of 1159 participants became seronegative for anti-NC IgG antibodies. Age above 70 years and cough were associated with persistent anti-NC IgG levels. Most anti-NC IgG negative sera were positive for anti-S IgG (77.9%). The neutralization assay showed high concordance with anti-S antibodies positivity.

Conclusion. In conclusion, a decline of anti-NC IgG values was recorded four months after the first evaluation. A high proportion of anti-NC seronegative individuals were positive for anti-spike IgG antibodies, which appear to persist longer and to better correlate with neutralization activity.

Key words

- Covid-19
- serology
- neutralizing antibodies

INTRODUCTION

Although cases of reinfection by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have been sporadically reported [1], a previous history of SARS-CoV-2 infection is associated with a greatly reduced risk of reinfection [2, 3]. As suggested by a recent modelling study, the presence of neutralizing antibodies induced by natural infection or by an effective vaccine is likely to be predictive of protection [4]. The duration of protection against infection with common human coronaviruses appears to be rather short [5, 6], however in the previous SARS epidemic, SARS-CoV specific IgG were

shown to remain detectable for at least two years [7]. Earlier data on the kinetics of IgG antibodies against SARS-CoV-2 among both symptomatic and asymptomatic individuals showed a rapid decline [8, 9]. More recent studies, however, tend to confirm that infection elicits durable serum antibody titers for several months [10-12]. Whether memory-B-cell and T-cell responses may still confer protection in individuals experiencing antibody decline to undetectable levels is unknown [12, 13].

The type of antibody response may also play a role. Experimental vaccination against SARS-CoV with nu-

cleocapsid protein (NC) can induce strong antibody responses that were found to be non-neutralizing [14]. While non-neutralizing antibodies might still exert antiviral activity, for example via the Fc-Fc receptor-based effector function, non-neutralizing NC antibodies may lead to enhanced disease for some vaccine candidates in animal models when neutralizing antibodies are absent [14]. Studies conducted on SARS-CoV-2 have shown that the spike (S) protein is the main target for neutralizing antibodies [15-17].

To evaluate the persistence of SARS-CoV-2 antibodies, we repeated a serosurvey in five municipalities of the Autonomous Province (AP) of Trento, North of Italy, recruiting those individuals who had resulted positive in a large population-based seroprevalence study conducted four months earlier [18]. In a subsample of seropositive participants, the antibody neutralizing titer was also evaluated.

METHODS

Study population and design

As already reported [18], the study was conducted in 5 municipalities of the AP of Trento with the highest incidence of COVID-19 confirmed cases.

The Department of Prevention of the Azienda Provinciale per i Servizi Sanitari (APSS) sent a letter of invitation to participate in a second study to all the citizens who tested positive for anti-SARS-CoV-2 antibodies in the serosurvey conducted 4 months before, between May 5 and 15, 2020.

Serum preparation and storage

Blood samples (5 ml) were collected in Serum Separator Tubes (BD Diagnostic Systems, Franklin Lakes, NJ, USA) and centrifuged at room temperature at 1600 rpm for 10 min. Aliquots were transferred to 2ml polypropylene, screw cap cryotubes (Sorfa, Zhejiang, China) and immediately frozen at -20 °C. Frozen sera were then shipped to the Istituto Superiore di Sanità (ISS) as national reference laboratory for COVID-19, in dry ice following biosafety shipment condition. Upon arrival serum samples were immediately stored at -80 °C [18].

SARS-CoV-2 IgG immunoassays for nucleocapsid (NC) and spike (S)

Two commercial chemiluminescent immunoassays (CLIA), employing either NC or S antigens and designed for high throughput in healthcare settings, were used. All the serum samples were evaluated by the Abbott SARS-CoV-2 IgG assay (Abbott Diagnostics, Chicago, IL, USA), using the NC antigen; sera resulting negative were retested with the DiaSorin Liaison SARS-CoV-2 IgG assay (DiaSorin, Italy), which uses S1/S2 antigen. The Abbott Diagnostics anti-NC IgG assay was performed on the Architect i2000SR automated analyser. The analyser automatically calculates SARS-CoV-2 NC IgG antibody concentration expressed as an index value. According to the manufacturer's instructions, the results were interpreted considering as positive an index of ≥ 1.4 and as negative an index of < 1.4 .

