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**Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.**

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**Title**

Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.

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e-mail: [zeno.bisoffi@sacrocuore.it](mailto:zeno.bisoffi@sacrocuore.it)**ABSTRACT****Introduction**

As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 has infected more than 3,172,000 individuals, killing over 224,000 people and spreading to more than 200 countries. Italy has been the most affected country in Europe and the third most affected in the world in terms of number of cases.

Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population of Verona; ii) to assess the accuracy (sensitivity, specificity and predictive values) of an ELISA serological test for the screening of SARS-CoV-2.

## Methods and analysis

The study will be carried out on a random sample of subjects aged at least 10 years old from the general population of Verona. Participants will undergo the measurement of vital parameters (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by laser thermometer), the administration of a COVID-19 related symptoms questionnaire, the collection of a blood sample, and a nasopharyngeal swab. Our evaluation will include the statistical technique of Latent Class Analysis, that will be the basis for the estimation of prevalence.

## Results

Results from the study will allow us to: i) estimate with a very low margin of error the prevalence of asymptomatic SARS-CoV-2 positive individuals and the prevalence of those who are negative for SARS-CoV-2; ii) better define phase 2 of Italy's outbreak management strategy; iii) better understand the symptoms of SARS-CoV-2 infection and what potential role they may have in disease prognosis; iv) evaluate the diagnostic accuracy and value of the ELISA serological test as a screening tool for the general population.

**Ethics and dissemination** The study protocol has been approved by the Ethics Committee of Verona and Rovigo provinces on April 15, 2020 (internal protocol number 2641CESC).

## Key words

SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class Analysis

## Article Summary section

### Strengths and limitations

Study based on random sample of a general population

Very low estimation standard error (max 1.5%)

Simultaneous comparison of symptoms, real-time PCR test and ELISA serological test

Results will depend on response rate

### Patient and Public Involvement statement:

PPI representatives worked with us to refine the research question, however it was difficult to involve patients in other areas of the study design due to the very technical methods required to do a data linkage analysis. PPI representatives will write a plain language summary and design a leaflet for dissemination to their peers and distributing to patient groups.

## INTRODUCTION

As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 has infected more than 3,172,000 individuals, killing over 224,000 people and spreading to more than 200 countries<sup>1</sup>. Italy has been the most affected country in Europe and the third most affected in the world in terms of number of cases.. The epidemic is posing an extremely difficult challenge to health care establishments, health workers and to the general population. The

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2  
3 identification of asymptomatic SARS-CoV-2 positive individuals is crucial in reducing the  
4 spread of the virus throughout the world. The frequency of such cases is unknown, despite  
5 asymptomatic cases regularly being referred to in the literature<sup>2,3,4</sup>.

6 Data from the cruise ship 'Diamond Princess' have shown that the percentage of  
7 asymptomatic SARS-CoV-2 positive cases among all passengers and crew members tested  
8 prior to disembarkation was about 50%. This rate has since been revised as 17.9%<sup>5</sup>.

9 A similar study focusing on a Japanese population returning from China, found the percentage  
10 of asymptomatic cases to be 33.3%<sup>6</sup>.

11 Finally, a small group of residents in a skilled nursing facility has also been screened: 13 of 23  
12 residents were defined as asymptomatic, but 10 of these developed symptoms over the  
13 subsequent 7 days<sup>7</sup>. Add that asymptomatic SARS-CoV-2 positive individuals are able to  
14 transmit the virus.

15 Currently, SARS-CoV-2 detection is based on accepted and standardised molecular methods,  
16 that require some hours to carry out. Samples are accumulating in many laboratories that are  
17 at risk of being overwhelmed. This causes further critical delays in managing SARS-CoV-2  
18 positive cases and obtaining a definitive COVID-19 diagnosis.

19 Serological tests would be extremely useful for identifying asymptomatic carriers of SARS-  
20 CoV-2. Despite this, to date the accuracy of these tests is insufficient to replace the current  
21 laboratory diagnosis. Serological tests focus on the detection of IgM and/or IgA and IgG. IgM  
22 can be identified in the blood after 3-6 days, while IgG can be detected after 8 days<sup>8</sup>. A recent  
23 publication has evaluated the median seroconversion time for antibody IgA, IgM and IgG. The  
24 authors evaluated 173 SARS-CoV-2 positive subjects and reported a median time of 11, 12 and  
25 14 days, respectively. Additionally, antibodies were found in > 40% of patients within 1 week  
26 of symptom onset, rapidly increasing to 100.0% (IgA), 94.3% (IgM) and 79.8% (IgG) from day  
27 15 after symptom onset. In contrast, RNA detectability decreased from 66.7% (58/87) in  
28 samples collected before day 7 to 45.5% (25/55) during days 15-39. Combining RNA and  
29 antibody detection significantly improved the sensitivity of COVID-19 diagnosis ( $p < 0.001$ ),  
30 even in the early phase during the first week of symptoms ( $p = 0.007$ ). Moreover, a higher titre  
31 of antibody was independently associated with a worse clinical classification ( $p = 0.006$ )<sup>9</sup>.  
32 Therefore, the aims of this study are:

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43 i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the  
44 general population of Verona;  
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47 ii. to assess the accuracy (sensitivity, specificity and predictive values) of ELISA serological  
48 test for the screening of SARS-CoV-2.  
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## METHODS AND ANALYSIS

### Study design

This will be an observational cross-sectional prevalence study and an observational prospective diagnostic study in Verona (Veneto, Italy). Samples will be collected and analysed at IRCCS Sacro Cuore Don Calabria Hospital.

### Study population

The study will be carried out on a random sample of the general population of Verona. Subjects eligible for the study must reside in Verona, be at least 10 years old, and provide consent for the participation to the study and for the donation of biological samples for study purposes.

Subjects will be selected at random from the municipality of Verona's registry and invited to participate.

### Procedures

Each randomly selected citizen will receive an invitation letter to their place of residence, outlining the reasons for the study and how to take part.

Those who decide to participate, will be invited to contact a dedicated telephone number, at which point general information will be given and their contact details collected.

Specially trained staff at IRCCS Sacro Cuore Don Calabria Hospital will contact citizens both to confirm participation by verbal consent and to arrange an appointment (according to a pre-established calendar, automatically managed by a suitable software that limits the formation of queues). During the same phone call, a COVID-19 related symptoms questionnaire (see annex 1) will also be administered and all information related to the logistics and implementation of the study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). This is to minimise the duration of physical contact and length of stay by participants in the centre, as well as to maximise protection against possible contagion.

In the case of participants under the age of 18, the phone call and questionnaire will be conducted with a parent (preferably the primary care giver), while the minor will be required for the sample collection only.

Each participant that attends the IRCCS Sacro Cuore Don Calabria Hospital will be asked to deliver signed informed consent forms (customised according to the age of the participant), which will be verified and countersigned by the principal investigator (PI) or by delegated staff. Participants who fail to bring their consent form can obtain another copy directly at the centre.

Participants will then undergo the measurement of vital parameters (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by laser thermometer), the collection of a blood sample, and a nasopharyngeal swab. All procedures will be performed by specialised personnel of the IRCCS Sacro Cuore Don Calabria Hospital in dedicated clinical settings with suitable anti-contagion equipment.

Auxiliary staff will monitor the movement of people externally and internally the Hospital.

So as not to potentially contaminate hospital rooms and to avoid the need for continual sanitisation, samples will be taken outdoors in a designated tented facility located inside the hospital grounds, so as to allow examinations to be carried out even in the event of adverse



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3 weather. For those arriving by motorised vehicle, swabs may be collected from participants  
4 while they remain seated inside it.

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6 Up to 280 samples per day are expected to be collected.

7 The collected samples will be stored in a refrigerator at 4°C until their transfer, within  
8 twenty-four hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital.  
9 Samples will be stored there until their processing and analysis.

10 Sample collection procedures will take place with 10-minute intervals between the end of one  
11 sample collection and the start of the next one to ensure privacy and to avoid any close  
12 contact between subjects.

13 To further guarantee the safety of participants, the route inside the centre will be one-way  
14 only eliminating the need to return to spaces already frequented. Visual indications such as  
15 strips on the ground, will allow all individuals to maintain the recommended safe distance  
16 from each other.

17 If a subject is unable to travel to the testing centre, home collection of the sample will be  
18 arranged during the initial phone call to confirm verbal consent.

19 The results of the examinations will be communicated to participants and in cases of a  
20 positive SARS-CoV-2 result, appropriate procedures will be activated.

