COVID-19 AND FRAGILE PATIENTS

Multicenter observational study to assess the incidence and features of COVID-19 and the response to COVID-19 vaccination in fragile patients

STUDY PROTOCOL

Connecting European Cohorts to Increase Common and Effective Response to SARS- CoV-2 Pandemic

History of Changes

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1.0	15.04.2021	Maddalena Giannella (UNIBO), Natascia Caroccia (UNIBO)
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3. PROTOCOL SUMMARY

3.1 Synopsis

Steering Committee	Maddalena Giannella, UNIBO Umberto Maggiore, AOU Parma Jesus Rodrìguez Baño, SAS Elisa Cordero, SAS Evelina Tacconelli, UNIVR Anna Maria Azzini, UNIVR Elda Righi, UNIVR Mauro Krampera, UNIVR Marco Cipolli, UNIVR Michele Milella, UNIVR Maurizio Nordio, REG VEN Fiorella Gastaldon, REG VEN Umberto Cillo, REG VEN Gino Gerosa, REG VEN Mara Cananzi, REG VEN Salvatore Piano, REG VEN Elisa Benetti, REG VEN Patrizia Burra, REG VEN Monica Loy, REG VEN Lucrezia Furian, REG VEN Antonella D'Arminio Monforte, ICONA Adriana Tami, UMCG, Netherlands Thomas Jaenisch, UKHD, Germany Rejko Krüger, LIH, Luxembourg Carlo Giaquinto, PENTA Isadora C. Siqueira, PENTA Francesco Lapi, PENTA Jeannie Collins, PENTA Cecilia Liberati, PENTA Alfredo Tagarro, PENTA Marthe Le Prevost, PENTA Gariel Levy Hara, UBA, Argentina Patrick Mallon, UCD, Ireland

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Study Rationale	Clinical spectrum and outcome of COVID-19 is extremely variable. Older age and multiple comorbidities have been associated with worse outcome. However, the vulnerability to the SARS-CoV-2 infection, the clinical spectrum of COVID-19 disease, and the long-term morbidity and mortality in several types of fragile populations have to be reported yet. In addition, patients with fragile conditions are normally excluded from pivotal studies assessing the safety and efficacy of vaccinations. It is therefore paramount, particularly in the current pandemic situation, to address the question of immunogenic response to vaccines and safety in settings at high risk for altered immune competence and/or safety profiles.
Study objectives	● To describe the characteristics of COVID-19 and its sequelae in several types of fragile populations. ● To describe the rate, the etiology (SARS-CoV-2 variants), the severity of COVID-19, and the rate of re-infections in several types of fragile patients. ● To evaluate the immunologic response to the COVID-19 vaccination in selected fragile populations. ● To evaluate the safety of COVID-19 vaccination in selected fragile populations.

	• Identify human genetic markers and epigenetic characteristics in frail patients during post-vaccination monitoring and in case of breakthrough infections as well as to analyse the gut microbiome in such patients
Study population	Patients of all age and any comorbidity included in an ORCHESTRA fragile population cohort
Inclusion criteria	• Any age • Any comorbidity • Person (or attorney or deputy who has been authorized to make the decision for patients who lack capacity) consent to participate or appropriate local waiver of consent.
Exclusion criteria	Patients did not agree to participate.
Study design	Multicenter, multinational observational study aimed at building several types of fragile populations in which to assess the rate and the clinical spectrum of COVID-19, with particular interest in long-term follow-up, and at monitoring clinical and immunological response after SARS-CoV-2 vaccination in fragile subjects. For long-term follow-up of fragile patients with COVID-19, a predefined schedule of assessment is planned in alignment with Work Package (WP) 2. Patients already diagnosed with COVID-19 can be included if baseline data according to WP2 protocol have been collected, and if follow-up visits are possible. For assessing the clinical and immunological response to vaccination, patients will be prospectively evaluated at baseline (first and

	second vaccination doses), and at multiple timepoints after vaccination with clinical and immunological assessments.
Vaccines	Approved COVID-19 vaccines
Follow-up	Patients will be followed-up for up to 18 months after the SARS-CoV-2 infection diagnosis or up to 12 months after vaccination.
ORCHESTRA Partners	Università degli Studi di Verona (UNIVR); Alma Mater Studiorum – Università di Bologna (UNIBO); Azienda Ospedaliera-Universitaria di Parma (AOU Parma); Institut National de la Sante et de la Recherche Medicale (INSERM); Servicio Andaluz de Salud (SAS); Consorzio Interuniversitario (CINECA); Luxembourg Institute of Health (LIH); Assistance Publique Hopitaux de Paris (AP-HP); Regione Emilia Romagna (RER-ASSR); Fundacion Privada Instituto de Salud Global Barcelona (ISGLOBAL); Ludwig-Maximilians-Universitaet Muenchen (LMU MUENCHEN); Universiteit Antwerpen (UANTWERPEN); Helmholtz Zentrum Muenchen Deutsches Forschungszentrumfuer Gesundheit und Umwelt GMBH (HMGU); Klinikum der Universitaet zu Koeln (UHC); Fondazione PENTA – for the treatment and care of children with HIV and related diseases - ONLUS (PENTA); Universitaet Stuttgart (USTUTT); Centre de Recherches Medicales DE Lambaréné (CERMEL);

	Regionalny Urad Verejneho Zdravotnictva so siidlom v Banskejbytrici(RAPH BB); Charité – Universitaetsmedizin Berlin (CHARITÉ); Academisch Ziekenhuis Groningen (UMCG); Centre Informatique National de l'Enseignement Superieur (CINES); Universidad de Buenos Aires (UBA); Institutul National de Sanatate Publica (INSP); Regione del Veneto (REG VEN); Translational Health Science and Technology Institute (THSTI), Faridabad, Haryana, India; Catholics Bishops Conference of India, Society for Medical Education (CBCI), Bangalore, India.

3.2 Schedule of assessments (SoA)

Table 1. Schedule of assessments for long-term follow-up in COVID-19 patients.

	COVID-19 (2 weeks[1] ± 2 weeks)	3 months[1] (± 1 month)	6 months[1] (± 1 month)	12 months[1] (± 2 months)	18 months[1] (± 3 months)
Screening/baseline					
Inclusion criteria[1]	X				
Demographics[2]	X				
Healthcare setting[3]	X				
Length of hospital stay, days	X				
ICU admission	X				
Medical history[4]	X				
Treatment					
Comorbidity management[5]	X	X	X	X	X
Anti-COVID therapy[6]	X				
Antibiotic therapy	X	X	X	X	X
Oxygen therapy[7]	X	X*	X*	X*	X*
SARS-CoV2[8] and other respiratory pathogen vaccination[9]	X	X	X	X	X
Clinical assessment					
Relevant medical new events[10]	X	X	X	X	X
COVID-19 symptom[11] onset	X				
COVID-19 symptom end	X	X*	X*	X*	X*
COVID severity[12]	X				
SOFA score	X				
Vital signs[13]	X	X	X	X	X
Physical examination[14]	X	X	X	X	X
12-lead electrocardiography	X	X	X	X	X
6-minute walking test	X	X	X	X	X
DLCO (diffusing capacity for carbon monoxide)	X	X	X	X	X
Pulmonary function test[15]	X	X	X	X	X
Questionnaires					
Functional status[16]	X	X	X	X	X
Respiratory impairment[17]	X	X	X	X	X
Mental health[18]	X	X	X	X	X
Risk perception and behaviour[19, 20]					
SARS-CoV-2 vaccination: acceptance/non-acceptance and reasons[21]		X		X	X
Socio-economic indicators		X		X	X
Use of resources (COVID-19 unrelated)		X		X	X
Imaging					
Lung ultrasound	X	X	X*	X	X*
X-ray	X	X	X*	X*	X*
High-resolution CT scan	X	X	X*	X*	X*
Cardiac ultrasound	X	X	X*	X	X*
Cardiac MRI [22]	X[21]	X[21]	X*	X[21]	X*
Biochemistry					

Blood tests[23]	X	X	X*	X	X*
Arterial blood gas test (pO2/pCO2/pH)	X	X	X*	X*	X*
Urine tests[24]	X	X	X*	X	X*
Immunology					
Immune - serology and type I IFNs autoantibodies	X	X	X	X	X
Immune - cytokine and chemokine	X	X [35]	X [35]	X [35]	X *
Immune - cellular	X	X [35]	X [35]	X [35]	X *
Microbiological tests-NP swabs					
Viral variant and metagenomics sequencing	X	X*,[34]	X*,[34]	X*,[34]	X*
EDTA whole blood					
Genetic and epigenetic analysis	X	X	X	X	X
Stool Sample (faeces or rectal swab)					
Metagenomic sequencing	X		X		X
Microbiological tests					
SARS-CoV-2 molecular test in nasopharyngeal swab or tracheal aspirate or bronchoalveolar lavage or saliva	X	X*	X*	X*	X*
Adjunctive variables for specific fragile populations					
HIV					
HIV-infection status[25]	X	X	X	X	X
HIV-Infection therapy[26]	X	X	X	X	X
Assessment of adherence to follow-up visits and antiretroviral therapy	X	X	X	X	X
Elderly					
Cognitive status[27]	X	X	X	X	X
Pregnant women/new born					
History of positive SARS-CoV-2 molecular test on amniotic fluid or breast milk[28]	X				
History of detection of microthrombotic disease on placenta tissue or umbelical cord tissue	X				
Children					
History of positive SARS-CoV-2 molecular test on amniotic fluid or breast milk[29]	X				
Biometric paramethers[30]	X	X	X	X	X
Transplant					
Transplant general information[31]	X				
Graft function[32]	X	X	X	X	X
Immunosuppressive regimen[33]	X	X	X	X	X

Onco-haematology					
Assessment of adherence to oncologic follow-up visits and therapy	X	X	X	X	X
Assessment of progression of the disease and relapse	X	X	X	X	X
Assessment of adverse events[34]	X	X	X	X	X

Footnotes

Modular data capture according to level of commitment (level I, level II, level III).

