COVID-19 in Pediatric Patients: clinical and immunological features (COPP-IMM)

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PROTOCOL TITLE ***'****COVID-19 in Pediatric Patients: clinical and immunological features'*

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Coordinating investigator/project leader	Dr. G. LugthartImmunologist Laboratory for Pediatric Immunology Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden g.lugthart@lumc.nl +31-71-5262802
Principal investigator(s) (in Dutch: hoofdonderzoeker/ uitvoerder)	Dr. E.P. Buddingh Pediatric infectiologist/immunologist PI COPP-IMM study Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden e.p.buddingh@lumc.nl +31-71-5262824
<Multicenter research: per site>	S. Hammer Pediatrician Amphia, Breda SHammer@amphia.nl J. van der Linden Bernhoven, Uden j.vanderlinden@bernhoven.nl Y. Thomasse Dijklander Ziekenhuis, Hoorn/ Purmerend y.e.m.thomasse@westfriesgasthuis.nl M. Breukels Elkerliek, Deurne mbreukels@elkerliek.nl S. W. J. Terheggen-Lagro AUMC, Amsterdam s.w.terheggenlagro@amsterdamumc.nl A. Oudshoorn, Gelre Ziekenhuizen, Apeldoorn a.oudshoorn@gelre.nl

	J. Bolt-Wieringa Haaglanden Medisch Centrum, Den Haag j.boltwieringa@haaglandenmc.nl J. Bekhof Isala, Zwolle j.bekhof@isala.nl M. Bannier Maastricht UMC, Maastricht michiel.bannier@mumc.nl K. van Aerde RadboudUMC, Nijmegen Koen.vanAerde@radboudumc.nl M. Jacobs Slingeland, Doetinchem m.jacobs@slingeland.nl M. van Houten Spaarne Gasthuis, Hoofddorp MvanHouten2@spaarnegasthuis.nl L. van der Aa Zaans Medisch Centrum, Zaandam Aa.L@zaansmc.nl P. Fraaij ErasmusMC-Sophia, Rotterdam p.fraaij@erasmusmc.nl G. Tramper Franciscus, Rotterdam G.Tramper@franciscus.nl K. Miedema Tergooi, Blaricum kmiedema@tergooi.nl
Sponsor (in Dutch: verrichter/opdrachtgever)	LUMC, E. Buddingh
Subsidising party	Bontius Stichting Leids Universiteits Fonds (#wakeuptocorona)

Independent expert	Drs. C. Meijer Department of Pediatrics Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden c.r.meijer-boekel@lumc.nl +31-71-5262824
Laboratory site	Laboratory for Pediatric Immunology Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden +31-71-5262802 **/** +31-71-5298792

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Head of Department: Prof. dr. E.H.H.M Rings Department of Pediatrics Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden		
Principal Investigator: Dr. E.P. Buddingh Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden		

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
ANOVA	**Analysis of Variance**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
COPP	**Observational cohort study 'Clinical features of COVID-19 in Pediatric Patients'**
COREON	**Commissie Regelgeving Onderzoek of The Federation of Dutch Medical Scientific Societies**
COVID-19	**Coronavirus Disease - 2019**
CV	**Curriculum Vitae**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
IC	**Informed Consent**
ICU	**Intensive Care Unit**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
MIS-C	**Multisystem Inflammatory Syndrome in Children**
PedsQL	**Pediatric Quality of Life Inventory**
PROMIS	**Patient-Reported Outcomes Measurement Information System**
RNAseq	**Ribonucleic acid sequencing**
(S)AE	**(Serious) Adverse Event**
SARS-CoV-2	**Severe Acute Respiratory Syndrome CoronaVirus-2**
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.**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: The immune response of children to infection with SARS-CoV-2 is markedly different to the immune response of adults with COVID-19. Children are less likely to develop severe COVID-19, but some children do need supplemental oxygen or intensive care. In rare cases, children who have been infected with SARS-CoV-2 develop a potentially life-threatening post-infectious inflammatory syndrome termed 'multisystem inflammatory syndrome in children' (MIS-C). To better understand and treat these severe sequelae of SARS-CoV-2 infection in children, comprehensive immunological analysis in parallel with the collection of detailed clinical information is needed.

Objective: Primary objective: To obtain a detailed immunological profile of children presenting to Dutch hospitals with acute SARS-CoV-2 infection or with a SARS-CoV-2 related post-infectious inflammatory syndrome. Secondary objectives: (1) To correlate the immunological profiles with detailed clinical parameters. We will collect clinical data in this COPP-IMM study in the same way as in our related observational cohort study 'Clinical features of COVID-19 in Pediatric Patients' (COPP-study). Clinical parameters include: severity of disease, underlying illnesses, age at presentation, clinical syndrome, laboratory parameters at diagnosis, outcome. (2) To identify immunological targets of therapy.

Study design: Multicenter prospective cohort study

Study population: Children age 0-17 years, in- or outpatient in Dutch hospitals with COVID-19 or MIS-C.