21  
22 All essential information, including completed questionnaires and selected laboratory  
23 findings, will be recorded in an electronic Case Report Form (e-CRF) using the platform  
24 OpenClinica.

### 25 26 27 **Measurements**

28 This protocol refers to STARD guidelines<sup>10</sup> for the reporting of diagnostic test accuracy. Based  
29 on an assessment methodology already used at the IRCCS in diagnostic studies<sup>11 12</sup>, the  
30 assessment will be carried out using an approved molecular test as the gold standard,  
31 assuming that the sensitivity will not be 100% (due to variances in nasopharyngeal swabbing  
32 technique). The evaluation will also include the statistical technique of Latent Class Analysis,  
33 that will be the basis for the estimation of prevalence.

34 Enzyme-linked immunosorbent assay (ELISA) will be performed according to the  
35 manufacturer's instructions, detecting SARS-Cov-2 antibodies of classes IgA (described as  
36 early markers of acute respiratory tract infections) and IgG (indicating a persisting or past  
37 infection). In a recent study<sup>13</sup> the value of specific IgA detection, in the early detection of acute  
38 SARS-CoV-2 infections, has been confirmed. The assay uses the S1 domain of the spike protein  
39 on the surface of SARs-CoV-2 as its antigen, which is considered to be more specific for the  
40 serological detection of SARS-CoV-2 antibodies. The Primary Reference Standard test is a real-  
41 time reverse transcription polymerase chain reaction (RT-PCR), executed at our department,  
42 that has been set up according to the procedures followed by the Regional Reference  
43 laboratory (Department of Microbiology, University Hospital of Padua) and cross-validated.



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3 RNA will be extracted from nasopharyngeal swabs in accordance with our routine laboratory  
4 practice. Briefly, total RNA will be extracted using a MagnaPure LC.2 instrument (Roche  
5 Diagnostic, Monza, Italy), and MagNA Pure LC RNA Isolation Kit - High Performance (Roche),  
6 according to the manufacturer's instructions for cell containing samples. Eluted RNA will be  
7 analysed following the routine in-house real-time RT-PCR protocol for the COVID-19  
8 diagnostic test. The remaining RNA aliquots will be stored at -80°C until they are required for  
9 further tests.  
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12 True positive subjects will be those with a positive real-time RT-PCR result as this indicates  
13 the presence of SARS-CoV-2 RNA. True negative subjects will be subjects with a negative real-  
14 time RT-PCR result that have no detectable SARS-CoV-2 RNA. The outcome for indeterminate  
15 results is outlined later.  
16

17  
18 In detecting SARS-CoV-2, RT-PCR revealing specific viral RNA sequences may be considered  
19 the gold standard, being a test with virtually 100% specificity and therefore acceptable as a  
20 gold standard for sensitivity of index tests. However, the sensitivity of RT-PCR cannot truly be  
21 considered to be 100% in detecting SARS-CoV-2 RNA, as is becoming clear to reference  
22 laboratories performing the test, due to the viral load being too low for the sequences to be  
23 revealed or a flawed swabbing technique. In cases that only use this gold standard,  
24 classification of discordant results (negative gold standard, positive index test) would be  
25 subject to error. Using a composite reference standard (CRS) is one of the alternative methods  
26 when a "perfect" gold standard is not available<sup>14,15</sup>. However, this method has its limitations  
27 too, as when a CRS is used, its accuracy cannot be assumed "a priori"<sup>16</sup>. Alternative methods to  
28 address a lack of a gold standard are latent class models<sup>17</sup>. Latent class analysis (LCA) is  
29 planned using the available tests for SARS-CoV-2 as well as other, selected, clinical and  
30 paraclinical variables.  
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33 Each test will be executed independently by experienced lab personnel. Lab professionals will  
34 not be aware of the clinical data of the subjects and will not know in advance the results of any  
35 other test.  
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#### 37 Subjects found positive.

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39 Subjects with a positive test result will be informed of the test result and managed according  
40 to the routine procedures for clinical assessment and isolation.  
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#### 45 **Sample size calculation**

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47 To the best of our knowledge, there is no information published in an accredited scientific  
48 journal that indicates the prevalence of asymptomatic SARS-CoV-2 positive individuals among  
49 the general population. In Italy, epidemiologists have reported a potential prevalence equal to  
50 5 or 10 times higher than the number of detected SARS-CoV-2 positive individuals. Other  
51 similar sources indicate that the prevalence of asymptomatic SARS-CoV-2 positive subjects is  
52 9-10% of the general population<sup>18,19</sup>.  
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Therefore, assuming an objective population of 235,034 inhabitants (residents in the municipality of Verona on 1 January 2020 that are aged at least 10 years - source ISTAT), a prevalence of asymptomatic SARS-CoV-2 positive individuals of 10.0% and an alpha value (first species error) of 5%, then a random sample of 1527 subjects is required to obtain a standard error of no more than 1.5%, i.e. a 95% confidence interval of lower amplitude or equal to 3%. Assuming a drop-out rate of 35% it will be necessary to enrol 2061 subjects. A systematic probabilistic sample technique will be used to perform the sample list.

### Data analysis plan

Demographic and clinical data will be summarised using descriptive statistics, measures of variability and precision, and plots. Statistical tests will be used based on the type of variables, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. Statistical models and estimations will be adjusted for covariates if necessary. Information criteria and likelihood ratio methods will be used to compare candidate models.

Test results will be displayed in contingency tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.

For the latent class analysis, we use latent class models (LCM) to boost the diagnostic accuracy of the gold standard test by adding to these models the results of multiple tests used in the same samples, as well as other key information from the CRF.

In LCM, it is assumed that the subject's true condition is unknown, and it is modelled by two latent classes. Outcomes of this model are interpreted as the probability that the subject has or does not have the condition (specificity and sensitivity) and the probability that the condition is present (prevalence)<sup>20</sup>. Observations with missing reference standard results will be excluded from the analysis.

### DISCUSSION AND CONCLUSIONS

Currently there are no published data in the literature that reliably estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals in Italy or indeed in any part of the world. SARS-CoV-2 cases are now reported worldwide but at differing incidences depending on the region. Developed regions with a temperate climate, and a medium to high population density, seem to be the most affected. However, the true prevalence of asymptomatic SARS-CoV-2 positive individuals is unknown, as is the prevalence of those who have never contracted the virus. Furthermore, it is not yet established whether now recovered, previously SARS-CoV-2 positive individuals, can become reinfected.

This study will be useful in understanding the spread of SARS-CoV-2 in a city with a total population of around 260,000 inhabitants and a population density of approximately 1,300 inhabitants per square kilometre (Istat source). Most importantly, the prevalence of asymptomatic SARS-CoV-2 positive individuals and the prevalence of those who are negative

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3 for SARS-CoV-2 can be estimated. This will allow phase 2 of Italy's outbreak management  
4 strategy, in which day-to-day activities will gradually be reintroduced and population contact  
5 will resume, to be planned more effectively.  
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8 In addition, the study will allow us to better understand the symptoms of SARS-CoV-2  
9 infection and what potential role they may have in disease prognosis. Finally, the study will  
10 allow us to evaluate the diagnostic accuracy and value of the ELISA serological test as a  
11 screening tool for the general population.  
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### 16 **Ethics and dissemination**

17 This protocol will be registered at <http://www.clinicaltrials.gov>. The full study protocol will  
18 be made accessible at a public repository upon publication.  
19

20 This study adheres to the National Health and Medical Research Council National Statement  
21 on Ethical Conduct in Human Research and the principles of the Declaration of Helsinki.