Level I	Assessments in level I are mandatory
Level II	Customized according to the feasibility of each cohort

* Reassessed only if outside the normal ranges at the previous assessment or if clinically indicated

1. Day 0: first positive SARS-CoV-2 test
2. Demographics: age (years), sex, ethnic group (African, Asian, European, Latin America...), education (no formal education, lower than college, college or higher), cigarette smoking (never-smoker, former smoker, current smoker), usual residence (home, long-term care facility, public dormitory, prison, homeless), current occupation (student, unemployed with no benefits, unemployed with benefits, employed, self-employed, informal worker)
3. Healthcare setting: (a) outpatient (b) non-intensive care unit (c) intensive care unit.
4. Medical history: cardiovascular diseases (hypertension, coronary artery disease, congestive heart failure), diabetes (without insulin, with insulin), chronic respiratory disease (asthma, chronic obstructive pulmonary disease, obstructive sleep apnoea, restrictive lung disease, pulmonary hypertension), kidney disease (chronic with/without dialysis), liver disease other than cancer (HBV/HCV/HDV chronic viral hepatitis, other chronic disease, cirrhosis), metabolic disease, immunosuppressive conditions (solid organ transplant recipient, auto-immune diseases), cancer (solid cancer, haematological malignancies, type of primitive cancer/haematological malignancies, presence of metastases, if ongoing chemotherapy), mental or neurological disorders (psychiatric illness, anxiety disorder, mood disorder, psychotic disorder, Alzheimer disease, dementia other than Alzheimer, Parkinson's disease, myasthenia gravis, epilepsy, stroke (with/without residual deficits, neuromuscular disease, multiple sclerosis), muscular dystrophy, amyotrophic lateral sclerosis); TB co-infection; other opportunistic co-infection (specify) for HIV population
5. Comorbidity management: drug name and dose (to include only treatments taken regularly)
6. Anti-COVID therapy: drug name, maintenance dose, and duration

7. Antibiotic therapy: drug name, dose, duration, and type of treated infection
8. Oxygen therapy: nasal prongs, face mask, face mask with reservoir, high-flow nasal cannula, non-invasive ventilation, mechanical ventilation; numbers of O_2 (L/min) provided (maximum reached) and fraction of inspired O_2 (FiO_2) provided (maximum reached)
9. SARS-CoV-2 vaccination: vaccine name, date of administration
10. Relevant new medical events or worsening of previous conditions, including deep venous thrombosis, pulmonary embolism, infections (including a new SARS-CoV-2-infection during follow-up), malignancies (type of cancer, overall stage).
11. Symptoms: abdominal pain, ageusia/dysgeusia, anosmia, balance impairment, behaviour disorder, chest pain or chest tightness, confusion, cough, delirium, diarrhoea, disrupted sleep, dizziness, dyspnoea, fatigue, fever (including low-grade fever), headache, hypothermia, impaired cognitive status, lethargy, loss of appetite, mood affective disorder, myalgia, nausea/vomiting, palpitation, phlegm, runny nose, sore throat, stuffed nose, syncope, wheeze.
12. WHO Clinical Progression Scale
13. Vital signs: dead/alive, blood pressure, body temperature, heart rate, respiratory rate, peripheral oxygen saturation
14. Physical examination: BMI, abdominal examination, pulmonary examination, cardiac examination, neurological examination, peripheral vascular examination
15. Pulmonary function test: FEV_1, FVC, FEV_1/FVC, TLC, FRC, RV
16. Questionnaires to address the functional status: Post-COVID-19 Functional Status (PCFS) Scale, Global Physical Activity, Questionnaire (GPAQ), Barthel Index, Medical Outcome Study Short Form (MOS SF)-36 Score, EuroQol five-dimension five-level (EQ-5D-5L) questionnaire, Clinical Frailty Scale (CFS), Basic Activity of Daily Living (BADL).
17. Questionnaires to address the respiratory impairment: Saint George Respiratory Questionnaire (SGRQ), Transition Dyspnoea Index (TDI), mMRC (Modified Medical Research Council) Dyspnea Scale
18. Questionnaires to address the mental health: Hospital Anxiety and Depression Scale (HADS), Kessler Psychological Distress Scale (K10), Impact of Event Scale – Revised (IES-R), Resilience Scale for Adults (RSA)
19. Perceived risk of re-infection on a scale 0-10 (no risk- very high risk); perceived risk of admission/re-admission on a scale 0-10 (no risk- very high risk)
20. Frequency mask-wearing (type of mask); frequency hand washing; respect of social distance; avoidance of social gathering

21. Was the vaccine accepted? Why not accepted (lack of trust in efficacy and/or safety; not useful in the specific case; prefer someone else gets it before me)
22. Cardiac MRI only if abnormal cardiac ultrasound
23. Blood tests: White blood cell count, lymphocyte count, neutrophil count, platelets, sodium, potassium, creatinine, glucose, bilirubin, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, albumin, lactate dehydrogenase, ferritin, creatine kinase, fibrinogen, INR, partial thromboplastin time, D-dimer, NT-pro-BNP, troponin, C-reactive protein (CRP), procalcitonin, venous lactate
24. Urine tests: pH, concentration, protein, glucose, red blood, white blood cell count
25. CD4 lymphocyte count; HIV-viral load; AIDS status
26. HIV-therapy: drug name and dose (only ongoing treatment); previous switch to other regimens for virological failure
27. Questionnaires to address cognitive status: Cognitive Failure Questionnaire (CFQ), Mini-Mental State Examination, Clinical Dementia rating Scale, Montreal Cognitive Assessment (MOCA).
28. Results of SARS-CoV-2 molecular test on amniotic fluid
29. Weight, height/length, cranial circumference, BMI
30. Type of transplant (hearth, lung, kidney, liver, pancreas); single-combined; year of transplantation
31. Graft function: good, impaired, failure, rejection acute-chronic, recurrence of underlying disease, other
32. Immunosuppressive regimen: drug name and dose
33. According to Common Terminology Criteria for Adverse Events (CTCAE)
34. At least one of three timepoints (month 3, month 6, month 12) is required to perform metagenomics analysis.
35. At least one of the three timepoints (month 3, month 6, month 12) is required.

Table 2. Schedule of post-vaccination assessments

	1st dose	2nd dose[2]	3 months (± 1 month)*	6 months (± 2 months)*	12 months (± 3 months)*
Baseline information[1]					
Underlying fragile condition	X[1]	X[1]			
Healthcare setting (*i.e.* LTCF)	X[1]	X[1]			
Comorbidities	X[1]	X[1]			
Concomitant medical treatments	X[1]	X[1]			
Past and/or new COVID-19 disease[3]	X[1]	X[1]	X	X	X
Clinical evaluation					
Relevant medical new events[4]	X	X	X	X	X
Clinical symptoms suggestive of COVID-19	X	X	X	X	X
Lab assessment					
Whole blood count	X	X			
CD4/CD8 count	X	X			
IgG level	X	X			
C3 level	X	X			
Immunology					
Immune - serology and type I IFNs autoantibodies	X	X	X	X	X
Immune - cytokine and chemokine	X	X[7]	X[7]	X[7]	X[7]
Immune - cellular	X	X[7]	X[7]	X[7]	X[7]
Microbiological tests-NP swabs					
Viral variant and metagenomics sequencing	X[5]	X[5]	X[6]	X[6]	X[6]
EDTA whole blood					
Genetic and epigenetic analysis	X		X	X	X
Stool Sample (faeces or rectal swab)					
Metagenomic sequencing	X		X		
Questionnaires[1]					
Risk perception and behavior	X[1]			X	X
Socio-economic indicators	X[1]			X	X
Use of resources (COVID-19 unrelated)	X[1]			X	X
Adverse Events dedicated questionnaire		X	X	X	X
Immune response					
Quantitative Ab anti-S IgG[5]	X	X	X	X	X
Qualitative Ab anti-N IgM+IgG[5]	X				
Anti-SARS-CoV-2 sero-neutralization	X	X	X	X	X
Cytokine / chemokines	X	X	X	X	X
Anti-SARS-CoV-2 cell immunity		X		X[6]	X[6]

Footnotes

Modular data capture according to level of commitment (level I, level II).

Level I	Assessments in level I are core and should be prioritized
Level II	Customized according to the feasibility of each cohort

*3, 6 and 12 months counted from the 1st dose.

