Genetic Risk factors for Multi-system Inflammatory Syndrome in Children and Pediatric Post COVID condition (GRIP)

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PROTOCOL TITLE 'Genetic Risk factors for Multi-system Inflammatory Syndrome in Children and Pediatric Post COVID condition'

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Principal investigator(s) (in Dutch: hoofdonderzoeker/ uitvoerder)	Dr. E.P. Buddingh Pediatric infectiologist/immunologist Willem-Alexander Children's Hospital Leiden University Medical Centre Albinusdreef 2, 2333 ZA Leiden e.p.buddingh@lumc.nl +31-71-5262824
Sponsor	LUMC, Leiden
Subsidising party	ZonMw
Independent expert	Drs. C. Meijer Pediatric gastroenterologist Willem-Alexander Children's Hospital Leiden University Medical Centre Albinusdreef 2, 2333 ZA Leiden c.r.meijer-boekel@lumc.nl +31-71-5262824
Laboratory sites	**GenomeScan** Plesmanlaan 1d 2333 BZ, Leiden **Pediatric Immunological Laboratory** Dr. M. van den Burg LUMC, Leiden **AUMC/Sanquin pediatric immunology** Prof. Dr. Taco Kuijpers, AUMC, Amsterdam **Clinical Genetic Laboratory** Dr. M van Gijn UMCG, Groningen **Center of Translational Immunology** Dr. S. Vastert UMC Utrecht, Utrecht

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Head of Department: Prof. dr. E.H.H.M Rings Head of the department of pediatrics, Willem-Alexander Children's Hospital Leiden University Medical Centre, Leiden		
Principal Investigator: Dr. E.P. Buddingh Pediatric infectiologist/immunologist 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**
API	**Application Programming Interface**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CADD	**Combined Annotation Dependent Depletion**
COPP	**'Clinical features of COVID-19 in Pediatric Patients' study**
COPP2	**'Clinical features of COVID-19 in Pediatric Patients, long term effects' study**
COPP-IMM	**'COVID-19 in Pediatric Patients: clinical and immunological features' study**
COVID-19	**COronaVIrus Disease 2019**
CRF	**Case Record Form**
CV	**Curriculum Vitae**
DNA	**DeoxyriboNucleic Acid**
DSMB	**Data Safety Monitoring Board**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
FDR	**False Discovery Rate**
Gb	**Gigabyte**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
gnomAD	**Genome Aggregation Database**
GoNL	**Genome Of the Netherlands**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IEI	**Inborn Error of Immunity**
IUIS	**International Union of Immunological Societies**
MAF	**Minor Allele Frequency**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
MIS-C	**Multi-system Inflammatory Syndrome in Children**
NGS	**Next Generation Sequencing**
PICU	**Pediatric Intensive Care Unit**
PROMs	**Patient Reported Outcome Measures**
QC	**Quality Control**
QV	**Qualifying Variants**
RNA	**RiboNucleic Acid**
RT-PCR	**Reverse Transcriptase Polymerase Chain Reaction**

(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.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WES	**Whole Exome Sequencing**
WHO	**World Health Organisation**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale:
Following infection with SARS-CoV-2, some children develop the potentially life-threatening disease Multi-System Inflammatory Syndrome in Children (MIS-C) and some children develop post-COVID condition (formerly 'long COVID'). It is unknown why some children develop severe or prolonged symptoms after SARS-CoV-2 infection, while most children have asymptomatic or mild disease. We hypothesize that rare variants in genes associated with the immune system predispose children to develop MIS-C or post-COVID condition after infection with SARS-CoV-2.

Objective:
Primary objective: To identify rare, high impact genetic variants in immunological genes and pathways in children with a history of MIS-C or pediatric post-COVID condition.
Secondary objectives: To analyze the clinical characteristics and long-term effects of pediatric COVID-19 and MIS-C. To characterize the functional and clinical impact of genetic variants in MIS-C and post-COVID condition and identify targets for therapy.

Study design:
We will do an observational study. We will perform Whole Exome Sequencing (WES) using Next Generation Sequencing (NGS) on DNA from blood or saliva. We will include: (1) MIS-C cases: Children with a history of MIS-C; (2) post-COVID condition cases: Children with post-COVID condition; and (3) Controls: SARS-CoV-2 exposed age-matched control group: children who were infected with SARS-CoV-2 but did not develop moderate to severe COVID-19, MIS-C or post-COVID condition. We will do immunological analyses to validate the results of the genetic study. We will evaluate if certain genetic risk factors aggregate in specific subgroups of patients.

Study population:
Children 0-19 years old with a history of MIS-C (n=100), post-COVID condition (n=100), or uncomplicated SARS-CoV-2 infection (n=200).

Main study parameters/endpoints:
- Individual analysis of immunological genes in cases only: We will restrict our analysis to pathogenic (class 5) or likely pathogenic (class 4) variants in genes known to be associated with monogenic inborn errors of immunity to diagnose previously unsuspected inborn errors of immunity (IEI) in MIS-C or post-COVID condition cases.
- Case-control study: We will evaluate if a larger proportion of cases with MIS-C or post-COVID condition have rare and presumably deleterious variants in immunological genes than children with an asymptomatic or mild infection.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:
Results of this study are related to the target group (i.e. children with SARS-CoV-2 infection). Children with MIS-C or post-COVID condition participating in this study may benefit directly, if an immunological condition is identified that warrants treatment or follow-up. Controls will not directly benefit from this study, since genetic data from the controls is analyzed at 'group level' in the case-control study. To minimize burden we will send participants a saliva DNA home collection kit. These can be returned by regular mail. Samples for the immunological studies have already been collected and stored.

