RESEARCH PROTOCOL

Sars-Cov-2 Immune Response

(SIR-study)

(April 2020)

SIR-study version 2.0 dated 28 April 2020

PROTOCOL TITLE 'Sars-Cov-2 Immune Response (SIR-study)'

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Short title	**Immune response against Sars-CoV-2**
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Date	**28 april 2020**
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Sponsor (in Dutch): verrichter/opdrachtgever	***N/A***
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PROTOCOL SIGNATURE SHEET

Name	Signature	Date
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Coordinating Investigator/Project leader / Principal Investigator:	5.1.2e, 5.1.2e Medical microbiologist	

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2. LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
AE	Adverse Event
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CV	Curriculum Vitae
DSMB	Data Safety Monitoring Board
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
GCP	Good Clinical Practice
IB	Investigator's Brochure
IC	Informed Consent
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
(S)AE	(Serious) Adverse Event
SPC	Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

3. SUMMARY

The newly discovered coronavirus SARS-CoV-2 emerged in the human population at the end of 2019 and is currently hitting the worldwide human population with great force leading to great morbidity and mortality, accompanied by huge societal and economic disruption. The disease caused by SARS-CoV-2 is called COVID-19 (coronavirus disease 2019). Patients most affected show signs of massive inflammation of the lung and often require artificial ventilation. Little is known about the development of specific B-cell and T-cell responses in infected patients. In addition, little is known about the contribution of the specific immune response on this clinical presentation in relation to the virus strain lineages. Scarce serological evidence shows that, in patients with mild disease, B-cell response is limited. Preliminary data on total IgG kinetics show that a substantial part of persons with mild symptoms do not seroconvert. Furthermore, there's no data on whether infected patients develop immunological memory that is protective against reinfection. Data on T-cell responses are lacking.

The objective of this study is to determine both T-cell responses to SARS-CoV-2 antigens (spike protein and nucleocapsid), by using an enzyme-linked immunospot (ELISpot) interferon gamma release assay, and B-cell responses by using serologic assays for detection of SARS-CoV-2 neutralizing, spike protein–specific, and nucleocapsid-specific antibodies in patients with reverse-transcription polymerase chain reaction (RT-PCR) based laboratory confirmed COVID-19. Immune responses will be evaluated in patients with severe disease admitted to the intensive care unit (ICU), patients with moderate disease treated on the pulmonary ward, non-hospitalised patients with mild disease and healthy volunteers. Patients will be subjected to a clinical follow up by a pulmonologist in the outpatient clinic and subjected to blood and saliva sampling at four time points after inclusion during a year.

Study design: descriptive evaluation of the immune responses in prospectively included COVID-19 patients.

Study population:

- 100 hospitalized patients with a RT-PCR confirmed COVID-19 infection with severe disease admitted to the ICU
- 100 hospitalized patients with a RT-PCR confirmed COVID-19 infection admitted to the pulmonary ward with moderate disease
- 100 non-hospitalised patients with a RT-PCR confirmed COVID-19 infection with mild disease
- 200 healthy volunteers, SARS-CoV-2-PCR negative.

Main study parameters/endpoints: to determine the natural history of the immune response in COVID-19 disease.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

Study subjects will be subjected to five blood sampling moments (T=0 (moment of blood and saliva sampling within 48 hours upon RT-PCR confirmation of COVID-19 infection), T=1 after 14 days (or at dismissal from the hospital), T=2 one month after T=1, T=3 two months after T=2 and T=4 after 1 year). The non-hospitalized patients with RT-PCR confirmed COVID-19 disease with mild disease will sampled four times (T=0 (moment of blood and saliva sampling within 48 hours upon RT-PCR confirmation of COVID-19 infection), T=1 one month after T=0, T=2 two months after T=1 and T=3 after 1 year). Healthy volunteers will only be sampled once. Study subjects will be asked to fill in a standard questionnaire measuring general health (SF-36) and at each visit a case record form will be filled in by the attending physician.

4. INTRODUCTION AND RATIONALE

The newly discovered human betacoronavirus SARS-CoV-2 has caused a pandemic, which started in 2019, and has hit the worldwide population with great force leading to many hospitalizations and casualties. The disease caused by SARS-CoV-2 is called COVID-19 (coronavirus disease 2019) (1, 2). According to the Center for Systems Science and Engineering (CSSE) at John Hopkins University, 1.602.885 COVID-19 cases have been confirmed worldwide. Numbers by country showed 21.910 confirmed cases in the Netherlands (https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6, assessed April 10th). The diagnosis cannot be definitively made without microbiologic testing and is confirmed by detection of SARS-CoV-2 RNA by reverse-transcription polymerase chain reaction (RT-PCR) in an oro/nasopharyngeal swab specimen.