[1]For centers able to follow patients since the administration of the first and second vaccination doses the baseline information and the risk perception behavior should be collected at one of these time-points, in the other cases such information should be collected during the first post-vaccination follow-up.

[2]The assessment at 2nd dose is mandatory in patients who will receive such dose within 8-12 weeks after fist dose (current AstraZeneca vaccination schedule).
[3]If COVID-19 diagnosis, assessments reported in Table1 are required.
[4]Relevant new medical events or worsening of previous conditions, including new malignancies (type of cancer, overall stage, Kaposi skin-visceral, Lymphoma localization-type, Leukemia lymphoid-myeloid acute-chronic), deep venous thrombosis, pulmonary embolism, infections (including a new SARS-CoV-2-infection during follow-up)
[5]At least one time-point at 1st and 2nd dose is required.
[6] At least one of the three timepoints (month 3, month 6, month 12) is required.
[7] At least one of the four timepoints (2nd dose, month 3, month 6, month 12) is required.

4. LIST OF ABBREVIATIONS

AOU – Azienda Ospedaliera-Universitaria
AP-HP - Public Assistance Hospital of Paris
BMI - Body Mass Index
CBCI - Catholics Bishops Conference of India, Society for Medical Education, Bangalore, India
CERMEL - Lambaréné Medical Research Center
CFQ - Cognitive Failure Questionnaire
CFS - Clinical Frailty Scale
CHARITÉ - University Medicine Berlin
CINECA - Interuniversity Consortium
COVID-19 - COronaVIrus Disease 19
CRF - Clinical research form
CRO - Clinical Research Associate
CRP C-reactive protein
CTCAE - According to Common Terminology Criteria for Adverse Events
DLCO - Diffusion Lung CO
ECG - Electrocardiograph
eCRF - Electronic clinical research form
EQ-5D-5L - EuroQol five-dimension five-level
EPPICC - European Pregnancy and Paediatric Infections Cohort Collaboration
FEV - Forced Expiratory Volume
FRC – Functional Residual Capacity
FVC - Forced Vital Capcity
GCP - Good Clinical Practice
GMBH German Research Center for Health and Environment
GPAQ - Global Physical Activity, Questionnaire
HADS - Anxiety and Depression Scale
HIV - Human Immunodeficiency Virus
HMGU - Helmholtz Zentrum Muenchen
ICF - Informal Consent Form
ICMJE -International Committee of Medical Journal Editors
ICU - Intensive Care Unit
IES-R - Impact of Event Scale – Revised
INR - International Normalized Ratio
INSERM - Institut National de la Santé et de la Recherche Médicale

INSP - National Institute of Public Health

IQR - Interquartile Range

IRB – Institutional Review Board

IRB/IEC – Institutional review board/inidipendent ethics

IRCCS – Italian scientific health institutions

ISGLOBAL-Barcelona Private Foundation Global Health Institut

K10 - Kessler Psychological Distress Scale

LCTF – Long term care facilities

LIH - Luxembourg Institute of Health

LMU MUENCHEN - Ludwig-Maximilians-University Munich

LTCF – Long Term Care Facilities

mMRC - Modified Medical Research Council

MOS SF - Medical Outcome Study Short Form (MOS SF)-36 Score

MRI - Magnetic Resonance Imaging

PCFS - Post-COVID-19 Functional Status Scale

PCR- polymerase chain reaction

PD – Parkinson Disease

PENTA foundation – for the treatment and care of children with HIV and related diseases - ONLUS

USTUTT - University of Stuttgart

PI – Principal Investigator

RAPH BB - Regional Office of Public Health with its seat in Banská Bystrica

REG VEN-Veneto region

RER-ASSR Emilia Romagna region

RT-PCR - reverse transcriptase–polymerase chain reaction

RV-Residual volume

SAS-Andalusian Health Service

SGRQ - Saint George Respiratory Questionnaire

SoA - Schedule of Assessment

SOFA-Sequential [Sepsis-Related] Organ Failure Assessment Score

TDI - Transition Dyspnoea Index

THSTI - Translational Health Science and Technology Institute, Faridabad, Haryana, India

TLC – Total Lung Capacity

UANTWERPEN - University of Antwerpen

UBA - Universidad de Buenos Aires;

UCD - University College Dublin, Ireland

UHC - Clinic of the University of Cologne

UKHD - Universitatsklinikum Heidelberg, Gemany

UMCG - University Medical Center Groningen

UNIBO - University of Bologna

UNIVR– University of Verona

WGS – Whole genome sequencing

WES – Whole exome sequencing

WP – Work Package

5. BACKGROUND

The present study is part of ORCHESTRA project, a three-year international research project aimed at tackling the coronavirus pandemic. ORCHESTRA provides an innovative approach to learn from the pandemic SARS-CoV-2 crisis, derive recommendations to further management of COVID-19 and be prepared for the possible future pandemic waves. The ORCHESTRA project aims to deliver sound scientific evidence for the prevention and treatment of the infections caused by SARS-CoV-2 assessing epidemiological, clinical, microbiological, and genotypic aspects of population, environment and socio-economic features. The project builds upon existing, and new largescale population cohorts in Europe (France, Germany, Spain, Italy, Belgium, Romania, Netherlands, Luxemburg, and Slovakia) and non-European countries (India, Perú, Ecuador, Colombia, Venezuela, Argentina, Brazil, Congo and Gabon) including SARS-CoV-2 infected and non-infected individuals of all ages and conditions. The primary aim of ORCHESTRA is the creation of a new pan-European cohort applying homogenous protocols for data collection, data sharing, sampling, and follow-up, which can rapidly advance the knowledge on the control and management of the COVID-19. ORCHESTRA will include SARS-CoV-2-negative individuals and thereby enable a prospective follow-up and an analysis of vaccination response. The cohort will involve four different populations: general population, COVID-19 patients, fragile individuals (children, elderly, transplanted, oncological, HIV infected, and those with Parkinson disease), and health-care workers. Each of these “perpetual” cohorts can answer different research questions and vaccine strategies.

Within the ORCHESTRA project, the Work Package 4 (WP4) will focus on the cohort of fragile patients including children, pregnant women and new-borns, elderly (≥ 65 year age), transplant recipients, cancer patients, patients with HIV infection, and patients with Parkinson Disease.

6. STUDY RATIONALE

Since the beginning of COVID-19 pandemic, several special settings have been identified regarding the susceptibility to SARS-CoV-2 infection, the associated clinical spectrum and outcome. They include pregnant women, pediatric patients, elderly in particular those whole live in long-term care facilities (LTCFs), and immunocompromised hosts including solid organ transplant recipients (SOT), hematopoietic stem cell transplant (HSCT) recipients and patients with cancer.

Among pregnant women and children, asymptomatic or mild diseases have been frequently reported, prompting controversial concerns about their role in the infection transmission in community and hospital settings (1, 2). On the other hand, a high impact of COVID-19 on morbidity and mortality has been described in elderly and immunocompromised hosts (3, 4). Thus, optimization of prevention strategies, screening practices and therapeutic management is strongly advocated in fragile patients (5, 6). Indeed, these settings have been established as priority groups for vaccines. However, safety and efficacy of vaccination in these populations should be careful

assessed. Thus, preliminary epidemiological data are strongly needed to design further intervention trials and health policies.

Besides, an increasing body of evidence suggests that the gut microbiota plays a role in determining the severity of COVID-19, possibly through the modulation of immune responses (8, 9). Furthermore, dysbiosis of the gut microbiota could contribute to the persistence of symptoms, even after the resolution of the disease (10). For the same reasons, the microbiota could be involved in the onset of adverse reactions induced by vaccination, especially in fragile populations, as recently discussed (11). Defining the impact of the microbiota on immunity to vaccination and therefore on its effectiveness is currently considered a priority in various clinical settings (12).

Moreover, early observations show that vaccines do not induce an immune response conferring protection to many fragile patients, resulting in severe Covid-19 cases. It is important to understand what cellular networks and molecular pathways are switched on/off by the administration of vaccines, and to identify the biological patterns that characterize responders and non-responders. DNA methylation and gene expression analyses may inform on the genomic patterns involved in response to vaccines and in the differences between responders and non-responders. Indeed, DNA sequencing may reveal that the perturbation detected at the regulatory levels may be influenced by alterations in the genome of the divergent subjects.

With this premise, we deem that a WP dedicated to fragile patients in the ORCHESTRA project is necessary to inform about the peculiarities of the fragile cohort as a whole, and of each subgroup as well, providing clinical and biological information useful to design targeted prevention and therapeutic strategies.