22 The study could involve vulnerable groups within the community, and so it is imperative that  
23 the study is conducted in a sensitive and culturally appropriate manner. On invitation to the  
24 study, subjects will be given the opportunity to review all study materials and ask any  
25 questions. Furthermore, subjects who feel overwhelmed or anxious at any point during study  
26 participation will be referred to an appropriate support service. Subjects will also be  
27 reassured that they are free to withdraw from the study at any time without reason or  
28 consequence. Results from the study will be disseminated through presentation at national  
29 and international conferences and publications in peer-reviewed journals.  
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36  
37

### 38 **Author Contributions**

39 MG CP conceived the study, MG CP ZB contributed to study design; MG CP contributed to the  
40 preparation of the manuscript. MG CP ZB contributed to the preparation of documents for  
41 ethical approval including study materials (questionnaire, participant information statement,  
42 etc). All authors have contributed to the review of manuscript and are directly involved in the  
43 study scientific committee.  
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45

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48

49 **Patient consent.** Required.  
50

51 **Competing interests.** None declared  
52  
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54 **Ethics approval.** This study was reviewed and approved by the CESC (Comitato Etico per le  
55 Sperimentazioni Cliniche - approval n° 28, 17/04/2020).  
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11 **Data statement section:** The authors confirm that they will have full access to all data and as  
12 such, take responsibility for the integrity of said data and the accuracy of all data analysis.  
13 Data will be publish in a data repository  
14

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## ANNEX 1

## QUESTIONNAIRE

**Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona**

1. Subject Id \_\_\_\_\_

2. Issuing informed consent

Yes  No

3. Enrollment date \_\_\_\_ / \_\_\_\_ / \_\_\_\_

4. Date of birth \_\_\_\_ / \_\_\_\_ / \_\_\_\_

5. Sex

Male  Female

7. Have you already been diagnosed with Covid-19 positivity?

0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling

7.1 If yes, on what date or how many days ago? \_\_\_\_\_

8. How often are you vaccinated for seasonal flu?

1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities

Yes  No

9.1 If yes, which diseases are these?

1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological  
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

**In the past two weeks**

10. Have you had loss of taste and / or smell?

Yes  No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?

Yes  No

12. Have you had a fever (> 37.5)?

Yes  No

13. Have you had dry cough and / or productive cough (phlegm)?

Yes  No

14. Have you suffered from general muscle pain?



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1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?

1Yes 0No

16. Have you had a headache?

1Yes 0No

17. Have you had a sore throat?

1Yes 0No

18. Did you have chills?

1Yes 0No

19. Have you had diarrhea?

1Yes 0No

20. Have you suffered from shortness of breath / or dyspnea?

1Yes 0No

21. Have you had nausea / vomiting?

1Yes 0No

From 10 March 2020

22. With whom do you live at home (or other residential facility)?

1=alone 2=with another person 3=with more than one other person

23. For any reason, did you leave the house (or other residential facility)?

1Yes 0No

24. Have you had direct contacts (for at least 15 continuous minutes) with people other than your potential cohabitants?

1=Yes 2=No 3=I don't have cohabitants

25. Did any of your cohabitants, if any, leave the house / other facility?

1=Yes 2=No 3=I don't have cohabitants

26. Are you aware that you have been in contact with a positive person at COVID-19?

1=Yes 2=No 3=I am not aware of it

27. Did you use protective equipment during the emergency?

1=Yes, masks and gloves 2=Yes, only masks 3=No

28. Do you currently use protective equipment?

1=Yes, masks and gloves 2=Yes, only masks 3=No



# BMJ Open

## Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.

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<b>Primary Subject Heading</b>:	Infectious diseases
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Keywords:	Public health < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

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**Journal**

BMJ Open

**Title**

Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.

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## 10 **ABSTRACT**

### 11 12 **Introduction**

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16 As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 (**Severe Acute Respiratory Syndrome**  
17 **Coronavirus 2**) had infected more than 3,172,000 individuals, killing over 224,000 people and  
18 spreading to more than 200 countries. Italy was the most affected country in Europe and the third  
19 most affected in the world in terms of number of cases.  
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24 Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2  
25 positive individuals among the general population of Verona; ii) to assess the accuracy (sensitivity,  
26 specificity and predictive values) of an ELISA serological test for the screening of SARS-CoV-2.  
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### 30 31 **Methods and analysis**

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34 The study will be carried out on a random sample of subjects aged at least 10 years old from the  
35 general population of Verona. Participants will undergo the measurement of vital parameters  
36 (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by  
37 laser thermometer), the administration of a COVID-19 related symptoms questionnaire, the  
38 collection of a blood sample, and a nasopharyngeal swab. Our evaluation will include the  
39 statistical technique of Latent Class Analysis, that will be the basis for the estimation of  
40 prevalence.  
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### 48 **Results**

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51 Results from the study will allow us to: i) estimate with a very low margin of error the prevalence  
52 of asymptomatic SARS-CoV-2 positive individuals and the prevalence of those who are negative for  
53 SARS-CoV-2; ii) better define phase 2 of Italy's outbreak management strategy; iii) better  
54 understand the symptoms of SARS-CoV-2 infection and what potential role they may have in  
55 disease prognosis; iv) evaluate the diagnostic accuracy and value of the ELISA serological test as a  
56 screening tool for the general population.  
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3 **Ethics and dissemination** The study protocol has been approved by the Ethics Committee of  
4 Verona and Rovigo provinces on April 15, 2020 (internal protocol number 2641CESC).  
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7

8 **Key words**

9  
10 SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class  
11 Analysis  
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16 **Article Summary section**

17 **Strengths and limitations**

18 Study based on random sample of a general population

19 Very low estimation standard error (max 1.5%)

20 Simultaneous comparison of symptoms, real-time PCR test and ELISA serological test

21 Results will depend on response rate  
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29 **Patient and Public Involvement statement:**

30 PPI representatives worked with us to refine the research question, however it was difficult to  
31 involve patients in other areas of the study design due to the very technical methods required to  
32 do a data linkage analysis. PPI representatives will write a plain language summary and design a  
33 leaflet for dissemination to their peers and distributing to patient groups.  
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39 **INTRODUCTION**

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41 As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 had infected more than 3,172,000  
42 individuals, killing over 224,000 people and spreading to more than 200 countries<sup>1</sup>. Italy was at the  
43 time the most affected country in Europe and the third most affected in the world in terms of  
44 number of cases. The epidemic was posing an extremely difficult challenge to health care  
45 establishments, health workers and to the general population. The identification of asymptomatic  
46 SARS-CoV-2 positive individuals is crucial in reducing the spread of the virus throughout the world.  
47 The frequency of such cases is unknown, despite asymptomatic cases regularly being referred to in  
48 the literature<sup>2,3,4</sup>.  
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3 Data from the cruise ship 'Diamond Princess' have shown that the percentage of asymptomatic  
4 SARS-CoV-2 positive cases among all passengers and crew members tested prior to  
5 disembarkation was about 50%. This rate has since been revised as 17.9<sup>5</sup>.

6  
7  
8 A similar study focusing on a Japanese population returning from China, found the percentage of  
9 asymptomatic cases to be 33.3%<sup>6</sup>.

10  
11  
12 Finally, a small group of residents in a skilled nursing facility has also been screened: 13 of 23  
13 residents were defined as asymptomatic, but 10 of these developed symptoms over the  
14 subsequent 7 days<sup>7</sup>.

15  
16  
17 Currently, SARS-CoV-2 detection is based on accepted and standardised molecular methods, that  
18 require about 4 hours to carry out in our lab, although more rapid molecular test are being made  
19 available. Samples are accumulating in many laboratories that are at risk of being overwhelmed.  
20 This causes further critical delays in managing SARS-CoV-2 positive cases and obtaining a definitive  
21 COVID-19 diagnosis.

22  
23  
24 Serological tests would be extremely useful for identifying asymptomatic carriers of SARS-CoV-2.  
25 Despite this, to date the accuracy of these tests is insufficient to replace the current laboratory  
26 diagnosis. Serological tests focus on the detection of IgM and/or IgA and IgG. As it was noted  
27 during the previous SARS epidemic, a possible problem with serologic tests may be a cross-  
28 reaction with other corona viruses<sup>8</sup>. A recent publication has evaluated the median  
29 seroconversion time for antibody IgA, IgM and IgG. The authors evaluated 173 SARS-CoV-2  
30 positive subjects and reported a median time of 11, 12 and 14 days, respectively. Additionally,  
31 antibodies were found in > 40% of patients within 1 week of symptom onset, rapidly increasing to  
32 100.0% (IgA), 94.3% (IgM) and 79.8% (IgG) from day 15 after symptom onset. In contrast, RNA  
33 detectability decreased from 66.7% (58/87) in samples collected before day 7 to 45.5% (25/55)  
34 during days 15-39. Combining RNA and antibody detection significantly improved the sensitivity of  
35 COVID-19 diagnosis ( $p<0.001$ ), even in the early phase during the first week of symptoms  
36 ( $p=0.007$ ). Moreover, a higher titre of antibody was independently associated with a worse clinical  
37 classification ( $p=0.006$ )<sup>9</sup>. Therefore, the aims of this study are:

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3 i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general  
4 population of Verona;  
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8 ii. to assess the accuracy (sensitivity, specificity and predictive values) of two, commercially  
9 available serological tests for the screening of SARS-CoV-2.  
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## 14 **METHODS AND ANALYSIS**

