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RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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ABSTRACT

Introduction: The SARS-CoV-2 pandemic remains a threat to public health. Soon after its outbreak, it became apparent that children are less severely affected. Indeed, opposing clinical manifestations between children and adults are observed for other infections. The SARS-CoV-2 outbreak provides the unique opportunity to study the underlying mechanisms. This protocol describes the methods of an observational study that aims to describe age dependent differences in immune responses to primary respiratory infections using SARS-CoV-2 as a model virus and to assess age differences in clinical outcomes including lung function.

Methods and Analysis: The study aims to recruit at least 120 children and 60 adults that are infected with SARS-CoV-2 and collect specimen for a multi-omics analysis, including single cell RNA sequencing of nasal epithelial cells and peripheral blood mononuclear cells, mass cytometry of whole blood samples and nasal cells, mass spectrometry-based serum and plasma proteomics, nasal epithelial cultures with functional *in vitro* analyses, SARS-CoV-2 antibody testing, sequencing of the viral genome and lung function testing. Data obtained from this multi-omics approach is correlated with medical history and clinical data. Recruitment started in October 2020 and is ongoing.

Ethics and dissemination: The study was reviewed and approved by the Ethics Committee of Charité – Universitätsmedizin Berlin (EA2/066/20). All collected specimens are stored in the central biobank of Charité – Universitätsmedizin Berlin and are made available to all participating researchers and on request.

The study is registered at the German Clinical Trials Register with number DRKS00025715.

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Key Words: SARS-CoV-2, children, multiple breath washout, immune response, resilience, multi-omics

Strengths and limitations of this study

- Initial sampling in the early phase of SARS-CoV-2 infection enables insights in the primary immune response of children compared to adults. Longitudinal sampling allows to detect long term effects.
- A multi-omics approach with state-of-the-art techniques permits a high resolution immune mapping of SARS-CoV-2 infections
- Data is completed with clinical information and lung function testing
- Since the recruitment period extends over more than 18 month there will be a chance to study the immune response against different variants of the SARS-CoV-2 virus

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INTRODUCTION

In December 2019, the novel coronavirus SARS-CoV-2 emerged as cause of acute pneumonia [1 2]. By August 2021, more than 200 million people were infected with SARS-CoV-2 [3]. Soon after the beginning of the pandemic, it became obvious that children have an increased resilience against the primary infection. The course of disease in children is more likely to be milder and severe or even fatal courses remain extremely rare [4-8]. Various hypotheses to explain the reduced susceptibility and mortality of children are currently discussed, including reduced virus entry via ACE-2 in children [9], pre-activated components of the immune system, such as cross-reactive T cells [10-13] and antibodies [14], or a more accentuated innate immunity in children [15 16] (table 1). Most of these findings are complementary in the explanation of the observed phenomenon, however some findings are in part contradictory and require further investigation. Opposing clinical manifestations between children and adults are also observed for other viral respiratory infections [17 18 19]. This points to major changes in the general immune response pattern during aging. In the past comparative immune response analyses to primary infections in various age groups were difficult to perform, as many adults had been already exposed to the pathogens. The SARS-CoV-2 outbreak provides the unique opportunity to study the age-dependent changes in immune responses in a controlled manner.

To understand the mechanisms behind the lower susceptibility of children compared to adults to develop severe COVID-19 disease, we have established the observational study RECAST (increased **RE**silience of **C**hildren compared to **A**dults in **S**ARS-CoV-2 infec**t**ion) focusing on the differences in the clinical presentation, lung function and the immune response to SARS-CoV-2 in children compared to adults.

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Table 1. *Hypotheses to explain the resilience of children in SARS-CoV-2 infections*

Hypothesis	Proposed explanation	Scientific findings
Reduced susceptibility for SARS-CoV-2 in children [20]	Reduced virus entry via ACE-2 in children	SARS-Cov-2 uses ACE-2 in the upper and lower airways for host cell entry [21] The age-dependency of ACE-2 expression is controversely discussed [22 23 24]
		Even though an increased expression of ACE-2 renders the individual more susceptible to viral infection, ACE-2 also initiates anti-inflammatory signaling and might contribute to a milder immune response [25]
Age-dependent differential immune activation pattern	Pre-activated immune components in children entail a milder immune response	In early childhood, infections of the upper respiratory tract are frequent. It has been proposed that previous infections with coronaviridae might contribute to a cross-reactive immunity [26]
		Pre-existing T cell reactivity to SARS-CoV-2 could affect the severity of COVID-19 [10-13 27]
		Cross-reactive antibodies entail a milder immune response to SARS-CoV-2 [14]. Of note, uninfected infants do not express cross-reactive antibodies [28]
		The polyclonality and polyreactivity of IgM naturally present in children recognizes SARS-CoV-2 particles [29]
	Children possess a stronger innate immunity than adults	Children display a higher basal expression of pattern recognition receptors than adults and a stronger innate antiviral response [15 30]
		The nasopharyngeal mucosa of children exhibits a stronger innate immunity and expresses more anti-viral cytokines than adults [16]
	Children expose a different cytokine response upon SARS-CoV-2 infection than adults	In COVID-19, a cytokine storm leads to acute respiratory distress syndrome [31 32]
		Certain cytokine patterns correlate with COVID-19 severity [33]
		Pro-inflammatory cytokine concentrations might be lower in children infected with SARS-CoV-2 than in adults [34]
	Co-infections lead to a milder immune reaction, e.g. because of virus competition or primed immune components	In COVID-19, Co-infection with other pathogens is not rare, especially in children [35-37]

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The complexity of immune responses requires a multi-level approach to display changes on various layers, including local immune cell composition, cytokine signaling and systemic response. It can be assumed that the combination of several mechanisms leads to the largely different phenotypes. At the same time, modern techniques allow to engage on an exploratory approach analyzing simultaneously the involvement of canonical and non-canonical immune response patterns. The multi-omics approach presented here allows deeply detailed characterization of the various layers of age dependent specific immune responses. Therefore, we believe that the presented study design will contribute to a further understanding even beyond COVID-19.

To meet these requirements adequately, we chose a multi-omics approach, including: i) single cell RNA sequencing of peripheral blood mononuclear cells (PBMC) and nasal epithelial cells, ii) mass spectrometry-based serum and plasma proteomics, which has been used to identify prognostic marker signatures for SARS-CoV-2 disease severity and devise risk-adapted treatment strategies [38], iii) mass cytometry (cytometry by time-of-flight, CyTOF) of whole blood samples and nasal cells, that has been used to elucidate the role of T cell cytotoxicity in COVID [27] and to identify a dysregulation of the myeloid cell compartment as hallmark of severe COVID [39], iv) highly differentiated nasal epithelial cultures and functional *in vitro* analyses, that have been used to display age-related differences in the nasal epithelium [40], v) antibody testing and vi) sequencing of the viral genome. Obtained data are complemented with anamnestic and clinical information, lung function testing, including spirometry and multiple breath washout, which is a standardized method that allows to assess the ventilation homogeneity of the lungs already in preschool children, as well as testing of smell and taste. Longitudinal sampling allows monitoring of the immune response over the

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course of disease and beyond. Due to the maturation of the immune system during childhood, age-specific immune response pattern against SARS-CoV-2 can be expected [41 42]. Thus participants of all age groups are enrolled.

Participant recruitment began in October 2020 and is ongoing. Of particular interest is the recruitment of children infected with various SARS-CoV-2 virus variants of concern.

Study objectives

This study aims to characterize and compare primary infections with SARS-CoV-2 in children and adults, and to identify age-related determinants of disease course and prognosis. The immune system is not only highly complex, but immune response patterns also vary depending on individual predisposition; moreover, it also matures throughout the ageing of an individual. Furthermore, of pivotal interest for the interpretation of these data is the correlation between immune response pattern and clinical outcome.

METHODS AND ANALYSIS

Study design

RECAST is a prospective observational cohort study at Charité – Universitätsmedizin Berlin in Berlin, Germany. It is a sub-study of the Pa-COVID study of the Charité [43], aiming to characterize the disease course of patients suffering from COVID-19.

Data is collected longitudinally from patients with confirmed COVID-19 at three time points, directly after the diagnosis and at follow-up visits after two weeks and four to six months, and from healthy age-matched controls.

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Study population

Inclusion criteria

Main inclusion criteria for the index person is a primary acute SARS-CoV-2 infection in a minor (< 18 years of age) with positive PCR or antigen testing (both will be confirmed by PCR testing).

Exclusion criteria

Subjects with preexisting conditions affecting the immune response, such as diseases requiring chemotherapy or syndromes with immunodeficiency and subjects with concomitant medication that affects the immune response, such as systemic steroids, biologicals or investigational therapeutics targeting SARS-CoV-2, are excluded.

Study procedures

Patient identification and recruitment

A network of participating pediatric outpatient practices (n=20) has been established as sentinels to provide access to a pool of >25.000 pediatric patients. All children who tested positive for SARS-CoV-2 by PCR or antigen testing as well as their household members are eligible for inclusion.

Healthy controls are recruited from clinical routine diagnostic settings if the diagnostic screening for SARS-CoV-2 was negative.

Medical history and clinical assessment

Assessed data include epidemiological and demographic parameters, medical history and potential risk factors, clinical course – including all diagnostic results of the present medical attendance – and household and family constellation. A complete list of all items is attached in the appendix (table E1).

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Data are collected at first contact and during the follow-up visits. Symptoms of post- / long-COVID [44] are documented and symptoms of myalgic encephalomyelitis / chronic fatigue syndrome are assessed with the Canadian consensus criteria [45 46], Chalder Fatigue Scale [47] and PedsQL Multidimensional Fatigue Scale [48-56]. Loss of smell and taste are assessed with the "U-Sniff" test, a 12-item odor identification, the "Sniffin' Sticks" olfactory threshold test and taste samples for sweet, sour, salty, and bitter tastes [57 58]. For adults, health status and quality of life are assessed with the St George's Respiratory Questionnaire [59] and health status and mental health are evaluated with PHQ-9 [60] and PCL-5 [61] questionnaires. For children, quality of life is assessed using the KINDL questionnaire [62].

Disease severity is classified according to clinical features using the criteria outlined in the WHO COVID-19 clinical management guideline [63] as asymptomatic, mild, moderate, severe or critical disease. Also, clinical progression is classified according to the WHO clinical progression scale [64]. Applied classification scales are shown in tables 2 and 3.

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Table 2. *COVID disease severity.*

Abbreviated criteria for COVID-19 disease severity according to WHO COVID-19 Clinical management guideline [63].

