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ORIGINAL ARTICLE

SARS-CoV-2 Antibody Testing - How to Increase the Positive Predictive Value

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SUMMARY

Background: **Despite increasing COVID-19 infection rates, low overall prevalence resulting in a poor positive predictive value (PPV) of serological tests requires strategies to increase specificity. We therefore investigated a dual diagnostic strategy and evaluated the correlation between the severity of a SARS-CoV-2 infection and the detectable immune-response.**
Methods: **Participants were systematically categorized into positive and control cohorts and a probability score of COVID-19 was calculated based on clinical symptoms. Six hundred eighty-two serum samples were analyzed using a highly specific high-throughput system. Combining the serological test result and probability score was performed as a dual diagnostic strategy.**
Results: **Specificity of 99.61% and sensitivity of 86.0% were the basis of our approach. A dual diagnostic strategy led to increased pre-test probability and thus to a test specificity of 100%. In a flu-like symptomatic population, we estimated a COVID-prevalence of 4.79%. Moreover, we detected significantly higher antibody values in patients with fever than without fever.**
Conclusions: **Based on sensitivity and specificity results of our study being in line with previous findings, we demonstrated a dual assessment strategy including a symptom-based probability score and serological testing to increase the PPV. Moreover, the presence of fever seems to trigger a stronger immune-response.**
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KEY WORDS

COVID-19, positive predictive value, probability score, SARS-CoV-2 immune test

LIST OF ABBREVIATIONS

ARDS - Acute Respiratory Distress Syndrome
CLIA - Chemiluminescent immunoassays
CLSI - Institute for Clinical and Laboratory Standards
COI - Cutoff index
COVID-19 - Coronavirus disease 2019
CVE - Coefficient of variation Envelope
ECLIA - Electrochemiluminescence immunoassay

ELISA - Enzyme-linked immunosorbent assay
K - Control cohort
N - Nucleocapsid
ORF - First open reading frame
PPV - Positive predictive value
qPCR - Quantitative polymerase chain reaction
RdRp - RNA-dependent RNA polymerase gene
RDT - Rapid diagnostic tests
RSV - Respiratory syncytial virus
SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2
V - Verum cohort
WHO - World Health Organization

INTRODUCTION

The novel, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified as a pathogen causing critical respiratory infections (COVID-19) in December 2019 in Wuhan, the capital of Hubei, China. The extent of the symptoms ranges from inapparent disease to pneumonia and even ARDS (Acute Respiratory Distress Syndrome) with lung failure and lethal outcome. Within a few months, SARS-CoV-2 spread through China and resulted in a global pandemic [1]. According to World Health Organization (WHO) recommendation, molecular genetic methods such as quantitative polymerase chain reaction (qPCR) are used for the detection of SARS-CoV-2 in order to identify viral nucleic acid from nasopharyngeal swab secretions, sputum or bronchoalveolar lavage samples. Based on the genomic sequence of SARS-CoV-2 (Wuhan-Hu-1, GenBank Accession Number MN908947) published in January 2020, specific primers could be designed which include the target genes ORF1a and 1b (first open reading frames), RdRp (RNA-dependent RNA polymerase gene), E (envelope) and N (nucleocapsid) [1,2].
Despite the sensitive detection by qPCR, there are two diagnostic gaps within the clinically inapparent incubation period and the period of symptom decline. In addition, no detection of a past SARS-CoV-2 infection is possible [2-4]. This is of particular interest, since a considerable number of unreported cases of asymptomatic infections have been reported [5]. Accordingly, there is an urgent need for further laboratory testing approaches that can safely identify an overcome SARS-CoV-2 infection and ideally indicates an anti-SARS-CoV-2 immunity. These test systems are aimed at detecting the humoral response of a SARS-CoV-2 infection including IgM, IgA, and IgG [6]. Regarding the current state of research, seroconversion is observed 9 - 11 days after onset of symptoms for total antibodies, 10 - 12 days for IgM antibodies, and 12 - 14 days for IgG antibodies, where IgG is sometimes positive before IgM [7-10]. The main antigen structures of coronavirus investigated so far are the nucleocapsid [N], envelope [E], membrane [M] or spike [S] proteins. Technical formats include rapid tests using lateral-flow immunoassay technology for the simultaneous detection of IgM and IgG antibodies or a sandwich enzyme linked immunosorbent assay (ELISA) for the differential detection of IgM and IgG antibodies [11-14].
Serological testing is a promising way for assessing and monitoring seroprevalence at population level as sensitivity and specificity of currently available test systems are good. For example, Roche Elecsys® Anti-SARS-CoV-2 provides overall sensitivity results 14 days after positive PCR diagnosis from 88.1 - 100% and specificity from 99.65 - 99.91% [15]. Nevertheless, these results should be verified within a representative large-scale study cohort, including patients with a broad spectrum of comorbidities.
Furthermore, the PPV of a test depends on the prevalence of a disease within the population. The prevalence of COVID-19 varies in Germany depending on the region between 0.12 - 1.86%. This low prevalence seriously compromises the PPV of even the high-performance tests available [16]. Indeed, the increase of pre-test probability is the single most critical parameter for testing seroconversion in the population, requiring the optimization of clinical data points for interpretation of test results.
Within this study we addressed the problems associated with anti-SARS-CoV-2 serological testing mentioned above. We have sought to increase the PPV of our antibody test results by stratifying for predictive symptoms in our study population that could be used to increase pre-test likelihood. For this purpose, we first needed to verify the sensitivity and specificity of the chosen highly specific high-throughput testing system in a cohort categorized by a standardized acquisition of clinical data. Furthermore, we examined whether there was a positive correlation between test results and clinical data in our study.