1. INTRODUCTION AND RATIONALE

Most children infected with SARS-CoV-2 have little or no symptoms, but some children develop life-threatening or long-term post-infectious complications. Among these post-infectious sequelae are multi-system inflammatory syndrome in children (MIS-C) and post-COVID condition (formerly also known as 'long COVID'). MIS-C is a potentially life-threatening hyperinflammatory post-infectious disease that occurs in 1:2000 to 1:5000 children three to six weeks after infection with SARS-CoV-2 (1, 2). MIS-C is predominantly a childhood disease, but occasionally, (young) adults develop multi-system inflammatory syndrome (MIS-A) (3). Patients with MIS-C present with a high fever, frequent cardiac and gastrointestinal involvement, neurological symptoms and show high C-reactive protein levels (4, 5). About fifty percent of children with MIS-C are admitted to pediatric intensive care units (PICUs) because of cardiogenic or distributive shock (6). Most children respond well to anti-inflammatory treatment, but 1-2% of children with MIS-C die (7, 8). Children with MIS-C have profound immune dysregulation, with lymphopenia, T-cell receptor skewing, increased plasmablasts, a broad range of auto-antibodies, very high cytokine levels and activated monocytes (9-14). A subset of children with MIS-C demonstrate polyclonal expansion of TRBV11-2 skewing, suggesting superantigenic stimulation (15-17). Although the immunological profile of MIS-C is being uncovered, it is still not understood why some children develop MIS-C after SARS-CoV-2 infection while most children don't.

In addition to the life-threatening post-infectious complication MIS-C, children can develop post-COVID condition following infection with SARS-CoV-2 (18-22). Symptoms include fatigue, headache, cough, dyspnea or 'brain fog' and may last for months following the initial infection. The etiology of post-COVID condition is unknown, but similar to MIS-C, it may be a result of immunological dysregulation following infection with SARS-CoV-2 (23, 24).

Many children have been infected with SARS-CoV-2 since the beginning of the pandemic, but both MIS-C and pediatric post-COVID condition are relatively rare diseases. **Our hypothesis is, that rare variants in genes associated with the immune system predispose children to develop MIS-C or post-COVID condition after infection with SARS-CoV-2** (25). Identifying rare inborn errors of immunity in these children will guide proper treatment and counsel the patients and their families. In addition, by comparing the prevalence of genetic immunological variants in children with a complicated *vs.* uncomplicated course of SARS-CoV-2 infection, we will gain insight into the pathogenesis of these diseases.

Our hypothesis is supported by several lines of evidence. Kawasaki Disease, a disease with resemblances to MIS-C, is more prevalent in Asian populations and is associated with variants in *FCGR2A* and *ITPKC* (26-28). Variants in immunological genes have been reported in 3/18 MIS-C patients (in *XIAP*, *CYBB* and *SOCS1*) (29, 30). Mutations in these genes cause different severe immunodeficiencies, suggesting that MIS-C may be the first sign of an inborn error of immunity in a significant proportion of children. Similarly, in a subset of adults with life-threatening COVID-19, inborn errors of type I interferon immunity have been found (31, 32). Although MIS-C and severe acute COVID-19 are different entities, both are characterized by a dysfunctional, massive hyperinflammatory response and both are apparently associated with previously unsuspected inborn errors of immunity.

1.1 OBJECTIVES

1.1.1 Primary objective:

To identify rare, high impact genetic variants in immunological genes and pathways in children with a history of MIS-C or pediatric post-COVID condition

1.1.2 Secondary objectives:

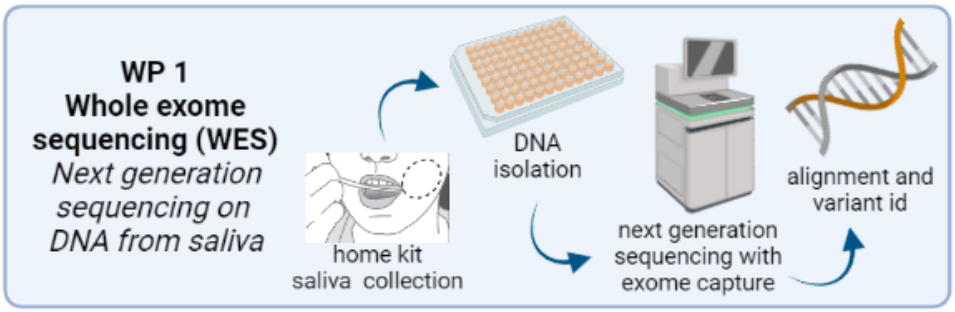
To analyze the clinical characteristics and long-term effects of pediatric COVID-19 and MIS-C

To characterize the functional and clinical impact of genetic variants in MIS-C and post-COVID condition and identify targets for therapy

2. STUDY DESIGN

To identify rare, high impact genetic variants in immunological genes in MIS-C and pediatric post-COVID condition we will do a single center **observational study**. Cases and controls will be recruited by the principal investigators of other studies on pediatric COVID and MIS-C. We will perform Whole Exome Sequencing (WES) using Next Generation Sequencing (NGS) on DNA from blood or saliva in:

(1) **MIS-C cases**: Children with a history of MIS-C (n=100);
(2) **post-COVID condition cases**: Children with post-COVID condition (n=100); and
(3) **controls**: SARS-CoV-2 exposed age-matched control group (n=200): children who were infected with SARS-CoV-2 but did not develop moderate to severe COVID-19, MIS-C or post-COVID condition

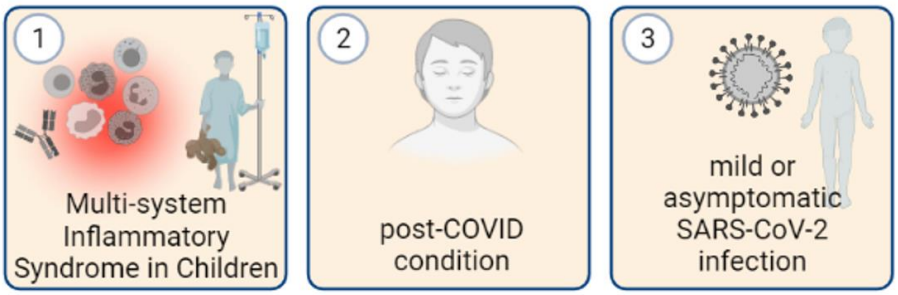


Since all patients and controls have already been included in other studies, we expect to be able to include all participants before January 2024.