The DiaSorin SARS-CoV-2 IgG was performed on the

LIAISON® XL fully automated chemiluminescence analyzer. The analyser automatically calculates SARS-CoV-2 S1/S2 IgG antibody concentrations expressed as arbitrary units (AU/mL). The assay range is up to 400 AU/mL. According to manufacturer's instructions, values ≥ 15 AU/mL were interpreted as positive, and values ≤ 12 AU/mL as negative, according to manufacturer's instructions; in case of results falling within an equivocal zone in between 12 AU/mL and 15 AU/mL, the test was repeated.

SARS-CoV-2 neutralizing antibody assay

In vitro neutralizing activity provides quantitative results as a measure of a functional humoral immune response against SARS-CoV-2. A known amount of SARS-CoV-2 (code 77III, isolated and cultivated at ISS, titer $1 \times 10^{3.4}$; GISAID accession ID: EPI_ISL_412973) was incubated with different dilutions of the serum sample to determine the dilution at which cytopathic effect on Vero E6 cells (ATCC® CRL-1586) is observed in 50% of infected wells (MN 50%). The detailed protocol is described below: two-fold serial dilutions of serum samples starting at 1:8 dilution up to 1:512 in cell culture medium EMEM (Sigma) supplemented with 1X pen/strep and 2% fetal bovine serum (FBS; Corning) were added to 96-well plates. The mixture of virus (100 TCID₅₀) and serum was incubated at 37 °C for 1 hour for a total volume of 100 μ l. After this incubation period, a solution of 22,000 cells per well in a total volume of 100 μ l was added and incubated at 37 °C for 5 days.

Finally, the neutralization titer was calculated and expressed as the serum dilution capable of reducing the cytopathic effect to 50% (MN 50%). Positive and negative sera samples and cell culture control together with the virus were added in each test.

Statistical analysis

The IgG levels were summarized by the median and by centiles (25th; 75th). The differences among IgG levels between the first and the second survey were evaluated by the Wilcoxon test. The differences among IgG levels between groups (positive versus negative in the second survey) in the first survey were assessed by Mann-Whitney test. The IgG levels observed in the first survey were categorised in tree classes: "weak positive" (between 1.4 and 3.0), "medium positive" (between 3.0 and 5.0), and "high positive" (> 5). The McNemar's test was used to compare frequency on paired data. The concordance between anti-NC, anti-S, and TICD50 was evaluated using the Kappa test [19] ($K < 0.20$ = "poor", $0.20-0.40$ = "fair", $0.40-0.60$ = "moderate", $0.60-0.80$ = "good", and $0.80-1.00$ = "very good").

A multivariable logistic regression model was used to determine the relationship between persistent anti-NC IgG in the second serosurvey (positive versus negative) and a set of explanatory variables. The following variables that were significantly associated ($p < 0.01$) at the univariate analysis were included in the multivariable model: gender, age group, geographical area, presence of symptoms, working in contact with the public and household size, IgG positivity group (weak, medium,

high) olfactory and gustatory dysfunctions, fever, weakness, cough, dyspnea, arthralgia, diarrhoea, and abdominal pain and vomit. The likelihood ratio test was used to compare different models.

A subset of anti-NC IgG positive samples was tested with the neutralization test. Assuming a positive proportion of 95% and precision of 4%, 106 samples are required with an alpha error of 5%.

In all the analyses a two-sided p-value <0.05 was considered statistically significant. Statistical analysis was performed by the STATA version 16.1 (STATA Corp., College Station, Texas, USA).

Ethical approval

Informed consent for blood collection was obtained from all the participants. The study was approved by the Ethical Committee of the ISS (Prot. PRE BIO CE n.15997, 04.05.2020).

RESULTS

Participation in the second survey

Overall, 1159 individuals of the 1402 individuals who resulted seropositive in the first survey (82.7%) were enrolled in the study (Figure 1). All age groups were well represented. The proportion of those who were retested ranged between 72.6% in the age group 20-29 years and 93.1% in the age group 60-69 years.

Changes in antibody levels against NC

Of the 1159 individuals who resulted initially seropositive, 480 (41.4%) seroreverted at the second evaluation. As shown in Figure 2, a statistically significant reduction in the median value was observed in the second survey, from a median of 5.7 (25th centile = 3.9; 75th centile = 7.4) to 1.9 (25th centile = 0.8; 75th centile = 3.6) (p-value <0.0001 using the non-parametric Wilcoxon signed-rank test).