Main study parameters/endpoints: We will perform detailed immunological analyses in a two-tiered approach. In the first tier we will do a rapid, more general assessment of the immune system. This will include a detailed phenotypical analysis of the cells of the innate and adaptive immune system using spectral flow cytometry, a multiplex analysis of inflammatory cytokines using Luminex bead arrays and a quantitative and qualitative analysis of anti-SARS-CoV-2 antibodies. Depending on the results of the investigations in the first tier, we will choose which in-depth immunological analyses will be done in the second tier. Investigations in the second tier will focus on the innate immune system, the adaptive immune system (either T-cell responses or B-cell responses) and/or biomarker discovery of serum proteins. The results from the immunological analyses will be correlated to clinical data as collected in the COPP-study.

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

Blood samples for this study will be collected after confirmation of the diagnosis COVID-19 or MIS-C and after the patient and/or the caregiver have consented for participation. The amount of blood drawn depends on the body weight of the patient, and ranges from 5 to 50 mL. To ensure minimal burden as possible, the collection of the blood sample will be combined with a blood sample collection for routine clinical care and/or will be drawn from an indwelling venous catheter, if possible. If this is not possible, a venepuncture will be done to collect material for this study. In this case, there will some burden to the patient. To ensure that this burden is minimal, we will apply a topical anaesthetic (lidocaine/prilocaine or lidocaine/tetracaine as per local guidelines) and will instruct local researchers on positive language before and during the procedure.

The risk of this study to the participants is negligible. There is no direct benefit of this study for the participants. The results from this study will benefit the target group, *i.e.* children with COVID-19 or MIS-C, by identifying immunological targets of therapy.

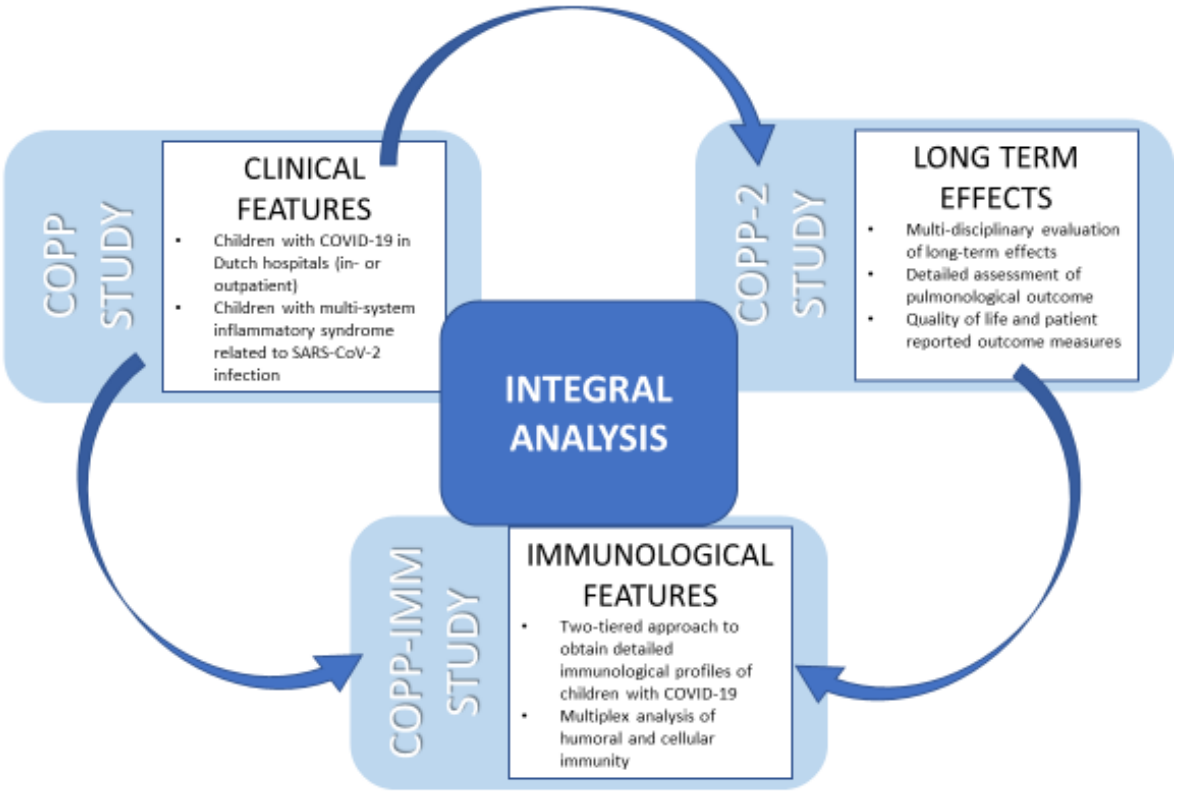
1. INTRODUCTION AND RATIONALE

Although in general children are less affected by SARS-CoV-2 infection, a subset of children infected with SARS-CoV-2 develops severe disease (1-5). From the beginning of the pandemic until April 2021, 223 children have been included in our prospective observational cohort study 'Clinical features of COVID-19 in Pediatric Patients' (COPP-study) (www.covidkids.nl/scientific-dashboard).