### 15 **Study design**

16 This will be an observational cross-sectional prevalence study and an observational prospective  
17 diagnostic study in Verona (Veneto, Italy). Samples will be collected and analysed at IRCCS Sacro  
18 Cuore Don Calabria Hospital.  
19  
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### 24 **Study population**

25 The study will be carried out on a random sample of the general population of Verona. Subjects  
26 eligible for the study must reside in Verona, be at least 10 years old, and provide consent for the  
27 participation to the study and for the donation of biological samples for study purposes.  
28  
29

30 Subjects will be selected at random from the municipality of Verona's registry and invited to  
31 participate.  
32  
33

34 According to official sources, the cumulative number of SARS-CoV-2 infections in Verona as of 25<sup>th</sup>  
35 May 2020 was 1528 cases (0.7% of the total population), of which 144 deaths, for a death rate of  
36 9.4%<sup>10</sup>  
37  
38  
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### 44 **Procedures**

45 Each randomly selected citizen will receive an invitation letter to their place of residence, outlining  
46 the reasons for the study and how to take part.  
47  
48

49 Those who decide to participate, will be invited to contact a dedicated telephone number, at  
50 which point general information will be given and their contact details collected.  
51  
52

53 Specially trained staff at IRCCS Sacro Cuore Don Calabria Hospital will contact citizens both to  
54 confirm participation by verbal consent and to arrange an appointment (according to a pre-  
55 established calendar, automatically managed by a suitable software that limits the formation of  
56 queues). During the same phone call, a COVID-19 related symptoms questionnaire (see annex 1)  
57 will also be administered and all information related to the logistics and implementation of the  
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3 study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). This is to  
4  
5 minimise the duration of physical contact and length of stay by participants in the centre, as well  
6  
7 as to maximise protection against possible contagion.

8  
9 In the case of participants under the age of 18, the phone call and questionnaire will be conducted  
10  
11 with a parent (preferably the primary care giver), while the minor will be required for the sample  
12  
13 collection only.

14  
15 Each participant that attends the IRCCS Sacro Cuore Don Calabria Hospital will be asked to deliver  
16  
17 signed informed consent forms (customised according to the age of the participant), which will be  
18  
19 verified and countersigned by the principal investigator (PI) or by delegated staff. Participants who  
20  
21 fail to bring their consent form can obtain another copy directly at the centre.

22  
23 Participants will then undergo the measurement of vital parameters (oxygen saturation measured  
24  
25 by oximeter, respiratory rate and body temperature detected by laser thermometer), the  
26  
27 collection of a blood sample, and a nasopharyngeal swab. All procedures will be performed by  
28  
29 specialised personnel of the IRCCS Sacro Cuore Don Calabria Hospital in dedicated clinical settings  
30  
31 with suitable anti-contagion equipment.

32  
33 Auxiliary staff will monitor the movement of people externally and internally the Hospital.

34  
35 So as not to potentially contaminate hospital rooms and to avoid the need for continual  
36  
37 sanitisation, samples will be taken outdoors in a designated tented facility located inside the  
38  
39 hospital grounds, so as to allow examinations to be carried out even in the event of adverse  
40  
41 weather. For those arriving by motorised vehicle, swabs may be collected from participants while  
42  
43 they remain seated inside it, as a final step, when they are going out after concluding the other  
44  
45 procedures including blood sampling).

46  
47 Up to 280 samples per day are expected to be collected.

48  
49 The collected samples will be stored in a refrigerator at 4°C until their transfer, within twenty-four  
50  
51 hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Blood samples  
52  
53 will be immediately stored there upon reception at -80°C, until their processing and analysis that  
54  
55 will be carried out in the following weeks, while swabs are processed upon reception, and then  
56  
57 also stored at -80°C.

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59 Sample collection procedures will take place with 10-minute intervals between the end of one  
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61 sample collection and the start of the next one to ensure privacy and to avoid any close contact  
62  
63 between subjects.

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65 To further guarantee the safety of participants, the route inside the centre will be one-way only

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3 eliminating the need to return to spaces already frequented. Visual indications such as strips on  
4 the ground, will allow all individuals to maintain the recommended safe distance from each other.

5  
6 If a subject is unable to travel to the testing centre, home collection of the sample will be arranged  
7 during the initial phone call to confirm verbal consent.

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10 The results of the examinations will be communicated to participants and in cases of a positive  
11 SARS-CoV-2 result, appropriate procedures will be activated.

12  
13  
14  
15 All essential information, including completed questionnaires and selected laboratory findings, will  
16 be recorded in an electronic Case Report Form (e-CRF) using the platform OpenClinica.

## 17 18 19 20 **Measurements**

21  
22 This protocol refers to STARD guidelines<sup>11</sup> for the reporting of diagnostic test accuracy. Based on  
23 an assessment methodology already used at the IRCCS in diagnostic studies<sup>12,13</sup>, the assessment  
24 will be carried out using an approved molecular test as the gold standard, assuming that the  
25 sensitivity will not be 100% (due to variances in nasopharyngeal swabbing technique). The  
26 evaluation will also include the statistical technique of Latent Class Analysis, that will be also the  
27 basis for the estimation of prevalence.

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35 Enzyme-linked immunosorbent assay (ELISA) Anti-SARS-CoV-2 ELISA IgA/IgG (Euroimmun,  
36 Germany), will be performed according to the manufacturer's instructions, detecting SARS-CoV-2  
37 antibodies of classes IgA (described as early markers of acute respiratory tract infections) and IgG  
38 (indicating a persisting or past infection). In a recent study<sup>14</sup> the value of specific IgA detection, in  
39 the early detection of acute SARS-CoV-2 infections, has been confirmed. The assay uses the S1  
40 domain of the spike protein on the surface of SARS-CoV-2 as its antigen, which is considered to be  
41 more specific for the serological detection of SARS-CoV-2 antibodies.

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47 The SARS-CoV-2 IgG assay (Abbott Laboratories Inc, USA) is a chemiluminescent microparticle  
48 immunoassay (CMIA) for the detection of IgG antibodies to SARS-CoV-2. This will be also  
49 performed according to the manufacturer's instructions.

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55 According to the most recently published study on both tests<sup>15</sup> the sensitivity and specificity of  
56 Euroimmun test were found to be 78.3% and 96.7% for IgG, and 86.7% and 82.7% for IgA; for  
57 Abbott test (IgG) they were 81.8% and 99.3%, respectively. Another study in preprint<sup>16</sup> found (for  
58 IgG) a sensitivity of Euroimmun test varying from 76.9% to 87.1%, and for Abbott from 96.2 to  
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3 97.1%, depending on the population group sampled; specificity was 97% for Euroimmun and 99%  
4 for Abbott. These figures were obtained on small groups of patients, some of whom with active or  
5 recent infection when they might have not yet developed detectable antibodies.  
6

7  
8 The Primary Reference Standard test is a real-time reverse transcription polymerase chain  
9 reaction (RT-PCR), executed at our department, that has been set up according to the procedures  
10 followed by the Regional Reference laboratory (Department of Microbiology, University Hospital  
11 of Padua) and cross-validated.  
12

13  
14 RNA will be extracted from nasopharyngeal swabs in accordance with our routine laboratory  
15 practice. Briefly, total RNA will be extracted using a MagnaPure LC.2 instrument (Roche Diagnostic,  
16 Monza, Italy), and MagNA Pure LC RNA Isolation Kit - High Performance (Roche), according to the  
17 manufacturer's instructions for cell containing samples. Eluted RNA will be analysed following the  
18 routine in-house real-time RT-PCR protocol for the COVID-19 diagnostic test. The remaining RNA  
19 aliquots will be stored at -80°C until they are required for further tests.  
20

21  
22 True positive subjects will be those with a positive real-time RT-PCR result as this indicates the  
23 presence of SARS-CoV-2 RNA. True negative subjects will be subjects with a negative real-time RT-  
24 PCR result that have no detectable SARS-CoV-2 RNA. The outcome for indeterminate results is  
25 outlined later.  
26

27  
28 In detecting SARS-CoV-2, RT-PCR revealing specific viral RNA sequences may be considered the  
29 gold standard, being a test with virtually 100% specificity and therefore acceptable as a gold  
30 standard for sensitivity of index tests. However, the sensitivity of RT-PCR cannot truly be  
31 considered to be 100% in detecting SARS-CoV-2 RNA, as is becoming clear to reference  
32 laboratories performing the test, due to the viral load being too low for the sequences to be  
33 revealed or a flawed swabbing technique. In cases that only use this gold standard, classification of  
34 discordant results (negative gold standard, positive index test) would be subject to error. Using a  
35 composite reference standard (CRS) is one of the alternative methods when a "perfect" gold  
36 standard is not available<sup>17,18</sup>. However, this method has its limitations too, as when a CRS is used,  
37 its accuracy cannot be assumed "a priori"<sup>19</sup>. Alternative methods to address a lack of a gold  
38 standard are latent class models<sup>20</sup>. Latent class analysis (LCA) is planned using the available tests  
39 for SARS-CoV-2 as well as other, selected, clinical and paraclinical variables.  
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42 Each test will be executed independently by experienced lab personnel. Lab professionals will not  
43 be aware of the clinical data of the subjects and will not know in advance the results of any other  
44 test.  
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### Subjects found positive.