7. OBJECTIVES

The primary objective of the ORCHESTRA fragile cohorts is to provide an extensive and harmonized collection of epidemiological and clinical data, and biological samples, to assess several features of COVID-19 in each cohort of fragile patients. These include:

- To assess the prevalence of SARS-CoV2 infection and COVID-19 disease in fragile patients;
- To describe the clinical spectrum of COVID-19 disease in different types of fragile patients;
- To analyze epidemiological, clinical and biological predictors of SARS-CoV-2 infection and prognosis in several types of fragile patients;
- To investigate the therapeutic management of COVID-19 and /or of the underlying condition and its impact on the outcome in fragile patients;

- To describe safety and efficacy of COVID-19 vaccination in selected cohorts of fragile patients;
- To describe the relationship between risk perception and adherence to preventive measures over time, including vaccine acceptance;
- To analyze the impact of COVID-19 pandemic on the use of health care services;
- To analyze the socio-economic determinants of infection and disease severity, and the socio-economic impact of the pandemic on cohort specific socio-economic indicators;
- To investigate the psychological consequences of lockdown in fragile patients.
- Identify human genetic markers and epigenetic characteristics in frail patients during post-vaccination monitoring and in the onset of breakthrough infections as well as to analyse the gut microbiome in such patients

8. STUDY DESIGN

This is an observational retrospective and prospective longitudinal cohort study of several types of fragile patients (see details below). All ages and comorbidities will be included. All the cohorts will be mixed, including patients with and without past or new diagnosis of SARS-CoV2 infection (COVID-19 positive and COVID-19 negative groups) during the study period.

Study will start soon after the approval by the Ethics Committee and will end in June 2023. For the baseline characteristics and past COVID-19 diagnosis, data will be recorded from February 2020. For the prospective part, patients will be recruited until June 2022. Minimum follow-up duration will be of: i) 12 months from the first vaccination dose for fragile patients who will receive anti-COVID-19 vaccination; ii) 18 months from diagnosis of SARS-CoV-2 infection for COVID-19 positive group, allowing a partial follow-up for participants whose SARS-COV-2 infection occur after January 2022; and iii) up to the end of recruitment for the other fragile patients (see Figure 1).

9. PATIENT COHORTS

The ORCHESTRA fragile population consists of 29 cohorts of 10 different fragile populations including pregnant women/new-born, children, patients with HIV infection, patients with autoimmune disease, solid organ transplant recipients, patients with oncological and hematological diseases, patients with cystic fibrosis, patients with Parkinson Disease and rheumatological diseases from from 14 countries (5 European and 9 non-European countries), with approximately 19784 subjects. Among these, 10.300 individuals are already registered in local databases and on active follow-up in the respective centers. A description of the cohorts is presented in the following sections.

9.1. University of Bologna (UNIBO) – Italy

UNIBO will participate in the recruitment of the following COVID-19 and non-COVID-19 fragile populations: HIV positive, solid organ transplant recipients (liver, kidney, heart, lung),

hematopoietic stem cell transplant recipients, patients with cancer and autoimmune disease patients.

9.2. Azienda-Ospedaliera Universitaria di Parma (AOU di Parma) – Italy

AOU Parma will participate in the recruitment of COVID19 and non-COVID-19 solid organ transplant (kidney).

9.3. Servicio Andaluz de Salud (SAS) – Spain

SAS will participate in the recruitment of the following COVID-19 and non-COVID-19 fragile populations: HIV positive subjects, solid organ transplant recipients, oncological and hemato-oncological patients, hemodialysis patients and rheumatological patients.

9.4. University of Verona (UNIVR) – Italy

UNIVR will participate in the recruitment of the following COVID-19 and non-COVID-19 fragile populations: HIV positive, solid organ transplant recipients, patients with cystic fibrosis, oncological and hematological patients.

9.5. Regione Veneto (REG VEN)– Italy

The Transplant Centers of Treviso, Vicenza and Padova will participate in the recruitment of COVID-19 and non-COVID-19 solid organ transplant recipients (kidney, liver, heart, lung).

9.6. Catholics Bishops Conference of India, Society for Medical Education (CBCI) – India

CBCI will participate in the recruitment of the following COVID-19 and non-COVID-19 fragile populations: HIV positive subjects and patients with auto-immune and rheumatological disorders.

9.7. Universidad de Buenos Aires (UBA) – Argentina

UBA will participate in the recruitment of COVID-19 and non-COVID-19 HIV positive subjects. The cohort, of approximately 100 patients, will be coordinated by the School of Medicine, involving sub-investigators that are already following HIV patients (some of them who already acquired COVID-19) since the beginning of the pandemic in March. Patients will be prospectively enrolled once the study protocol is approved by the Ethics Committee.

9.8. PENTA Foundation ONLUS – Italy

9.8.1 Italian cohorts

PENTA will participate with the following Italian children cohorts: a large cohort of children followed by primary care paediatricians involved in the “Pedianet” network (COVID-19 and non-COVID-19 subjects) and children attending the clinic for follow-up visits after intra-family COVID-19 infection. Pedianet is an organised network of more than 400 family paediatricians in Italy aimed to collect anonymous data from the electronic system they use in their daily activities to be used for clinical and epidemiological research.

9.8.2 EPPICC cohort

European Pregnancy and Paediatric Infections Cohort Collaboration (EPPICC) is a multi-country cohort of children and adolescents living with HIV across Europe and attending routine HIV care. The initial purpose of the cohort was to address key research questions related to long term outcomes of HIV infection and treatment in the paediatric population. The sample size is ~10,000 children/adolescents EVER in follow up included in the database, of which approximately 3000 were in active follow up during 2020.

Pregnant women cohort coordinated by Instituto Gonçalo Moniz- Fiocruz- Bahia, hospital partner: Maternidade de Referência Prof José Maria de Magalhães Neto (Maternidade RPJ). In April 2020, a pregnancy surveillance study that involves pregnant women admitted to Maternidade RPJ with suspected COVID-19 was started, with the aim to increase understanding of the risks and impact of COVID-19 during pregnancy and birth outcomes. The study population will be composed of women with confirmed COVID-19 as well as those with COVID-19-compatible symptoms who test negative for SARS-CoV-2 (control group). At present, Maternidade RPJ has recorded over 300 pregnancies with confirmed or suspected COVID-19 since March 2020

9.8.3 EPICO cohort

EPICO is a Spanish cohort of currently 1035 children attended in hospitals with COVID-19 with respiratory symptoms or suspected COVID-19 with infection by SARS-CoV-2 confirmed by PCR or serology, from March 2020.

9.8.4 Bahia cohort

The Instituto Gonçalo Moniz- Fiocruz- Bahia, partnered with the Maternidade de Referência Prof José Maria de Magalhães Neto (Maternidade RPJMMN), will also participate in the

ORCHESTRA project with a pregnancy surveillance study that involves pregnant women admitted to Maternidade RPJMMN with suspected COVID-19. The study population will be composed of women with confirmed COVID-19 as well as those with COVID-19-compatible symptoms who test negative for SARS-CoV-2 (control group).

At present, Maternidade RPJMMN has recorded over 300 pregnancies with confirmed or suspected COVID-19 since March 2020.

9.9. University College Dublin (UCD) – Ireland

The All Ireland Infectious Diseases (AIID) Cohort study was initially established at a single hospital site (Mater Misericordiae University Hospital) in 2013. It has since expanded to five hospital sites across Ireland. The aim of the cohort is to create a data-rich prospective dataset derived from patients who present with suspected Infectious Diseases focusing on HIV and Hepatitis and now includes COVID-19. UCD will participate in this protocol recruiting HIV positive subjects including COVID-19 and non-COVID-19 patients.

9.10. Luxembourg Institute of Health (LIH) – Luxembourg

LIH will participate in the recruitment of COVID-19 and non-COVID-19 subjects with Parkinson disease (PD). The Luxembourg Parkinson's Study is a nation-wide and comprehensive clinical, molecular and device-based cohort comprising more than 1,600 participants from Luxembourg and the Greater Region. The cohort includes patients with typical PD and atypical parkinsonism, irrespective of their disease stage, age, comorbidities, or linguistic background (followed-up yearly) and matching control subjects (followed-up every 4 years).

9.11. Translational Health Science and Technology Institute (THSTI) – India

THSTI will participate in the recruitment of a large cohort of COVID-19 and non-COVID-19 pregnant women participating in the GARBH-Ini (interdisciplinary Group for Advanced Research on BirtH outcomes - DBT India Initiative) program. GARBH-Ini Cohort was initiated in May 2015 at the Civil hospital in Gurugram (GCH), Haryana, India. Women are enrolled within 20 weeks of gestation and are followed until delivery and once at postpartum. The enrolled women are followed up at 4-5 time-points across 3 trimesters of pregnancy to document extensive clinical & epidemiological information, varied maternal and neonatal biospecimens and for serial ultrasonographic examination. Accounting for a cohort design to detect epidemiological risk factors & nested case-control design for identifying biomarkers, an apriori sample size of 8000 was estimated. More than 8534 pregnant mothers have been

enrolled till February 10, 2020 since May 2015 with documentation of 7260 pregnancy outcomes.

9.12. ZIKAlliance (ORCHESTRA partners: UMCG & UKHD)

ZIKAlliance is a multi-country network coordinated by the ORCHESTRA Partners Academisch Ziekenhuis Groningen (UMCG), Netherlands, and Universitatsklinikum Heidelberg (UKHD), Germany. The initial purpose of this cohort was the evaluation of risks of congenital malformations and other adverse pregnancy outcomes after Zika virus infection in pregnant women and their children. Further, ZIKAlliance has integrated the investigation of SARS-CoV-2 infection and COVID-19 disease into the on-going Zika pregnant women cohort study. ZIKAlliance will participate in this Project with a SARS-CoV-2 substudy including the cohorts of pregnant women and their newborns from Venezuela, Colombia, Ecuador and Peru. Mother and children are followed from birth up to 2 years of age.