In adults, severe COVID-19 is characterized by a hyperinflammatory response and cytokine storm. Typically, this hyperinflammatory response does not occur when children are infected with SARS-CoV-2. However, a subset of children is admitted to hospital with COVID-19, and a subset requires supplemental oxygen or even intensive care. In adults with severe COVID-19, treatment with dexamethasone results in better outcomes, but is unknown if children with severe COVID-19 have a similar immunological profile and thus if they would theoretically also benefit from immunosuppression by high-dose corticosteroids.

In rare cases, children develop a post-infectious hyperinflammatory syndrome some weeks after infection with SARS-CoV-2, termed 'multisystem inflammatory syndrome in children' (MIS-C) (6). This potentially life-threatening syndrome can result in myocardial dysfunction and shock, and typically responds well to anti-inflammatory treatment (7). As of April 2021, 60 children have been included in the COPP-study with an inflammatory syndrome suggestive of MIS-C. There are marked differences between the immunological profiles of children with MIS-C and adults with severe COVID-19 (6). However, there has not yet been any study evaluating if the immunological profile MIS-C also differs from the immunological profile of children with severe COVID-19.

In the COPP-study we aim to describe **which** children are at the highest risk of developing severe COVID-19 or MIS-C and **what the exact course of illness** is in these children. In the follow-up study COPP-2, the **late effects** of COVID-19 and MIS-C are evaluated. In the current COPP-IMM study, we will correlate these detailed clinical findings with a **comprehensive and integral analysis of the immune response** of children with COVID-19 or MIS-C.


COPP STUDY
CLINICAL FEATURES
Children with COVID-19 in Dutch hospitals (in- or outpatient)
Children with multi-system inflammatory syndrome related to SARS-CoV-2 infection
COPP-2 STUDY
LONG TERM EFFECTS
Multi-disciplinary evaluation of long-term effects
Detailed assessment of pulmonological outcome
Quality of life and patient reported outcome measures
INTEGRAL ANALYSIS
COPP-IMM STUDY
IMMUNOLOGICAL FEATURES
Two-tiered approach to obtain detailed immunological profiles of children with COVID-19
Multiplex analysis of humoral and cellular immunity

2. OBJECTIVES

Primary Objective:
To obtain a detailed immunological profile of children presenting to Dutch hospitals with acute SARS-CoV-2 infection or with a SARS-CoV-2 related post-infectious inflammatory syndrome (MIS-C).

Secondary Objectives:

(1) To correlate the immunological profiles with detailed clinical parameters as obtained in our related cohort study 'Clinical features of COVID-19 in Pediatric Patients' (COPP-study). Clinical parameters include:

- severity of disease
- underlying illnesses
- age at presentation
- clinical syndrome
- laboratory parameters as obtained in routine clinical care
- clinical outcome: days of hospitalization, life-saving interventions, ICU admissions, death, patient reported outcome measures at 6 weeks follow-up.

(2) To determine if there are immunological targets of therapy for children with severe COVID-19 or MIS-C

(3) To determine if hyperinflammation in MIS-C is different from the inflammatory response in severe pediatric COVID-19.

3. STUDY DESIGN

This study is a multicenter, observational, prospective cohort study in hospital-setting in the Netherlands.

Duration of the study: we will collect samples during 18 months. If during this time, we will not have reached the desired number of inclusions (at least 30 children for the immunological part of the study) and SARS-CoV-2 is still prevalent, we will continue our study for another 18 months.

4. STUDY POPULATION

4.1 Population (base)

Currently, 53 hospitals are participating in the COPP-study. From April through December 2020, there have been 157 inclusions (with some children included twice if they were transferred between hospitals). Of the 48 COPP-sites, 13 have currently expressed interest in participating in the COPP-IMM study, including five academic centers. The exact number of children that will be included in COPP-IMM depends on the course of the epidemic, but based on this data, we expect to be able to include about 60 children for the observational part of the study. We expect to be able to obtain informed consent for blood withdrawal in ~50% of these patients, so 30 patients.

4.2 Inclusion criteria

The same inclusion criteria will be used for this COPP-IMM study as for our ongoing COPP-study.

The inclusion criteria of the COPP-study are:
- Age 0-17 years AND
- Present to an emergency department or outpatient department of a Dutch hospital and/or be admitted to hospital
AND
- Have at least one positive real-time RT-PCR test on nasopharyngeal, oropharyngeal, sputum or fecal sample for SARS-CoV-2 OR
- Proof of a (recent) infection with SARS-CoV-2 by positive serology test (IgG/IgM) OR
- Fulfil a clinical diagnosis(*) of COVID-19, should testing of SARS-CoV-2 yield inconclusive results and/or if testing is no longer possible due to lack of reagents. In these cases there must be an epidemiological link to COVID-19.