3. STUDY POPULATION

3.1 Population (base)

In this study we will include children until 19 years of age.



1. Children in group (1): MIS-C, will be recruited from the ongoing COPP, COPP-IMM, WKZ and Kawasaki studies.
2. Children in group (2): post-COVID condition, will be recruited from the COPP follow-up study 'COPP2', the PoCoCoChi study (Post Corona Complaints in Children), the VINCERO/VINCERE study (vaccination study in 'long COVID') and the POCOS (Post COVID) clinical observational study.
3. Children in group (3): 'Exposed' control group, will be recruited from the COPP-study, the CoKIDS study, PoCoCoChi study, de CORONAthuisstudie and the SARSLIVA study.

We will recruit subjects from the respective studies based on prior consent to be approached for additional studies.

3.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria, depending on group:

1. Children (<19 years) with a history of MIS-C: as defined according to WHO criteria [36]
2. Children (<19 years) with post-COVID condition: as defined according to the WHO case definition [37].
   This includes a history of probable or confirmed prior SARS-CoV-2 infection, with signs and symptoms (including fatigue, shortness of breath, cognitive dysfunction) that are present after 12 weeks, last at least 2 months, have an impact on daily functioning and are not explained by an alternative diagnosis.
3. 'Exposed' control group: children (<19 years of age): a history of proven SARS-CoV-2 infection (RT-PCR, antigen test or serology positive)

3.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

1. Group 1 (MIS-C): no specific exclusion criteria

2. Group 2 (post-COVID condition): other plausible cause of symptoms AND/OR a history compatible with chronic fatigue syndrome prior to infection with SARS-CoV-2. Children with a history of MIS-C who suffer prolonged signs and symptoms will be included in the MIS-C group.
3. Group 3 ('exposed' control group): MIS-C or post-COVID condition; AND/OR Moderate or severe course of COVID-19, as defined in the COPP-study (N20.043) (need for supplemental oxygen and/or intensive care admission because of COVID-19 and/or death).

3.4 Sample size calculation

Our study is powered to answer the main research question: "Do more children with MIS-C or post-COVID condition have deleterious variants in immunological genes than exposed controls?" To analyze the genetic data from the case-control study, we will use rare-variant collapsing analyses, as reviewed by Povysil, et al [35] (see Chapter 9 (Statistical Analysis) for details).

Power analysis using Monte Carlo simulation:

In a previous study in MIS-C, 17% of patients (3/18) had deleterious variants in immunological genes. In controls, the prevalence of (predicted) loss of function variants in our gene-set is about 3.7% (according to gnomAD v. 2.1.1 data, using a minor allele frequency of <0.00001). This yields an odds ratio of 5.2 (3/18 * 0.963 / (0.037 * 15/18)). **Therefore, for the power analysis, we conservatively assume that cases have more qualifying variants than healthy controls, with an odds ratio of 4.** We found the 'normal variation' in controls to be present in all the clusters. We assume that the 'background variation' in cases is similarly spread out over the clusters.

The simulations have been run 1000x, with 100 cases and 200 controls.

Causative variants should cluster together

We hypothesize that causative genetic variants that are more prevalent in cases are functionally related. They underly a pathogenic mechanism and should cluster together. Therefore, we assume that excess variants in cases cluster together, either in one or in two clusters. See Chapter 9 (Statistical Analysis) for details on the cluster analysis.

Power of 80% assuming an odds ratio of 4 and one cluster affected

Individuals are positive if they have at least one qualifying variant in at least one gene. The **overall positive rate** is the proportion of simulations in which the Fisher exact P value comparing positive cases and controls is <0.05. A genecluster-by-individual collapsing matrix is constructed. For every simulated case or control it is assessed if at least one of the clusters is affected. A Fisher exact test is calculated for each simulation. The **cluster true positive rate** is the proportion of simulations in which both the overall Fisher exact P-value and the cluster-specific Q-value (using Benjamini-Hochberg FDR) are <0.05.

Cluster	Overall positive rate	Cluster true positive rate	Cluster false positive rate
1	0.823	0.807	0.014
2	0.814	0.791	0.008
3	0.812	0.803	0.014
4	0.833	0.816	0.012
5	0.806	0.772	0.002

Assuming an odds ratio of 4 the power for an overall positive result is above 80%. The power to detect the responsible cluster is around 80%. The false positive rate is below α= 0.05.

Detection of at least one cluster assuming two clusters are affected

Assuming an odds ratio of 4 and two clusters affected, the power for an overall positive result is above 80%. The power to detect at least one of the two responsible clusters is around 70%. The false positive rate is below α = 0.05.

Clusters	Overall positive rate	One cluster positive rate	Two cluster positive rate	Cluster false positive rate
1 + 2	0.819	0.710	0.409	0.009
1 + 3	0.835	0.726	0.426	0.013
1 + 4	0.842	0.698	0.416	0.004
1 + 5	0.837	0.714	0.35	0.005
2 + 3	0.814	0.692	0.363	0.003
2 + 4	0.823	0.665	0.362	0.004
2 + 5	0.844	0.638	0.292	0.000
3 + 4	0.833	0.700	0.387	0.010
3 + 5	0.809	0.659	0.304	0.005
4 + 5	0.832	0.66	0.307	0.003

4. TREATMENT OF SUBJECTS

Not applicable

5. INVESTIGATIONAL PRODUCT

Not applicable

6. NON-INVESTIGATIONAL PRODUCT

We will use self-collection kits to collect the saliva:
https://www.dnagenotek.com/ROW/products/collection-human/oragene-dna/500-series/OG-500.html

For children unable to self-collect saliva, a kit for assisted collection will be used:
https://www.dnagenotek.com/ROW/products/collection-human/oragene-dna/500-series/OG-575.html

7. METHODS

7.1 Study parameters/endpoints

7.1.1 Main study parameter/endpoint: WES to detect rare, high impact genetic variants

First, we will evaluate if some children with MIS-C or post-COVID condition have a previously unidentified inborn error of immunity (IEI)

We will use existing diagnostic algorithms to diagnose monogenic inborn errors of immunity in MIS-C or post-COVID condition cases. This means that here, **we will restrict our analysis to pathogenic (class 5) or likely pathogenic (class 4) variants (**as established by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guideline (33)) **in genes known to be associated with monogenic inborn errors of immunity (IUIS IEI gene set).**

Next, we will do a case-control rare variant association study.