9.13. Centre de Recherches Medicales de Lambaréné (CERMEL) – Gabon

CERMEL will participate in the recruitment of children including COVID-19 and non-COVID-19 subjects.

10. INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria are:

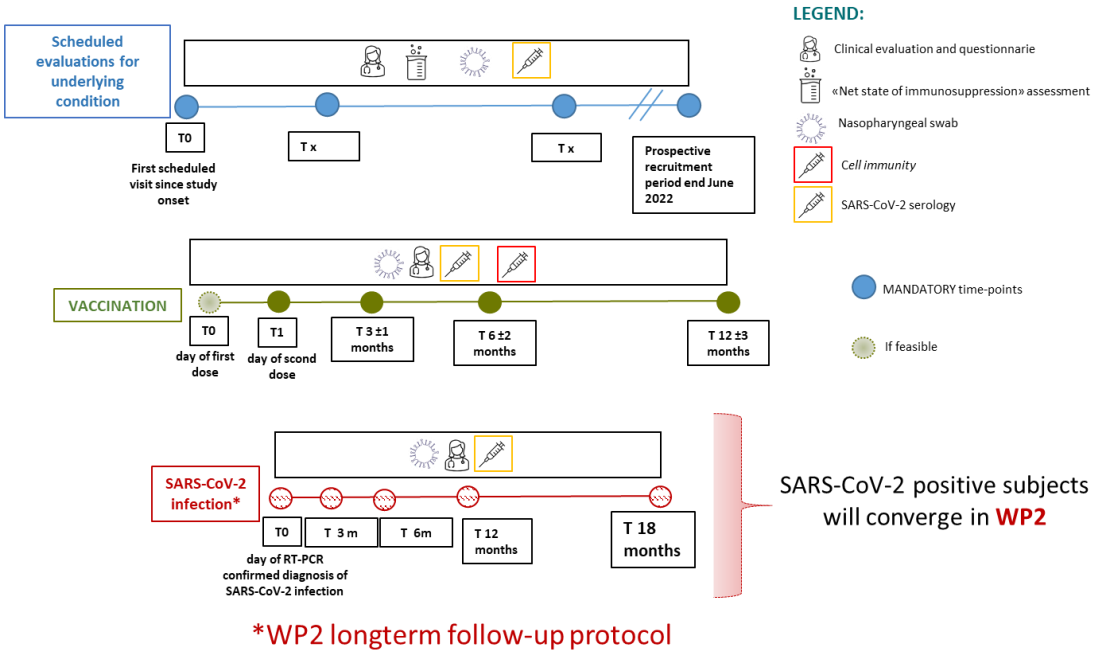
- Any age
- Person (or attorney or deputy who has been authorized to make the decision for patients who lack capacity) consent to participate, or appropriate local waiver of consent.

The only exclusion criteria will be the lack of consent to participate to the study.

11. STUDY PROCEDURES

In Figure 1, a study work flow is shown.

Figure 1: study work flow



All included patients will be evaluated during the scheduled visits for the underlying condition. During such visits, the following actions are required:

- Distribution of a questionnaire to capture the impact of COVID-19 pandemic on the socio-economic indicators, on the access to health care services, and to investigate risk perception and behavior towards preventing infection and COVID-19 disease, including vaccine acceptance. This questionnaire should be administered at two different time-points with an interval of at least 6 months to capture changes in behavior over the study period (Annex 1a or Annex 1b in case of children cohorts).

- Distribution of psychological scale questionnaires to investigate the impact of lockdown measures on mental health (Annex 2).
- Screening for past and/or current COVID-19 disease by medical interview, serology determination and nasopharyngeal swab or saliva specimens' collection.
- Collection, processing and storage of plasma for cytokine and chemokine analysis.
- Collection, processing and storage of serum for the subsequent sero-neutralization and type I IFNs autoantibodies detection.
- Collection, processing and storage of PBMCs for the subsequent anti-COVID-19 cellular immunity analysis.
- Collection, processing and storage of whole blood for genetic and epigenetic analysis.
- Collection, processing and storage of nasopharyngeal samples for SARS-CoV-2 detection and variant identification if clinically relevant.
- Collection, processing and storage of stool samples for metagenomic sequencing.
- In immunocompromised fragile cohorts, laboratory tests to assess the baseline "net state of immunosuppression" including whole blood count, lymphocyte differentiation, immunoglobulin levels, and complement (C3) levels should be done at the baseline (first clinical evaluation).

For patients with a past and/or new diagnosis of SARS-CoV2 infection/COVID-19 disease a pre-established long-term follow-up including multiple diagnostic levels in order to adapt to all the participating cohorts is requested. A detailed overview of the schedule of the study visits and the clinical parameters to be recorded is shown in **Table 1**. Patients who have already had COVID, and new patients being diagnosed with COVID, who can be followed up to at least 12 months, and for whom data can be collected according to **Table 1** should be included. In specific fragile cohorts, such as children cohorts, virtual assessments are allowed.

For patients who have been or will be vaccinated against COVID-19 during the study period, a pre-established monitoring of safety and efficacy in terms of immunological response will be performed. A detailed overview of the schedule of the clinical and laboratory parameters to be recorded is shown in **Table 2**. Patients who have already been vaccinated, or will be vaccinated, who can be followed for 12 months, and for whom data can be collected according to Table 2 should be included. The monitoring should start from the days of first and second dose administration when possible (i.e. vaccination administered in the same center where the patient is followed for the fragile condition), alternatively patients will be monitored at 3±1, 6±2 and 12±3 months after the first vaccination dose.

- Vaccination safety will be assessed by a dedicated questionnaire (Annex 3) distributed to patients at baseline or during the first monitoring visit.

- Vaccination efficacy will be established analyzing the immune response at the predefined time-points. The humoral immune response to vaccination will be assessed by a serological assay able to detect anti-Spike and anti-Nucleocapsid antibodies as well as sero-neutralization assays in sera samples. The latter will be useful to exclude possible natural infection at the baseline (1st/2nd dose, or first post-vaccination monitoring visit). The cytokine and cell-mediated immune response to vaccination will be assessed in a subgroup of patients by centralizing samples to a referral laboratory, according to local ethic rules and budget. The number of SARS-CoV-2 paucisymptomatic or symptomatic infection despite vaccination will be also assessed by SARS-CoV-2 detection in respiratory/saliva sample.

During the post-vaccination monitoring and in the onset of a breakthrough infection after one or two doses of the anti-SARS-CoV-2 vaccine, several analyses (genomic, transcriptomic, cytokine, viral, genetic and epigenetic) will be performed in a local or centralised laboratory according to the type of testing realized (see Table 3):

- PBMCs for characterisation of T-cell immune response will be sent to University of Antwerpen
- Plasma for cytokinome analysis will be sent to University of Antwerpen
- Serum for autoantibodies against type I IFNs detection will be sent to INSERM or University of Antwerpen
- Whole blood samples for epigenetic and genetic analysis will be sent to UNIBO, INSERM and HMGU. In-depth human genetic analysis will be conducted using WGS or whole exome sequencing (WES) followed by functional analyses of the most promising variants.
- Nasal swabs for characterization of viral markers and respiratory microbiome dynamics wiill be sent to University of Antwerpen or INSERM.
- Stool samples for intestinal microbiome profiling will be sent to University of Bologna.

Detailed instructions on sample collection, processing, storage, shipment and destination sites are provided in the WP6 protocol (WP6, D 6.1) attached as Annex 4.

Table 3 – Overview of samples collection, processing, storage and destination sites.

Sample	Aliquot	Sample type	Volume	Storage solution	Storage temp. (°C)	Shipping temp. (°C)	Task	Comment	Partner
PBMC	1 or 2 samples	PBMC		0.5 ml FBS/DMSO 20%	-70°C or below or liquid nitrogen	Dry ice	Characterisation of T-cell immune response		UANTWERPEN
Blood	1	EDTA plasma, but heparin plasma or serum can also be used, if EDTA plasma is absolutely unavailable	350 µL	EDTA plasma has to be processed according to the protocol provided before freezing (preferably at -80°C directly)	Short term at -20 °C, long term at -70°C or below	Dry ice	Cytokinome analysis	Please process and freeze within 2 hours.	UANTWERPEN
	2	Serum/plasma	200 µL	NA	-20°C, long term at -70°C or below	Dry ice	Auto-antibodies against type I IFNs	If available	INSERM
	3	Serum	100 µL	NA	-20°C, long term at -70 ° or below	Dry ice	Antibodies detection		INSERM; UANTWERPEN
Whole blood	1	Extracted DNA or whole blood	4 µg if DNA, 2 ml if whole blood	NA	-20°C/ -80°C	Dry ice	NGS of COVID-19 cohorts	DNA could be extracted locally or at HMGU.	INSERM/UNIBO
	2	DNA or whole blood	750 ng in 45 µL if extracted DNA; otherwise 1 aliquot	TE buffer or water if extracted DNA	-20°C	Dry ice	Illumina EPIC DNA methylation	DNA could be extracted locally or at HMGU.	HMGU
NP swab	1	NP swab	400 µL	TRIzol; RNA later; DNA/RNA shield	-70°C or below	Dry ice	Characterization of viral markers Respiratory microbiome dynamics		UANTWERPEN- INSERM
Stool sample or rectal swab	1	Stool (faecal swab if stool is unavailable)	1-2 g	RNA later if possible, otherwise frozen.	+4°C (up to 24 h) long term at -70°C or below	Dry ice	Intestinal microbiome profiling		UNIBO

12. STUDY VARIABLES

Endpoint variables:

- Infection with SARS-CoV2 detected by RT-PCR on respiratory/saliva specimens;
- Reinfection and/or clinical relapse defined according to recent proposed definitions (7);
- Clinical spectrum of COVID-19 according to WHO mild, moderate, severe, and critical criteria (https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-1);
- Duration of viable viral shedding in fragile patients diagnosed with COVID-19;
- Immune response in patients diagnosed with and/or vaccinated against COVID-19 assessed according to study protocol procedures (see above);
- All-cause mortality during the follow-up period;
- Change in basal clinical condition assessed by questionnaire on functional status and/or according to specific parameters of underlying fragile condition (i.e. graft function, cancer stage etc).