MATERIALS AND METHODS

Patient recruitment, sample collection, and detection of anti-SARS-CoV-2 IgM and IgG

From April to July 2020, we recruited subjects for the IMMUNITOR study to examine various commercial anti-SARS-CoV-2 antibody tests as described in our previous work [17]. The study was approved by the Institutional Review Board (2020-556N) in accordance with the Declaration of Helsinki. Informed written consent was obtained from each subject prior to sample collection and analysis. A systematic questionnaire served to categorize the study participants comprising four different control cohorts, a group of COVID-19 convalescent subjects, and a further collective of patients with pauci-symptomatic unspecific flu-like symptoms and no positive SARS-CoV-2 PCR result (Table 1). The questionnaire was assessed on REDCap, a secure web platform for online databases and surveys supported by the University of Heidelberg (University V. REDCap https://redcap.umm.uni-heidelberg.de/redcap/ [updated 22.12.

2020.]). The survey consisted of 3 parts (assessing of contact with SARS-CoV-2, general medical history, and symptoms of the past two weeks) with a total of 36 questions. Study participants were instructed to fill out the questionnaire online. If necessary, further questions about the survey were discussed with the medical staff on the day of sample collection and, if needed, responses were adjusted. The clinical data were documented in pseudonymized form. In each case 7.5 mL serum were collected by peripheral venipuncture. In total, 682 serum samples were used for the analysis of this study. After one hour incubation to ensure adequate clotting, serum samples were centrifuged at 2,000 g for 10 minutes at 4°C, serum was aliquoted and stored at -80°C until use. For the measurements, serum samples were thawed and 300 µL each were transferred into a 1.5 mL Safe-Lock Tube (Eppendorf AG, Hamburg, Germany). The samples were centrifuged at 2,000 g for 5 minutes at 20°C to minimize the existence of precipitates. A heat-inactivation was not recommended by the manufacturer's instructions. The samples were analyzed on the cobas e 411. All samples were analyzed with the Elecsys® Anti-SARS-CoV-2 immunoassay (ROCHE, Mannheim, Germany). Cutoff index (COI) results with a value above 1.0 can be considered positive for SARS-CoV-2 total anti-N Ig antibodies according to the manufacturer's instructions.

Data analysis

The analysis of the data included the correlation with the clinical data and the determination of the specificity and sensitivity of the Elecsys® kit. Clinical data were systematically acquired via the REDCap platform. Surveys assessed for implementation of the REDCap Platform were checked according to the dual control principle in Microsoft Office Excel 2010 and REDCap. To assess the influence of clinical parameters on the presence of antibodies against SARS-CoV-2, further statistical analyses were performed in SPSS version 26 (e.g., ROC-curves, *t*-test for independent samples, Spearman's rank correlation coefficient). For all statistical analyses, p-values < 0.05 were considered statistically significant.