Disease severity	Definition	Criteria
Asymptomatic		
Mild		Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia
Moderate	Pneumonia	Clinical signs of (non-severe) pneumonia Adolescent or adult: Fever, cough, dyspnoea, fast breathing Child: Cough or difficulty breathing + fast breathing and/or chest indrawing Diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications.
Severe	Severe pneumonia	Adolescent or adult: Plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO2 < 90% on room air Child: Plus at least one of the following: Central cyanosis or SpO2 < 90%; severe respiratory distress (e.g. fast breathing, grunting, very severe chest indrawing); general danger sign: inability to breastfeed or drink, lethargy or unconsciousness, or convulsions
Critical disease	Acute respiratory distress syndrome (ARDS) OR sepsis / septic shock	Oxygenation impairment, invasive ventilation or bilevel NIV / CPAP (≥ 5 cmH_2O) required OR Infection and ≥ 2 Systemic Inflammatory Response Syndrome (SIRS) criteria

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Table 3. *WHO clinical progression scale*.

Modified from WHO working group [64].

Patient State	Descriptor	Score
Uninfected	Uninfected; no viral RNA detected	0
Ambulatory: mild disease	Asymptomatic; viral RNA detected	1
	Symptomatic; independent	2
	Symptomatic; assistance needed	3
Hospitalized: moderate disease	Hospitalized; no oxygen therapy	4
	Hospitalized; oxygen by mask or nasal prongs	5
Hospitalized: severe disease	Hospitalized; oxygen by NIV or high flow	6
	Intubation and mechanical ventilation, pO2/pFiO2 ≥ 150 or SpO2/FiO2 ≥ 200	7
	Mechanical ventilation, pO2/pFiO2 < 150 (SpO2/FiO2 < 200) or vasopressors	8
	Mechanical ventilation, pO2/pFiO2 < 150 and vasopressors, dialysis or ECMO	9
Dead	Dead	10

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Sample collection

Nasal and pharyngeal swab samples are collected for a SARS-CoV-2-PCR, single-cell RNA sequencing and establishment of air-liquid cell cultures. In addition, peripheral blood mononuclear cells (PBMCs) are collected for single-cell sequencing, whole blood for mass cytometry and plasma and serum for mass-spectrometry based proteomics, and SARS-CoV-2-specific antibody testing.

Study database

For Pa-COVID-19 a study protocol was established that harmonizes clinical, molecular, and immunological phenotyping assessment in COVID-19 patients [43]. All data are added to an electronic case report form (eCRF; SecuTrial). Participants included in RECAST are part of Pa-COVID-19. All participants are assigned a pseudonym consisting of a six- or seven-digit alphanumerical participant code. A separate log allows to match each participant and their code. Access to SecuTrial requires username and password. All local data are secured by password.

Sample description

Patients recruited in RECAST are grouped into six age categories (table 4). Due to the nature of observatory studies and the lack of pre-existing data, it is not possible to predict the extent of assumptive differences. Preliminary findings suggest that for most planned analyses a sample size of 15 is sufficient. The outlined sample sizes should suffice even for comparisons between children of different age groups.

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Table 4. *Age categories of the RECAST participants*.

Participants in the RECAST study are grouped according to their age and their disease state. The projected minimum number of participants for each group as well as the timepoints for samplings are stated.

Age group		Disease state	Number	Timepoints
Children	Nursery (0-3 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Kindergarten (3-6 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Primary school (6-12 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Secondary school (13-18 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
Adults	<60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	>60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
TOTAL			**≥ 360**	**≥ 720**

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PLANNED ANALYSES AND OUTCOMES OF INTEREST

We propose a multi-omics workup for all patients. A synopsis of the planned analyses is depicted in figure 1.

SARS-CoV-2-specific PCR and antibody testing

All participants are screened for an active SARS-CoV-2 infection with RT-qPCRs targeting E and N genes [65]. Antibody testing is conducted for all serum and saliva samples with SARS-CoV-2-specific IgG- and IgA-ELISAs. In case of a reactive screening result, confirmatory testing with a recombinant Immunofluorescence assay (IFA) and a plaque reduction neutralization test [66] are conducted.

Nasal epithelial culture and functional *in-vitro* analyses

Conditional reprogramming allows for the generation of long-term cultures of primary airway epithelial cells [67-71]. Without the need of genetic modification or clone selection, conditional reprogramming enables cell expansion, while re-differentiated cultures retain their organ-specific phenotype [72]. We establish highly differentiated polarized *in-vitro* air-liquid interface cultures that reproduce and allow for the analysis of physiological *in-vivo* conditions, such as heterogeneous cell composition with preserved lineage [73] as well as functional characteristics, including production of airway surface liquid [74] [75] and mucociliary clearance [76] [77]. For material collection, FLOQswabs (Copan, Italy) are used. Swabs are transferred into DMEM/F12 medium (Gibco, USA) and transported to our laboratory within two hours.

Mass cytometry of whole blood samples

Whole blood is fixed with a proteomic stabilizer for preservation of surface and intracellular markers. Blood samples are stored at -80 °C until batch-based analysis. Thawed samples are stained in batches of nine patient and one anchor reference

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sample. Upon barcoding of individual samples, they are pooled and stained with metal isotype conjugated anti-human antibodies as described previously [27] [39]. CyTOF technology allows for the detection of more than 40 different barcodes simultaneously to identify cell populations in a high-throughput setting [78] [39].

Mass spectrometry-based serum and plasma proteomics

A platform technology with semi-automated sample preparation to allow for ultra-high-throughput liquid chromatography and mass-spectrometry based analyses of the proteome has recently been established by members of our group [79]. In a directed approach, we characterize the immune response-related serum and plasma proteome, with focus on the acute phase response and the complement system. However, the plethora of proteome signals that are generated per sample also allows for an undirected approach, delivering predictive proteome signatures. To facilitate the computation of such extensive bulk data, a deep neural network is employed [80].

Single-cell sequencing of nasal epithelial cell samples and PBMCs

The nasopharynx is the entry point for an infection with SARS-CoV-2 [81] and as such of distinguished concern in the exploration of the individual immune response pattern. Using single-cell RNA sequencing (scRNAseq) of nasal and bronchial samples, we were previously able to identify cell types and states that correlate with a severe disease course of COVID-19 [82]. Here, scRNAseq will be applied to define the composition and transcriptional activity of immune and epithelial cells in the nasal environment of children and adults throughout the various states of SARS-CoV-2 infection. Nasal swaps (FLOQswabs, Copan, Italy) are used for sample collection. Following sample collection, swabs are directly transferred into cold DMEM/F12 medium (Gibco, USA) and transported to our biosafety laboratory within one hour for

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further processing. Library preparation is performed according to manufacturer's protocol (10x) and sequencing is performed using the Illumina NextSeq 6000 platform.

In addition to the analysis of cells in the respiratory environment, PBMCs are isolated to study the transcriptional activity of blood cell populations. Cell separation, library preparation and comparative single cell transcriptome analyses are conducted according to the manufacturer's protocol. Differential transcriptome profiles of immune cells of the blood will help us to characterize the distinctive features of the systemic and localized immune response to SARS-CoV-2 infections in children and adults.

Lung function testing and multiple breath washout

SARS-CoV-2 infections cause severe lung damage in adults [31] [32]. In a large review with 2135 children, 45% (951 children) were classified with a severity of moderate, severe or critical, all with lung involvement per definition [7]. There is evidence that children with acute lung injury experience the same lung pathologies as adults [83]. To assess the extent of transient and permanent functional lung impairment, we investigate the lung function with spirometry and multiple breath washout (MBW). MBW measures the lung ventilation homogeneity [84] [85]. This technique is already feasible without sedation in children from 2 years of age [85]. The technical MBW procedures are in accordance with the American Thoracic Society Technical Statement [86]. Measurements are conducted by certified personnel (ECFS-CTN certified) and for study measurements Exhalyzer D (Ecomedics, Dürnden, Switzerland) will be used [87]. N_2 washout is used as tracer gas to determine the lung clearance index as outcome measure. The lung clearance index (LCI) increases with lung ventilation inhomogeneity.

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Biobanking

Collected material is processed and stored at the central biobank of Charité (ZeBanC, https://biobank.charite.de). Material that is not immediately used is subjected to cryopreservation.

ETHICS AND DISSEMINATION

All procedures in this study are in compliance with the principles of the 1964 Declaration of Helsinki and its amendments. We act in adherence to the principles of Good Clinical Practice (International Council for Harmonization, ICH 1996). The study was reviewed and approved by the Charité Ethics Committee (EA2/066/20). All participants enrolled give written informed consent in person, for participants minor of age the written informed consent of the legal guardian is also required.

Study procedures never interfere with the medical management of participants. Samples required for medical management always have priority. There is no direct benefit for patients participating in the study. Results from the study might improve our understanding of the disease and benefit the public health.

Data is monitored regularly. Informed consent forms are audited by a monitor appointed by the Charité Clinical Trial Management Unit. Data monitoring of collected data is performed in the course of the study.

As established for Pa-COVID [43], we reiterate the fundamental principle in this study that all contributors and researchers who have access to samples commit to unrestricted data sharing. In accordance with FAIR data principles, all data collected shall be findable, accessible, interoperable, and re-usable [88]. Each participating research group will publish their findings individually and in correlation with each other.

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Patient and public involvement

We will disseminate all findings in an appropriate and understandable manner to all participants, including children. We welcome the collaboration of participants and public in the interpretation and dissemination of all findings.

DISCUSSION

The SARS-CoV-2 pandemic has accelerated scientific research in the field of virology and related immunobiology for nearly two years, yet many crucial questions remain unanswered. Soon after the emergence of the virus it became apparent that, while children are just as likely to be infected with SARS-CoV-2 as adults, they are less severely affected [4-8 89]. RECAST is an observational study that aims to elucidate the differences between children and adults in primary SARS-CoV-2 infections using a multi-omics approach. Revealing age-dependent differences will help to develop better suited therapeutics and vaccination strategies beyond SARS-CoV-2 infections.

Previous multi-omics approaches conducted with specimen from adult donors served to elucidate the immune response in COVID [82 90], to identify predictors of severe disease courses [91 92] and to isolate possible targets for therapy [82 92 93]. Multi-omics-based studies focusing on SARS-CoV-2 infections in children remain rare and are limited to small participant numbers and only analyze a limited number of –omics dimensions: A study including 24 infected children analyzed the single-cell transcriptional landscape in the upper airways [15]; with single-cell multi-omic profling of matched nasal, tracheal, bronchial and blood samples of 19 infected children, a study characterized the immune landscape with focus on the upper airways [30]; the plasma proteomic and metabolomic data of 18 infected children was analyzed in another study [94]; clinical characteristics and serum markers were analyzed in a larger group that

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summarized children and young adults and T cell response in a pediatric subgroup of 11 participants was examined [95]; and a study with 24 infected children analyzed the T cell response and specific antibody response [96]. Even though these studies contributed greatly to a better understanding of age-related immune response patterns in COVID-19, there is still a substantial demand for research. Especially studies analyzing the immune response over the whole age and severity spectrum applying a multi-omics approach are needed. In addition, mechanistic investigations, revealing the causal relationship between the different immune defense layers, are missing. In RECAST, we will conduct a full multi-omics workup with at least 120 infected children, a larger number of participants than in previously published multi-omics studies. Moreover, we will conduct follow-up visits for 6 months to profile the development of age-specific immune response patterns over the course of time. Biobanking and long-term storage of samples will be used to perform subsequent mechanistic studies upon first data collection and hypotheses formulation. The recruitment of family members, both infected and non-infected, allows to assess the effect of genetic relationships. The combination of high resolution multi-dimensional immunologic methods with clinical endpoints in the RECAST study will enable us to contribute to the understanding of the increased resilience of children to SARS-CoV-2 infections.