Subjects with a positive test result will be informed of the test result and managed according to the routine procedures for clinical assessment and isolation.

### **Sample size calculation**

To the best of our knowledge, there is no information published in an accredited scientific journal that indicates the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population. In Italy, epidemiologists have reported a potential prevalence equal to 5 or 10 times higher than the number of detected SARS-CoV-2 positive individuals. Other similar sources indicate that the prevalence of asymptomatic SARS-CoV-2 positive subjects is 9-10% of the general population<sup>21,22</sup>.

Therefore, assuming an objective population of 235,034 inhabitants (residents in the municipality of Verona on 1 January 2020 that are aged at least 10 years - source ISTAT), a prevalence of asymptomatic SARS-CoV-2 positive individuals of 10.0% and an alpha value (first species error) of 5%, then a random sample of 1527 subjects is required to obtain a standard error of no more than 1.5%, i.e. a 95% confidence interval of lower amplitude or equal to 3%. Assuming a drop-out rate of 35% it will be necessary to enrol 2061 subjects.

A systematic probabilistic sample technique will be used to perform the sample list.

### **Data analysis plan**

Demographic and clinical data will be summarised using descriptive statistics, measures of variability and precision, and plots. Statistical tests will be used based on the type of variables, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. Statistical models and estimations will be adjusted for covariates if necessary. Information criteria and likelihood ratio methods will be used to compare candidate models.

Test results will be displayed in contingency tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.

For the latent class analysis, we use latent class models (LCM) to boost the diagnostic accuracy of the gold standard test by adding to these models the results of multiple tests used in the same samples, as well as other key information from the CRF.

The basis of LCA is that each subject belongs to one of a finite number of classes; in our study we

1  
2  
3 have two classes: with and without Covid-19. Each class is described by a set of parameters that  
4 define the statistical distribution of outcomes; here the probability that the subject has or does not  
5 have the condition Covid-19 (specificity and sensitivity) and the probability that the condition  
6 Covid-19 is present (prevalence)<sup>20</sup>. Observations with missing reference standard results will be  
7 excluded from the analysis.  
8  
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11

## 12 13 **DISCUSSION AND CONCLUSIONS**

14  
15  
16 Currently there are no published data that reliably estimate the prevalence of asymptomatic SARS-CoV-2  
17 positive individuals in Italy. SARS-CoV-2 cases are now reported worldwide but at differing incidences  
18 depending on the region. Developed regions with a temperate climate, and a medium to high population  
19 density, seem to be the most affected. However, the true prevalence of asymptomatic SARS-CoV-2 positive  
20 individuals is unknown, as is the prevalence of those who have never contracted the virus. Furthermore, it is  
21 not yet established whether now recovered, previously SARS-CoV-2 positive individuals, can become  
22 reinfected.  
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30 This study will be useful in understanding the spread of SARS-CoV-2 in a city with a total population of  
31 around 260,000 inhabitants and a population density of approximately 1,300 inhabitants per square  
32 kilometre (ISTAT source). Most importantly, the prevalence of asymptomatic SARS-CoV-2 positive  
33 individuals and the prevalence of those who are negative for SARS-CoV-2 can be estimated. This will allow  
34 phase 2 of Italy's outbreak management strategy, in which day-to-day activities will gradually be  
35 reintroduced and population contact will resume, to be planned more effectively.  
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41 In addition, the study will allow us to better understand the symptoms of SARS-CoV-2 infection and what  
42 potential role they may have in disease prognosis. Finally, the study will allow us to evaluate the diagnostic  
43 accuracy and value of the ELISA serological test as a screening tool for the general population.  
44  
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48

## 49 **Ethics and dissemination**

50 This protocol will be registered at <http://www.clinicaltrials.gov>. The full study protocol will be  
51 made accessible at a public repository upon publication.  
52  
53

54 This study adheres to the National Health and Medical Research Council National Statement on  
55 Ethical Conduct in Human Research and the principles of the Declaration of Helsinki.  
56

57 The study could involve vulnerable groups within the community, and so it is imperative that the  
58 study is conducted in a sensitive and culturally appropriate manner. On invitation to the study,  
59  
60

1  
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3 subjects will be given the opportunity to review all study materials and ask any questions.  
4  
5 Furthermore, subjects who feel overwhelmed or anxious at any point during study participation  
6  
7 will be referred to an appropriate support service. Subjects will also be reassured that they are  
8  
9 free to withdraw from the study at any time without reason or consequence. Results from the  
10  
11 study will be disseminated through presentation at national and international conferences and  
12  
13 publications in peer-reviewed journals.  
14

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21

### 22 23 **Author Contributions**

24  
25 MG CP conceived the study, MG CP ZB AP CM contributed to study design; MG CP ZB contributed  
26  
27 to the preparation of the manuscript. MG CP ZB contributed to the preparation of documents for  
28  
29 ethical approval including study materials (questionnaire, participant information statement, etc).  
30  
31 All authors have contributed to the review of manuscript and are directly involved in the study  
32  
33 scientific committee.  
34

35  
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37  
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39

40  
41 **Patient consent.** Required.  
42

43  
44  
45 **Competing interests.** None declared  
46

47  
48  
49 **Ethics approval.** This study was reviewed and approved by the CESC (Comitato Etico per le  
50  
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52

53  
54  
55 **Provenance and peer review.** Not commissioned; externally peer reviewed.  
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11  
12 **Word count: 2,807.**

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16 **Data statement section:** The authors confirm that they will have full access to all data and as such,  
17 take responsibility for the integrity of said data and the accuracy of all data analysis. Data will be  
18 publish in a data repository

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## ANNEX 1

## QUESTIONNAIRE

**Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona**

1. Subject Id \_\_\_\_\_

2. Issuing informed consent

1Yes 0No

3. Enrollment date \_\_\_\_ / \_\_\_\_ / \_\_\_\_\_

4. Date of birth \_\_\_\_ / \_\_\_\_ / \_\_\_\_\_

5. Sex

1 Male 0 Female

7. Have you already been diagnosed with Covid-19 positivity?

0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling

7.1 If yes, on what date or how many days ago? \_\_\_\_\_

8. How often are you vaccinated for seasonal flu?

1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities

1Yes 0No

9.1 If yes, which diseases are these?

1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological  
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

**In the past two weeks**

10. Have you had loss of taste and / or smell?

1Yes 0No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?

1Yes 0No

12. Have you had a fever (> 37.5)?

1Yes 0No

13. Have you had dry cough and / or productive cough (phlegm)?

1Yes 0No

14. Have you suffered from general muscle pain?

1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?