In the subsequent case-control analysis we will focus on determining if a larger proportion of cases with MIS-C or post-COVID condition have rare and presumably deleterious variants in immunological genes than children with an asymptomatic or mild infection.

7.1.2 Secondary study parameters/endpoints: Clinical characteristics and long-term effects of pediatric COVID-19 and MIS-C

We will describe the clinical characteristics of children hospitalized with COVID-19 or MIS-C. Also, we will evaluate the long-term effects of pediatric COVID-19 and MIS-C. A detailed description of the clinical course of MIS-C and post-COVID condition will enable us to make subgroups of patients. For example, children with MIS-C who need inotropic support vs. those who do not. For this purpose, we will use available data from our ongoing nation-wide cohort study of children with COVID-19 or MIS-C in Dutch hospitals (Clinical features of COVID-19 in Pediatric Patients; COPP-study and COPP-IMM study; WHO-CRF with additions). Inclusion in this study is ongoing in 53 hospitals.

To evaluate the long-term effects of pediatric COVID-19 and MIS-C, we will analyze data from the ongoing follow-up study COPP2 and the PICU follow-up data. Children included in the COPP-study are invited at six to twelve months for a multi-disciplinary study visit. Patient reported outcome measures (PROMs) will be collected, as well as an assessment of exercise tolerance, lung function, smell and taste testing, neurocognitive testing and an immunological assessment. Children referred to our clinic because of post-COVID condition will be assessed in a similar manner. We will compare this with data from the PoCoCoChi study (“Post Corona Complaints in Children”), in which children tested for SARS-CoV-2 at a GGD test location Kennemerland with a negative or positive test result are prospectively evaluated for long-term effects. Other reference groups of children will be identified from the ongoing home studies including COKids, CORNAthuis and SARSLIVA.

7.1.3 Other study parameters: integrated analysis and aim to identify therapeutic targets

1. We will do targeted immunological studies to validate the results from the genetic study. For example, if we find that a child has a novel variant that is classified as class IV (likely pathogenic) in a specific gene normally present in T-cells, we will first evaluate if this gene is expressed (RNA and/or protein) in T-cells of the patient. If there is no RNA/protein expression, this further supports the pathogenicity of the novel variant. If we find that more cases have predicted loss of function variants in cluster 1 (complement activation), we will measure the levels of complement proteins in serum. This can be done in: a) the cases with predicted loss of function variants and compare this with controls; and b) also be evaluated in cases without genetic perturbations in complement genes to evaluate if these are functionally impaired (by acquired or intronic variants).
2. We will evaluate if genetic risk factors aggregate in specific subgroups of patients. For example, we will compare if more MIS-C cases who have cardiac involvement (more severe phenotype) have genetic variants in immunological genes than MIS-C cases who do not need inotropic support.
3. Identification of therapeutic targets: For example, if we find more cases than controls have variants in cluster 2 (intracellular signaling pathways), we will test pharmacological agents that influence these pathways in cells of cases and controls (for example JAK inhibitors).

7.2 Randomisation, blinding and treatment allocation

Not applicable

7.3 Study procedures

7.3.1 Recruitment and sampling

Recruitment: Invitation by email

- Participants will be invited by email through the study in which they have participated before
- The email will the patient information forms and will contain a link to a secure formdesk form
- The link to the formdesk form will be unique so we will be able to trace from which group (MIS-C, post-COVID condition, controls) the potential participant belongs

Formdesk form

- "Dank voor uw aanmelding. Wij nemen binnenkort contact met u op"
- Form: name, birthdate, address, verify email-address, phone number

Phone call

- Explanation about the study
- Answer questions from the potential participant
- Cases (MIS-C or post-COVID condition): General practitioner.
- Controls: we will ask some questions about prior SARS-CoV-2 to verify that the participant does not exclude as a control
- For cases and controls: country in which the parents and grandparents of the child were born.
- Determine which Saliva Collection Kit is most suitable for their child (assisted collection or self-collection).

Confirmation by email

- "Dank dat u wilt meedoen met de GRIP studie. U ontvangt binnenkort het speeksel-afname setje met het toestemmingsformulier"
- Links to the instruction materials and again to patient information form.

GRIP Saliva Collection Kit by regular mail

- A Saliva collection kit
- A manual on how to use the kit and where to find additional information
- The consent form that parents and/or children need to sign to participate in the GRIP study
- A return envelope

Reminder

- We will send a reminder by email if we have not received the consent form and saliva kit within three weeks
- If we have not received the kit within two to three weeks after the reminder by email, we will phone the potential participants to check if all the materials have been received and verify they still wish to participate

Please read below for additional information on each of the steps.

Recruitment: invitation by email

For this study, we will ask the study coordinators of the already existing studies to approach their participants about participation in the GRIP study (see chapter 3). This recruitment will take place by e-mail. The e-mail will contain;

- A link to the Patient Information Form about participation in the GRIP study. There will be different forms for the MIS-C/Post-COVID group, and the 'exposed control group';
- A link to our website (www.covidkids.nl/grip) where the participant can find information and a video about the procedures in the GRIP study;
- A link that will refer the participant to a short questionnaire in Formdesk. The link to the formdesk form will be unique so we will be able to trace from which group (MIS-C, post-COVID condition, controls) the potential participant belongs

Formdesk form

If the subject wants to participate in the GRIP study, he/she/the parents can fill out their home address, e-mail address and phone number in this questionnaire. By filling out and returning the questionnaire, the subject consents with our research team contacting the subject by phone for a further explanation on the GRIP study.