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received payments for his participation in an advisory board for vaccination against SARS-CoV-2. VC has a patent pending in the diagnosis of SARS-CoV-2 infections. LS was involved in further studies relating to SARS-CoV-2 that were funded by national grants.

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Figure 1. Performed analyses in the RECAST study

The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

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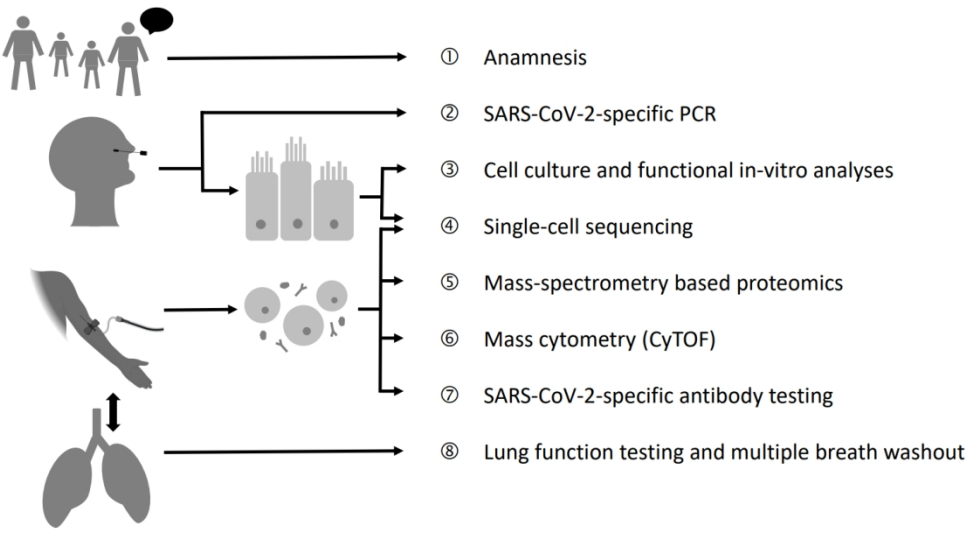

Figure 1. Performed analyses in the RECAST study
The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

413x226mm (96 x 96 DPI)

Appendix, table E1. *Assessed data*.

Assessed data	Item list
Family constellation	
Biometric information, including sex at birth, body height, body weight, ethnicity, number of children and grand-children and profession	
Tests for SARS-CoV-2 in the last 4 weeks, inlcuding type, date and result	
Vaccination against SARS-CoV-2, including manufacturer, date(s) and symptoms after vaccination	
Temporal course of infection in the family	
Index case and assumed point of infection	
Information about household, including number of household members, pet animals and information about smoking (including smoked substances)	
Paediatric anamnesis, including biological parents and legal parents, gestational week at birth, problems during pregnancy, birth and perinatal period, childcare or daycare or school or training, previous RSV infections	
Medical conditions and therapy, including smoking (including substance and length), atopic disposition, allergies, asthma and previous hospitalizations	
Symptoms (including duration) as listed and symptom severity according to WHO scales	Fever or elevated temperature, exhaustion and fatigue, loss of appetite, dizziness, diarrhea, nausea, vomiting, impaired sense of taste or smell, rhinorrhea, congested nose, sneezing, unproductive cough, productive cough, shortness of breath, headache, abdominal pain, sore throat, muscle ache, chest pain, ear pain, joint pain, conjunctivitis, lymphadenitis, skin efflorescence, shivering, paresis, impaired consciousness
Hygiene measures to prevent spreading of disease	
Ability to smell, including parosmia	
Consequences of SARS-CoV-2 infection, including hospitalization, concomitant disease(s) and treatment	
Symptoms of Post-COVID or fatigue as listed, including their duration, whether dependent in occurence or intensity on physical or mental stress or posture	Pain, nausea, vomiting, lymphadenitis, loss of appetite, abdominal complaint(s), loss of smell or taste, palpitation, shortness of breath, coughing, fever, skin efflorescence, conjunctivitis, hair loss, reddening or swelling of hand or feet, reddening or swelling of tongue, lips or cheeks, exhaustion or fatigue, reduced physical and exercise capacity, sleep ineffectivity, disturbed sleeping rhythm, disturbed sleep duration, mood disturbance, cognitive impairment, behavioral changes, sadness or depression, restlessness or excitement, orthostatic dysregulation, increased sensibility to trigges, such as light, noise, smell, touch or others, impaired adaptability to cold, heat or others

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	observational study
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5, 6	
Objectives	3	State specific objectives, including any prespecified hypotheses	7	
Methods				
Study design	4	Present key elements of study design early in the paper	8	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8 - 10	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	9 - 10	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		

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Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	10 - 13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10 - 13
Bias	9	Describe any efforts to address potential sources of bias	NA study not terminated, no data interpretation
Study size	10	Explain how the study size was arrived at	NA study not terminated, no data interpretation
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	NA study not terminated, no data interpretation
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	NA study not terminated, no data interpretation
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	NA study not terminated, no data interpretation	
		(b) Give reasons for non-participation at each stage		
		(c) Consider use of a flow diagram		
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders		
		(b) Indicate number of participants with missing data for each variable of interest		
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)		
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time		
		Case-control study—Report numbers in each exposure category, or summary measures of exposure		
		Cross-sectional study—Report numbers of outcome events or summary measures		
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included		
		(*b*) Report category boundaries when continuous variables were categorized		
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses		
Discussion				
Key results	18	Summarise key results with reference to study objectives	14	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14 - 15	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15	
Generalisability	21	Discuss the generalisability (external validity) of the study results	NA study not terminated, no data interpretation	

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Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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Manuscripts

RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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ARTICLE SUMMARY

Introduction: The SARS-CoV-2 pandemic remains a threat to public health. Soon after its outbreak, it became apparent that children are less severely affected. Indeed, opposing clinical manifestations between children and adults are observed for other infections. The SARS-CoV-2 outbreak provides the unique opportunity to study the underlying mechanisms. This protocol describes the methods of an observational study that aims to characterize age dependent differences in immune responses to primary respiratory infections using SARS-CoV-2 as a model virus and to assess age differences in clinical outcomes including lung function.

Methods and Analysis: The study aims to recruit at least 120 children and 60 adults that are infected with SARS-CoV-2 and collect specimen for a multi-omics analysis, including single cell RNA sequencing of nasal epithelial cells and peripheral blood mononuclear cells, mass cytometry of whole blood samples and nasal cells, mass spectrometry-based serum and plasma proteomics, nasal epithelial cultures with functional *in vitro* analyses, SARS-CoV-2 antibody testing, sequencing of the viral genome and lung function testing. Data obtained from this multi-omics approach is correlated with medical history and clinical data. Recruitment started in October 2020 and is ongoing.

Ethics and dissemination: The study was reviewed and approved by the Ethics Committee of Charité – Universitätsmedizin Berlin (EA2/066/20). All collected specimens are stored in the central biobank of Charité – Universitätsmedizin Berlin and are made available to all participating researchers and on request.

The study is registered at the German Clinical Trials Register with number DRKS00025715.

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Key Words: SARS-CoV-2, children, multiple breath washout, immune response, resilience, multi-omics

Strengths and limitations of this study

- Sample collection from children and adults with primary SARS-CoV-2 infection at multiple time points, however samples from severely ill patients are not included
- Mass cytometry, single-cell RNA sequencing and mass spectrometry-based serum and plasma proteomics display the local and systemic immune response
- Air-liquid interface cultures reproduce *in-vivo* conditions and will be used for functional studies
- Analysis of clinical data and lung function testing complement the multi-omics approach

INTRODUCTION

In December 2019, the novel coronavirus SARS-CoV-2 emerged as cause of acute pneumonia [1 2]. By August 2021, more than 200 million people were infected with SARS-CoV-2 [3]. Soon after the beginning of the pandemic, it became obvious that children have an increased resilience against the primary infection. The course of disease in children is more likely to be milder and severe or even fatal courses remain extremely rare [4-8]. Various hypotheses to explain the reduced susceptibility and mortality of children are currently discussed, including reduced virus entry via ACE-2 in children [9], pre-activated components of the immune system, such as cross-reactive T cells [10-13] and antibodies [14], or a more accentuated innate immunity in children [15, 16] (table 1). Most of these findings are complementary in the explanation of the observed phenomenon, however some findings are in part contradictory and require further

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investigation. Opposing clinical manifestations between children and adults are also observed for other viral respiratory infections [15] [16] [17]. This points to major changes in the general immune response pattern during aging. In the past comparative immune response analyses to primary infections in various age groups were difficult to perform, as many adults had already been exposed to the pathogens. The SARS-CoV-2 outbreak provides the unique opportunity to study the age-dependent changes in immune responses in a controlled manner.

To understand the mechanisms behind the lower susceptibility of children compared to adults to develop severe COVID-19 disease, we have established the observational study RECAST (increased **RE**silience of **C**hildren compared to **A**dults in **S**ARS-CoV-2 infec**t**ion) focusing on the differences in the clinical presentation, lung function and the immune response to SARS-CoV-2 in children compared to adults.

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Table 1. *Hypotheses to explain the resilience of children in SARS-CoV-2 infections*

Hypothesis	Proposed explanation	Scientific findings
Reduced susceptibility for SARS-CoV-2 in children [18]	Reduced virus entry via ACE-2 in children	SARS-Cov-2 uses ACE-2 in the upper and lower airways for host cell entry [19] The age-dependency of ACE-2 expression is controversely discussed [20 21 22]
		Even though an increased expression of ACE-2 renders the individual more susceptible to viral infection, ACE-2 also initiates anti-inflammatory signaling and might contribute to a milder immune response [23]
Age-dependent differential immune activation pattern	Pre-activated immune components in children entail a milder immune response	In early childhood, infections of the upper respiratory tract are frequent. It has been proposed that previous infections with coronaviridae might contribute to a cross-reactive immunity [24]
		Pre-existing T cell reactivity to SARS-CoV-2 could affect the severity of COVID-19 [10-13 25]
		Cross-reactive antibodies entail a milder immune response to SARS-CoV-2 [14]. Of note, uninfected infants do not express cross-reactive antibodies [26]
		The polyclonality and polyreactivity of IgM naturally present in children recognizes SARS-CoV-2 particles [27]
	Children possess a stronger innate immunity than adults	Children display a higher basal expression of pattern recognition receptors than adults and a stronger innate antiviral response [28 29]
		The nasopharyngeal mucosa of children exhibits a stronger innate immunity and expresses more anti-viral cytokines than adults [30]
	Children expose a different cytokine response upon SARS-CoV-2 infection than adults	In COVID-19, a cytokine storm leads to acute respiratory distress syndrome [31 32]
		Certain cytokine patterns correlate with COVID-19 severity [33]
		Pro-inflammatory cytokine concentrations might be lower in children infected with SARS-CoV-2 than in adults [34]
	Co-infections lead to a milder immune reaction, e.g. because of virus competition or primed immune components	In COVID-19, Co-infection with other pathogens is not rare, especially in children [35-37]

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The complexity of immune responses requires a multi-level approach to display changes on various layers, including local immune cell composition, cytokine signaling and systemic response. It can be assumed that the combination of several mechanisms leads to the largely different phenotypes. At the same time, modern techniques allow to engage on an exploratory approach analyzing simultaneously the involvement of canonical and non-canonical immune response patterns. The multi-omics approach presented here allows deeply detailed characterization of the various layers of age dependent specific immune responses. Therefore, we believe that the presented study design will contribute to a further understanding even beyond COVID-19.