PPV was calculated with following formula according to Bayes' theorem [18]:

PPV = (sensitivity x prevalence)/[sensitivity x prevalence + (1-specificity) x (1-prevalence)].

An adapted probability score of a COVID-19 infection was calculated according to the score reported by Menni et al. [19]. Light modifications were applied because "skipped meals" and "fatigue" were not assessed in our questionnaire. With regard to gender, the code 1 was used for male and 0 for female. A score above 0.5 was considered as "elevated risk" for a COVID-19 infection. Adjusted Menni probability score = $1/(1 + \exp(-(X)))$ with X= -1.32 - (0.01 * age) + (0.48 * gender) + (1.75 * loss of smell/taste) + (0.31 * cough) + (0.49 * muscle pain) + (0.39 * nausea).

RESULTS

Demographics and test results

In our study, 682 samples were included in the analysis. Demographic data and test results are displayed in the flowchart (Figure 1A). V1 and the whole control cohort (K-groups) were included in the sensitivity and specificity analysis. The K1 group contains participants with confirmed infections with pathogens other than SARS-CoV-2, where cross-reactivity could have been expected. Unfortunately, this group was underrepresented in our IMMUNITOR-cohort (n = 4). In order to depict a significant analysis, we therefore added 30 pre-corona sera of hospitalized patients with serious pulmonary diseases for whom no survey was filled out. Within K4 (n = 65), K3 (n = 28), and K1 (n = 34) groups, there were no positive results. In the K2 group (n = 129), we found one positive result without medical history, contact with COVID-19 patients, or any suspicious symptoms. In total, our analysis revealed a specificity of 99.61% and a sensitivity of 86.00% in our study cohort (Figure 1B). In a previous study, it has already been demonstrated that adapting the manufacturer-defined cutoff for assessing positive or negative results may enhance the sensitivity of the test. It was shown, that the sensitivity of 91.7% (95% CI: 81.6% - 97.2%), which was calculated at the recommended cutoff COI 1.0, was increased to 100% (95% CI: 94.0% - 100%) by using the cutoff COI 0.165. This cutoff was determined with a ROC curve and applied to a cohort whose specimens were obtained 14 days after receiving a positive qPCR result [22]. In our study, all subjects of the V1 group had a positive qPCR result and were included in the study not earlier than three weeks after recovery. To improve sensitivity, we analyzed the COI data of V1 and all control cohorts (K1 - 4) in a ROC curve to adjust the decision cutoff as represented in Figure 2. The ROC analysis presents two values for a possible adjustment of the positivity cutoff. These potential cutoff values are 0.163 and 2.515. Their effects on sensitivity and specificity are furthermore illustrated in the Figure 2.

Probability score within symptomatic patients

As a prerequisite for using a test for screening purposes, it is necessary to achieve test specificity of 100% in case of low disease prevalence, explained by the calculation of the PPV (cf. Materials and Methods).

In order to increase the COVID-19 prevalence in the test cohorts, we have calculated the resulting PPV using a COVID-19 probability score adapted from Menni et al. [19]. The combination of both, our adjusted probability score with a cutoff of 0.5 and Roche COI results with a positivity cutoff of 1.0, leads to a specificity of 100% and a sensitivity of 68%. The results obtained by combining the Roche result and probability score are presented in Table 2 and Figure 3.

Furthermore, a comparison of COI results and probability scores was performed in order to examine the relationship and to determine a suitable cutoff for the ad-

Table 1. Systematic classification of subjects included in the IMMUNITOR-study.