(*) This includes:
- signs, symptoms and imaging results indicative of COVID-19 pneumonia without evidence for other causes;
- inflammatory syndrome with a history consistent with recent COVID-19 AND/OR laboratory results consistent with current COVID-19 OR recent COVID-19 (e.g. positive SARS-CoV2-PCR and/or positive serology). This multisystem inflammatory disorder in children and adolescents (MIS-C) is characterized by: fever lasting at least three days, AND at least two of the following: rash or bilateral non-purulent conjunctivitis or muco-cutaneous inflammation signs, hypotension or shock, features of myocardial dysfunction, pericarditis, valvulitis, or coronary abnormalities, evidence of coagulopathy, acute gastrointestinal problems, AND elevated markers of inflammation AND no other obvious microbial cause of inflammation (WHO case definition).

In addition to these general inclusion criteria for the clinical part of the study, a minimum body weight of 3 kg is required for blood sampling.

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:
- No evidence of current or recent COVID-19
- No consent from guardians and/or patient
- For the blood sampling: body weight less than 3 kg.

4.4 Sample size calculation

A formal sample size calculation cannot be done, since it is currently unknown what the variation in immunological response is children with COVID-19 or MIS-C and how this relates to outcome measures. Based on the number of participating sites and the inclusion rate in the first wave of the epidemic, we expect to be able to include at least 60 children in total, and 30 children for the immunological part of the study.

We expect the rate of consent for blood withdrawal to be higher in patients with more severe disease (since the blood can be taken during routine blood withdrawal and no separate venapuncture will be needed). Therefore, based on inclusion rates for the related COPP-study, we estimate we will be able to include at least 10 patients with MIS-C, 10 patients with moderate to severe COVID-19 and 10 patients with mild COVID-19. Clinical information will be available for all of the patients included in the immunological part of the study.
If we will have reached 60 inclusions for the immunological part of the study within the frist 18 months of the study, we will do an interim analysis to determine if more inclusions are needed. Since this is an exploratory study, no formal maximal number of inclusions can be given, but based on previous inclusions in de COPP-study and the expected course of the epidemic we do not expect to reach a higher inclusion than 60 in this timeframe.

5. TREATMENT OF SUBJECTS

Not applicable

6. INVESTIGATIONAL PRODUCT

Not applicable

7. NON-INVESTIGATIONAL PRODUCT

Not applicable

8. METHODS

8.1 Study parameters/endpoints

The COPP-IMM study is closely related to the ongoing observational clinical cohort study COPP (N20.043). The COPP-IMM study consists of two parts:

a) a clinical cohort study, identical to the COPP-study (the data of children inlcuded in COPP-IMM will be analysed together with the data of children included in the COPP-study). A summary of this analysis is shown on the website https://covidkids.nl/scientific-dashboard/
b) detailed immunological profiling, in relation to clinical phenotype

As of April 2021, 53 hospitals are participating in the COPP study. When the COPP-IMM study starts, 17 of these will participate in the COPP-IMM study instead of the COPP study. The remaining 36 hospitals will continue to participate in the COPP study. Clinical data from COPP and COPP-IMM will be collected using the same database (using eCRFs in Castor) and will be analysed as one dataset.

For the COPP-IMM study, patients/parents/guardians can choose if they wish to participate in:

- Only the clinical observational part of the study, with or without questionnaires (similar to the COPP-study)
- Both the clinical observational part of the study **AND** the immunological part of the study (one blood sample at diagnosis)

8.1.1 Main study parameter/endpoint

For the clinical observational part of the study (analyzed together with data from the COPP-study) the main study parameters are:

- To describe the clinical features of the COVID-19 in hospitalized and outpatient pediatric patients in the Netherlands.
- To describe the clinical course of the COVID-19 in hospitalized and outpatient pediatric patients.
- To describe the response to treatment, including supportive care.
- To determine risk factors for severe disease in children with COVID-19.

For the immunological part of the COPP-IMM study, we will obtain a detailed immunological profile of children presenting to Dutch hospitals with acute SARS-CoV-2 infection or with a SARS-CoV-2 related post-infectious inflammatory syndrome. Since clinically relevant pediatric COVID-19 is rare and we will have a limited amount of material available from each patient, we will do this in a two-tiered approach. This will enable us to choose which will be the most informative in-depth immunological analysis following a more general screening of the innate and adaptive immune system.

In the FIRST TIER we will do a general assessment of the immune system. In this general assessment we will do the following investigations:

1. Detailed phenotypical analysis of the cells of the innate and adaptive immune system using multicolor flow cytometry. We will use the EuroFlow protocol PID Orientation