Baseline variables will include:

- Demographic data (sex, date of birth, ethnicity, blood group);
- Underlying conditions;
- Vaccination status against respiratory infectious diseases (i.e. Influenza, pneumococcus, BCG) if available.

13. STATISTICAL ANALYSIS

We will carry out comprehensive descriptive analyses taking into account sociodemographic factors and clinical courses. The frequency distributions of the characteristics will be given in absolute and relative numbers, median plus interquartile range (IQR) or mean values plus 95% confidence interval (CIs). Associations with specific epidemiological features, laboratory results, therapeutic and preventive (including vaccination) strategies will be analysed using chi-square tests, t-tests or Mann-Whitney tests, depending on the data. To evaluate potential risk factors, multivariate regression models will be carried out. Outcome time analyses using Cox proportional-hazards regression models with time-dependent covariates will be performed to examine factors associated with each endpoint (including death). In addition, we will use cumulative incidence functions, such as the Fine-Gray sub-distribution hazard regression model, to account for competing events (i.e., death in evaluating graft function etc.). For missing values, a different strategy to understand the causes and the significance for the analysis will be developed and a graduated procedure for dealing with censorship and imputations via linked regressions will be developed. The significance level is defined with a p-value <0.05. Risk perception and socio-economic factors will be analysed using quasi-

experimental techniques (*eg*: propensity score matching, difference-in-differences) by exploiting several sources of variation over time and across individuals. All statistical analyses will be carried out with STATA, Python and/or R statistics software by trained staff (epidemiologists, statisticians) using the latest analysis methods.

14. REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

14.1 Regulatory and ethical aspects

The study protocol is designed and will be conducted to ensure adherence to the principles and procedures of Good Clinical Practice and to comply with Italian laws, as described in the following documents and accepted, with their signature, by the study investigators: 1. ICH harmonized tripartite guidelines for good clinical practice 1996.2. Directive 91/507 / EEC, The Rules Governing Medicinal Products in the European Community. 3. Legislative Decree No. 211 of 24 June 2003.4. Legislative Decree n.200 November 6, 2007.5. D.M. 21 December 2007.6. AIFA Determination March 20, 2008. All essential clinical documents will be kept to demonstrate the validity of the study and the integrity of the data collected.

All the document e protocol, protocol amendments, ICF, and other relevant documents must be submitted to an IRB/IEC by the investigator and reviewed and approved by the IRB/IEC before the study is initiated.

14.2 Financial disclosure

Finance and insurance are addressed in the Investigator and/or CRO agreements, as applicable.

14.3 Informed consent process

Participant's informed consent/assent (ICF) must be obtained and documented in accordance with local regulations, ICH-GCP requirements, and the ethical principles that have their origin in the principles of the Declaration of Helsinki. Prior to obtaining informed consent, information should be given in a language and at a level of complexity understandable to the participant in both oral and written form by the Investigator (or designee). Each participant will have the opportunity to discuss the study and its alternatives with the Investigator. Prior to participation in the study, the ICF should be signed and personally dated by the participant, or his/her legal representative.

The participant or his/her legal representative must receive a copy of the signed and dated Informed Consent form. As part of the consent process, each participant must consent to direct access to his/her medical records for study-related monitoring, auditing, IRB/IEC review, and regulatory inspection. If the ICF is amended during the study, the Investigator must follow all applicable

regulatory requirements pertaining to the approval of the amended Informed Consent form by the IRB/IEC and use of the amended form.

The participant may withdraw his/her consent to participate in the study at any time. Consent may be waived with documented approval as per local guidelines, for example in the case where only routinely collected data from medical records are collected for this study.

14.4 Data protection

The participant must be informed that his/her personal study-related data will be used by the local Principal Investigator in accordance with local data protection law. The level of disclosure must also be explained to the participant who will be required to give consent for their data to be used as described in the informed consent.

Participants will be assigned a unique identifier by the Promotor. Any participant records or datasets that are transferred to the sponsor will contain the identifier only; participant names or any information which would make the participant identifiable will not be transferred.

The electronic case report form (eCRF) will be provided using RedCap® software of the University of Bologna/CINECA. Investigators from participating study sites log into the system with username and a safe password including letters, numbers, and symbols.

Investigators will be informed about handling of their personal data and their IP and location during the registration process

The data collection should be performed also retrospectively after a patient case has been completed (treatment or follow-up is finished or patient's death). This process will be compliant with all applicable European and German federal data protection regulations, including EU directive 2016/679 and the German DS-GVO.

14.5 Description of the data collected

Only data that is strictly necessary and relevant to meet the objectives of the research are collected.

The data collected via the medical file as part of the research falls into the categories following:

Demographics data: age, sex, race, education, cigarette smoking, usual residence.

Detailed clinical data, including about possible previous and concomitant illnesses, medical findings and therapies in the context of SARS-CoV-2 infection.

Data collected by self-administered questionnaires: questionnaires to address the functional status, respiratory impairment, mental health, and socio-economic issues with related behavior.

Data will be collected by a standardized electronic case report form (eCRF) and managed using REDCap capture tool. Collected data will be periodically checked for accuracy by an investigator of the coordinating unit (UNIBO). Queries for incongruous or missing data will be submitted to

investigators. There will be 12 mailing cycles a year in which baseline and post-COVID-19 follow-up and/or post vaccination monitoring information will be requested. This policy will ensure that the cohort statistics will be based on a data set that is current, complete and as accurate as possible.

14.6 Data quality assurance

All participant data relating to the study will be recorded on the electronic-CRF. The Investigator is responsible for verifying that data entries are accurate and correct by physically or electronically signing the eCRF or paper CRF. The local investigator must maintain accurate documentation (source data) that supports the information entered in the eCRF or paper CRF. The local investigator must permit study-related monitoring, audits, IRB/IEC review, and regulatory agency inspections and provide direct access to source data documents. The promotor is responsible for the data management of this study including quality checking of the data as described above in study procedures' section.

14.7 Source Document

All source documents must be accurate, clear, unambiguous, permanent, and capable of being audited. They should be made using some permanent form of recording (typing, printing, optical disc). They should not be obscured by correction fluid or have temporary attachments (such as removable self-stick notes). Source documents are original records in which raw data are first recorded. These may include hospital/clinic/general practitioner records, charts, diaries, x-rays, laboratory results, pharmacy records, care records, ECG or other printouts, questionnaires, or video, for example. Source documents should be kept in a secure, limited access area.

14.8 Study and site closure

The study will start after the approval by the ethics committee approximately on April 2021 and it will end 12 months after the last patient enrollment. The overall duration of the study is 26 months.

14.9 Publication policy

The PI is responsible for the final publication of data. Authors must satisfy all of the following ICMJE authorship criteria: 1. Substantial contributions to conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND 2. Drafting the work or revising it critically for important intellectual content; AND 3. Final approval of the version to be published; AND 4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Data collection, general supervision of the research group, or overseeing the conduct of the study alone

does not justify authorship. Publications will be planned by the PI and the scientific and statistical committees. Publication of partial or local data must be approved by the PI.

14.10 Amendments or any other modification

Modifications to the protocol will be made as amendment. No other modality is allowed. Any modification will be recorded in the "Clinical Study Report" Archiving documents. The principal investigator is responsible for archiving and storing the essential documents during all the period of study according by current legislation and GCP.

15. ADD-ON SUBSTUDIES

15.1 Pregnant Women (PW) Cohort for evaluation of risks of congenital malformations and other adverse pregnancy outcomes after Zika virus infection (part of ZIKAlliance) - SARS-CoV-2 sub study.

15.2 Children (CH) cohort for the evaluation of developmental and neurological abnormalities in infants born to mothers residing in areas with Zika virus transmission during pregnancy (part of ZIKALLIANCE) - SARS-CoV-2 sub study.