Comparing the median values in the positive and negative groups, those who seroreverted started from a lower average value (median = 3.6; 25th centile = 2.7;

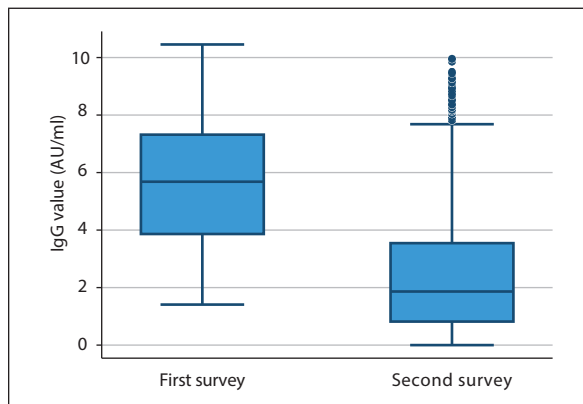


Figure 2 Distribution of the IgG values against SARS-CoV-2 nucleocapsid in the first and in the second serosurvey.

75th centile = 4.6) compared with those who remained positive (median = 7.0; 25th centile = 5.9; 75th centile = 8.2) at the second survey; the difference was statistically significant (Mann-Whitney test; p <0.0001).

As shown in Figure 3, when the participants were stratified into three groups in accordance with their anti-NC IgG level at the baseline (i.e., weak positive, with a value between 1.4 and 3; moderate positive, between 3 and 5; and high positive, greater than 5), the median value of the weakly and moderately positive groups decreased below the assay cut-off after 4 months, while the median of the highly positive remained above the cut-off.

Correlation between anti-SARS-CoV-2 IgG against NC and S proteins and neutralization activity

The samples resulting negative for antibodies against NC in the second study were tested to evaluate the presence of antibodies against the S protein. Since for one sample the available amount of serum was not sufficient for the analysis, 479 available serum samples

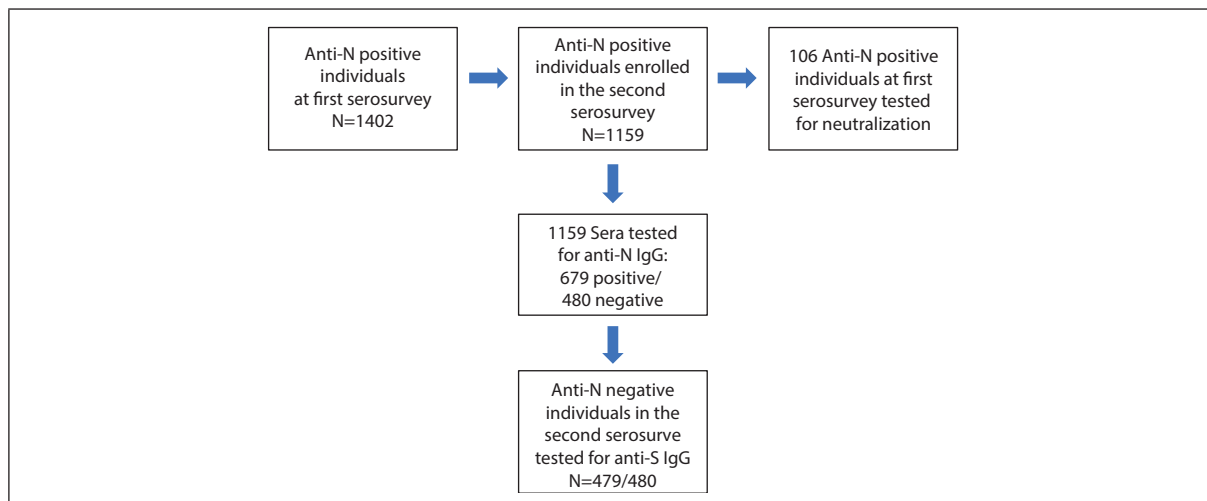


Figure 1 Study flow diagram of the sample collection and testing process.