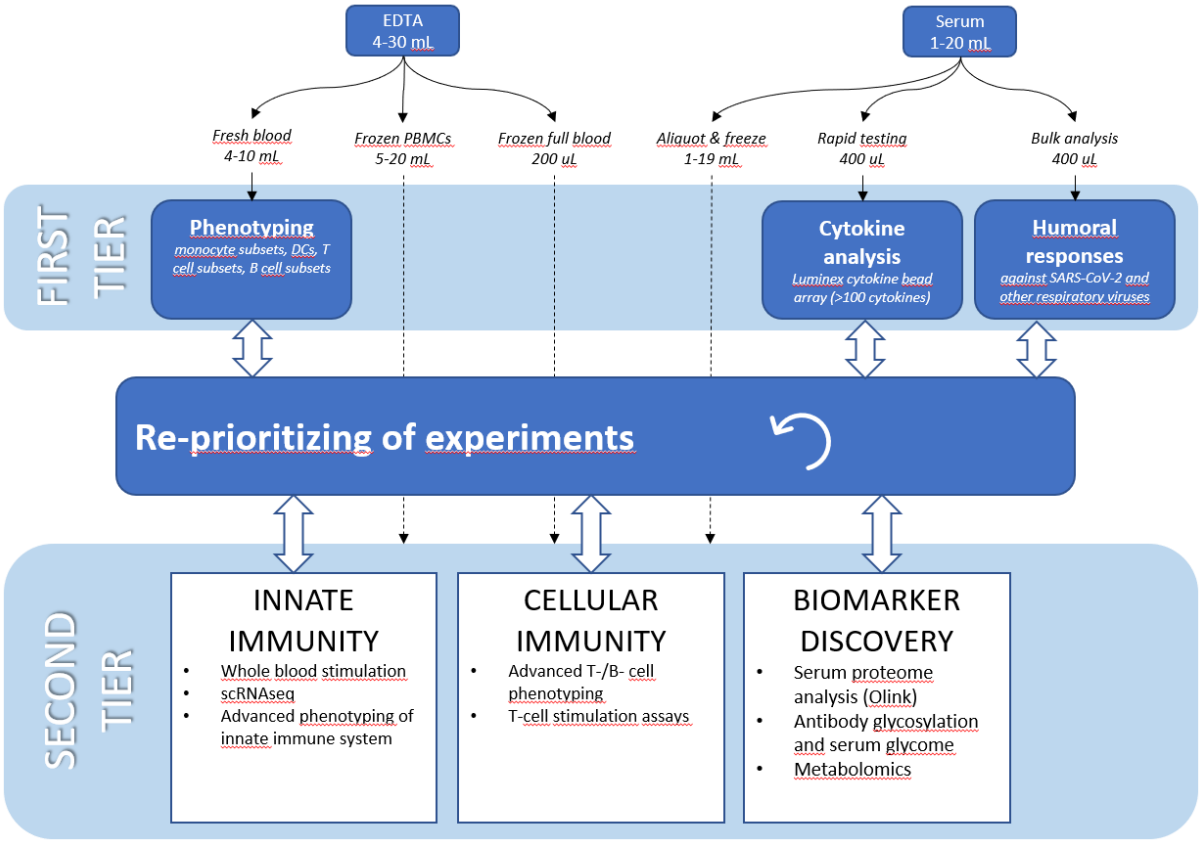
tube to be able to identify and quantify the main leukocyte and lymphocyte subsets, using 12 markers: CD27, CD45RA, CD8, IgD, CD16, CD56, CD4, IgM, CD19, CD3, CD45, and TCRγδ. This will enable us to identify B-cells (including B-cell subsets), T-cells (including T-cell subsets), NK-cells, monocytes (including non-classical CD16+ monocytes), dendritic cells, basophils, neutrophils and eosinophils (minimum of 4 mL of EDTA needed).
2. Multiplex analysis of inflammatory cytokines in serum using Luminex bead arrays (in excess of 100 analytes, to determine Th1/Th2 and innate responses) (200-400 uL).
3. Quantitative and qualitative (affinity) analysis of anti-SARS-CoV-2 antibodies in serum and potential cross-reactive antibodies to other coronaviruses (200-400 uL).

Depending on the results of the investigations in the first tier, we will choose one of the following in-depth immunological analyses for the SECOND TIER:
1. The innate immune system (whole blood stimulation assays with cytokine production, single cell RNAseq or advanced flow cytometric phenotyping of the innate immune system).
2. The adaptive immune system; with either a focus on T-cell responses (T-cell stimulation assays to evaluate effector responses against SARS-CoV-2 peptides), or B-cell responses (in-depth B cell subset analysis).
3. Biomarker discovery of serum proteins using multiplex assays such as Olink or mass spectrometry.

In almost all children we expect to be able to perform all analyses from the FIRST TIER, even in the youngest children (4 mL of EDTA-blood and 1 mL of blood in serum tube). For the analyses in the SECOND TIER, we will have to prioritise the analyses, especially in those patients for whom only a limited amount of blood is available. The exact priority of analyses in the SECOND TIER depend on the results obtained in the FIRST TIER.

Examples:
- If FIRST TIER analysis will demonstrate marked changes in CD4/CD8 T cell distributions in children with severe COVID-19 as compared to non-severe COVID-19, the SECOND TIER investigations will focus on detailed T cell analysis (advanced T cell phenotyping and functional assays) in children with severe and non-severe COVID-19.
- If FIRST TIER analyses show high levels of cytokines associated with inflammasome activity (IL-1beta, IL-18) in children with MIS-C as compared to children with COVID-19, the SECOND TIER analysis will focus on *in vitro* stimulation of monocytes with inflammasome activating agents and in-depth innate immune phenotyping in children with MIS-C and COVID-19.

If less than 2*10e6 mononuclear cells are isolated from the EDTA blood sample, then these will not be used for flow cytometry in the FIRST TIER, but will be stored to be used in SECOND TIER analyses that require small amounts of cells (such as single cell RNA sequencing).