15.3 Epidemiologic study of respiratory infections by novel coronavirus (SARS-CoV-2) in the paediatric population

15.4 UNIVR: Rete Oncologica Veneta (ROV)

15.5 UNIBO: CONTRAST study

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ANNEX 1a and ANNEX 1b – Time points for the socio-economic questionnaires

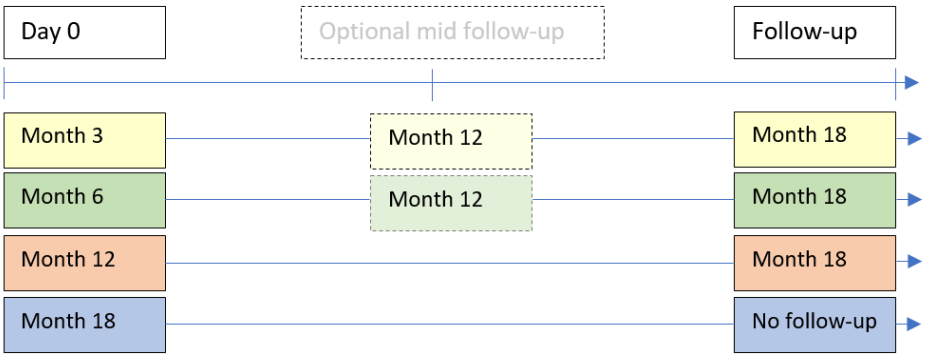
Day 0 of this questionnaire refers to the first contact with the patient. Day 0 may be allocated in different time points depending on the cohort and study. **For WP2** patients, Day 0 is the first prospective follow-up point, which could be month 3, month 6, month 12 or month 18, depending on the time of infection diagnosis. **For WP4**, Day 0 corresponds to either the first contact for an underlying condition visit or to vaccine administration. If Day 0 cannot correspond to the time of vaccine administration then Day 0 will correspond to the first post-vaccination follow-up.
Information will be collected at least at two time points for each patient, unless Day 0 corresponds to month 18 in WP2 (in this case it is unfeasible to interview the patient again). The two time points will be ≈12 months apart unless not feasible, that is when Day 0 is at month 12 or 18 for WP2.
The second time point will be:

- **For WP2**, month 18. For example, if the first prospective data collection point corresponded to the follow-up visit at month 12, then the second and last contact with such patient would take place at month 18.
- **For WP4**, month 12, which corresponds to either the 12 month vaccination follow-up or the visit of end of study.

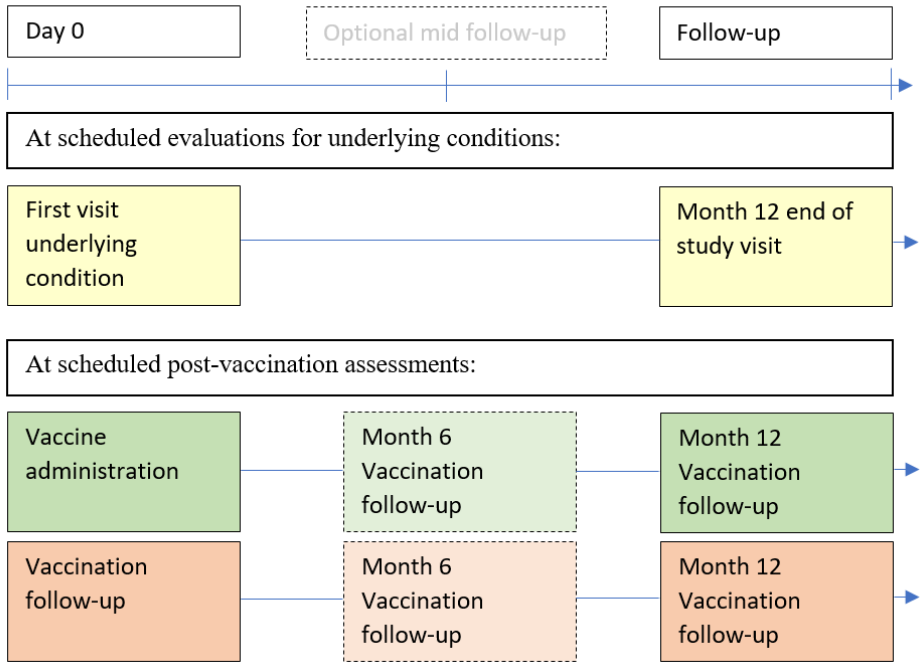
In addition to the two time points, information could also be collected ≈6 months after Day 0 whenever possible (although not mandatory).

WP2

In the context of the scheduled SARS-CoV-2 infection follow-up assessments.



WP4



ANNEX 1a – Socio-economic questionnaire

To be asked on Day 0 ONLY:

Day 0 refers to the first contact with the patient, independently of whether they have been vaccinated, whether they are positive or negative, or of the time of infection, i.e. all cohort patients should be interviewed. It is crucial to record the date of the contact/interview.

1. (This is the ID that allows to identify the patient as part of ORCHESTRA and/or the original cohort): Patient ID _____ / Date of interview _____

Demographic information:

2. What is the highest level of education that you have achieved to date?
    a. No formal school
    b. Primary school
    c. Secondary school
    d. Some university
    e. Undergraduate degree
    f. Post-graduate degree

3. What is your current marital status?
    a. Married
    b. Cohabitation
    c. Divorced/separated
    d. Widowed
    e. Single

Defining household as the family unit,

4. what is the total number of household members?

5. what is the number of household members below age 14?

Use of health care resources (COVID-19 UNRELATED/RELATED TO YOUR UNDERLYING CONDITIONS):

Since the beginning of the pandemic (February/March 2020), have you experienced:

6. any disruption in your routine visits (these are related to underlying conditions) and/or periodic check-ups (these may be, for example, regular gynecological visits for preventative reasons)?

    - Cancellation Yes/No/NA

    - Postponement Yes/No/NA

7. any interruption to the treatment you regularly follow?

- Problems accessing medication Yes/No/NA
- Postponement of your treatment session Yes/No/NA
- Cancellation of your treatment session Yes/No/NA

Socio-economic indicators

8. Which one of the following status best describes your current labour situation?
    a. Paid Employee (in a firm/office/other)
    b. Formal and remunerated self-employed
    c. Informal/occasional work (with no contract)
    d. Volunteer or other non-remunerated work
    e. Housekeeping/look after family members
    f. Permanent inability to work (with benefits)
    g. Permanent inability to work (without benefits)
    h. Temporary inability to work (with benefits): e.g., maternity leave
    i. Unemployed without benefits
    j. Unemployed with benefits
    k. Retired
    l. Student
    m. Other

Question 9 is only for those replying a, b, c or d to question 8:

9. How many total hours a week do you work on average?

10. Thinking about your income and the income of everyone who lives in your household and contributes to the household budget, what is your current total monthly household net income (after taxes)?

    a. Less than 100€
    b. 100€ to 299€
    c. 300€ to 499€
    d. 500€ to 999€
    e. 1,000€ to 1,999€
    f. 2,000€ to 2,999€
    g. 3,000€ to 3,999€
    h. 4,000€ to 4,999€
    i. 5,000€ to 5,999€
    j. More than 6,000€

Since the beginning of the pandemic (February/March 2020), have you:

11. had financial or liquidity problems (e.g. problems with paying rent, school fees, loan, utility bills, etc.)? Yes/No

12. received any financial or material aid (e.g. public assistance, cash-transfers, food banks, etc)? Yes/No

Knowledge, Risk perception, Behaviour

13. How often have you recently looked for information on COVID19 (eg: efficacy and safety of vaccines, development of vaccination plans in your country, number of new cases) in the newspapers, television, radio, social networks, etc?
    a. Several times each day
    b. Once a day
    c. 2-3 times a week
    d. Once a week
    e. Less than once a week

14. On a theoretical scale from 0 to 10 (where 0 means no risk at all and 10 means maximum risk), what do you think is your current risk of infection/ re-infection?

15. On a theoretical scale from 0 to 10 (where 0 means no risk at all and 10 means maximum risk), what do you think is your current risk that, in case of infection/re-infection, there will be severe complications or even death?

16. What do you do to protect yourself against infection or re-infection, and how often? (provide an answer on frequency for all measures specified)

	Never	Rarely	Often	Very often
Wash my hands with hand-soap / hydro-alcoholic solutions	☐	☐	☐	☐
Keep a distance of at least 2 meters from others	☐	☐	☐	☐
Stay at home / Avoid gathering with friends and relatives not belonging to my *bubble*	☐	☐	☐	☐
Use mask	☐	☐	☐	☐

17. Have you been offered a vaccine by the public health system? Yes/No/NA
18. Have you accepted or will you accept to be vaccinated once you get offered a vaccine? Yes/No/NA
19. IF NOT (for those who have not accepted or would not accept to get vaccinated): Why did/would you not accept the vaccine? (multiple replies allowed)
    a. Lack of trust in its efficacy.
    b. Fear of adverse effects.
    c. Having passed COVID-19 already, I am afraid that adverse effects will be stronger than the disease itself.
    d. Not helpful in my case as I adopt many other measures (eg: I always wear a mask).

e. I already had COVID-19 and I am sure I will not get it again.
f. I prefer someone else gets it before me.
g. I believe there are other (better) ways to prevent COVID-19 than with a vaccine.
h. Other

To be asked at the next contacts (6, 12, 18 months, whatever applies):
Follow-up interviews after Day 0.

1. (This is the ID that allows to identify the patient as part of ORCHESTRA and/or the original cohort): Patient ID _____ / Date of interview _____

Demographic information:

Defining household as the family unit,

2. what is the total number of household members?