To meet these requirements adequately, we chose a multi-omics approach, including: i) single cell RNA sequencing of peripheral blood mononuclear cells (PBMC) and nasal epithelial cells, ii) mass spectrometry-based serum and plasma proteomics, which has been used to identify prognostic marker signatures for SARS-CoV-2 disease severity and devise risk-adapted treatment strategies [38], iii) mass cytometry (cytometry by time-of-flight, CyTOF) of whole blood samples and nasal cells, that has been used to elucidate the role of T cell cytotoxicity in COVID [25] and to identify a dysregulation of the myeloid cell compartment as hallmark of severe COVID [39], iv) highly differentiated nasal epithelial cultures and functional *in vitro* analyses, that have been used to display age-related differences in the nasal epithelium [40], v) antibody testing and vi) sequencing of the viral genome. Obtained data are complemented with anamnestic and clinical information, lung function testing, including spirometry and multiple breath washout, which is a standardized method which allows to assess the ventilation homogeneity of the lungs already in preschool children as well as smell and taste. Longitudinal sampling allows monitoring of the immune response over the course of disease and beyond. Due to the maturation of the immune system during childhood, age-specific immune response

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pattern against SARS-CoV-2 can be expected [41] [42]. Thus participants of all age groups are enrolled.

Participant recruitment began in October 2020 and is ongoing. Of particular interest is the recruitment of children infected with various SARS-CoV-2 virus variants of concern.

Study objectives

This study aims to characterize and compare primary infections with SARS-CoV-2 in children and adults, and to identify age-related determinants of disease course and prognosis. The immune system is not only highly complex, but immune response patterns also vary depending on individual predisposition; moreover, it also matures throughout the ageing of an individual. Furthermore, of pivotal interest for the interpretation of these data is the correlation between immune response pattern and clinical outcome.

METHODS AND ANALYSIS

Study design

RECAST is a prospective observational cohort study at Charité – Universitätsmedizin Berlin in Berlin, Germany. It is a sub-study of the Pa-COVID study of the Charité [43], aiming to characterize the disease course of patients suffering from COVID-19.

Data is collected longitudinally from patients with confirmed COVID-19 at three time points, directly after the diagnosis and at follow-up visits after two weeks and four to six months, and from healthy age-matched controls.

Recruitment started in October 2020 and is planned to end in October 2023.

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Study population

Inclusion criteria

Main inclusion criteria for the index person is a primary acute SARS-CoV-2 infection in a minor (< 18 years of age) with positive PCR or antigen testing (both will be confirmed by PCR testing).

Exclusion criteria

Subjects with preexisting conditions affecting the immune response, such as diseases requiring chemotherapy or syndromes with immunodeficiency and subjects with concomitant medication that affects the immune response, such as systemic steroids, biologicals or investigational therapeutics targeting SARS-CoV-2, are excluded.

Study procedures

Patient identification and recruitment

A network of participating pediatric outpatient practices (n=20) has been established as sentinels to provide access to a pool of >25.000 pediatric patients. All children who tested positive for SARS-CoV2 by PCR or antigen testing as well as their household members are eligible for inclusion.

Healthy controls are recruited from clinical routine diagnostic settings if the diagnostic screening for SARS-CoV-2 was negative.

Medical history, clinical assessment and functional testing

Assessed data include epidemiological and demographic parameters, medical history and potential risk factors, clinical course – including all diagnostic results of the present medical

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attendance – and household and family constellation. A complete list of all items is attached in the appendix (table E1).

Data are collected at first contact and during the follow-up visits. Symptoms of post- / long-COVID [44] are documented and symptoms of myalgic encephalomyelitis / chronic fatigue syndrome are assessed with the Canadian consensus criteria [45 46], Chalder Fatigue Scale [47] and PedsQL™ Multidimensional Fatigue Scale [48-56]. Loss of smell and taste are assessed with the "U-Sniff" test, a 12-item odor identification, the "Sniffin' Sticks" olfactory threshold test and taste samples for sweet, sour, salty, and bitter tastes in children aged six years or older [57 58]. For adults, health status and quality of life are assessed with the St George's Respiratory Questionnaire [59] and health status and mental health are evaluated with PHQ-9 [60] and PCL-5 [61] questionnaires. For children, quality of life is assessed using the KINDL questionnaire [62].

Disease severity is classified according to clinical features using the criteria outlined in the WHO COVID-19 clinical management guideline [63] as asymptomatic, mild, moderate, severe or critical disease. Also, clinical progression is classified according to the WHO clinical progression scale [64]. Applied classification scales are shown in tables 2 and 3.

Functional testing, including lung function testing and multiple breath washout, will be conducted at the follow-up visits after two weeks and four to six months.

Patient and public involvement

We will disseminate all findings in an appropriate and understandable manner to all participants, including children. We welcome the collaboration of participants and public in the interpretation and dissemination of all findings.

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Table 2. *COVID disease severity.*

Abbreviated criteria for COVID-19 disease severity according to WHO COVID-19 Clinical management guideline [63].

Patient State	Descriptor	Score
Uninfected	Uninfected; no viral RNA detected	0
Ambulatory: mild disease	Asymptomatic; viral RNA detected	1
	Symptomatic; independent	2
	Symptomatic; assistance needed	3
Hospitalized: moderate disease	Hospitalized; no oxygen therapy	4
	Hospitalized; oxygen by mask or nasal prongs	5
Hospitalized: severe disease	Hospitalized; oxygen by NIV or high flow	6
	Intubation and mechanical ventilation, pO2/pFiO2 ≥ 150 or SpO2/FiO2 ≥ 200	7
	Mechanical ventilation, pO2/pFiO2 < 150 (SpO2/FiO2 < 200) or vasopressors	8
	Mechanical ventilation, pO2/pFiO2 < 150 and vasopressors, dialysis or ECMO	9
Dead	Dead	10

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Table 3. *WHO clinical progression scale*.

Modified from WHO working group [64].

Disease severity	Definition	Criteria
Asymptomatic		
Mild		Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia
Moderate	Pneumonia	Clinical signs of (non-severe) pneumonia Adolescent or adult: Fever, cough, dyspnoea, fast breathing Child: Cough or difficulty breathing + fast breathing and/or chest indrawing Diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications.
Severe	Severe pneumonia	Adolescent or adult: Plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO2 < 90% on room air Child: Plus at least one of the following: Central cyanosis or SpO2 < 90%; severe respiratory distress (e.g. fast breathing, grunting, very severe chest indrawing); general danger sign: inability to breastfeed or drink, lethargy or unconsciousness, or convulsions
Critical disease	Acute respiratory distress syndrome (ARDS) OR sepsis / septic shock	Oxygenation impairment, invasive ventilation or bilevel NIV / CPAP (≥ 5 cmH_2O) required OR Infection and ≥ 2 Systemic Inflammatory Response Syndrome (SIRS) criteria

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Sample collection

Samples will be collected from SARS-CoV-2 positive participants at each of three time points, directly after the diagnosis and at follow-up visits after two weeks and four to six months, and once from healthy age-matched controls.

Nasal and pharyngeal swab samples are collected for a SARS-CoV-2-PCR, single-cell RNA sequencing and establishment of air-liquid cell cultures. In addition, peripheral blood mononuclear cells (PBMCs) are collected for single-cell sequencing, whole blood for mass cytometry and plasma and serum for mass-spectrometry based proteomics, and SARS-CoV-2-specific antibody testing.

Study database

For Pa-COVID-19 a study protocol was established that harmonizes clinical, molecular, and immunological phenotyping assessment in COVID-19 patients [43]. All data are added to an electronic case report form (eCRF; SecuTrial®). Participants included in RECAST are part of Pa-COVID-19. All participants are assigned a pseudonym consisting of a six- or seven-digit alphanumerical participant code. A separate log allows to match each participant and their code. Access to SecuTrial® requires username and password. All local data are secured by password.

Sample description

Patients recruited in RECAST are grouped into six age categories (table 4). Due to the nature of observatory studies and the lack of pre-existing data, it is not possible to predict the extent of assumptive differences. Preliminary findings suggest that for most planned analyses a sample size of 15 is sufficient. The outlined sample sizes should suffice even for comparisons between children of different age groups.

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Table 4. *Age categories of the RECAST participants*.

Age group		Disease state	Number	Timepoints
Children	Nursery (0-3 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Kindergarten (3-6 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Primary school (6-12 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Secondary school (13-18 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
Adults	<60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	>60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
TOTAL			≥ **360**	≥ **720**

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PLANNED ANALYSES AND OUTCOMES OF INTEREST

We propose a multi-omics workup for all patients. A synopsis of the planned analyses is depicted in figure 1.

SARS-CoV-2-specific PCR and antibody testing

All participants are screened for an active SARS-CoV-2 infection with RT-qPCRs targeting E and N genes [65]. Antibody testing is conducted for all serum and saliva samples with SARS-CoV-2-specific IgG- and IgA-ELISAs. In case of a reactive screening result, confirmatory testing with a recombinant Immunofluorescence assay (IFA) and a plaque reduction neutralization test [66] are conducted.

Nasal epithelial culture and functional *in-vitro* analyses

Conditional reprogramming allows for the generation of long-term cultures of primary airway epithelial cells [67-71]. Without the need of genetic modification or clone selection, conditional reprogramming enables cell expansion, while re-differentiated cultures retain their organ-specific phenotype [72]. We establish highly differentiated polarized *in-vitro* air-liquid interface cultures that reproduce and allow for the analysis of physiological *in-vivo* conditions, such as heterogeneous cell composition with preserved lineage [73] as well as functional characteristics, including production of airway surface liquid [74 75] and mucociliary clearance [76 77]. For material collection, FLOQswabs (Copan, Italy) are used. Swabs are transferred into DMEM/F12 medium (Gibco, USA) and transported to our laboratory within two hours.