Information by telephone

Once we receive a filled out questionnaire, the potential participant of the GRIP study will be contacted by phone. During this phone call:

- We will shortly explain the study
- Subjects can ask additional questions

If the parents/patients wish to participate, we will collect the following information during this phone call:

- For cases (MIS-C or post-COVID condition): General practitioner. This is needed to be able to give feedback on the genetic results.
- For controls: we will ask some questions to verify that the participant does not exclude as a control: (approximate) date of prior SARS-CoV-2 infection(s); how the infection was determined; clinical course of prior SARS-CoV-2 infection(s): exclusion as a control if any of the following apply: admission to hospital; occurrence of MIS-C; persistent health problems related to SARS-CoV-2 infection after 12 weeks
- For cases and controls: country in which the parents and grandparents of the child were born. This information is needed for the interpretation of rare genetic variants.

We will discuss with the parents which type of Saliva Collection Kit is most suitable for their child (assisted collection or self-collection, see next paragraph on saliva collection).

If the subject wants to participate in the GRIP study, we will send him/her/the parents an confirmation e-mail again containing the 'Patient Information Form' and links to the instruction materials. We will send them the 'GRIP Saliva Collection Kit' containing the saliva collection kit and the consent form by regular mail (see next paragraph).

Confirmation email

We will send a confirmation email. In this email, we will again link to the information form and instruction materials.

GRIP Saliva Collection Kit

When a subject want to participate in the GRIP study, we will send the 'GRIP Saliva Collection Kit' to their home address. This package will contain the following materials:

- A Saliva collection kit
- A manual on how to use the kit and where to find additional information (using a QR code and a link to refer them to the instruction video), see also Appendix A.
- The consent form that parents and/or children need to sign to participate in the GRIP study
- A return envelope

Children under the age of four years will receive a saliva collection kit for assisted collection, which contains an absorbant sponge. Sample saliva volume for this kit is 0.75 mL.

- advantage of the assisted collection kit: ideal if the donor is not able to spit
- disadvantages of the assisted collection kit: longer collection time (it may take up to 15 minutes to collect enough saliva); more expensive; DNA yield is lower (median yield 17.3 µg)

For children over the age of four years, we will provide a collection kit for self-collection of saliva. Children will need to spit into a funnel to collect the saliva. Sample saliva volume for this kit is 2 mL.

- advantages of the self-collection kit: shorter collection time (2-5 minutes), less expensive, higher DNA yield (median yield 110 µg)
- disadvantage of the self-collection kit: the participant needs to be able to spit into the funnel until the fill line is reached

In the introductory phone call, we will ask the parents of children over the age of four years if they think their child is able to spit into the funnel. If not, the parents/participant will receive the assisted-collection kit.

When the subject has returned the collected saliva and a completely signed consent form, he or she is officially a participant in the GRIP study.

7.3.2 Whole exome sequencing and data analysis

The saliva collection kits can be returned via regular mail and will be stored at room temperature (15-30°C) until analysis.

The collected saliva samples will be sent to GenomeScan (an ISO/IEC 17025 accredited laboratory). The samples will only contain the study specific number of the participant. GenomeScan will do the DNA isolation. DNA sequencing will be done using the Agilent SureSelect Human All Exon V7 kit, using Illumina

NovaSeq6000 sequencing, Paired-End, 150 bp. Per sample we will obtain ~12 Gb, 40 million Paired-End reads.

In a NEN-EN-ISO 15189 accredited genome diagnostic center existing bio-informatic pipelines and databases will be used. Sequenced reads will be mapped to the Genome Reference Consortium Human Build 37 (GRCh37 or hg19) reference genome. Variants are classified according the guidelines as established by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guideline (33), similar to the approach in our Genetics first – primary immunodeficiencies study (34, 35).

We will make use of publicly available reference exome data sets (including GnomAD database, GoNL database) to determine the frequency of genetic variants in reference populations (36). We will include only protein-altering variation with a presumed high impact on protein function, including protein-truncation (stop or frameshift mutations) or disturbance of canonical splice-sites. In-frame insertions/deletions and missense mutations will be scored, and variants with a 'likely' estimated impact on protein functioning (CADD>15) will be included in the analysis (37, 38).

Class	**Description**	**Probability of being Pathogenic**
5	Definitely Pathogenic	>0.99
4	Likely Pathogenic	0.95–0.99
3	Uncertain	0.05–0.949
2	Likely Not Pathogenic or of Little Clinical Significance	0.001–0.049
1	Not Pathogenic or of No Clinical Significance	<0.001

Data collection

Clinical data:

- Clinical data of the MIS-C and post-covid condition patients has already been collected in the existing studies. We will ask consent of the participants to use this data to be able to compare genetic and clinical data.
- For the 'exposed' controls we will need to verify that prior SARS-CoV-2 infection(s) was/were uncomplicated. This needs to be the case in order for the subject to be elligible for inclusion in the present study. This we will verify in the introductory phone call. Also, to assess whether cases and controls have different genetic backgrounds, we will ask what the birth place is of the parents and grandparents of the subject (this is important for the interpretation of rare genetic variants). Age (in years) and gender will be collected from the cases since MIS-C and post-COVID condition occur mainly in school-aged children. Information on prior infections, age and birthplace of (grand)parents of the healthy 'exposed' controls will be entered in Castor EDC.

Genetic data:

- GenomeScan will provide us with FASTQ files containing the sequencing reads.