Group	Characteristics
V1	Recovered patients from COVID-19: Patients with preliminary positive SARS-CoV-2 qPCR test result, at least 3 weeks after positive qPCR result
V2	Possibly unrecognized SARS-CoV-2 infections: Currently symptom-free participants who had atypical respiratory illness or flu symptoms since December 2019 without positive testing for SARS-CoV-2 and no other proven infection
K1	Possible cross reactions with other infections: Subjects with evidence of other pathogens since December 2019 (influenza, RSV, etc.)
K2	Healthy cohort: Clinically healthy participants with no cold or flu symptoms since December 2019 and no relevant chronic comorbidities. No contact to SARS-CoV-2 positive people
K3	Contact to SARS-CoV-2 positive people: Healthy participants with no cold or flu symptoms and no relevant chronic comorbidities since December 2019, but had contact with a patient diagnosed with COVID-19 more than 3 weeks ago. This group exceeded an incubation period and may represent a group with a recovered and clinically inapparent SARS-CoV-2 infection
K4	Chronic diseases: Clinically healthy participants with no cold or flu symptoms since December 2019. This group has immunologically relevant comorbidities (e.g., autoimmune diseases, therapies with monoclonal antibodies, cirrhosis of the liver, other chronic infections, etc.)

Abbreviations: V - verum cohort, K - control cohort, RSV - Respiratory syncytial virus.

Table 2. Results determined by the combination of Roche result and probability score.

	COI < 1 OR negative predictive value	COI ≥ 1 AND positive predictive value	Total
V1-group	16	34	50
K-groups	256	0	256
Total	272	34	306

Abbreviations: COI - cutoff index, K - control cohort, V - verum cohort.

justed Menni score. ROC analysis revealed 0.5 as a suitable cutoff for the adjusted Menni score. Subsequently, we applied the dual assessment strategy to the symptomatic V2 cohort whose status regarding SARS-CoV-2 infection is uncertain. Regarding the V2 cohort, there were 18/376 with a positive probability score and a COI ≥ 1. This would correspond to a COVID-19 prevalence of nearly 4.79% in a flu-like symptomatic population. For a population of flu-like symptoms, a positive adjusted Menni score would result in a PPV of antibody testing of 92%.

To exclude temporary interference, cross-reactivity or sample mix-up, we confirmed unexpected positive serological answers in the antibody testing for the V2 and K2 group by repeating the blood-draw and the antibody test. Out of the positive results in the V2 and K group (n = 23), 17 agreed to undergo a second antibody testing. The positive result was confirmed in every case. The COI test result was not significantly different in the first and the second analysis (mean COI-T1: 36.8; mean COI-T2: 47.4, p = 0.353).

Testing and clinical data correlation

Elecsys® is a qualitative assay with a COI < 1 considered to be negative and a COI ≥ 1 evaluated as positive. However, a numerical test result is obtained, showing a wide range above the cutoff of 1 in our data (span of positive test results: min. 1.15; max. 105.00; mean value: 31.95 ± 28.70). To evaluate whether there is a correlation between the quantitative test result and the severity of the clinical presentation, the symptom severity was calculated for each patient within the study cohort.

The scale for symptom severity ranged from 0 - 7 and was calculated from assessed symptoms in relation to COVID-19 disease or the reported flu-like infection. Within positive Elecsys® results (n = 66), there was no significant correlation between symptom severity and quantitative test results (Spearman's rho: 0.118; p =