Mass cytometry of whole blood samples

Whole blood is fixed with a proteomic stabilizer for preservation of surface and intracellular markers. Blood samples are stored at -80 °C until batch-based analysis. Thawed samples are stained in batches of nine patient and one anchor reference sample. Upon barcoding of

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individual samples, they are pooled and stained with metal isotype conjugated anti-human antibodies as described previously [25 39]. CyTOF technology allows for the detection of more than 40 different barcodes simultaneously to identify cell populations in a high-throughput setting [19, 75].

Mass spectrometry-based serum and plasma proteomics

A platform technology with semi-automated sample preparation to allow for ultra-high-throughput liquid chromatography and mass-spectrometry based analyses of the proteome has recently been established by members of our group [78]. In a directed approach, we characterize the immune response-related serum and plasma proteome, with focus on the acute phase response and the complement system. However, the plethora of proteome signals that are generated per sample also allows for an undirected approach, delivering predictive proteome signatures. To facilitate the computation of such extensive bulk data, a deep neural network is employed [79].

Single-cell sequencing of nasal epithelial cell samples and PBMCs

The nasopharynx is the entry point for an infection with SARS-CoV-2 [80] and as such of distinguished concern in the exploration of the individual immune response pattern. Using single-cell RNA sequencing (scRNAseq) of nasal and bronchial samples, we were previously able to identify cell types and states that correlate with a severe disease course of COVID-19 [81]. Here, scRNAseq will be applied to define the composition and transcriptional activity of immune and epithelial cells in the nasal environment of children and adults throughout the various states of SARS-CoV-2 infection. Nasal swabs (FLOQswabs, Copan, Italy) are used for sample collection. Following sample collection, swabs are directly transferred into cold DMEM/F12 medium (Gibco, USA) and transported to our biosafety laboratory within one hour

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for further processing. Library preparation is performed according to manufacturer's protocol (10x) and sequencing is performed using the Illumina NextSeq 6000 platform.

In addition to the analysis of cells in the respiratory environment, PBMCs are isolated to study the transcriptional activity of blood cell populations. Cell separation, library preparation and comparative single cell transcriptome analyses are conducted according to the manufacturer's protocol. Differential transcriptome profiles of immune cells of the blood will help us to characterize the distinctive features of the systemic and localized immune response to SARS-CoV-2 infections in children and adults.

Lung function testing and multiple breath washout

SARS-CoV-2 infections cause severe lung damage in adults [31 32]. In a large review with 2135 children, 45% (951 children) were classified with a severity of moderate, severe or critical, all with lung involvement per definition [7]. There is evidence that children with acute lung injury experience the same lung pathologies as adults [82]. To assess the extent of transient and permanent functional lung impairment, we investigate the lung function with spirometry and multiple breath washout (MBW). MBW measures the lung ventilation homogeneity [83 84]. This technique is already feasible without sedation in children from 2 years of age [84]. Spirometry depends on the cooperation of the participant and may usually be conducted with children aged six years or older. The technical MBW procedures are in accordance with the American Thoracic Society Technical Statement [85]. Measurements are conducted by certified personnel (ECFS-CTN certified) and for study measurements Exhalyzer D (Ecomedics, Dürnden, Switzerland) will be used [86]. N_2 washout is used as tracer gas to determine the lung clearance index as outcome measure. The lung clearance index (LCI) increases with lung ventilation inhomogeneity.

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Biobanking

Collected material is processed and stored at the central biobank of Charité (ZeBanC, https://biobank.charite.de). Material that is not immediately used is subjected to cryopreservation.

ETHICS AND DISSEMINATION

All procedures in this study are in compliance with the principles of the 1964 Declaration of Helsinki and its amendments. We act in adherence to the principles of Good Clinical Practice (International Council for Harmonization, ICH 1996). The study was reviewed and approved by the Charité Ethics Committee (EA2/066/20). All participants enrolled give written informed consent in person, for participants minor of age the written informed consent of the legal guardian is also required.

Study procedures never interfere with the medical management of participants. Samples required for medical management always have priority. There is no direct benefit for patients participating in the study. Results from the study might improve our understanding of the disease and benefit the public health.

Data is monitored regularly. Informed consent forms are audited by a monitor appointed by the Charité Clinical Trial Management Unit. Data monitoring of collected data is performed in the course of the study.

As established for Pa-COVID [43], we reiterate the fundamental principle in this study that all contributors and researchers who have access to samples commit to unrestricted data sharing. In accordance with FAIR data principles, all data collected shall be findable, accessible, interoperable, and re-usable [87]

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Results originating from nasal epithelial culture and functional in-vitro analyses, mass cytometry, mass spectrometry-based proteomics, single-cell sequencing and lung function testing and multiple breath washout as well as clinical data will be will be disseminated separately or in context in a variety of ways including abstracts, posters and presentations at conferences and published manuscripts in peer-reviewed journals. As soon as all analyses are completed, a comprehensive review will be published to put the findings in context of each other.

DISCUSSION

The SARS-CoV-2 pandemic has accelerated scientific research in the field of virology and related immunobiology for nearly two years, yet many crucial questions remain unanswered. Soon after the emergence of the virus it became apparent that, while children are just as likely to be infected with SARS-CoV-2 as adults, they are less severely affected [4-8 88]. RECAST is an observational study that aims to elucidate the differences between children and adults in primary SARS-CoV-2 infections using a multi-omics approach. Revealing age-dependent differences will help to develop better suited therapeutics and vaccination strategies beyond SARS-CoV-2 infections.

Previous multi-omics approaches conducted with specimen from adult donors served to elucidate the immune response in COVID [81 89], to identify predictors of severe disease courses [90 91] and to isolate possible targets for therapy [81 91 92]. Multi-omics-based studies focusing on SARS-CoV-2 infections in children remain rare and are limited to small participant numbers and only analyze a limited number of –omics dimensions: A study including 24 infected children analyzed the single-cell transcriptional landscape in the upper airways [28]; with single-cell multi-omic profling of matched nasal, tracheal, bronchial and blood samples of 19

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infected children, a study characterized the immune landscape with focus on the upper airways [29]; the plasma proteomic and metabolomic data of 18 infected children was analyzed in another study [93]; clinical characteristics and serum markers were analyzed in a larger group that summarized children and young adults and T cell response in a pediatric subgroup of 11 participants was examined [94]; and a study with 24 infected children analyzed the T cell response and specific antibody response [95]. Even though these studies contributed greatly to a better understanding of age-related immune response patterns in COVID-19, there is still a substantial demand for research. Especially studies analyzing the immune response over the whole age and severity spectrum applying a multi-omics approach are needed. In addition, mechanistic investigations, revealing the causal relationship between the different immune defense layers, are missing. In RECAST, we will conduct a full multi-omics workup with at least 120 infected children, a larger number of participants than in previously published multi-omics studies. Moreover, we will conduct follow-up visits for 6 months to profile the development of age-specific immune response patterns over the course of time. Biobanking and long-term storage of samples will be used to perform subsequent mechanistic studies upon first data collection and hypotheses formulation. The recruitment of family members, both infected and non-infected, allows to assess the effect of genetic relationships. The combination of high resolution multi-dimensional immunologic methods with clinical endpoints in the RECAST study will enable us to contribute to the understanding of the increased resilience of children to SARS-CoV-2 infections.

Author Contributions: BS, JR, MM, VC, MR and LS initiated the project and led the BMBF grant proposal. All authors contributed to the design of the study. SS, JR, NZ, MK, TB, HSB, JM and ER are collecting data biological material and are conducting functional testing. BS, MM, VC, MR, LS, IL, RE, JR and VS lead their respective research field and supervise the conduction of experiments and the

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interpretation of results. CDH, PMB and AB are conducting experiments. SS and JR produced the first draft of the protocol. All authors provided critical review of the manuscript and have approved the final version.

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Competing interests statement: SS, NZ, MK, HSB, JM, ER, AB, PMB, CDH, VAS, RE, IL, MR, MM, BS and JR have no conflict of interest related to this study. TB received payments for his participation in an advisory board for vaccination against SARS-CoV-2. VC has a patent pending in the diagnosis of SARS-CoV-2 infections. LS was involved in further studies relating to SARS-CoV-2 that were funded by national grants.

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Figure 1. Performed analyses in the RECAST study

The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

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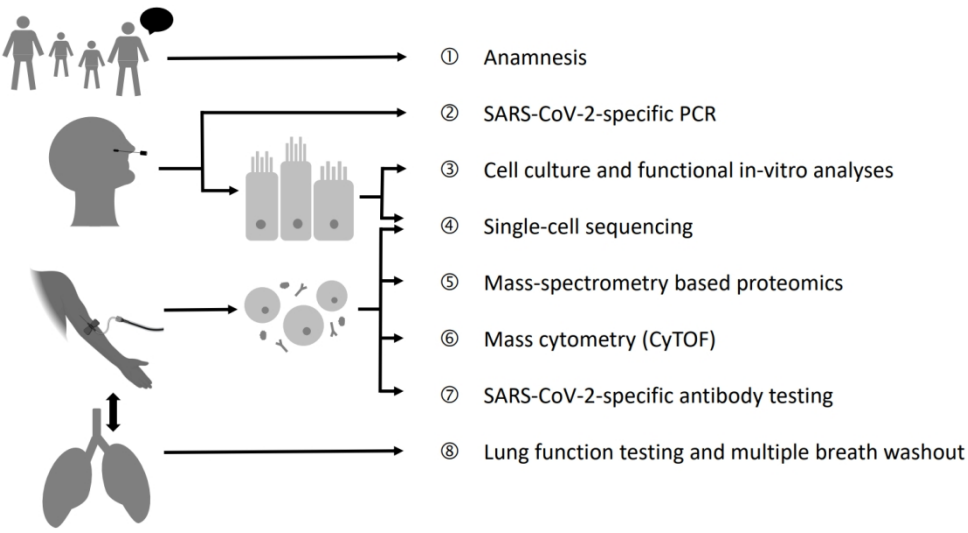

Figure 1. Performed analyses in the RECAST study
The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

413x226mm (96 x 96 DPI)

Appendix, table E1. *Assessed data*.