Reporting of individual genetic results to the participant

Cases (MIS-C or 'post-COVID condition'):

We will do individual genetic analyses of whole exome sequencing results of genes associated with inborn errors of immunity. Cases with class 4 or 5 variants in these IEI genes (also taking into account the mode of inheritance, e.g. autosomal recessive, X-linked etc.) have a likely diagnosis of an inborn error of immunity. Since this can have clinical implications, we will refer cases with a likely diagnosis of an IEI to a clinical geneticist for counseling. Genetic results will be reported back according to the VKGN consensus-based guideline (39):

Cases (MIS-C or 'post-COVID condition'):

1. There is a diagnosis of IEI which according to current knowledge explains why the child had MIS-C or post-COVID condition. This we will report back to the participant regardless of whether the diagnosis of IEI will lead to a change in treatment or follow-up.
2. There is a diagnosis of a genetic disease, but according to current knowledge it does not explain why the child had MIS-C or post-COVID condition (incidental finding). We will report incidental findings back to the participant, if :
    - according to current knowledge the diagnosis would lead to a change in management (treatment or follow-up) of the participant during childhood (until the age of 16 years)
    - according to current knowledge the diagnosis would lead to a change in management (treatment or follow-up) of the participant after the age of 16 years. Participants can choose to 'opt out' to receive this information.
    - according to current knowledge the diagnosis does **not** lead to change in management (treatment or follow-up): we will **not** report these findings. Participants older than 16 years of age can choose to 'opt in' to receive these results.
    - according to current knowledge there is no genetic diagnosis in the participant, but the chance that potential future off-spring of the participant is born with the condition is higher than 25%. Participants can choose to 'opt out' to receive this information.

Controls

- In the case-control part of the study, data on genetic variants are analyzed at 'group level' and not traced back to the individual participants. Therefore, controls (healthy, SARS-CoV-2 exposed children) will not receive feedback on their own genetic data.

Reporting of overall results

Eventually, overall results will be published on our website www.covidkids.nl and in scientific journals.

7.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences.

7.5 Replacement of individual subjects after withdrawal

When an individual withdraws from the study, we will continue inviting other possible participants until we reach the number of participants that are needed in this study.

7.6 Follow-up of subjects withdrawn from treatment

Not applicable

7.7 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.

8. SAFETY REPORTING

8.1 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 jeopardize 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.

8.2 AEs, SAEs and SUSARs

8.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the study procedure. For this study, we will not register adverse events, since the study procedure is a one-time very low risk procedure, for which we do not expect study related adverse events to occur.

8.2.2 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 hospitalization or prolongation of existing inpatients' hospitalization;
- 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 SAE's will be reported immediately. This means all SAE's related to participation in this study protocol, meaning from the saliva collection, will be reported. All other SAE'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.

8.3 Annual safety report

Not applicable.

8.4 Follow-up of adverse events

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

8.5 Data Safety Monitoring Board (DSMB)

Not applicable

9. STATISTICAL ANALYSIS

9.1 Primary study parameter(s)

The first steps in the analysis will be to **identify the qualifying variants**, as previously reviewed by Povisyl *et al.* (40).

1) Sample QC: Obtained genotypes will be used to investigate the relatedness between samples. Samples that are too interrelated, possibly due to unknown relatedness, are excluded for further analysis as this violates the assumption of independence between samples. Likewise, samples that are too unrelated, possibly due to an unknown admixture, are excluded for further analysis as this would yield an inflated estimate of rare variants, that are in fact mostly benign population-specific variants.
2) Variant QC: For each contrast, variants are scrutinized for differences in coverage and other variant QC measures. Variants with significant differences in QC between cases and controls are discarded.
3) Variant filtering: This will enrich the subsequent gene-collapsing analyses for rare and presumably deleterious variation that confers disease risk, while minimizing the impact of neutral background variation. The goal is to optimize statistical power to test for involvement in the pathophysiology of MIS-C. For this purpose, we will filter for rare variation in external reference cohorts (gnomAD, MAF<0.00001). In addition, we will include only protein-altering variation with a presumed high impact on protein function, including protein-truncation (stop or frameshift mutations) or disturbance of canonical splice-sites. In-frame insertions/deletions and missense mutations will be scored, and variants with a 'likely' estimated impact on protein functioning (CADD>15) will be included in the analysis (37, 38).

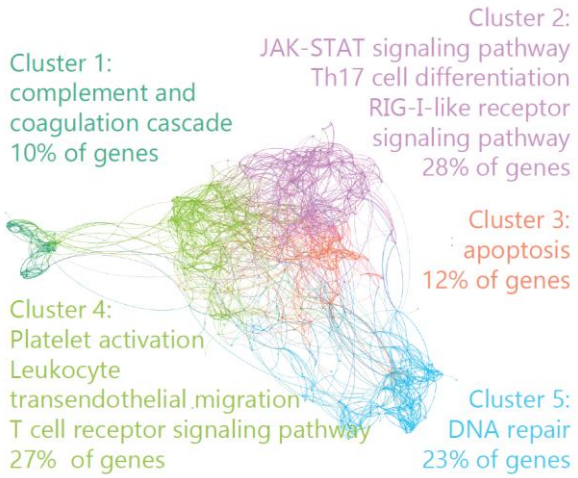
Qualifying variant selection					
			filters		
variant	gene	sample	variant quality	variant annotation	external MAF
V1	G1	S1	ü	ü	ü
V1	G1	S2	û	ü	ü
V3	G2	S3	ü	ü	û
V4	G3	S3	ü	ü	ü
V5	G3	S4	ü	û	ü
V6	G4	S5	ü	ü	ü
...				...	
Vn	G453	S1	ü	ü	ü

Example of Qualifying Variant selection table, adapted from Povysil et al,(40)

Cluster analysis of IEI gene-set

We have done a cluster analysis on the IEI-gene set. Leading Eigen cluster analysis was done based on known and predicted protein-protein interactions, using STRINGdb version 11.5 (41, 42). The interactions include direct (physical) and indirect (functional) associations. We included all interactions of medium confidence (cutoff = 0.4) and subsequently assigned

isolated genes to the best fitting cluster using label propagation. Enrichment of KEGG-pathways within these clusters was assessed using WebGestalt (43). A force-atlas graph algorithm was used for visualization.



Collapsing analysis of qualifying variants

All qualifying variants (QV) will be collapsed. The goal of collapsing is to optimize signal detection by reducing contamination from neutral background variation. Also, collapsing increases the power to answer our main question: "Do children with MIS-C or post-COVID condition have more deleterious variants in immunological genes than exposed controls?"

A cluster is affected in an individual if there is at least one gene with a QV.