1  
2  
3 1Yes 0No  
4

5 16. Have you had a headache?  
6

7 1Yes 0No  
8

9 17. Have you had a sore throat?  
10

11 1Yes 0No  
12

13 18. Did you have chills?  
14

15 1Yes 0No  
16

17 19. Have you had diarrhea?  
18

19 1Yes 0No  
20

21 20. Have you suffered from shortness of breath / or dyspnea?  
22

23 1Yes 0No  
24

25 21. Have you had nausea / vomiting?  
26

27 1Yes 0No  
28

29 From 10 March 2020  
30

31 22. With whom do you live at home (or other residential facility)?  
32

33 1=alone 2=with another person 3=with more than one other person  
34

35 23. For any reason, did you leave the house (or other residential facility)?  
36

37 1Yes 0No  
38

39 24. Have you had direct contacts (for at least 15 continuous minutes) with people other than  
40 your potential cohabitants?  
41

42 1=Yes 2=No 3=I don't have cohabitants  
43

44 25. Did any of your cohabitants, if any, leave the house / other facility?  
45

46 1=Yes 2=No 3=I don't have cohabitants  
47

48 26. Are you aware that you have been in contact with a positive person at COVID-19?  
49

50 1=Yes 2=No 3=I am not aware of it  
51

52 27. Did you use protective equipment during the emergency?  
53

54 1=Yes, masks and gloves 2=Yes, only masks 3=No  
55

56 28. Do you currently use protective equipment?  
57

58 1=Yes, masks and gloves 2=Yes, only masks 3=No  
59  
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# BMJ Open

**Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.**

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2020-040036.R2
Article Type:	Protocol
Date Submitted by the Author:	09-Sep-2020
Complete List of Authors:	Guerriero, Massimo; University of Verona, Bisoffi, Zeno; University of Verona, Department of Diagnostics and Public Health; IRCCS Sacro Cuore Don Calabria Hospital, Department of Infectious - Tropical Diseases and Microbiology Poli, Albino; University of Verona, Department of Diagnostics and Public Health Micheletto, Claudio ; Azienda Ospedaliera Universitaria Integrata Verona, Pomari, Carlo; IRCCS Sacro Cuore Don Calabria Hospital, Department of Internal Medicine, Unit of Pneumology
<b>Primary Subject Heading</b>:	Infectious diseases
Secondary Subject Heading:	Immunology (including allergy), Epidemiology, Diagnostics
Keywords:	Public health < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, EPIDEMIOLOGY, Molecular diagnostics < INFECTIOUS DISEASES

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**Journal**

BMJ Open

**Title**

Prevalence of asymptomatic SARS-CoV-2 positive individuals in the general population of northern Italy and evaluation of a diagnostic serological ELISA test: a cross sectional study protocol.

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## 10 **ABSTRACT**

### 11 12 **Introduction**

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16 As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 (**Severe Acute Respiratory Syndrome**  
17 **Coronavirus 2**) had infected more than 3,172,000 individuals, killing over 224,000 people and  
18 spreading to more than 200 countries. Italy was the most affected country in Europe and the third  
19 most affected in the world in terms of number of cases.  
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23  
24 Therefore, the aims of this study are: i) to estimate the prevalence of asymptomatic SARS-CoV-2  
25 positive individuals among the general population of Verona; ii) to assess the accuracy (sensitivity,  
26 specificity and predictive values) of an ELISA serological test for the screening of SARS-CoV-2.  
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### 30 31 **Methods and analysis**

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34 The study will be carried out on a random sample of subjects aged at least 10 years old from the  
35 general population of Verona. Participants will undergo the measurement of vital parameters  
36 (oxygen saturation measured by oximeter, respiratory rate and body temperature detected by  
37 laser thermometer), the administration of a COVID-19 related symptoms questionnaire, the  
38 collection of a blood sample, and a nasopharyngeal swab. Our evaluation will include the  
39 statistical technique of Latent Class Analysis, that will be the basis for the estimation of  
40 prevalence.  
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48 **Ethics and dissemination** The study protocol has been approved by the Ethics Committee of  
49 Verona and Rovigo provinces on April 15, 2020 (internal protocol number 2641CESC). The study  
50 results will be submitted for publication in international, peer reviewed journals and the complete  
51 data set will be deposited in a public repository. Most relevant data will be made available to  
52 policy-makers as well as disseminated to stakeholders and to the community.  
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### 58 59 **Key words** 60

1  
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3 SARS-CoV-2, COVID-19, ELISA serological test, Real-time PCR, Survey Random Sample, Latent Class  
4  
5 Analysis  
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7

## 8 **Article Summary section**

### 9 **Strengths and limitations**

10 Study based on random sample of a general population

11 Very low estimation standard error (max 1.5%)

12 Simultaneous comparison of symptoms, real-time PCR test and ELISA serological test

13 Results will depend on response rate  
14  
15

### 16 **Patient and Public Involvement statement:**

17 PPI representatives worked with us to refine the research question, however it was difficult to  
18 involve patients in other areas of the study design due to the very technical methods required to  
19 do a data linkage analysis. PPI representatives will write a plain language summary and design a  
20 leaflet for dissemination to their peers and distributing to patient groups.  
21  
22

## 23 **INTRODUCTION**

24 As of 30<sup>th</sup> April 2020, the novel betacoronavirus SARS-CoV-2 had infected more than 3,172,000  
25 individuals, killing over 224,000 people and spreading to more than 200 countries<sup>1</sup>. Italy was at the  
26 time the most affected country in Europe and the third most affected in the world in terms of  
27 number of cases. The epidemic was posing an extremely difficult challenge to health care  
28 establishments, health workers and to the general population. The identification of asymptomatic  
29 SARS-CoV-2 positive individuals is crucial in reducing the spread of the virus throughout the world.  
30 The frequency of such cases is unknown, despite asymptomatic cases regularly being referred to in  
31 the literature<sup>2,3,4</sup>.  
32

33 Data from the cruise ship 'Diamond Princess' have shown that the percentage of asymptomatic  
34 SARS-CoV-2 positive cases among all passengers and crew members tested prior to  
35 disembarkation was about 50%. This rate has since been revised as 17.9<sup>5</sup>.  
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3 A similar study focusing on a Japanese population returning from China, found the percentage of  
4 asymptomatic cases to be 33.3%<sup>6</sup>.

5  
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7 Finally, a small group of residents in a skilled nursing facility has also been screened: 13 of 23  
8 residents were defined as asymptomatic, but 10 of these developed symptoms over the  
9 subsequent 7 days<sup>7</sup>.

10  
11  
12 Currently, SARS-CoV-2 detection is based on accepted and standardised molecular methods, that  
13 require about 4 hours to carry out in our lab, although more rapid molecular test are being made  
14 available. Samples are accumulating in many laboratories that are at risk of being overwhelmed.  
15  
16 This causes further critical delays in managing SARS-CoV-2 positive cases and obtaining a definitive  
17 COVID-19 diagnosis.

18  
19  
20 Serological tests would be extremely useful for identifying asymptomatic carriers of SARS-CoV-2.  
21  
22 Despite this, to date the accuracy of these tests is insufficient to replace the current laboratory  
23 diagnosis. Serological tests focus on the detection of IgM and/or IgA and IgG. As it was noted  
24 during the previous SARS epidemic, a possible problem with serologic tests may be a cross-  
25 reaction with other corona viruses<sup>8</sup>. A recent publication has evaluated the median  
26 seroconversion time for antibody IgA, IgM and IgG. The authors evaluated 173 SARS-CoV-2  
27 positive subjects and reported a median time of 11, 12 and 14 days, respectively. Additionally,  
28 antibodies were found in > 40% of patients within 1 week of symptom onset, rapidly increasing to  
29 100.0% (IgA), 94.3% (IgM) and 79.8% (IgG) from day 15 after symptom onset. In contrast, RNA  
30 detectability decreased from 66.7% (58/87) in samples collected before day 7 to 45.5% (25/55)  
31 during days 15-39. Combining RNA and antibody detection significantly improved the sensitivity of  
32 COVID-19 diagnosis ( $p<0.001$ ), even in the early phase during the first week of symptoms  
33 ( $p=0.007$ ). Moreover, a higher titre of antibody was independently associated with a worse clinical  
34 classification ( $p=0.006$ )<sup>9</sup>. Therefore, the aims of this study are:

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48 i. to estimate the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general  
49 population of Verona;  
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53 ii. to assess the accuracy (sensitivity, specificity and predictive values) of two, commercially  
54 available serological tests for the screening of SARS-CoV-2.  
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## METHODS AND ANALYSIS

### Study design

This will be an observational cross-sectional prevalence study and an observational prospective diagnostic study in Verona (Veneto, Italy). Samples will be collected and analysed at IRCCS Sacro Cuore Don Calabria Hospital.

### Study population

The study will be carried out on a random sample of the general population of Verona. Subjects eligible for the study must reside in Verona, be at least 10 years old, and provide consent for the participation to the study and for the donation of biological samples for study purposes.

Subjects will be selected at random from the municipality of Verona's registry and invited to participate.

According to official sources, the cumulative number of SARS-CoV-2 infections in Verona as of 25<sup>th</sup> May 2020 was 1528 cases (0.7% of the total population), of which 144 deaths, for a death rate of 9.4%<sup>10</sup>

### Procedures

Each randomly selected citizen will receive an invitation letter to their place of residence, outlining the reasons for the study and how to take part.

Those who decide to participate, will be invited to contact a dedicated telephone number, at which point general information will be given and their contact details collected.