Assessed data	Item list
Family constellation	
Biometric information, including sex at birth, body height, body weight, ethnicity, number of children and grand-children and profession	
Tests for SARS-CoV-2 in the last 4 weeks, inlcuding type, date and result	
Vaccination against SARS-CoV-2, including manufacturer, date(s) and symptoms after vaccination	
Temporal course of infection in the family	
Index case and assumed point of infection	
Information about household, including number of household members, pet animals and information about smoking (including smoked substances)	
Paediatric anamnesis, including biological parents and legal parents, gestational week at birth, problems during pregnancy, birth and perinatal period, childcare or daycare or school or training, previous RSV infections	
Medical conditions and therapy, including smoking (including substance and length), atopic disposition, allergies, asthma and previous hospitalizations	
Symptoms (including duration) as listed and symptom severity according to WHO scales	Fever or elevated temperature, exhaustion and fatigue, loss of appetite, dizziness, diarrhea, nausea, vomiting, impaired sense of taste or smell, rhinorrhea, congested nose, sneezing, unproductive cough, productive cough, shortness of breath, headache, abdominal pain, sore throat, muscle ache, chest pain, ear pain, joint pain, conjunctivitis, lymphadenitis, skin efflorescence, shivering, paresis, impaired consciousness
Hygiene measures to prevent spreading of disease	
Ability to smell, including parosmia	
Consequences of SARS-CoV-2 infection, including hospitalization, concomitant disease(s) and treatment	
Symptoms of Post-COVID or fatigue as listed, including their duration, whether dependent in occurence or intensity on physical or mental stress or posture	Pain, nausea, vomiting, lymphadenitis, loss of appetite, abdominal complaint(s), loss of smell or taste, palpitation, shortness of breath, coughing, fever, skin efflorescence, conjunctivitis, hair loss, reddening or swelling of hand or feet, reddening or swelling of tongue, lips or cheeks, exhaustion or fatigue, reduced physical and exercise capacity, sleep ineffectivity, disturbed sleeping rhythm, disturbed sleep duration, mood disturbance, cognitive impairment, behavioral changes, sadness or depression, restlessness or excitement, orthostatic dysregulation, increased sensibility to trigges, such as light, noise, smell, touch or others, impaired adaptability to cold, heat or others

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	observational study
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5, 6	
Objectives	3	State specific objectives, including any prespecified hypotheses	7	
Methods				
Study design	4	Present key elements of study design early in the paper	8	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8 - 10	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	9 - 10	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		

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Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	10 - 13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10 - 13
Bias	9	Describe any efforts to address potential sources of bias	NA study not terminated, no data interpretation
Study size	10	Explain how the study size was arrived at	NA study not terminated, no data interpretation
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	NA study not terminated, no data interpretation
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	NA study not terminated, no data interpretation
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	NA study not terminated, no data interpretation	
		(b) Give reasons for non-participation at each stage		
		(c) Consider use of a flow diagram		
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders		
		(b) Indicate number of participants with missing data for each variable of interest		
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)		
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time		
		Case-control study—Report numbers in each exposure category, or summary measures of exposure		
		Cross-sectional study—Report numbers of outcome events or summary measures		
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included		
		(*b*) Report category boundaries when continuous variables were categorized		
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses		
Discussion				
Key results	18	Summarise key results with reference to study objectives	14	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14 - 15	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15	
Generalisability	21	Discuss the generalisability (external validity) of the study results	NA study not terminated, no data interpretation	

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Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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Manuscripts

RECAST: An observational study for the understanding of the increased REsilience of Children compared to Adults in SARS-CoV-2 infection

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ARTICLE SUMMARY

Introduction: The SARS-CoV-2 pandemic remains a threat to public health. Soon after its outbreak, it became apparent that children are less severely affected. Indeed, opposing clinical manifestations between children and adults are observed for other infections. The SARS-CoV-2 outbreak provides the unique opportunity to study the underlying mechanisms. This protocol describes the methods of an observational study that aims to characterize age dependent differences in immune responses to primary respiratory infections using SARS-CoV-2 as a model virus and to assess age differences in clinical outcomes including lung function.

Methods and Analysis: The study aims to recruit at least 120 children and 60 adults that are infected with SARS-CoV-2 and collect specimen for a multi-omics analysis, including single cell RNA sequencing of nasal epithelial cells and peripheral blood mononuclear cells, mass cytometry of whole blood samples and nasal cells, mass spectrometry-based serum and plasma proteomics, nasal epithelial cultures with functional *in vitro* analyses, SARS-CoV-2 antibody testing, sequencing of the viral genome and lung function testing. Data obtained from this multi-omics approach is correlated with medical history and clinical data. Recruitment started in October 2020 and is ongoing.

Ethics and dissemination: The study was reviewed and approved by the Ethics Committee of Charité – Universitätsmedizin Berlin (EA2/066/20). All collected specimens are stored in the central biobank of Charité – Universitätsmedizin Berlin and are made available to all participating researchers and on request.

The study is registered at the German Clinical Trials Register with number DRKS00025715.

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Key Words: SARS-CoV-2, children, multiple breath washout, immune response, resilience, multi-omics

Strengths and limitations of this study

- Sample collection from children and adults with primary SARS-CoV-2 infection at multiple time points, points, however samples from severely ill patients are not included
- Mass cytometry, single-cell RNA sequencing and mass spectrometry-based serum and plasma proteomics display the local and systemic immune response
- Air-liquid interface cultures reproduce *in-vivo* conditions and will be used for functional studies
- Analysis of clinical data and lung function testing complement the multi-omics approach

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INTRODUCTION

In December 2019, the novel coronavirus SARS-CoV-2 emerged as cause of acute pneumonia [1 2]. By August 2021, more than 200 million people were infected with SARS-CoV-2 [3]. Soon after the beginning of the pandemic, it became obvious that children have an increased resilience against the primary infection. The course of disease in children is more likely to be milder and severe or even fatal courses remain extremely rare [4-8]. Various hypotheses to explain the reduced susceptibility and mortality of children are currently discussed, including reduced virus entry via ACE-2 in children [9], pre-activated components of the immune system, such as cross-reactive T cells [10-13] and antibodies [14], or a more accentuated innate immunity in children [15, 16] (table 1). Most of these findings are complementary in the explanation of the observed phenomenon, however some findings are in part contradictory and require further investigation. Opposing clinical manifestations between children and adults are also observed for other viral respiratory infections [15 16 17]. This points to major changes in the general immune response pattern during aging. In the past comparative immune response analyses to primary infections in various age groups were difficult to perform, as many adults had already been exposed to the pathogens. The SARS-CoV-2 outbreak provides the unique opportunity to study the age-dependent changes in immune responses in a controlled manner.

To understand the mechanisms behind the lower susceptibility of children compared to adults to develop severe COVID-19 disease, we have established the observational study RECAST (increased **RE**silience of **C**hildren compared to **A**dults in **S**ARS-CoV-2 infec**t**ion) focusing on the differences in the clinical presentation, lung function and the immune response to SARS-CoV-2 in children compared to adults.

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Table 1. *Hypotheses to explain the resilience of children in SARS-CoV-2 infections*

Hypothesis	Proposed explanation	Scientific findings
Reduced susceptibility for SARS-CoV-2 in children [18]	Reduced virus entry via ACE-2 in children	SARS-Cov-2 uses ACE-2 in the upper and lower airways for host cell entry [19] The age-dependency of ACE-2 expression is controversely discussed [20 21 22]
		Even though an increased expression of ACE-2 renders the individual more susceptible to viral infection, ACE-2 also initiates anti-inflammatory signaling and might contribute to a milder immune response [23]
Age-dependent differential immune activation pattern	Pre-activated immune components in children entail a milder immune response	In early childhood, infections of the upper respiratory tract are frequent. It has been proposed that previous infections with coronaviridae might contribute to a cross-reactive immunity [24]
		Pre-existing T cell reactivity to SARS-CoV-2 could affect the severity of COVID-19 [10-13 25]
		Cross-reactive antibodies entail a milder immune response to SARS-CoV-2 [14]. Of note, uninfected infants do not express cross-reactive antibodies [26]
		The polyclonality and polyreactivity of IgM naturally present in children recognizes SARS-CoV-2 particles [27]
	Children possess a stronger innate immunity than adults	Children display a higher basal expression of pattern recognition receptors than adults and a stronger innate antiviral response [28 29]
		The nasopharyngeal mucosa of children exhibits a stronger innate immunity and expresses more anti-viral cytokines than adults [30]
	Children expose a different cytokine response upon SARS-CoV-2 infection than adults	In COVID-19, a cytokine storm leads to acute respiratory distress syndrome [31 32]
		Certain cytokine patterns correlate with COVID-19 severity [33]
		Pro-inflammatory cytokine concentrations might be lower in children infected with SARS-CoV-2 than in adults [34]
	Co-infections lead to a milder immune reaction, e.g. because of virus competition or primed immune components	In COVID-19, Co-infection with other pathogens is not rare, especially in children [35-37]

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The complexity of immune responses requires a multi-level approach to display changes on various layers, including local immune cell composition, cytokine signaling and systemic response. It can be assumed that the combination of several mechanisms leads to the largely different phenotypes. At the same time, modern techniques allow to engage on an exploratory approach analyzing simultaneously the involvement of canonical and non-canonical immune response patterns. The multi-omics approach presented here allows deeply detailed characterization of the various layers of age dependent specific immune responses. Therefore, we believe that the presented study design will contribute to a further understanding even beyond COVID-19.

To meet these requirements adequately, we chose a multi-omics approach, including: i) single cell RNA sequencing of peripheral blood mononuclear cells (PBMC) and nasal epithelial cells, ii) mass spectrometry-based serum and plasma proteomics, which has been used to identify prognostic marker signatures for SARS-CoV-2 disease severity and devise risk-adapted treatment strategies [38], iii) mass cytometry (cytometry by time-of-flight, CyTOF) of whole blood samples and nasal cells, that has been used to elucidate the role of T cell cytotoxicity in COVID [25] and to identify a dysregulation of the myeloid cell compartment as hallmark of severe COVID [39], iv) highly differentiated nasal epithelial cultures and functional *in vitro* analyses, that have been used to display age-related differences in the nasal epithelium [40], v) antibody testing and vi) sequencing of the viral genome. Obtained data are complemented with anamnestic and clinical information, lung function testing, including spirometry and multiple breath washout, which is a standardized method which allows to assess the ventilation homogeneity of the lungs already in preschool children as well as smell and taste. Longitudinal sampling allows monitoring of the immune response over the course of disease and beyond. Due to the maturation of the immune system during childhood, age-specific immune response

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pattern against SARS-CoV-2 can be expected [41] [42]. Thus participants of all age groups are enrolled.

Participant recruitment began in October 2020 and is ongoing. Of particular interest is the recruitment of children infected with various SARS-CoV-2 virus variants of concern.

Study objectives

This study aims to characterize and compare primary infections with SARS-CoV-2 in children and adults, and to identify age-related determinants of disease course and prognosis. The immune system is not only highly complex, but immune response patterns also vary depending on individual predisposition; moreover, it also matures throughout the ageing of an individual. Furthermore, of pivotal interest for the interpretation of these data is the correlation between immune response pattern and clinical outcome.

METHODS AND ANALYSIS

Study design

RECAST is a prospective observational cohort study at Charité – Universitätsmedizin Berlin in Berlin, Germany. It is a sub-study of the Pa-COVID study of the Charité [43], aiming to characterize the disease course of patients suffering from COVID-19.

Data is collected longitudinally from patients with confirmed COVID-19 at three time points, directly after the diagnosis and at follow-up visits after two weeks and four to six months, and from healthy age-matched controls.