How the immune response against SARS-CoV-2 develops remains largely unknown, emerging data show that antibodies to the receptor-binding domain of the spike protein and the nucleocapsid protein were detected by enzyme-linked immunosorbent assay (ELISA) in most patients by 14 days following the onset of symptoms (3, 4, 7, 9, 10). Data on specific T-cell responses are lacking.

The Centre for Infectious Diseases Research, part of the National Institute for Public Health and the Environment (RIVM), has developed a serologic assay for detection of SARS-CoV-2 antibodies against spike protein and the nucleocapsid antigen and antibodies against alphacoronavirus species such as Human coronavirus 229E, Human coronavirus NL63 and other betacoronavirus such as Human coronavirus OC43. In addition, at the RIVM, antibodies can be measured by using a virus neutralisation assay to evaluate serum samples for their neutralization capacity against SARS-CoV-2.

In addition to antibody testing, interferon-γ (IFN-γ) ELISpot assays can be used to quantify the antigen-specific T-cell response (11). The Diakonessenhuis Utrecht has developed an ELISpot against both the spike protein end the nucleocapsid antigen. Preliminary data show a robust T-cell response against SARS-CoV-2 proteins in patients recovered from COVID-19. The method involves the isolation of peripheral blood mononuclear cells (PBMCs) and, subsequently, addition of a set number of PBMCs to a plate coated with anti-IFN-γ capture antibodies. Cells are then stimulated with a pre-determined concentration of SARS-CoV-2 specific antigens. Upon recognition of the stimulus, antigen-specific T-cells will secrete cytokine (IFN-γ) which can be

captured by the antibody coated on the plate. Following 16-20 hour stimulation, the bound IFN-γ is detected by using a chromogenic substrate, allowing enumeration of the number of SARS-CoV-2 specific T cells through calculation of the number of spot forming units. The laboratory for medical microbiology and immunology in the Diakonessen Hospital Utrecht has extensive experience with this procedure to test T-cell responses in tuberculosis and Lyme borreliosis cases (12, 13).
Insights in the development of B- and T-cell responses to SARS-CoV-2 and the evaluation of the effectiveness of the specific antibodies are necessary for correct assessment of the immune status of (asymptomatic) patients and may help to predict whether the immune response is protective and how long any protective effect will last. Currently, in the Diakonessen Hospital, 194 patients have been diagnosed with COVID-19. The first COVID-19 diagnosis was made on 28 February. Because of the lack of a vaccine, we expect still many more patients will present themselves for treatment in our hospital, which will enable us to enrol the various cohorts of patients in the study proposed here.

5. OBJECTIVES

Primary Objective:
To determine both T- and B-cell responses in COVID-19 patients.

Secondary objective:

- To determine the capability of the SARS-CoV-2 ELISpot to detect patients with a wide range of clinical manifestations with COVID-19
- To determine the dynamics in T-cell and B-cell responses in time in COVID-19 patients
- To determine the differences in immune responses cases with severe, moderate and mild disease
- To relate strain diversity to disease and immune response
- To determine biomarkers such as cytokines, for example IL-2, IL-6 and TNFα, linked to the development of severe, moderate or mild disease
- To determine factors for (the development) of immunity to COVID-19

6. STUDY DESIGN

Descriptive study of the evaluation of the immune responses in prospectively collected COVID-19 patients. Patients with RT-PCR proven SARS-CoV-2 infections with various disease severity will be included in the study, as well as patients with negative oro/nasopharyngeal SARS-COV-2 RT-PCR and clinically strong suspicion of COVID-19 disease, and healthy volunteers. Patients included in the RT-PCR negative cohort, who become RT-PCR positive, will switch to the RT-PCR proven COVID-19 cohort.
Informed consent will be obtained from all healthy controls and patients included in the study. In case of an emergency situation, there is the option for "deferred consent" as described by the Centrale Commissie Mensgebonden Onderzoek. If the patient dies before deferred consent, the patient will be excluded from the study. Patients will be subjected to a clinical follow up by a