Specially trained staff at IRCCS Sacro Cuore Don Calabria Hospital will contact citizens both to confirm participation by verbal consent and to arrange an appointment (according to a pre-established calendar, automatically managed by a suitable software that limits the formation of queues). During the same phone call, a COVID-19 related symptoms questionnaire (see annex 1) will also be administered and all information related to the logistics and implementation of the study will be provided (e.g. mask and gloves use, methods of sample collection, etc.). This is to minimise the duration of physical contact and length of stay by participants in the centre, as well as to maximise protection against possible contagion.

In the case of participants under the age of 18, the phone call and questionnaire will be conducted

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3 with a parent (preferably the primary care giver), while the minor will be required for the sample  
4 collection only.

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6 Each participant that attends the IRCCS Sacro Cuore Don Calabria Hospital will be asked to deliver  
7 signed informed consent forms (customised according to the age of the participant), which will be  
8 verified and countersigned by the principal investigator (PI) or by delegated staff. Participants who  
9 fail to bring their consent form can obtain another copy directly at the centre.

10  
11 Participants will then undergo the measurement of vital parameters (oxygen saturation measured  
12 by oximeter, respiratory rate and body temperature detected by laser thermometer), the  
13 collection of a blood sample, and a nasopharyngeal swab. All procedures will be performed by  
14 specialised personnel of the IRCCS Sacro Cuore Don Calabria Hospital in dedicated clinical settings  
15 with suitable anti-contagion equipment.

16  
17 Auxiliary staff will monitor the movement of people outside and inside the Hospital.

18  
19 So as not to potentially contaminate hospital rooms and to avoid the need for continual  
20 sanitisation, samples will be taken outdoors in a designated tented facility located inside the  
21 hospital grounds, so as to allow examinations to be carried out even in the event of adverse  
22 weather. For those arriving by motorised vehicle, swabs may be collected from participants while  
23 they remain seated inside it, as a final step, when they are going out after concluding the other  
24 procedures including blood sampling.

25  
26 Up to 280 samples per day are expected to be collected.

27  
28 The collected samples will be stored in a refrigerator at 4°C until their transfer, within twenty-four  
29 hours, to the testing laboratories of the IRCCS Sacro Cuore Don Calabria Hospital. Blood samples  
30 will be immediately stored there upon reception at -80°C, until their processing and analysis that  
31 will be carried out in the following weeks, while swabs are processed upon reception, and then  
32 also stored at -80°C.

33  
34 Sample collection procedures will take place with 10-minute intervals between the end of one  
35 sample collection and the start of the next one to ensure privacy and to avoid any close contact  
36 between subjects.

37  
38 To further guarantee the safety of participants, the route inside the centre will be one-way only  
39 eliminating the need to return to spaces already frequented. Visual indications such as strips on  
40 the ground, will allow all individuals to maintain the recommended safe distance from each other.

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42 If a subject is unable to travel to the testing centre, home collection of the sample will be arranged  
43 during the initial phone call to confirm verbal consent.  
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3 The results of the examinations will be communicated to participants and in case of a positive  
4 SARS-CoV-2 result, appropriate procedures will be activated.  
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8 All essential information, including completed questionnaires and selected laboratory findings, will  
9 be recorded in an electronic Case Report Form (e-CRF) using the platform OpenClinica.  
10  
11

## 12 **Measurements**

13  
14 This protocol refers to STARD guidelines<sup>11</sup> for the reporting of diagnostic test accuracy. Based on  
15 an assessment methodology already used at the IRCCS in diagnostic studies<sup>12,13</sup>, the assessment  
16 will be carried out using an approved molecular test as the gold standard, assuming that the  
17 sensitivity will not be 100% (due to variances in nasopharyngeal swabbing technique). The  
18 evaluation will also include the statistical technique of Latent Class Analysis, that will be also the  
19 basis for the estimation of prevalence.  
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26  
27 Enzyme-linked immunosorbent assay (ELISA) Anti-SARS-CoV-2 ELISA IgA/IgG (Euroimmun,  
28 Germany), will be performed according to the manufacturer's instructions, detecting SARS-CoV-2  
29 antibodies of classes IgA (described as early markers of acute respiratory tract infections) and IgG  
30 (indicating a persisting or past infection). In a recent study<sup>14</sup> the value of specific IgA detection, in  
31 the early detection of acute SARS-CoV-2 infections, has been confirmed. The assay uses the S1  
32 domain of the spike protein on the surface of SARS-CoV-2 as its antigen, which is considered to be  
33 more specific for the serological detection of SARS-CoV-2 antibodies.  
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40 The SARS-CoV-2 IgG assay (Abbott Laboratories Inc, USA) is a chemiluminescent microparticle  
41 immunoassay (CMIA) for the detection of IgG antibodies to SARS-CoV-2. This will be also  
42 performed according to the manufacturer's instructions.  
43  
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47 According to the most recently published study on both tests<sup>15</sup> the sensitivity and specificity of  
48 Euroimmun test were found to be 78.3% and 96.7% for IgG, and 86.7% and 82.7% for IgA; for  
49 Abbott test (IgG) they were 81.8% and 99.3%, respectively. Another study in preprint<sup>16</sup> found (for  
50 IgG) a sensitivity of Euroimmun test varying from 76.9% to 87.1%, and for Abbott from 96.2 to  
51 97.1%, depending on the population group sampled; specificity was 97% for Euroimmun and 99%  
52 for Abbott. These figures were obtained on small groups of patients, some of whom with active or  
53 recent infection when they might have not yet developed detectable antibodies.  
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3 The Primary Reference Standard test is a real-time reverse transcription polymerase chain  
4 reaction (RT-PCR), executed at our department, that has been set up according to the procedures  
5 followed by the Regional Reference laboratory (Department of Microbiology, University Hospital  
6 of Padua) and cross-validated.  
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10 RNA will be extracted from nasopharyngeal swabs in accordance with our routine laboratory  
11 practice. Briefly, total RNA will be extracted using a MagnaPure LC.2 instrument (Roche Diagnostic,  
12 Monza, Italy), and MagNA Pure LC RNA Isolation Kit - High Performance (Roche), according to the  
13 manufacturer's instructions for cell containing samples. Eluted RNA will be analysed following the  
14 routine in-house real-time RT-PCR protocol for the COVID-19 diagnostic test. The remaining RNA  
15 aliquots will be stored at -80°C until they are required for further tests.  
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20 True positive subjects will be those with a positive real-time RT-PCR result as this indicates the  
21 presence of SARS-CoV-2 RNA. True negative subjects will be subjects with a negative real-time RT-  
22 PCR result that have no detectable SARS-CoV-2 RNA. The outcome for indeterminate results is  
23 outlined later.  
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28 In detecting SARS-CoV-2, RT-PCR revealing specific viral RNA sequences may be considered the  
29 gold standard, being a test with virtually 100% specificity and therefore acceptable as a gold  
30 standard for sensitivity of index tests. However, the sensitivity of RT-PCR cannot truly be  
31 considered to be 100% in detecting SARS-CoV-2 RNA, as is becoming clear to reference  
32 laboratories performing the test, due to the viral load being too low for the sequences to be  
33 revealed or a flawed swabbing technique. In cases that only use this gold standard, classification of  
34 discordant results (negative gold standard, positive index test) would be subject to error. Using a  
35 composite reference standard (CRS) is one of the alternative methods when a "perfect" gold  
36 standard is not available<sup>17,18</sup>. However, this method has its limitations too, as when a CRS is used,  
37 its accuracy cannot be assumed "a priori"<sup>19</sup>. Alternative methods to address a lack of a gold  
38 standard are latent class models<sup>20</sup>. Latent class analysis (LCA) is planned using the available tests  
39 for SARS-CoV-2 as well as other, selected, clinical and paraclinical variables.  
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50 Each test will be executed independently by experienced lab personnel. Lab professionals will not  
51 be aware of the clinical data of the subjects and will not know in advance the results of any other  
52 test.  
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55

#### 56 Subjects found positive.

57

58 Subjects with a positive test result will be informed of the test result and managed according to  
59 the routine procedures for clinical assessment and isolation.  
60



## Sample size calculation

To the best of our knowledge, there is no information published in an accredited scientific journal that indicates the prevalence of asymptomatic SARS-CoV-2 positive individuals among the general population. In Italy, epidemiologists have reported a potential prevalence equal to 5 or 10 times higher than the number of detected SARS-CoV-2 positive individuals. Other similar sources indicate that the prevalence of asymptomatic SARS-CoV-2 positive subjects is 9-10% of the general population<sup>21,22</sup>.