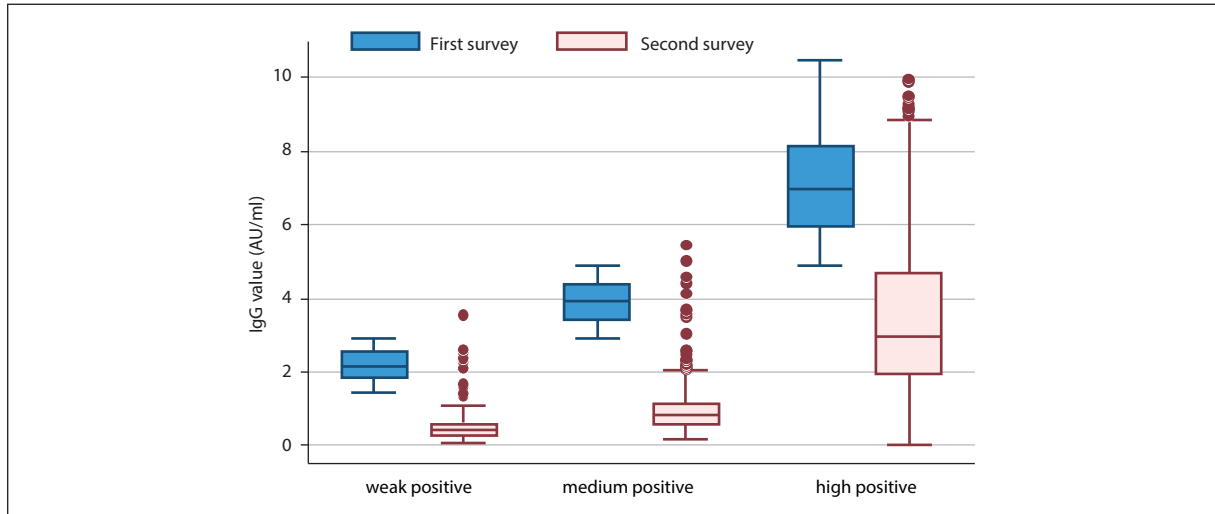


Figure 3
Median of the IgG values against SARS-CoV-2 nucleocapsid in the first and second survey by IgG positivity groups.

were tested, and 373 of them (77.9%) resulted positive (Figure 4).

Comparison between serology and functional neutralization assay

One-hundred-six sera from a subgroup of individuals who tested positive in the initial study were selected for testing anti-NC IgG, anti-S IgG, and *in vitro* neutralizing activity 4 months after the baseline. Comparable numbers of weak, moderate, and high positive sera were selected. Of the 106 sera, 97 (91.5%) showed neutralizing activity (TCID₅₀ ≥ 1/8), and 9 sera (8.5%) had a TCID₅₀ titer < 1/8; 57 (53.8%) were anti-NC positive and 93 (87.7%) were anti-S positive.

As shown in Table 1, only 53 sera showing neutralizing activity were anti-NC IgG positive (54.6%) versus 92 (94.8%) which were anti-S IgG positive. Most of the anti-NC IgG negative sera (41 out of 49) were anti-S positive (83.7%) and 44 had neutralizing activity (89.8%). Of the 57 anti-NC IgG positive sera, 52 were also anti-S positive (91.2%). Of 93 anti-S positive sera, 92 showed neutralizing activity. Overall, these data confirmed that despite a decline in anti-NC IgG levels below the positivity threshold, most of the sera are positive for anti-S IgG. A high concordance between anti-S positivity and neutralization activity, as calculated by

McNemar's test was found, showing that neutralizing activity relied on anti-S positivity.

High and significant agreement (94.3%) was found between anti-S and TCID₅₀ ($k = 0.70$; $p < 0.0001$) (Table 1). To further confirm the concordance, when IgG levels were considered, a good correlation between anti-S and TCID₅₀ was observed (rho-Spearman: 0.84, $p < 0.0001$) compared with anti-NC/anti-S (rho-Spearman: 0.61, $p < 0.0001$) and anti-NC/TCID₅₀ (rho-Spearman: 0.56, $p < 0.0001$).

Factors associated with persistent anti-NC IgG after 4 months

The multivariable logistic regression model showed that age group, gender, anti-NC IgG level in the first serosurvey, and cough were factors associated with the persistence of anti-NC seropositivity (Table 2). In particular, the individuals with high anti-NC IgG levels in the first serosurvey had the highest probability to be seropositive after four months (OR = 69.2). Age above 70 years and cough, as reported during the first survey, were also strongly associated with persistent anti-NC IgG levels, this association may be explained since those above 70 years of age and those with cough included a greater proportion of highly positive individuals.