8.1.2 Secondary study parameters/endpoints (if applicable)

We will correlate the immunological profiles with detailed clinical outcome measures of the patients. We will collect these clinical outcome measures in the same manner as in our related cohort study 'Clinical features of COVID-19 in Pediatric Patients' (COPP-study). Clinical parameters collected include: severity of disease (need of supplemental oxygen); underlying illnesses; age at presentation; clinical syndrome; laboratory parameters at diagnosis and during illness; response to treatment; clinical outcome: days of hospitalization, life-saving interventions, ICU admissions, death, patient reported outcome measures at 6 weeks follow-up.

Also, we will explore if there are potential immunological targets of therapy for children with severe COVID-19 or MIS-C, and if immunological profiles differ between MIS-C and severe pediatric COVID-19.

8.1.3 Other study parameters (if applicable)

Immunological analyses will be done in cooperation with research-teams investigating adult patients with COVID-19 (eg in the BEAT-COVID1 study), so as to be able to compare hospitalized children and adults with COVID-19. Also, where possible, immune responses in hospitalized children will be compared with results from pediatric population-based studies.

We will perform similar immunological analyses in children included in the follow-up study COPP-2 (multi-center study in which children included in the COPP-study will be invited 6-12 months after presentation for a multi-disciplinary follow-up assessment).

The PedsQL and TAPQOL questionnaires measure Health Related Quality of Life at 12 weeks after diagnosis. To measure overall health and specific domains of functioning (anxiety, depression, peer relationships, sleep problems, anger, and neurocognitive functioning, anger and fatigue), we will use the PROMIS items in children older than 8 years of age. Patients admitted to the intensive care unit undergo neuropsychological evaluation as standard care in most Dutch academic centres, 3-6 months after admittance to the intensive care unit. We ask parents and patient for permission to retrieve these results.
The outcome of these psychosocial and Quality of Life evaluations will be analysed together with the detailed clinical parameters of our participants in the COPP and COPP-IMM study to examine possible risk factors for reduced quality of life or neuropsychological outcomes after COVID-19 or MIS-C.

8.2 Randomisation, blinding and treatment allocation

not applicable

8.3 Study procedures

1. **Patients will be included** in the study in case of SARS-CoV-2 positive test, or fulfilling a clinical diagnosis of COVID-19, or fulfilling a diagnosis of MIS-C; and after obtaining informed consent (IC) from both guardians and/or patient. Because only one parent can accompany the hospitalized child (due to COVID-19 restrictions), initially it is only one guardian who will sign the consent form. If applicable, the signature of the second guardian will be collected as soon as possible. Consent can be given for just the clinical part of the study (with or without consent for follow-up studies); or for both the clinical and immunological part of the study.

2. **Clinical data** from the subject at time of presentation in the hospital will be noted in a case report form. In case of a diagnosis of COVID-19 after hospital presentation the data will be collected from the electronic medical record. Summarized this data will contain:
    a. Patient characteristics and risk factors: age, gender, height, weight, exposure to known COVID-19 cases, prior medical history, medication.
    b. Clinical features at presentation: symptoms, vital signs, physical examination, imaging results, laboratory results.
    c. Course of illness: clinical symptoms, vital signs, physical examination, imaging results, laboratory result, the need for respiratory support, the need for IC treatment, medication, aerosol treatment, co-morbidity and outcome.
    Clinical data will be recorded in Castor. For details regarding the datamanagement plan see **Appendix A**.

    When patients are being admitted to the IC, additional in information about the neurological tests that have been performed (standard care) will be collected for

this study. Guardians and/or children can give consent for this data collection in our standard consent form.

3. **Blood samples** for this study will be collected after confirmation of the diagnosis COVID-19 or MIS-C and after the patient and/or the caregiver have consented for participation in this study. If possible, the collection of the blood sample will be combined with a blood sample collection for routine clinical care and/or will be drawn from an indwelling venous catheter. In this case, there will be no additional burden to the patient.

   If after confirmation of the diagnosis and obtainment of informed consent, no blood sample collection is scheduled for routine clinical care and there is no venous catheter present from which the blood can be drawn, a venepuncture will be done to collect material for this study. In this case, there will some burden to the patient. To ensure that this burden is minimal, we will apply a topical anaesthetic (lidocaine/prilocaine or lidocaine/tetracaine as per local guidelines) prior to the procedure. Also, we will instruct local researchers to engage in “positive language” before and during the procedure (See **Appendix B**).

   The amount of blood drawn depends on the body weight of the patient. With these amounts, we remain well below the recommended maximum of 3% of total blood volume in all weight categories (8). If the local investigator does not succeed in obtaining the required amount of blood, a smaller sample will be collected. In that case, immunological investigations will need to be prioritized (see 8.1.1 for details regarding priorities).

body weight	EDTA (mL)	serum (mL)	total volume (mL)
3-5 kg	4	1	5
5-10 kg	5	2	7
10-20 kg	10	5	15
20-40 kg	20	10	30
>/= 40 kg	30	10	40

Scarce material from blood samples will be stored in local centers. They will be used to retrospectively analyze additional laboratory parameters associated with severity of disease and outcome, such as cytokine levels (particularly IL-6) and medication concentrations (particularly chloroquine).