Therefore, assuming an objective population of 235,034 inhabitants (residents in the municipality of Verona on 1 January 2020 that are aged at least 10 years - source ISTAT), a prevalence of asymptomatic SARS-CoV-2 positive individuals of 10.0% and an alpha value (type 1 error) of 5%, then a random sample of 1527 subjects is required to obtain a standard error of no more than 1.5%, i.e. a 95% confidence interval of lower amplitude or equal to 3%. Assuming a drop-out rate of 35% it will be necessary to enrol 2061 subjects.

A systematic probabilistic sampling technique will be used to perform the sample list.

## Data analysis plan

Demographic and clinical data will be summarised using descriptive statistics, measures of variability and precision, and plots. Statistical tests will be used based on the type of variables, tests assumptions and sample dimension. All parameters will be reported with 95% confidence intervals. Statistical models and estimations will be adjusted for covariates if necessary. Information criteria and likelihood ratio methods will be used to compare candidate models.

Test results will be displayed in contingency tables of which sensitivity, specificity and predictive values will be calculated. Estimations will be reported along with the 95% confidence intervals.

For the latent class analysis, we will use latent class models (LCM) to boost the diagnostic accuracy of the gold standard test by adding to these models the results of multiple tests used in the same samples, as well as other key information from the CRF.

The basis of LCA is that each subject belongs to one of a finite number of classes; in our study we have two classes: with and without Covid-19. Each class is described by a set of parameters that define the statistical distribution of outcomes; here the conditional probability that the subject has or does not have the condition Covid-19 (specificity and sensitivity) and the probability that the

1  
2  
3 condition Covid-19 is present (prevalence)<sup>20</sup>. Observations with missing reference standard results  
4 will be excluded from the analysis.  
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## 7 **DISCUSSION AND CONCLUSIONS**

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10  
11 Currently there are no published data that reliably estimate the prevalence of asymptomatic SARS-  
12 CoV-2 positive individuals in Italy. SARS-CoV-2 cases are now reported worldwide but at differing  
13 incidences depending on the region. Developed regions with a temperate climate, and a medium  
14 to high population density, seem to be the most affected. However, the true prevalence of  
15 asymptomatic SARS-CoV-2 positive individuals is unknown, as is the prevalence of those who have  
16 never contracted the virus. Furthermore, it is not yet established whether now recovered,  
17 previously SARS-CoV-2 positive individuals, can become reinfected.  
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26 This study will be useful in understanding the spread of SARS-CoV-2 in a city with a total population  
27 of around 260,000 inhabitants and a population density of approximately 1,300 inhabitants per  
28 square kilometre (ISTAT source). Most importantly, the prevalence of asymptomatic SARS-CoV-2  
29 positive individuals and the prevalence of those who are negative for SARS-CoV-2 can be  
30 estimated. This will allow phase 2 of Italy's outbreak management strategy, in which day-to-day  
31 activities will gradually be reintroduced and population contact will resume, to be planned more  
32 effectively.  
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40 In addition, the study will allow us to better understand the symptoms of SARS-CoV-2 infection and  
41 what potential role they may have in disease prognosis. Finally, the study will allow us to evaluate  
42 the diagnostic accuracy and value of the ELISA serological test as a screening tool for the general  
43 population.  
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### 49 **Ethics and dissemination**

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51 This protocol will be registered at <http://www.clinicaltrials.gov>. The full study protocol will be  
52 made accessible at a public repository upon publication.  
53

54  
55 This study adheres to the National Health and Medical Research Council National Statement on  
56 Ethical Conduct in Human Research and the principles of the Declaration of Helsinki.  
57

58  
59 The study could involve vulnerable groups within the community, and so it is imperative that the  
60 study is conducted in a sensitive and culturally appropriate manner. On invitation to the study,

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3 subjects will be given the opportunity to review all study materials and ask any questions.  
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5 Furthermore, subjects who feel overwhelmed or anxious at any point during study participation  
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7 will be referred to an appropriate support service. Subjects will also be reassured that they are  
8  
9 free to withdraw from the study at any time without reason or consequence. Results from the  
10  
11 study will be disseminated through presentation at national and international conferences and  
12  
13 publications in peer-reviewed journals.  
14

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17  
18 The authors thank Nicoletta De Santis for the editing, Elinor Julie Rae Anderson for language  
19  
20 editing and Elvia Malo that contributed to the preparation of documents for ethical approval.  
21

### 22 23 **Author Contributions**

24  
25 MG CP conceived the study, MG CP ZB AP CM contributed to study design; MG CP ZB contributed  
26  
27 to the preparation of the manuscript. MG CP ZB contributed to the preparation of documents for  
28  
29 ethical approval including study materials (questionnaire, participant information statement, etc).  
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31 All authors have contributed to the review of manuscript and are directly involved in the study  
32  
33 scientific committee.  
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35  
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37  
38 Linea 3” to IRCCS Sacro Cuore Don Calabria Hospital.  
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40  
41 **Patient consent.** Required.  
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45 **Competing interests.** None declared  
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49 **Ethics approval.** This study was reviewed and approved by the CESC (Comitato Etico per le  
50  
51 Sperimentazioni Cliniche – approval n° 28, 17/04/2020).  
52

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55 **Provenance and peer review.** Not commissioned; externally peer reviewed.  
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59 **Open access.** This is an open access article distributed in accordance with the Creative Commons  
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12 **Word count: 2,796.**

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16 **Data statement section:** The authors confirm that they will have full access to all data and as such,  
17 take responsibility for the integrity of said data and the accuracy of all data analysis. Data will be  
18 published in a data repository  
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## ANNEX 1

## QUESTIONNAIRE

**Estimate of the prevalence of asymptomatic subjects affected by COVID-19 in residents of the municipality of Verona**

1. Subject Id \_\_\_\_\_

2. Issuing informed consent

1Yes 0No

3. Enrollment date \_\_\_\_ / \_\_\_\_ / \_\_\_\_\_

4. Date of birth \_\_\_\_ / \_\_\_\_ / \_\_\_\_\_

5. Sex

1 Male 0 Female

7. Have you already been diagnosed with Covid-19 positivity?

0=No 1=Yes, with swab 2=Yes, with rapid test 3=Yes, with venous sampling

7.1 If yes, on what date or how many days ago? \_\_\_\_\_

8. How often are you vaccinated for seasonal flu?

1=Regularly 2=Occasionally 3=Never

9. Presence of comorbidities

1Yes 0No

9.1 If yes, which diseases are these?

1=Pulmonary 2=Cardiological 3=Hypertension 4=Oncological 5=Renal 6=Immunological  
7=Metabolic 8=Rheumatological 9=Hepatic 10=Depression and / or anxiety 11=Other

**In the past two weeks**

10. Have you had loss of taste and / or smell?

1Yes 0No

11. Did you experience burning or feeling of sand in your eyes (conjunctivitis)?

1Yes 0No

12. Have you had a fever (> 37.5)?

1Yes 0No

13. Have you had dry cough and / or productive cough (phlegm)?

1Yes 0No

14. Have you suffered from general muscle pain?

1Yes 0No

15. Have you had an unjustified feeling of tiredness / general asthenia?



1  
2  
3 1Yes 0No  
4

5 16. Have you had a headache?  
6

7 1Yes 0No  
8

9 17. Have you had a sore throat?  
10

11 1Yes 0No  
12

13 18. Did you have chills?  
14

15 1Yes 0No  
16

17 19. Have you had diarrhea?  
18

19 1Yes 0No  
20

21 20. Have you suffered from shortness of breath / or dyspnea?  
22

23 1Yes 0No  
24

25 21. Have you had nausea / vomiting?  
26

27 1Yes 0No  
28

29 From 10 March 2020  
30

31 22. With whom do you live at home (or other residential facility)?  
32

33 1=alone 2=with another person 3=with more than one other person  
34

35 23. For any reason, did you leave the house (or other residential facility)?  
36

37 1Yes 0No  
38

39 24. Have you had direct contacts (for at least 15 continuous minutes) with people other than  
40 your potential cohabitants?  
41

42 1=Yes 2=No 3=I don't have cohabitants  
43

44 25. Did any of your cohabitants, if any, leave the house / other facility?  
45

46 1=Yes 2=No 3=I don't have cohabitants  
47

48 26. Are you aware that you have been in contact with a positive person at COVID-19?  
49

50 1=Yes 2=No 3=I am not aware of it  
51

52 27. Did you use protective equipment during the emergency?  
53

54 1=Yes, masks and gloves 2=Yes, only masks 3=No  
55

56 28. Do you currently use protective equipment?  
57

58 1=Yes, masks and gloves 2=Yes, only masks 3=No  
59  
60