Recruitment started in October 2020 and is planned to end in October 2023.

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Study population

Inclusion criteria

Main inclusion criteria for the index person is a primary acute SARS-CoV-2 infection in a minor (< 18 years of age) with positive PCR or antigen testing (both will be confirmed by PCR testing).

Exclusion criteria

Subjects with preexisting conditions affecting the immune response, such as diseases requiring chemotherapy or syndromes with immunodeficiency and subjects with concomitant medication that affects the immune response, such as systemic steroids, biologicals or investigational therapeutics targeting SARS-CoV-2, are excluded.

Study procedures

Patient identification and recruitment

A network of participating pediatric outpatient practices (n=20) has been established as sentinels to provide access to a pool of >25.000 pediatric patients. All children who tested positive for SARS-CoV2 by PCR or antigen testing as well as their household members are eligible for inclusion.

Healthy controls are recruited from clinical routine diagnostic settings if the diagnostic screening for SARS-CoV-2 was negative.

Medical history, clinical assessment and functional testing

Assessed data include epidemiological and demographic parameters, medical history and potential risk factors, clinical course – including all diagnostic results of the present medical

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attendance – and household and family constellation. A complete list of all items is attached in the appendix (table E1).

Data are collected at first contact and during the follow-up visits. Symptoms of post- / long-COVID [44] are documented and symptoms of myalgic encephalomyelitis / chronic fatigue syndrome are assessed with the Canadian consensus criteria [45 46], Chalder Fatigue Scale [47] and PedsQL™ Multidimensional Fatigue Scale [48-56]. Loss of smell and taste are assessed with the "U-Sniff" test, a 12-item odor identification, the "Sniffin' Sticks" olfactory threshold test and taste samples for sweet, sour, salty, and bitter tastes in children aged six years or older [57 58]. For adults, health status and quality of life are assessed with the St George's Respiratory Questionnaire [59] and health status and mental health are evaluated with PHQ-9 [60] and PCL-5 [61] questionnaires. For children, quality of life is assessed using the KINDL questionnaire [62].

Disease severity is classified according to clinical features using the criteria outlined in the WHO COVID-19 clinical management guideline [63] as asymptomatic, mild, moderate, severe or critical disease. Also, clinical progression is classified according to the WHO clinical progression scale [64]. Applied classification scales are shown in tables 2 and 3.

Functional testing, including lung function testing and multiple breath washout, will be conducted at the follow-up visits after two weeks and four to six months.

Patient and public involvement

We will disseminate all findings in an appropriate and understandable manner to all participants, including children. We welcome the collaboration of participants and public in the interpretation and dissemination of all findings.

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Table 2. *COVID disease severity.*

Abbreviated criteria for COVID-19 disease severity according to WHO COVID-19 Clinical management guideline [63].

Patient State	Descriptor	Score
Uninfected	Uninfected; no viral RNA detected	0
Ambulatory: mild disease	Asymptomatic; viral RNA detected	1
	Symptomatic; independent	2
	Symptomatic; assistance needed	3
Hospitalized: moderate disease	Hospitalized; no oxygen therapy	4
	Hospitalized; oxygen by mask or nasal prongs	5
Hospitalized: severe disease	Hospitalized; oxygen by NIV or high flow	6
	Intubation and mechanical ventilation, pO2/pFiO2 ≥ 150 or SpO2/FiO2 ≥ 200	7
	Mechanical ventilation, pO2/pFiO2 < 150 (SpO2/FiO2 < 200) or vasopressors	8
	Mechanical ventilation, pO2/pFiO2 < 150 and vasopressors, dialysis or ECMO	9
Dead	Dead	10

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Table 3. *WHO clinical progression scale*.

Modified from WHO working group [64].

Disease severity	Definition	Criteria
Asymptomatic		
Mild		Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia
Moderate	Pneumonia	Clinical signs of (non-severe) pneumonia Adolescent or adult: Fever, cough, dyspnoea, fast breathing Child: Cough or difficulty breathing + fast breathing and/or chest indrawing Diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications.
Severe	Severe pneumonia	Adolescent or adult: Plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO2 < 90% on room air Child: Plus at least one of the following: Central cyanosis or SpO2 < 90%; severe respiratory distress (e.g. fast breathing, grunting, very severe chest indrawing); general danger sign: inability to breastfeed or drink, lethargy or unconsciousness, or convulsions
Critical disease	Acute respiratory distress syndrome (ARDS) OR sepsis / septic shock	Oxygenation impairment, invasive ventilation or bilevel NIV / CPAP (≥ 5 cmH_2O) required OR Infection and ≥ 2 Systemic Inflammatory Response Syndrome (SIRS) criteria

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Sample collection

Samples will be collected from SARS-CoV-2 positive participants at each of three time points, directly after the diagnosis and at follow-up visits after two weeks and four to six months, and once from healthy age-matched controls.

Nasal and pharyngeal swab samples are collected for a SARS-CoV-2-PCR, single-cell RNA sequencing and establishment of air-liquid cell cultures. In addition, peripheral blood mononuclear cells (PBMCs) are collected for single-cell sequencing, whole blood for mass cytometry and plasma and serum for mass-spectrometry based proteomics, and SARS-CoV-2-specific antibody testing.

Study database

For Pa-COVID-19 a study protocol was established that harmonizes clinical, molecular, and immunological phenotyping assessment in COVID-19 patients [43]. All data are added to an electronic case report form (eCRF; SecuTrial®). Participants included in RECAST are part of Pa-COVID-19. All participants are assigned a pseudonym consisting of a six- or seven-digit alphanumerical participant code. A separate log allows to match each participant and their code. Access to SecuTrial® requires username and password. All local data are secured by password.

Sample description

Patients recruited in RECAST are grouped into six age categories (table 4). Due to the nature of observatory studies and the lack of pre-existing data, it is not possible to predict the extent of assumptive differences. Preliminary findings suggest that for most planned analyses a sample size of 15 is sufficient. The outlined sample sizes should suffice even for comparisons between children of different age groups.

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Table 4. *Age categories of the RECAST participants*.

Age group		Disease state	Number	Timepoints
Children	Nursery (0-3 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Kindergarten (3-6 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Primary school (6-12 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	Secondary school (13-18 years)	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
Adults	<60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
	>60 years	SARS-CoV2 -	≥ 30	1
		SARS-CoV2 +	≥ 30	3 (day 0, 14, 180)
TOTAL			≥ **360**	≥ **720**

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PLANNED ANALYSES AND OUTCOMES OF INTEREST

We propose a multi-omics workup for all patients. A synopsis of the planned analyses is depicted in figure 1.

SARS-CoV-2-specific PCR and antibody testing

All participants are screened for an active SARS-CoV-2 infection with RT-qPCRs targeting E and N genes [65]. Antibody testing is conducted for all serum and saliva samples with SARS-CoV-2-specific IgG- and IgA-ELISAs. In case of a reactive screening result, confirmatory testing with a recombinant Immunofluorescence assay (IFA) and a plaque reduction neutralization test [66] are conducted.

Nasal epithelial culture and functional *in-vitro* analyses

Conditional reprogramming allows for the generation of long-term cultures of primary airway epithelial cells [67-71]. Without the need of genetic modification or clone selection, conditional reprogramming enables cell expansion, while re-differentiated cultures retain their organ-specific phenotype [72]. We establish highly differentiated polarized *in-vitro* air-liquid interface cultures that reproduce and allow for the analysis of physiological *in-vivo* conditions, such as heterogeneous cell composition with preserved lineage [73] as well as functional characteristics, including production of airway surface liquid [74 75] and mucociliary clearance [76 77]. For material collection, FLOQswabs (Copan, Italy) are used. Swabs are transferred into DMEM/F12 medium (Gibco, USA) and transported to our laboratory within two hours.

Mass cytometry of whole blood samples

Whole blood is fixed with a proteomic stabilizer for preservation of surface and intracellular markers. Blood samples are stored at -80 °C until batch-based analysis. Thawed samples are stained in batches of nine patient and one anchor reference sample. Upon barcoding of

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individual samples, they are pooled and stained with metal isotype conjugated anti-human antibodies as described previously [25 39]. CyTOF technology allows for the detection of more than 40 different barcodes simultaneously to identify cell populations in a high-throughput setting [19, 75].

Mass spectrometry-based serum and plasma proteomics

A platform technology with semi-automated sample preparation to allow for ultra-high-throughput liquid chromatography and mass-spectrometry based analyses of the proteome has recently been established by members of our group [78]. In a directed approach, we characterize the immune response-related serum and plasma proteome, with focus on the acute phase response and the complement system. However, the plethora of proteome signals that are generated per sample also allows for an undirected approach, delivering predictive proteome signatures. To facilitate the computation of such extensive bulk data, a deep neural network is employed [79].

Single-cell sequencing of nasal epithelial cell samples and PBMCs

The nasopharynx is the entry point for an infection with SARS-CoV-2 [80] and as such of distinguished concern in the exploration of the individual immune response pattern. Using single-cell RNA sequencing (scRNAseq) of nasal and bronchial samples, we were previously able to identify cell types and states that correlate with a severe disease course of COVID-19 [81]. Here, scRNAseq will be applied to define the composition and transcriptional activity of immune and epithelial cells in the nasal environment of children and adults throughout the various states of SARS-CoV-2 infection. Nasal swabs (FLOQswabs, Copan, Italy) are used for sample collection. Following sample collection, swabs are directly transferred into cold DMEM/F12 medium (Gibco, USA) and transported to our biosafety laboratory within one hour

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for further processing. Library preparation is performed according to manufacturer's protocol (10x) and sequencing is performed using the Illumina NextSeq 6000 platform.

In addition to the analysis of cells in the respiratory environment, PBMCs are isolated to study the transcriptional activity of blood cell populations. Cell separation, library preparation and comparative single cell transcriptome analyses are conducted according to the manufacturer's protocol. Differential transcriptome profiles of immune cells of the blood will help us to characterize the distinctive features of the systemic and localized immune response to SARS-CoV-2 infections in children and adults.

Lung function testing and multiple breath washout

SARS-CoV-2 infections cause severe lung damage in adults [31 32]. In a large review with 2135 children, 45% (951 children) were classified with a severity of moderate, severe or critical, all with lung involvement per definition [7]. There is evidence that children with acute lung injury experience the same lung pathologies as adults [82]. To assess the extent of transient and permanent functional lung impairment, we investigate the lung function with spirometry and multiple breath washout (MBW). MBW measures the lung ventilation homogeneity [83 84]. This technique is already feasible without sedation in children from 2 years of age [84]. Spirometry depends on the cooperation of the participant and may usually be conducted with children aged six years or older. The technical MBW procedures are in accordance with the American Thoracic Society Technical Statement [85]. Measurements are conducted by certified personnel (ECFS-CTN certified) and for study measurements Exhalyzer D (Ecomedics, Dürnden, Switzerland) will be used [86]. N_2 washout is used as tracer gas to determine the lung clearance index as outcome measure. The lung clearance index (LCI) increases with lung ventilation inhomogeneity.