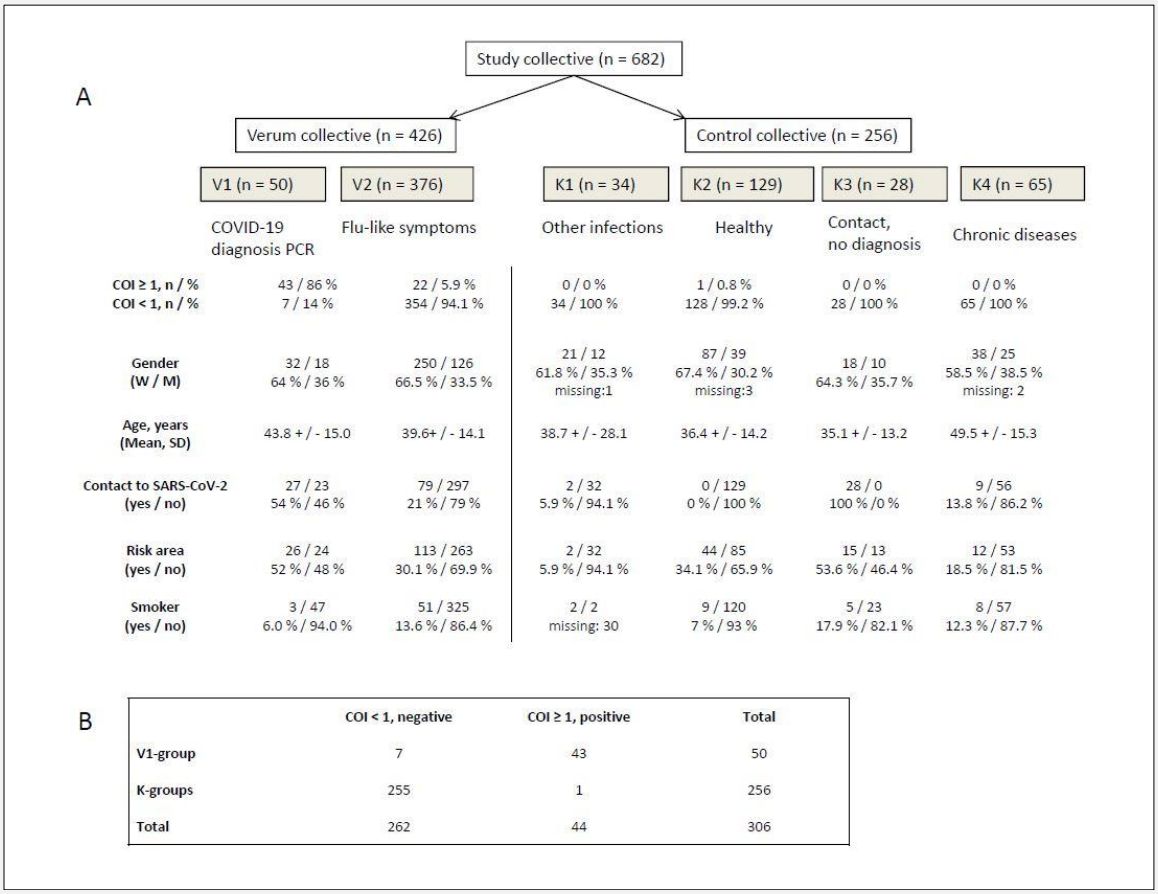

Figure 1. A. Flowchart of the study cohort including demographical data and distribution of test results. B. Overview of COI-results for V1- and K- groups as basis for calculation of sensitivity and specificity of Elecsys ®.

Abbreviations: COI - cutoff index, K - control cohort, V - verum cohort.

0.346).

Nevertheless, a significantly higher Elecsys®-COI was observed in subjects with fever compared to those without fever. In detail, the COI on average was 39.4 ± 31.0 for subjects with fever compared to a mean COI of 21.2 ± 21.3 in subjects without fever (p = 0.01) as illustrated in Figure 4.

It is unclear if patients with pauci-symptomatic COVID-19 disease develop an immune response [23]. Within the V1 group with positive qPCR result 7/51 had a negative test for antibodies. The symptom severity score within patients with no positive immune response was not significantly lower than in patients with positive immune response (mean symptom severity score in negative test results: n = 7: 3.29 ± 2.10; mean symptom severity score in positive test results: n = 43: 3.24 ± 1.51; p = 0.68). However, people with negative test results did not report significantly less the symptomatic fever than people with a positive test result (29% vs. 60%, p = 0.12).

DISCUSSION

This study assessed SARS-CoV-2 antibodies in a large, standardized cohort of 682 participants divided in six predefined subgroups. As a foundation for our further analyses, we assessed diagnostic specificity and sensitivity using a standardized, well-designed control cohort and the verum 1 group. The availability of serological tests for COVD-19 has increased enormously in recent months and some of these tests are currently in clinical application. These are in demand in COVID-19 diagnostics, in the assessment of seroconversion and immunity, in the appraisal of suitability as a convalescent plasma donor, and to clarify epidemiological issues