4. **Timing of sampling**

We aim to collect the sample *as soon as possible* after inclusion in the study, preferably before any *immune modulatory medication* has been administered. This will enable us to determine the immunological profile in the *acute phase* of the illness. The acute phase of the illness is defined as: at presentation of the patient (in case of screening, asymptomatic patient or ambulatory patient) OR ongoing symptoms (in case of acute COVID-19) OR ongoing signs of hyperinflammation (in case of MIS-C). The local investigator will note the exact date and time of blood sampling in the case report form.

For a Standard Operating Procedure regarding the blood draw and logistics, see **Appendix C**.

5. If available, locally **stored samples** will be collected to retrospectively analyze the immunological parameters of the patients in this study, particularly if no consent was obtained for additional sampling for the COPP-IMM study, if an extra blood draw was not feasible for logistic reasons, or if longitudinal analyses are informative.

6. **Follow-up questionnaire** at 12 weeks after start of symptoms (See **Appendix D**). The questionnaires are age-appropriate and contain the following topics: general complaints, remaining symptoms, recovery to normal life, overall health, and quality of life using validated surveys: PedsQL (Pediatric Quality of Life Inventory) and PROMIS (Patient-Reported Outcomes Measurement Information System). PROMIS outcomes are: anxiety, depression, anger, peer relations, sleeping problems, overall health and fatigue. These surveys have been evaluated by the patient support group (Kind & Ziekenhuis). The flow diagram of the follow-up surveys is displayed in **Appendix E**.

7. A separate 2 minutes questionnaire will be sent to children eligible for vaccination against SARS-CoV-2.This questionnaire consists of questions about if a child was vaccinated, and if so, when and if there were any side effects. We will send this questionnaire once a year during the course of this study.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.5 Replacement of individual subjects after withdrawal

Subjects will not be replaced.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

The study will be prematurely terminated in case of problems regarding the inclusion or participation of patients.. Also, the study will be terminated if it is temporarily suspended for reasons of subjects' safety and the accredited METC gives a negative decision after assessing the reasons that led to the temporary suspension.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

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

9.2.2 Serious adverse events (SAEs)

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

- 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; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

Only study related (S)AE's will be reported immediately. This means all (S)AE's related to participation in this study protocol, meaning from the venepuncture, will be reported. All other (S)AE's will not be reported, since the study procedure is considered to be of very low risk.

The sponsor will report these SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs 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. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

10. STATISTICAL ANALYSIS

10.1 Primary study parameter(s)

Statistical analysis of the primary objective: immunological profiling of children with COVID-19 or MIS-C:

1. Relative distributions of immune cell subsets will be obtained by multicolor flow cytometry. Data will be imported in R for statistical analysis. Cytosplore will be used for clustering and dimensionality reduction (9). Distributions will be compared to available reference values (normal distribution of immune cell subsets in children, adjusted for age) and will be compared across subgroups (mild COVID-19, moderate to severe COVID-19 and MIS-C).
2. Cytokine levels will be analyzed in R and SPSS. Levels will likely be non-normally distributed, in which case non-parametric Kruskal-Wallis tests will be done for comparison across groups. To adjust for multiple testing, we will use the two-stage step-up method of Benjamini *et al* (10). Cytokine levels will be compared to normal values as already available for healthy adults, and across the subgroups(mild COVID-19, moderate to severe COVID-19 and MIS-C).
3. Levels of neutralizing antibodies will be compared across subgroups (mild COVID-19, moderate to severe COVID-19 and MIS-C) using ANOVA in SPSS.

10.2 Secondary study parameter(s)

Secondary objectives:
(1) To correlate the immunological profiles with detailed clinical parameters
Adjusted relative risks for specific outcome measures (such as the need for supplemental oxygen, ICU admission, duration of hospital stay) in association with immunological profiles will be calculated using logistic regression.
(2) To determine if there are immunological targets of therapy for children with severe COVID-19 or MIS-C: Evaluate the levels of certain drug-targetable cytokines using Kruskal-Wallis tests in SPSS (eg IFN-gamma, IL-1beta, TNF-alpha) and perform agglomerative hierarchical clustering using average linking (Between Groups) with squared Euclidean distance to determine subgroups of patients with specific inflammatory profiles..
(3) To determine if hyperinflammation in MIS-C is different from the inflammatory response in severe pediatric COVID-19: non-parametric Kruskal-Wallis tests will be done for comparison across groups (in R and SPSS).