pulmonologist in the outpatient clinic and to blood and saliva sampling at four time points after inclusion during a year. In total, COVID-19 patients will be sampled for blood and saliva at five moments; T=0 =moment of blood and saliva sampling within 48 hours upon RT-PCR confirmation of COVID-19 infection; T=1 = 14 days after T=1 or at dismissal from the hospital); T=2 = one month after T=1; T=3 = two months after T=2; and T=4 = after 1 year). The non-hospitalized patients with RT-PCR confirmed COVID-19 disease with mild disease will sampled four times (T=0 (moment of blood and saliva sampling within 48 hours upon RT-PCR confirmation of COVID-19 infection), T=1 one month after T=0, T=2 two months after T=1 and T=3 after 1 year). Healthy volunteers will be sampled only at T=0. The samples of these volunteers will be used to check the validity of the assays used in this study; the positivity rate among this group should not be significantly higher than the sero-prevalence in the Dutch population. Without disease symptoms there is no need for follow-up. Each subject will be asked to fill out a questionnaire about his or her health (SF36). At each visit, the attending physician will fill in a case record form. The saliva and the blood will be subjected to various serological assays, including the RIVM in-house microarray and virus neutralization assay and the Diakonessenhuis in-house ELISpot assay. The blood will also be subjected to human leucocyte antigen (HLA) typing using fluorescence-activated cell sorting (FACS) analysis. This will allow a comparison of the B-cell and T-cell immune responses for the various patient groups and controls, and identification of possible markers for the development and severity of disease. The investigation of the saliva samples will provide insight into the development of the mucosal immunity against SARS-CoV-2. The prolonged follow-up up to a year enables the investigation of the development of immunity. To investigate whether the Sars-COV-2 strain influences the severity of disease and/or the course of the immuneresponse, the viral strains will also be sequenced. Blood and saliva samples will be stored at minus 80 degrees Celsius to enable biomarker analysis.

7. STUDY POPULATION

7.1 Population (base)

- COVID-19 patients and controls will be recruited at the Diakonessenhuis hospital.

Group	Study participants	Number
1	Hospitalized patients with a proven COVID-19 infection with severe disease on the ICU	100
2	Hospitalized patients with a proven COVID-19 infection on the pulmonary ward with moderate disease	100
3	non-hospitalized patients with RT-PCR confirmed COVID-19 disease with mild disease	100
4	Healthy volunteers, SARS-CoV-2 PCR negative	200

7.2 Inclusion criteria

- (group1) Hospitalized patients with a RT-PCR confirmed COVID-19 admitted to the ICU
- (group 2) Hospitalized patients with a RT-PCR confirmed COVID-19 admitted to the pulmonary ward
- (group 3) Non-hospitalized patients with RT-PCR proven COVID-19 infection with mild disease. It is envisaged that most of these patients will be recruited from hospital personnel that will be tested in the so-called "test street" in Zeist city and emergency ward patients with proven COVID-19 infection who will not be admitted to the hospital. Patients and personnel will be contacted by the investigators and asked for study participation.
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- (group 4) Healthy volunteers will be recruited from the hospital personnel and/or personnel from the RIVM. Upon enrolment SARS-CoV-2 PCR status will be determined.

Study subjects will be included in the study when they have submitted the following;

- A signed informed consent
- Blood samples, taken at least at T=0

7.3 Exclusion criteria

- < 18 years

7.4 Sample size calculation

Sample size calculation is not possible in this explorative study since the diagnostic characteristics of this new test are not known. Sample size is based on a pragmatic approach.

8. TREATMENT OF SUBJECTS

Subjects in this study with an active disease will be treated according to our national guidelines and their treatment will not be influenced by our study.

9. INVESTIGATIONAL MEDICINAL PRODUCT

N/A

10. METHODS

10.1 Study parameters/endpoints

10.1.1 Main study parameters/endpoints

Results of the SARS-CoV-2 serology and ELISpot assay for all the included subjects.
Biomarker analysis of the different groups.
Sequencing of the viral strains isolated from patients of the various groups.

10.1.2 Secondary study parameters/endpoints (if applicable)

Patient wellbeing will be assessed by using SF36 questionnaires and they will be followed up clinically.

10.1.3 Other study parameters (if applicable)

Association between HLA geno-typing and SARS-CoV-2 infection and/or disease severity.