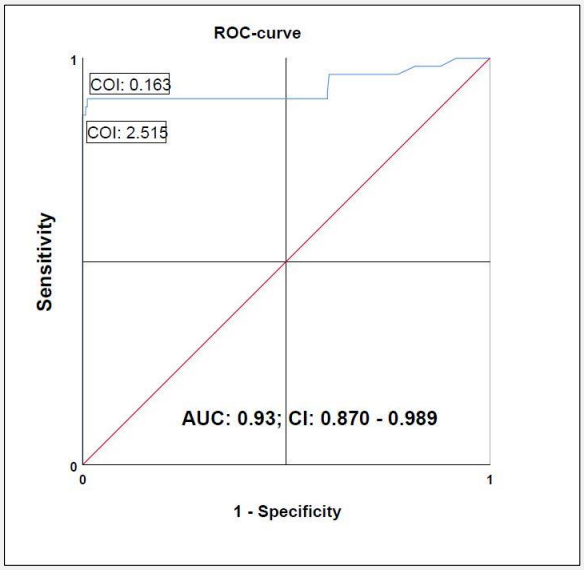

Figure 2. Depiction of ROC analysis for a potential adjustment of the positivity cutoff.

Both a COI of 0.163 or 2.515 could be defined as new cutoffs. A cutoff of 0.163 would improve the test sensitivity to 90.0%, but would decrease the test-specificity to 98.7%. A COI of 2.515 would increase the specificity of the test to 100% without lowering the sensitivity.

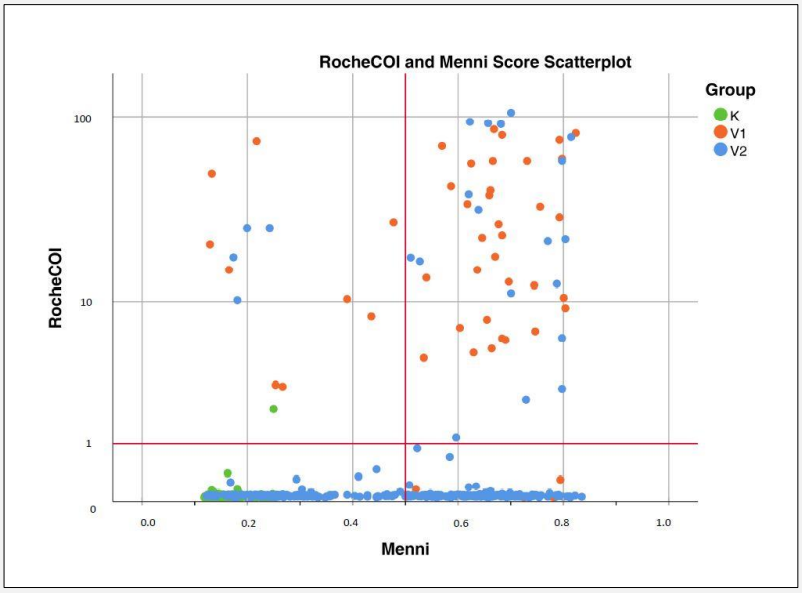

Figure 3. Relationship of adjusted Menni score and COI results.

Results of the adjusted Menni score are shown on the x-axis and COI values are displayed on the y-axis. All control groups are shown as green dots, subjects from group V1 are shown in red, and the symptomatic V2 cohort including volunteers without molecularly confirmed SARS-CoV-2 detection is visualized as blue dots. Selected cutoffs are illustrated as red lines.
Abbreviations: COI - cutoff index, K - control cohort, V - verum cohort.