10.3 Other study parameters

Comparison between immunological profiles of hospitalized children with COVID-19 and other cohorts (eg adults with severe COVID-19, non-admitted children with COVID-19) will be done using parametric (ANOVA) or non-parametric (Kruskal-Wallis) tests were applicable, depending on whether the data is normally distributed or not. Logistic regression will be used to determine associations between clinical parameters and the results from the questionnaires on Quality of Life and functioning.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This prospective cohort study is subject to the Medical Research Involving Human Subjects Act (WMO). This study will be conducted according to the principles of the Declaration of Helsinki (version 2013, 19-10-2013, (www.wma.net) and in accordance with the Medical Research Involving Human Subjects Act (WMO), the General Data Protection Regulation (AVG), the Dutch Act on implementation of the General Data Protection Regulation (uitvoeringswet AVG) and other guidelines, regulations and acts. We will comply with the principles enshrined in the Council of Europe Convention on human rights and biomedicine – known as the Bioethics Convention Oviedo). Its main purpose is to protect individuals against exploitation.

11.2 Recruitment and consent

The recruitment and informed consent procedures will be done by local researcher in the hospital. Local researchers will be recorded in a researcher log. Subjects will be approached for consent preferably within at least 24h after presentation in the hospital. They will have at least 24h to consider their decision. This time facilitates the subject to make a considered decision, yet will ensure reliable prospective data collection.

Both parents/caregivers need to sign the consent form before the child can be included in the study. In case only one parent is present during the hospital visit/hospitalization (for example, due to COVID-19 restrictions), is it obligated to collect the second signature as soon as possible. This can be done, for example:

- During the next time the second parent visits the hospital,
- By sending the patient information and the consent form to the second parent; the signed form can be returned with a reply envelop,
- The second parent returns a signed consent form by e-mail.

In case of study subjects age 12-16 years of age, we will require their written consent, as well as at least one parent or caregiver.
In case of study subjects age 16 and older, we will only require their written consent and not from their parents or caregivers.

See attachment for patient information letter and informed consent.

11.3 Objection by minors or incapacitated subjects (if applicable)

The research will be conducted according to the Code of Conduct for Medical Research by the Federation of Dutch Medical Scientific Societies (Federa) and the code of conduct relating to expressions of objection by minors participating in medical research by the Netherlands Association for paediatric research.
(https://www.ccmo.nl/onderzoekers/publicaties/publicaties/2001/06/01/gedragscode-verzet-bij-minderjarigen).

https://english.ccmo.nl/investigators/legal-framework-for-medical-scientific-research/codes-of-conduct/code-of-conduct-for-medical-research

11.4 Benefits and risks assessment, group relatedness

Subjects do not benefit personally from study participation. This non-therapeutic research with minors will have negligible risks and minimal burden. This study can only be performed in minors, as it will provide age-specific data that cannot be obtained otherwise.

11.5 Compensation for injury

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

We request dispensation by the METC for the statutory obligation to provide insurance for damage to research subjects, because participating in this study is without risks.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Patients will receive a COPP-IMM study number. The key to the code is safeguarded by the research team at the local site, by placing this in a secured datasafe managed by the 'Trialbureau' of the 'Willem-Alexander Kinderziekenhuis' (WAKZ).

Documentation belonging to the study will be archived for 15 years in the "Investigator File". Biological material will be labelled with the COPP-IMM study number and date of sampling. The local researcher will note in the COPP-Castor database that the participant has consented for COPP-IMM study and will note the date of sampling. Castor is a LUMC approved data storage program.

The LUMC COPP / COPP-IMM team (Buddingh, Mooij, Von Asmuth, Lugthart) only has access to the coded data and not to patient-identifiable data.
Only the treating physician from the various participating centers will have access to the source data. This means that during the study, within the LUMC Dr. E. Buddingh will have access to both coded and source data. After completion of the study, the key to the code will be safeguarded with the Trialbureau WAKZ, an independent location. The LUMC COPP/COPP-IMM team won't have access to the key to the code when analyzing the collected data.

Biological samples will be stored for 15 years for additional investigations that are related to this study. As soon as the samples are no longer necessary, the material will be destroyed.

12.2 Monitoring and Quality Assurance

MultiCenter: Monitoring in all sites in the Netherlands will be executed by (internal) monitors of the LUMC according to the monitor plan (see **Appendix G**).

12.3 Amendments

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

12.4 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.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor 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 time when the last included patient has completed the follow-up survey at six weeks.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, 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.

12.6 Public disclosure and publication policy

The results of this study will be published in peer-reviewed journals, initiated by the sponsor. Temporary results will be published on a dashboard on the website www.covidkids.nl. The investigators of the participating sites will form part of the publishing consortium.

13. STRUCTURED RISK ANALYSIS

As this research does not involve a medicinal product, food product, medical device or other (as described in chapter 6 and 7) this paragraph is not applicable.

14. REFERENCES

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15. APPENDICES

APPENDIX A: Data Management Plan

APPENDIX B: Instruction in "Positive language" during procedures, courtesy of Stichting Kind en Ziekenhuis

APPENDIX C: Standard Operating Procedure (SOP) blood collection

APPENDIX D: Questionnaire at 12 weeks follow-up

- Algemene vragenlijst
- Surveys PedsQL
- Surveys PROMIS

APPENDIX E: Flow chart follow up questionnaire after 12 weeks

APPENDIX F: Statement patient data in observative cohort studies in emergency situations and COVID-19

APPENDIX G: Monitor plan

APPENDIX H: Yearly Questionnaire corona vaccination