DISCUSSION

Hereby, we report the results of a repeated serosurvey conducted in five municipalities in the AP of Trento, located in Northern Italy [18]. One of the main findings of the second survey, conducted on a large population of initially seropositive individuals, consisted in the rapid decrease of antibodies against the SARS-CoV-2 nucleocapsid. Of the 1159 participants, 41.1% resulted seronegative by 4 months after the first evaluation. Surprisingly, when we tested the NC-negative serum samples for antibodies directed against the S protein, we found different results, with most patients still showing seropositivity. To better understand and explain these findings, we evaluated the presence of neutralizing

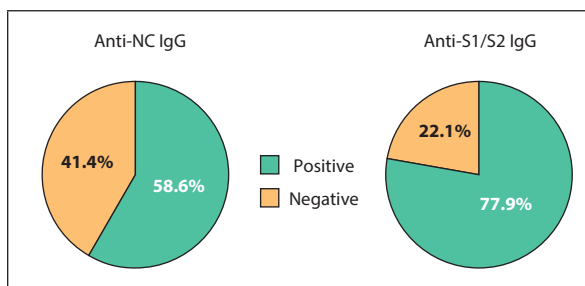


Figure 4
Percentage of anti-spikes (S1/S2) IgG antibodies on retested anti-NC IgG negative sera.

Table 1
Concordance between IgG against NC and S proteins and neutralization activity

	Anti-S +	Anti-S -	p-value*	Agreement Kappa; p-value	TCID50≥1/8	TCID50<1/8	p-value*	Agreement Kappa; p-value
Anti-NC +	52	5	<0.0001	56.6%	53	4	<0.0001	54.7%
Anti-NC -	41	8		K=0.08; p=0.1186	44	5		K=0.03; p=0.2787
Anti-S +					92	1	0.1025	94.3%
Anti-S -					5	8		K=0.70; p<0.0001

* McNemar test.

antibodies in a subgroup of previously anti-NC seropositive individuals and found that almost all the sera positives for antibodies against the S protein were able to neutralize the virus entry into cell lines *in vitro*. The key role played by neutralizing antibodies in recipients of anti-SARS-CoV2 vaccines has been recently highlighted [20].

Correlates of protection have been identified for many viral infections. These correlates are usually based on a specific level of antibodies induced by vaccination or natural infection that significantly reduces the risk of (re-)infection. For some viral infections and vaccines, the kinetic of the antibody response is also known, allowing for a prediction of how long protection will persist [21]. Studies on SARS-CoV2 had shown con-

flicting results. Studies conducted on a smaller number of individuals and/or clinical series reported a decay of neutralizing antibody levels 2 months after infection [8, 9]. These results appear to be consistent with those obtained for other human coronaviruses, such as NL63, 229E, OC43, and HKU1, showing a rapid decay of antibodies directed against the nucleocapsid protein [22]. However, other studies showed different results, with high IgG levels after several months [10-12, 23]. The inconsistency in the results of previous studies could be explained by differences in the study populations (i.e., patients with mild vs moderate or severe disease) or by methodological heterogeneity across different studies (i.e., detection of antibodies directed against the NC vs whole S or the receptor binding domain of the spike) [8].

The rapid decay of anti-NC IgG observed in the present study is likely related to the fact that individuals with severe disease or who were institutionalized in nursing homes were excluded from the serosurvey, hence only asymptomatic and paucisymptomatic infections were evaluated. In a longitudinal study of RT-PCR confirmed COVID-19 cases, the participants showed a wide range of antibody responses, and a decline in antibodies levels and virus neutralization was observed within three months of the onset of symptoms [24]. For those who developed a low neutralizing antibody response the titers could return to baseline over a relatively short period, whereas those who developed a robust neutralizing antibody response maintained high titers despite the initial decline [24]. Although the persistence of protective antibodies might be explained, to some extent, by the sporadic COVID-19 reinfection that have been reported [25-27], a consensus is growing on a slow waning of antibody responses in the late convalescent period. In this regard, recent data show that SARS-CoV-2 infection protect from reinfection up to one year [3, 4].