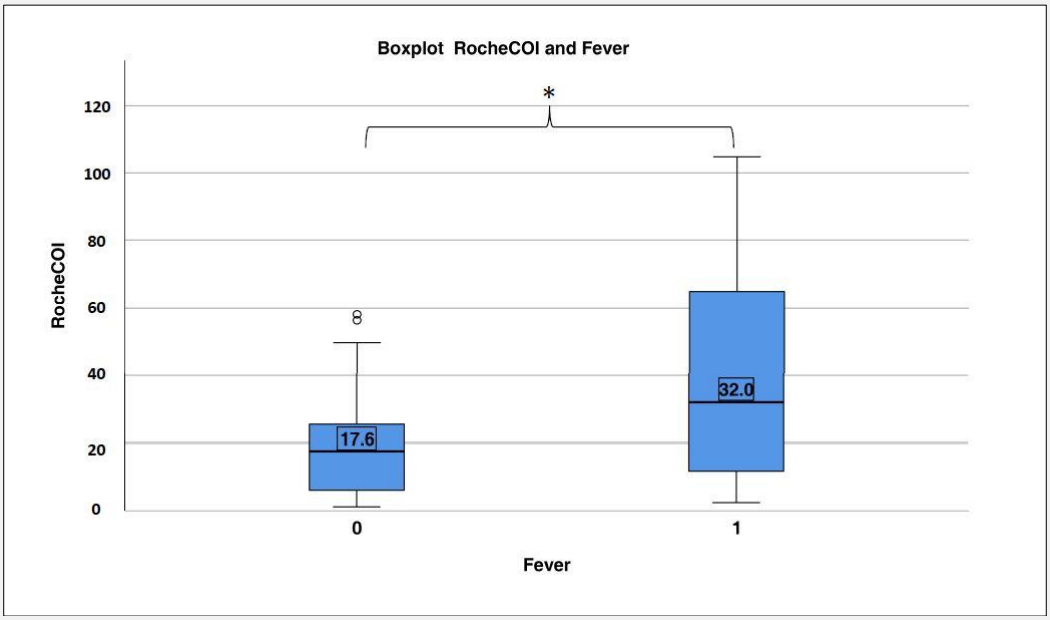

Figure 4. Boxplot representing COI in subjects with fever and those without fever.

Among all positive test results (n = 66), there was a significantly higher COI for those with fever showing a median COI of 32.0 compared to subjects without fever having a median COI of 17.6 (p = 0.01).
Abbreviations: COI - cutoff index.

such as regional exposure and spread [22-25]. However, this enormous increase in commercial systems requires validation with a sufficiently large study population [22]. Since a false positive result represents a potential hazard for the test person due to false assumption of immunity, the verification of specificity is of particular importance. The Institute for Clinical and Laboratory Standards (CLSI) recommends a minimum sample size of 50 to determine the diagnostic specificity of a test [22]. In our study, we included 306 subjects from five different categories to assess specificity. This included both, subjects with molecular genetics confirmed SARS-CoV-2 infection (V1), as well as asymptomatic control groups with a low probability of pretesting (K2), and control groups with potential cross-reactivity (K1, K4). The diagnostic specificity of 99.61% obtained in our cohort is slightly lower than the expected Elecsys® test specificity of 99.65% - 99.91% [15]. The sensitivity found in our collective of proven qPCR within recovered patients and tested in every case at least 21 days after positive qPCR results was 86.0%, likewise a little below the expected test sensitivity. Both specificity and sensitivity results are lower than previously reported results [17,26] and the first external validation of the Elecsys® test [20]. Favresse et al. proposed to lower positivity cutoff to 0.165 since it improved the sensitivity without lowering specificity. We also identified a possible COI of 0.163 leading to a sensitivity of 90.0%, but this would decrease the test specificity. However, this would significantly reduce the PPV. We have therefore decided to maintain the COI positivity cutoff of 1.0.

Serological test methods are a promising way to retrospectively determine the COVID-19 seroprevalence of a population, but since the PPV of a test system is calculated on the basis of prevalence, even a high specificity test will still result in a low PPV in Germany (e.g., a PPV from 48.8% for the city Mannheim with a COVID-prevalence of 0.430 at end of sample collection) [16]. The PPV of a test system could be raised if the pre-test probability is increased. This could be achieved if only patients with typical symptoms of COVID-19 were tested. In our study 18/376 participants with flu-like symptoms since the start of the corona pandemic had a positive immune answer in combination with typical COVID-19 symptoms and a high probability score. We can assume that these 18 people (4.79% of participants with flu like-symptoms) are unregistered COVID-19 cases. Contrary to assumptions raised at the beginning of pandemic, a much lower number of inapparent SARS-CoV-2 infections could be deduced, since only these 18 participants of the symptomatic cohort actually showed a positive antibody result. Among the 256 test persons of

all control groups, only one person showed a positive result, which we even classified as false negative due to the complete exclusion of COVID-19 contact. Although an absence of exposure can never be proven completely, even in the case of one positive person presenting no symptoms, the number of unreported cases would remain lower than expected.

Instructions of Elecsys® do not allow a titer specification as it is a qualitative test system, but our results revealed a high range above the cutoff 1. Weidner et al. described a positive and significant correlation in several antibody assays' results with neutralizing antibody titer [27]. In their study, Elecsys® showed the lowest but nevertheless significant correlation with the neutralization test antibody titer ($\rho = 0.457$) indicating the quantitative applicability of the test system, although other studies found a limited association between Elecsys® and neutralization test antibody titer [27].