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Biobanking

Collected material is processed and stored at the central biobank of Charité (ZeBanC, https://biobank.charite.de). Material that is not immediately used is subjected to cryopreservation.

ETHICS AND DISSEMINATION

All procedures in this study are in compliance with the principles of the 1964 Declaration of Helsinki and its amendments. We act in adherence to the principles of Good Clinical Practice (International Council for Harmonization, ICH 1996). The study was reviewed and approved by the Charité Ethics Committee (EA2/066/20). All participants enrolled give written informed consent in person, for participants minor of age the written informed consent of the legal guardian is also required.

Study procedures never interfere with the medical management of participants. Samples required for medical management always have priority. There is no direct benefit for patients participating in the study. Results from the study might improve our understanding of the disease and benefit the public health.

Data is monitored regularly. Informed consent forms are audited by a monitor appointed by the Charité Clinical Trial Management Unit. Data monitoring of collected data is performed in the course of the study.

As established for Pa-COVID [43], we reiterate the fundamental principle in this study that all contributors and researchers who have access to samples commit to unrestricted data sharing. In accordance with FAIR data principles, all data collected shall be findable, accessible, interoperable, and re-usable [87]

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Results originating from nasal epithelial culture and functional in-vitro analyses, mass cytometry, mass spectrometry-based proteomics, single-cell sequencing and lung function testing and multiple breath washout as well as clinical data will be will be disseminated separately or in context in a variety of ways including abstracts, posters and presentations at conferences and published manuscripts in peer-reviewed journals. As soon as all analyses are completed, a comprehensive review will be published to put the findings in context of each other.

DISCUSSION

The SARS-CoV-2 pandemic has accelerated scientific research in the field of virology and related immunobiology for nearly two years, yet many crucial questions remain unanswered. Soon after the emergence of the virus it became apparent that, while children are just as likely to be infected with SARS-CoV-2 as adults, they are less severely affected [4-8 88]. RECAST is an observational study that aims to elucidate the differences between children and adults in primary SARS-CoV-2 infections using a multi-omics approach. Revealing age-dependent differences will help to develop better suited therapeutics and vaccination strategies beyond SARS-CoV-2 infections.

Previous multi-omics approaches conducted with specimen from adult donors served to elucidate the immune response in COVID [81 89], to identify predictors of severe disease courses [90 91] and to isolate possible targets for therapy [81 91 92]. Multi-omics-based studies focusing on SARS-CoV-2 infections in children remain rare and are limited to small participant numbers and only analyze a limited number of –omics dimensions: A study including 24 infected children analyzed the single-cell transcriptional landscape in the upper airways [28]; with single-cell multi-omic profling of matched nasal, tracheal, bronchial and blood samples of 19

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infected children, a study characterized the immune landscape with focus on the upper airways [29]; the plasma proteomic and metabolomic data of 18 infected children was analyzed in another study [93]; clinical characteristics and serum markers were analyzed in a larger group that summarized children and young adults and T cell response in a pediatric subgroup of 11 participants was examined [94]; and a study with 24 infected children analyzed the T cell response and specific antibody response [95]. Even though these studies contributed greatly to a better understanding of age-related immune response patterns in COVID-19, there is still a substantial demand for research. Especially studies analyzing the immune response over the whole age and severity spectrum applying a multi-omics approach are needed. In addition, mechanistic investigations, revealing the causal relationship between the different immune defense layers, are missing. In RECAST, we will conduct a full multi-omics workup with at least 120 infected children, a larger number of participants than in previously published multi-omics studies. Moreover, we will conduct follow-up visits for 6 months to profile the development of age-specific immune response patterns over the course of time. Biobanking and long-term storage of samples will be used to perform subsequent mechanistic studies upon first data collection and hypotheses formulation. The recruitment of family members, both infected and non-infected, allows to assess the effect of genetic relationships. The combination of high resolution multi-dimensional immunologic methods with clinical endpoints in the RECAST study will enable us to contribute to the understanding of the increased resilience of children to SARS-CoV-2 infections.

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Data availability statement

The original data sets collected during the current study are available upon reasonable request that is of scientific nature and aims to achieve the goals described in this publication. This includes all individual participant data collected during the trial, after deidentification. The informed consent forms are available for monitoring at our study center for entitled personnel. Data will be available immediately, until 10 years after the study has concluded. Proposals should be directed to jobst.roehmel@charite.de (https://orcid.org/0000-0002-1535-8852).

Author Contributions:

BS, JR, MM, VC, MR and LS initiated the project and led the BMBF grant proposal. All authors contributed to the design of the study. SS, JR, NZ, MK, TB, HSB, JM and ER are collecting data biological material and are conducting functional testing. BS, MM, VC, MR, LS, IL, RE, JR and VS lead their respective research field and supervise the conduction of experiments and the interpretation of results. CDH, PMB and AB are conducting experiments. SS and JR produced the first draft of the protocol. All authors provided critical review of the manuscript and have approved the final version.

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Competing interests statement:

SS, NZ, MK, HSB, JM, ER, AB, PMB, CDH, VAS, RE, IL, MR, MM, BS and JR have no conflict of interest related to this study. TB received payments for his participation in an advisory board for vaccination against SARS-CoV-2. VC has a patent pending in the diagnosis of SARS-CoV-2

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infections. LS was involved in further studies relating to SARS-CoV-2 that were funded by national grants.

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Figure 1. Performed analyses in the RECAST study

The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

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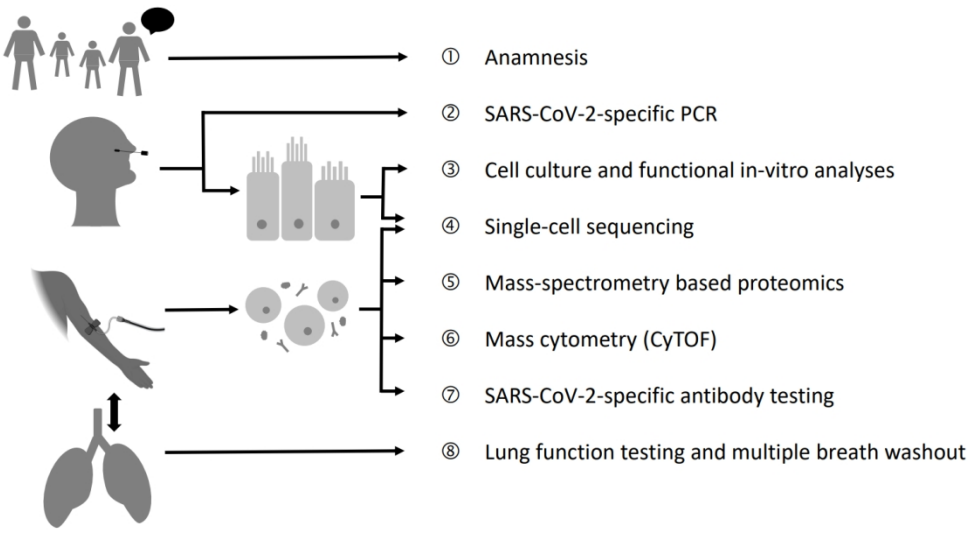

Figure 1. Performed analyses in the RECAST study
The RECAST study consist of 8 elements, including the anamnesis and clinical data, a comprehensive multi-omics workup of the immune landscape of the blood and the upper airways and lung function testing.

413x226mm (96 x 96 DPI)

Appendix, table E1. *Assessed data*.

Assessed data	Item list
Family constellation	
Biometric information, including sex at birth, body height, body weight, ethnicity, number of children and grand-children and profession	
Tests for SARS-CoV-2 in the last 4 weeks, inlcuding type, date and result	
Vaccination against SARS-CoV-2, including manufacturer, date(s) and symptoms after vaccination	
Temporal course of infection in the family	
Index case and assumed point of infection	
Information about household, including number of household members, pet animals and information about smoking (including smoked substances)	
Paediatric anamnesis, including biological parents and legal parents, gestational week at birth, problems during pregnancy, birth and perinatal period, childcare or daycare or school or training, previous RSV infections	
Medical conditions and therapy, including smoking (including substance and length), atopic disposition, allergies, asthma and previous hospitalizations	
Symptoms (including duration) as listed and symptom severity according to WHO scales	Fever or elevated temperature, exhaustion and fatigue, loss of appetite, dizziness, diarrhea, nausea, vomiting, impaired sense of taste or smell, rhinorrhea, congested nose, sneezing, unproductive cough, productive cough, shortness of breath, headache, abdominal pain, sore throat, muscle ache, chest pain, ear pain, joint pain, conjunctivitis, lymphadenitis, skin efflorescence, shivering, paresis, impaired consciousness
Hygiene measures to prevent spreading of disease	
Ability to smell, including parosmia	
Consequences of SARS-CoV-2 infection, including hospitalization, concomitant disease(s) and treatment	
Symptoms of Post-COVID or fatigue as listed, including their duration, whether dependent in occurence or intensity on physical or mental stress or posture	Pain, nausea, vomiting, lymphadenitis, loss of appetite, abdominal complaint(s), loss of smell or taste, palpitation, shortness of breath, coughing, fever, skin efflorescence, conjunctivitis, hair loss, reddening or swelling of hand or feet, reddening or swelling of tongue, lips or cheeks, exhaustion or fatigue, reduced physical and exercise capacity, sleep ineffectivity, disturbed sleeping rhythm, disturbed sleep duration, mood disturbance, cognitive impairment, behavioral changes, sadness or depression, restlessness or excitement, orthostatic dysregulation, increased sensibility to trigges, such as light, noise, smell, touch or others, impaired adaptability to cold, heat or others

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1	observational study
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5, 6	
Objectives	3	State specific objectives, including any prespecified hypotheses	7	
Methods				
Study design	4	Present key elements of study design early in the paper	8	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8 - 10	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	9 - 10	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		

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Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	10 - 13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10 - 13
Bias	9	Describe any efforts to address potential sources of bias	NA study not terminated, no data interpretation
Study size	10	Explain how the study size was arrived at	NA study not terminated, no data interpretation
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	NA study not terminated, no data interpretation
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	NA study not terminated, no data interpretation
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	NA study not terminated, no data interpretation	
		(b) Give reasons for non-participation at each stage		
		(c) Consider use of a flow diagram		
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders		
		(b) Indicate number of participants with missing data for each variable of interest		
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)		
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time		
		Case-control study—Report numbers in each exposure category, or summary measures of exposure		
		Cross-sectional study—Report numbers of outcome events or summary measures		
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included		
		(*b*) Report category boundaries when continuous variables were categorized		
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses		
Discussion				
Key results	18	Summarise key results with reference to study objectives	14	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14 - 15	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15	
Generalisability	21	Discuss the generalisability (external validity) of the study results	NA study not terminated, no data interpretation	

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Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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