10.2 Randomisation, blinding and treatment allocation

N/A

10.3 Study procedures

Procedures:

- Venapunction and saliva sampling
- Tests performed on venous blood drawing and saliva samples:
  - COVID-19 serology by using an in-house SARS-corona microarray and at least one commercially available enzyme-linked immunosorbent assay. Dubious or positive serology results will be confirmed by testing of SARS-CoV-2 neutralization capacity of serum (blood) (8)
  - COVID-19 in-house ELISpot (supernatants of the ELISpot assay test will be stored for additional assessment of various cytokines) (blood).
  - Biomarker analysis (blood, saliva)
  - Whole genome sequencing on viral strains in patient material such as saliva
  - Immunologic monitoring of lymphocyte subsets on blood
  - HLA genotyping on blood

T=0	1x Li-Hep (7 ml) 1x EDTA (5 ml) 2x stolbuis (7 ml) 1x saliva sampling
T=1 (10 days)	1x Li-Hep (7 ml) 1x EDTA (5 ml) 2x stolbuis (7 ml) 1x saliva sampling
T=2 (1 month)	1x Li-Hep (7 ml) 1x EDTA (5 ml) 2x stolbuis (7 ml) 1x saliva sampling
T=3 (3 months)	1x Li-Hep (7 ml) 1x EDTA (5 ml)

	2x stolbuis (7 ml) 1x saliva sampling
T=4 (1 year)	1x Li-Hep (7 ml) 1x EDTA (5 ml) 2x stolbuis (7 ml) 1x saliva sampling

10.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so, without any consequences. In addition, the investigator can decide to withdraw a subject from the study for urgent medical reasons. Data already obtained will remain available for analysis.

10.4.1 Specific criteria for withdrawal (if applicable)

N/A

10.5 Replacement of individual subjects after withdrawal

N/A

10.6 Follow-up of subjects withdrawn from treatment

N/A

10.7 Premature termination of the study

N/A

11. SAFETY REPORTING

11.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

11.2 Adverse and serious adverse events

N/A

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to [the investigational product / the experimental treatment]. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
- is a new event of the trial likely to affect the safety of the subjects, such as an unexpected outcome of an adverse reaction, lack of efficacy of an IMP used for the treatment of a life-threatening disease, major safety finding from a newly completed animal study, etc.

All SAEs will be reported through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reactions. SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

11.2.1 Suspected unexpected serious adverse reactions (SUSAR)

N/A

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are adverse reactions, of which the nature, or severity, is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved IMP or Summary of Product Characteristics (SPC) for an authorised medicinal product).

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical trial that was assessed by the METC;
- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.

The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

11.2.2Annual safety report

N/A

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, Medicine Evaluation Board and competent authorities of the concerned Member States.
This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

11.3 Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

11.4 Data Safety Monitoring Board (DSMB)

N/A

12. STATISTICAL ANALYSIS

12.1 Descriptive statistics

All quantitative data will be presented as mean±SE if normally distributed, otherwise median values (with range) are reported.
The sensitivity, specificity, positive predictive value (PPV) and the negative predictive value (NPV) will be calculated for the various parameters

12.2 Univariate analysis

Testing for differences between groups will performed by using the Fisher's exact (categorical data) or the Mann–Whitney test (numerical data) when appropriate. SPSS (version 23) and GraphPad Prism 5.0 will be used for data analysis.
Confidence intervals will be calculated for the sensitivity, specificity, PPV and NPV.

12.3 Multivariate analysis

N/A

12.4 Interim analysis (if applicable)

N/A

13. ETHICAL CONSIDERATIONS

13.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (Brasil October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

13.2 Recruitment and consent

Patients are asked by their own physician if they are interested in participating in this study using the informed consent and patient information letter. The investigator will inform them subsequently and ask for their written consent. Healthy subjects will have at least 1 week to consider.

13.3 Objection by minors or incapacitated subjects (if applicable)

Incapacitated patients will not be recruited.

13.4 Benefits and risks assessment, group relatedness

This research projects provides insight into the immunological response to COVID-19 by using only venous blood samples and saliva (both to be obtained with minimally invasive procedures without significant complications), the immune response of this recently emerged infectious disease will be elucidated.

13.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7, subsection 6 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 450.000,-- (i.e. four hundred and fifty thousand Euro) for death or injury for each subject who participates in the research;
2. € 3.500.000,-- (i.e. three million five hundred thousand Euro) for death or injury for all subjects who participate in the research;
3. € 5.000.000,-- (i.e. five million Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within four years after the end of the study.

13.6 Incentives (if applicable)

N/A

14. ADMINISTRATIVE ASPECTS AND PUBLICATION

14.1 Handling and storage of data and documents

Patients are coded in order of inclusion. All materials will be coded. Only the investigators will have access to the source data. For all patients and controls, all human material will be kept for 15 years.

14.2 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion. All substantial amendments will be notified to the METC and to the competent authority.
Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

14.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

14.4 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.
In case the study is ended prematurely, the investigator will notify the accredited METC, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

14.5 Public disclosure and publication policy

All data will be published unreservedly.

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