Genecluster-by-individual collapsing matrix

Cluster	With affected gene(s)		Without affected gene(s)	
	cases	controls	cases	controls
C1	5	1	95	199
C2	1	2	99	198
C3	5	1	95	199
C4	1	1	99	199
C5	1	3	99	197

Gene-by-individual collapsing matrix

Gene	With QV		Without QV	
	cases	controls	cases	controls
G1	1	0	99	200
G2	0	1	100	199
G3	2	0	98	200
G4	0	0	100	200
G5	1	1	98	199
...				
G453	1	0	99	200

9.2 Secondary study parameter(s)

Analyses for the secondary study parameters are descriptive. Clinical and follow-up data for pediatric hospitalized COVID and MIS-C patients have been collected since the beginning of the pandemic. Data collection is ongoing. Using R and the Castor API, we continuously report our findings online, plotting an epidemic curve comparing COVID and MIS-C, and analyze baseline characteristics, clinical course and laboratory values, using Kruskal-Wallis, Chi-square or Fisher Exact depending on the variables (see our website www.covidkids/nl/scientific-dashboard). We will expand these analyses to include laboratory values, treatment and long-term outcome (including patient reported outcomes measures). We will do multi-variate analyses to determine which factors are independent predictors of outcome.

10. ETHICAL CONSIDERATIONS

10.1 Regulation statement

This 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, 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), the Code of Conduct for Responsible Use of Human Tissue (Gedragscode Goed gebruik van lichaamsmateriaal) 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.

10.2 Recruitment and consent

See also 7.3.1 Recruitment and sampling

Recruitment

Subjects will first be informed about the GRIP study through an e-mail they receive from the study coordinator of the study in which they already participate. In this e-mail, we will link to the Patient Information Form of the GRIP study. By filling out the questionnaire in Formdesk, the subjects consent for sharing their home address, phone number and e-mail address with us, so we can contact them about participation in the GRIP study.

Information

After receiving the contact information, the GRIP study team will contact potential participants by phone to inform them about the GRIP study, answer the questions the subjects might have and collect additional data. This team consists of the principal investigator, the research coordinator of the departmental Trialbureau and a research supporter.

Consent

If the subject is interested in participating in the GRIP study, the GRIP study team will send the subject the saliva collection kid and an informed consent form with a retour envelop. Only when we receive a completely signed consent form, the subject participates in the GRIP study. If, after several reminders, we do not receive a signed informed consent form, we will destroy all data that we have collected in the phone call.

Time path

We expect to be able to call the subject within one week after receiving their contact details. We expect to be able to send the collection kit and consent form within one week after the phone call. These two weeks between first contact and signing the informed consent form are ample time for subjects to consider their participation.

10.3 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 pediatric research.
https://www.ccmo.nl/onderzoekers/publicaties/publicaties/2001/06/01/gedragscodeverzet-bii-minderiarigen
https://english.ccmo.nl/investigators/legal-framework-for-medical-scientificresearch/codes-of-conduct/code-of-conduct-for-medical-research

10.4 Benefits and risks assessment, group relatedness

Children with MIS-C or 'post-COVID condition' may benefit from this study since they might receive a genetic diagnosis that explains why they have had a complicated clinical course following infection with SARS-CoV-2. This may benefit them by ensuring they will receive proper care targeted at the underlying condition. At the same time, there is a risk that the genetic diagnosis does not have therapeutic consequences. This may be a burden for the participant/family. Also, a genetic diagnosis in the participant may have implications for other family members.

Controls do not benefit personally from study participation.

The procedure to collect the DNA (saliva collection) 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. MIS-C is a condition that mainly occurs in children (MIS-A in adults is very rare).
Regarding children with post-COVID condition: Since the immunological and clinical response to SARS-CoV-2 is markedly different between adults and children, results obtained in studies on post-COVID condition or 'long COVID' in adults do not automatically apply to children with this condition.

10.5 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.

10.6 Incentives (if applicable)

Not applicable.

11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

11.1 Handling and storage of data and documents

Participants will receive a GRIP 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". Signed informed consent forms will be stored in a secured physical location in the LUMC. Biological material will be labelled with the GRIP study number and date of sampling. Clinical information of healthy controls will be collected using Castor EDC. Castor EDC is a LUMC approved data storage program.

During the study, within the LUMC Dr. E. Buddingh and the research supporter 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 GRIP team won't have access to the key to the code when analyzing the collected data.

We will ask the patients or their caregivers for consent to request information regarding the clinical information collected in the existing studies on MIS-C or 'post-COVID condition'. We will link the GRIP study number to the clinical data using a unique identifier in the recruitment email.

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.

11.2 Monitoring and Quality Assurance

Monitoring will be executed by (internal) monitors of the LUMC according to the monitor plan.

11.3 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.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

11.4 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, other problems, and amendments.

11.5 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 receiving the results of the DNA analyses of the last included participant.

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.

11.6 Public disclosure and publication policy

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

12. STRUCTURED RISK ANALYSIS

12.1 Potential issues of concern

For the assisted collection kit (with a sponge to collect saliva): As per manufacturer guidance, we will instruct the parents/caregivers to check the sponge for damage each time before inserting into donor's mouth. Use second sponge if first sponge shows any signs of wear or tear.

12.2 Synthesis

With proper use, there is a negligible risk to use the saliva collection kits.