When investigating the humoral response to a SARS-CoV-2 infection, it is of further interest whether the extent of an antibody titer correlates with the severity of symptoms during the acute phase of COVID-19 [24]. Moreover, it is still not certain if oligosymptomatic COVID-19 patients develop seroconversion [4,21]. A previous study by Grzelak et al. has already investigated the prevalence of symptomatic and asymptomatic COVID-19 patients and their serological immune response. On average, only 32% of oligosymptomatic patients showed a humoral immune response in this study. This indicates that minor symptoms are based on a smaller viral load resulting in a lower specific immune response. In our study cohort, we did not find a significant correlation between symptom severity and COI result. Even within the V1 group, participants with proven COVID-19 infection and negative antibody testing did not have significantly lower symptom severity scores than people with positive test results. These results suggest that a correlation between overall COVID-19 disease severity and COI value is not directly possible. However, Weidner et al. found that the presence of the symptom fever correlated positively with neutralization test [28]. In our data, the hypothesis that fever results in a more intense immune response could be replicated as people with fever had a significantly higher Elecsys® test result than patients without fever ($p = 0.01$).

One of our study limitations is that assessing survey data relied on participants' correct statements and was not done under supervision. Of course, each participant had a personal talk with medical staff and could discuss unclear answers in the survey or answer comprehension questions related to the survey. In this way, erroneous answers due to misunderstandings could be resolved. Furthermore, the K1 group contains participants with confirmed infections with other pathogens than SARS-CoV-2, where cross-reactivity could have been expected. Unfortunately, this group was underrepresented in our cohort ($n = 4$). Since systematic testing for pathogens of flu-like symptoms is not necessary therapy relevant, this could explain why only a few subjects had a proven infection with other pathogens. We have addressed this under-representation by including 30 sera with a confirmed non-corona pathogen of hospitalized patients with serious pulmonary diseases.

Another aspect regarding false positivity is cross-reactivity. Due to the affiliation of SARS-CoV-2 to the *Betacoronavirus* family, a false positive result may emerge in the case of a recovered MERS-CoV infection as well as other non-SARS coronaviruses such as OC43, 229E, HKU1, and NL63 [6,23]. Since we could not include a participant with a recovered infection caused by a different pathogen of the corona family, an evaluation of possible cross-reactivity in this area remains unresolved. On top of that, the diversity of diseases within the K4 group was high and no positive result was observed in this group. Even assuming a positive result in the K4 group, a clear assignment to a disease possibly associated with cross-reactivity would be questionable due to the mentioned diversity. To address this question, further studies should focus on autoimmune diseases with possible auto-antibodies, where cross-reactivity is more likely.

In conclusion, this study assessed SARS-CoV-2 antibodies in a standardized large-scale study cohort of 682 participants. Concerning the assessment of specificity and sensitivity, our results are slightly lower than previously reported. However, considering the detailed characterization of our control groups and the fact that only one false positive result was reported by Elecsys® in this cohort, a high specificity of 99.61% could be calculated and a good test quality can be obtained. Nevertheless, because of the currently still low overall prevalence, the PPV is still low. In this study, we have shown an approach that allows an increase in PPV without 100% test specificity. Using the adapted Menni score, we were able to propose a dual diagnostic strategy and applied it to a symptomatic cohort without molecular genetics COVID-19 affirmation and increased the PPV up to 92%. However, a general correlation between the severity of symptoms and the COI value could not be identified. Nevertheless, the presence of fever seems to trigger a stronger immune response and reporting fever correlated with a higher COI. In conclusion, the feasibility of the Elecsys® test system to assess anti-SARS-CoV-2 immune response was confirmed and approaches to increase the PPV by restricting serological tests to patients with COVID-associated symptoms have been introduced. This dual-test strategy should be verified within a large study cohort to evaluate its suitability for screening purposes.

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Ethical Approval:
The study was approved by the Institutional Review Board (2020-556N) in accordance with the Declaration of Helsinki.

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Declaration of Interest:
The authors have declared no conflicts of interest.

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