The type of antibody response to infection or vaccination may also play a role. Atyeo *et al.* [28], showed that a predominant humoral response to NC protein is associated with poor outcome in patients admitted to hospital, compared to response to S protein. Accordingly, Wajnberg *et al.* showed that antibody responses to the S protein correlate significantly with SARS-CoV-2 neutralization [22], a finding confirmed by the present study.

Table 2
Factors associated with seropositivity (multivariable logistic regression model)

Variables	OR	95% CI	
Gender			
Female	Ref		
Male	1.80	1.26	2.56
Age group (years)			
<20	Ref		
20-29	0.61	0.30	1.26
30-39	0.70	0.33	1.47
40-49	0.99	0.52	1.90
50-59	1.29	0.68	2.44
60-69	1.30	0.68	2.48
70+	5.09	2.30	11.24
Anti-NC IgG positivity group in the first serosurvey			
Weak	Ref		
Moderate	2.29	1.16	4.55
High	69.23	35.84	133.72
Cough			
No	Ref		
Yes	2.05	1.16	3.63

The observation that anti-NC IgG persist less than anti-S IgG has an important practical implication, in fact the use of anti-NC assays in seroepidemiological studies may cause an underestimation of the real prevalence. On the other hand, anti-NC IgG assays are best candidates for distinction between natural infection and vaccination, as current vaccines are S-based. In this context, it is important to keep in mind that a negative anti-NC IgG result months after infection should consider the possibility of seroreversion rather than the lack of evidence for natural infection.

Before drawing conclusions, strengths and limits should be mentioned. Firstly, only 17.3% of individuals did not participate in the survey, thus the refusal rate was low, and the possibility of a selection bias was minimized. Secondly, the study was repeated approximately 4 months after the first test; however, a proportion of the participants was apparently asymptomatic and others reported having had symptoms suggestive of COVID-19 sometime before the survey. Thus, the 4 months represent the minimum interval of time elapsed between the virtual date of infection and the second test. Thirdly, although the serological assay we used is assumed to have high sensitivity and specificity, the occurrence of some false positive or false negative results influencing the reliability and consistency of the results could not be completely ruled out.

In conclusion, we found a general antibody decay over time, with a relatively high proportion of initially SARS-CoV-2 seropositive individuals losing their anti-NC antibodies by 4 months after the first positive test. However, most of these individuals still had neutralizing anti-S IgG antibodies, suggesting a potential long-term duration of protective immune response even in those individuals with an asymptomatic or paucisymptomatic infection. This finding may have important implications in the choice of the target for antibodies persistence over the time together with the potential effectiveness and long-term protection of immune responses induced by vaccines and on herd immunity. Further studies are needed to understand whether persistence of anti-S,

potentially neutralizing antibodies, is actual correlate of long-term protection.

Authors contribution

PS, AB together with AF were responsible for the conception and design of the study; GF and PS coordinated the analysis on sera; PL, PV, AN, AC, IS, MS, IS, SF, EB, CF performed the analysis on sera; SP, MGZ, GB, RM, PPB, organized the samples and data collection; AB performed the statistical analysis; SB and GR help in the discussion of data; GR revised critically the manuscript; GF and PS wrote the manuscript. All the authors revised and approved the manuscript.

Conflict of interest disclosure

The Authors declare no conflict of interest related to this study.

Funding

This work was supported by APSS Trento which sustained the expenses for the IgG assays on collected sera.

Acknowledgments

The Authors would like to thank the study's participants.

The Authors thank the Serosurvey Study Group for COVID-19 in the AP of Trento: Elisabetta Mattevi, Lorenza Dalla Valle, Luca Endrizzi, Lucia Pecoraro, Andrea Varesco, Elisa Brida, Alessia Daldoss, Claudia Sebis, Bruno Zanon, Mauro Bandera, Laboratory Dept., APSS Trento; Flavia Riccardo and Patrizio Pezzotti, Dept. of Infectious Diseases, Istituto Superiore di Sanità; Massimo Pocher, Renzo Sannicolò, Andrea Manica, Angela Fogarolli, E-healthcare Solution Service, APSS Trento; Veronica Maroni, Elisa Chizzola, Privacy Office, APSS Trento; Simona Sforzin, Primary Care Director APSS Trento, and the entire staff of primary care involved in the study.

Received on 28 June 2021.

Accepted on 8 September 2021.

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