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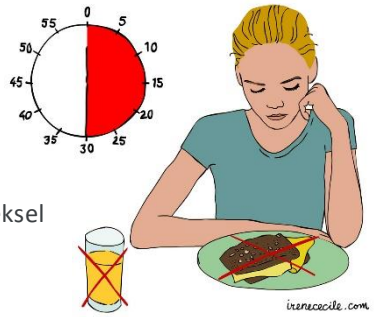
14. Appendix A: Instruction saliva collection

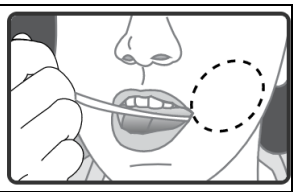
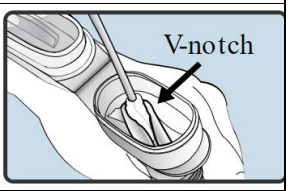
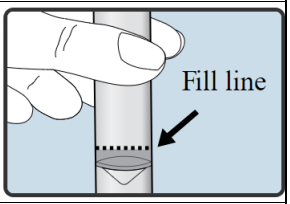
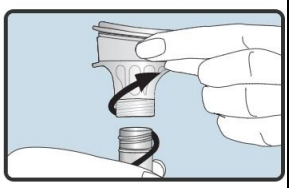
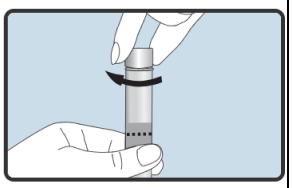
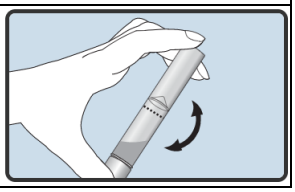
14.1 Speeksel-wat: voor kinderen die nog niet zelf in een buisje kunnen spugen.

https://www.dnagenotek.com/ROW/products/collection-human/oragene-dna/500-series/OG-575.html

- Let op: Uw kind mag 30 minuten voor het afnemen van het speeksel niet eten of drinken.
- Uw kind moet tijdens de afname rechtop zitten.
- Zorg ervoor dat u de plastic folie op het bovenste gedeelte van het buisje (''de trechter'') **NIET** verwijdert.
- Het kan ongeveer 15 minuten duren om het speeksel af te nemen. Zorg ervoor dat u rustig de tijd neemt om het speeksel af te nemen.

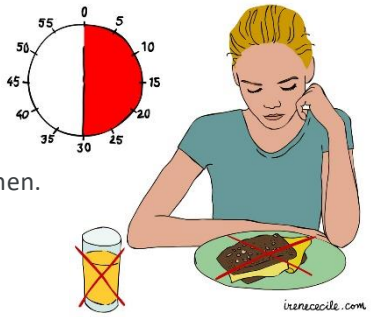


Volg de stappen in de instructievideo zo goed mogelijk:

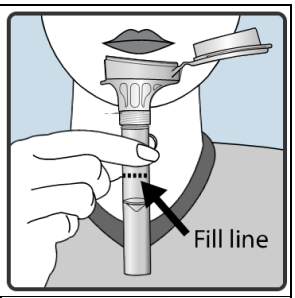
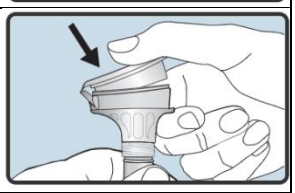
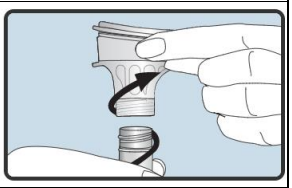
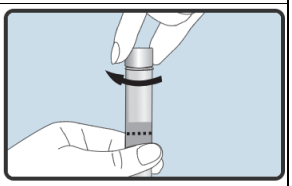
1.	Plaats de spons binnenin de wang. Beweeg de spons gedurende 30 seconde voorzichtig langs het tandvlees en de binnenkant van de wangen om zoveel mogelijk speeksel op te nemen.	
2.	Zodra de spons vol zit met speeksel, plaatst u de spons in de V-inkeping (V-notch) van de trechter. Om het speeksel in het buisje te verzamelen, moet u de spons met een draaiende en duwende beweging tegen de binnenkant van de V-inkeping houden	
3.	Herhaal stap 1 en stap 2 met hetzelfde sponsje totdat het speeksel tot het streepje op het buisje komt (“Fill line”). Zorg ervoor dat er zo min mogelijk belletjes in het speeksel zitten. Het is belangrijk om te controleren of de spons kapot gaat. Als de spons kapot gaat moet u een tweede spons gebruiken om verder te gaan met de speekselverzameling.	
4.	Als er genoeg speeksel in het buisje zit, sluit u de deksel door er stevig op te drukken. Als u een harde klik hoort zit het goed dicht. Zorg ervoor dat u het buisje rechtop houdt en dat de deksel goed gesloten is.	
5.	Schroef het bovenste gedeelte (''de trechter'') los van het buisje.	
6.	Sluit het buisje door de kleine dop stevig op het buisje te draaien.	
7.	Schud het buisje met het dopje erop gedurende 5 seconde. Gooi het bovenste gedeelte (''de trechter'') in de vuilnisbak. Ook het gebruikte sponsje of de gebruikte sponsjes kunt u weggooien.	

14.2 Speeksel-spuug setje: voor grotere kinderen die zelf in een buisje kunnen spugen
https://www.dnagenotek.com/ROW/products/collection-human/oragene-dna/500-series/OG-500.html

- Let op: Uw kind mag 30 minuten voor het afnemen van het speeksel niet eten of drinken.
- Uw kind moet tijdens de afname rechtop zitten.
- Zorg ervoor dat u de plastic folie op het bovenste gedeelte van het buisje (''de trechter'') **NIET** verwijdert.
- Het duurt ongeveer 2-5 minuten om het speeksel af te nemen. Zorg ervoor dat u rustig de tijd neemt op het speeksel af te nemen



Volg de stappen in de instructievideo zo goed mogelijk:

1.	Laat uw kind in het gedeelte boven het buisje (''de trechter'') spugen totdat het speeksel tot het streepje op het buisje komt ("Fill line"). Zorg ervoor dat er zo min mogelijk belletjes in het speeksel zitten.	
2.	Als er genoeg speeksel in het buisje zit, sluit u de deksel door er stevig op te drukken. Als u een harde klik hoort zit het goed dicht. Zorg ervoor dat u het buisje rechtop houdt en dat de deksel goed gesloten is.	
3.	Schroef het bovenste gedeelte (''de trechter'') los van het buisje.	
4.	Sluit het buisje door de kleine dop stevig op het buisje te draaien.	
5.	Schud het buisje met het dopje erop gedurende 5 seconde. Gooi het bovenste gedeelte (''de trechter'') in de vuilnisbak.