3. what is the number of household members below age 14?

Use of health care resources (COVID-19 UNRELATED/RELATED TO YOUR UNDERLYING CONDITIONS):

Over the last 6 or 12 months, have you experienced:

4. any disruption in your routine visits (these are related to underlying conditions) and/or periodic check-ups (these may be, for example, regular gynecological visits for preventative reasons)?

   - Cancellation Yes/No/NA
   - Postponement Yes/No/NA

5. any interruption to the treatment you regularly follow?

   - problems accessing medication Yes/No/NA
   - Postponement of your treatment session Yes/No/NA
   - Cancellation of your treatment session Yes/No/NA

Socio-economic indicators

6. Which one of the following status best describes your current labour situation?
   a. Paid Employee (in a firm/office/other)
   b. Formal and remunerated self-employed
   c. Informal/occasional work (with no contract)
   d. Volunteer or other non-remunerated work
   e. Housekeeping/look after family members
   f. Permanent inability to work (with benefits)
   g. Permanent inability to work (without benefits)

h. Temporary inability to work (with benefits): e.g., maternity leave
i. Unemployed without benefits
j. Unemployed with benefits
k. Retired
l. Student
m. Other

Questions 7 is only for those replying a, b, c or d to question 6:

7. How many total hours a week do you work on average?

8. Thinking about your income and the income of everyone who lives in your household and contributes to the household budget, what is your current total monthly household net income (after taxes)?

    a. Less than 100€
    b. 100€ to 299€
    c. 300€ to 499€
    d. 500€ to 999€
    e. 1,000€ to 1,999€
    f. 2,000€ to 2,999€
    g. 3,000€ to 3,999€
    h. 4,000€ to 4,999€
    i. 5,000€ to 5,999€
    j. More than 6,000€

Over the last 6 or 12 months, have you:

9. had financial or liquidity problems (e.g. problems with paying rent, school fees, loan, utility bills, etc.)? Yes/No

10. received any financial or material aid (e.g. public assistance, cash-transfers, food banks, etc)? Yes/No

<u>Knowledge, Risk perception, Behaviour</u>

11. How often have you recently looked for information on COVID19 (eg: efficacy and safety of vaccines, development of vaccination plans in your country, number of new cases) in the newspapers, television, radio, social networks, etc?
    a. Several times each day
    b. Once a day
    c. 2-3 times a week
    d. Once a week
    e. Less than once a week

12. On a theoretical scale from 0 to 10 (where 0 means no risk at all and 10 means maximum risk), what do you think is your current risk of infection/ re-infection?

13. On a theoretical scale from 0 to 10 (where 0 means no risk at all and 10 means maximum risk), what do you think is your current risk that, in case of infection/re-infection, there will be severe complications or even death?

14. What do you do to protect yourself against infection or re-infection, and how often? (provide an answer on frequency for all measures specified)

	Never	Rarely	Often	Very often
Wash my hands with hand-soap / hydro-alcoholic solutions	☐	☐	☐	☐
Keep a distance of at least 2 meters from others	☐	☐	☐	☐
Stay at home / Avoid gathering with friends and relatives not belonging to my *bubble*	☐	☐	☐	☐
Use mask	☐	☐	☐	☐

15. Have you been offered a vaccine by the public health system? Yes/No/NA
16. Have you accepted or will you accept to be vaccinated once you get offered a vaccine? Yes/No/NA
17. IF NOT (for those who have not accepted or would not accept to get vaccinated): Why did/would you not accept the vaccine? (multiple replies allowed)
    a. Lack of trust in its efficacy
    b. Fear of adverse effects
    c. Having passed COVID-19 already, I am afraid that adverse effects will be stronger than the disease itself
    d. Not helpful in my case as I adopt many other measures (eg: I always wear a mask)
    e. I already had COVID-19 and I am sure I will not get it again
    f. I prefer someone else gets it before me
    g. I believe there are other (better) ways to prevent COVID-19 than with a vaccine.
    h. Other

SCHOOL AGE CHILDREN COHORTS WP4 - Socio-economic questions for the prospective protocol (from WP8)

In the case of children, questions will be asked to the caretaker.

To be asked on Day 0:

Day 0 refers to the first contact with the patient, independently of whether they have been vaccinated, whether they are positive or negative, or of the time of infection, i.e. all cohort patients should be interviewed. It is crucial to record the date of the contact/interview.

1. (This is the ID that allows to identify the patient as part of ORCHESTRA and/or the original cohort): Patient ID _____ / Date of interview _____

Demographic information:

2. What is the school year you are attending?

Use of health care resources (COVID-19 UNRELATED/RELATED TO YOUR UNDERLYING CONDITIONS):

Since the beginning of the pandemic (February/March 2020), have you experienced:

3. any disruption in your routine visits (for eventual underlying conditions) and/or periodic check-ups (such as pediatric visits, routine vaccination schedules)?
   - Cancellation Yes/No/NA
   - Postponement Yes/No/NA
4. any interruption to the treatment you regularly follow?
   - problems accessing medication Yes/No/NA
   - Postponement of your treatment session Yes/No/NA
   - Cancellation of your treatment session Yes/No/NA

Since the beginning of the pandemic (February/March 2020),

5. Were classes suspended at your school? Yes/No

If yes:

6. For how long were classes suspended?

7. Were you able to follow some forms of distance learning? Yes/No

If not:

8. Why could you not follow distance learning?
    a. distance learning was not organised at my school
    b. I was not interested/I was bored
    c. I could not rely on good technical means (*e.g.* computer or wifi)
    d. Other

9. In total, how many weeks of school can you estimate you have lost?

To be asked at month 6 or/and 12:
Follow-up interviews after Day 0.

1. (This is the ID that allows to identify the patient as part of ORCHESTRA and/or the original cohort): Patient ID _____ / Date of interview _____

Use of health care resources (COVID-19 UNRELATED/RELATED TO YOUR UNDERLYING CONDITIONS):

Over the last 6/12 months, have you experienced:

2. any disruption in your routine visits and/or periodic check-ups?

    - Cancellation Yes/No/NA
    - Postponement Yes/No/NA

3. any interruption to the treatment you regularly follow?

    - Problems accessing medication Yes/No/NA
    - Postponement of your treatment session Yes/No/NA
    - Cancellation of your treatment session Yes/No/NA

Over the last 6/12 months:

4. Were classes suspended at your school? Yes/No

If yes:

5. For how long were classes suspended?

6. Were you able to follow some forms of distance learning? Yes/No

If not:

7. Why could you not follow distance learning?
    a. distance learning was not organised at my school
    b. I was not interested/I was bored
    c. I could not rely on good technical means (*e.g.* computer or wifi)
    d. Other

8. In total, how many weeks of school can you estimate you have lost?

ANNEX 2 – Psychological scale Questionnaires

0. **Time log for synchronizing analyses across cohorts and countries**
1. **Generalized Anxiety Disorder 7-item (GAD-7) scale**
2. **Perceived Stress Scale - 4 Item version**
3. **UCLA Loneliness Scale-short version**
4. **Brief Resilience Scale (BRS)**
5. **Depression Scale (CES-D Scale)**

0. Time log for simplifying analyses across cohorts and countries [BASELINE][FOLLOW-UP]

Rational for this part: In order to compare effects of pandemic measures across different countries, we need to synchronize and include these questions about the prevention measures in place at the time assessment.

0.1 During the last two weeks have you been in a lockdown?

1	Yes
2	No

0.2 During the last two weeks have the schools been closed?

1	Yes
2	No
3	Not relevant for me, as I do not have children or I'm not working in a school

0.3 During the last two weeks did you need to do home office (either completely or only on several days/week)?

1	Yes
2	No

0.4 During the last two weeks were you obliged to wear masks in closed public areas (e.g. theaters, restaurants, cinema ,…)?

1	Yes
2	No

0.5 During the last two weeks were you obliged to wear masks in open public areas (e.g. parks, playgrounds,)?

1	Yes
2	No

0.6 During the last two weeks were you obliged to follow the 'vaccinated-tested-recovered' rule to:

1	go to work
2	go to a restaurant/bar
3	go to a shopping center
4	go to the cinema
5	go to a museum
6	go to sports events
7	go to any other place/event not mentioned before

0.7 If '7 - go to any other place/event not mentioned before' in 0.6, please specify – open field.

0.8 During the last two weeks did you have to keep a minimum distance of 1.5 or 2 meters when contacting people outside your household?

1	Yes
2	No

1. Generalized Anxiety Disorder 7-item (GAD-7) scale [BASELINE][FOLLOW-UP]

Over the last 2 weeks, how often have you been bothered by the following problems?

	Not at all	Several days	More than half the days	Nearly every day
Feeling nervous, anxious, or on edge	❍	❍	❍	❍
Not being able to stop or control worrying	❍	❍	❍	❍
Worrying too much about different things	❍	❍	❍	❍
Trouble relaxing	❍	❍	❍	❍
Being so restless that it is hard to sit still	❍	❍	❍	❍
Becoming easily annoyed or irritable	❍	❍	❍	❍
Feeling afraid as if something awful might happen	❍	❍	❍	❍

2. Perceived Stress Scale - 4 Item version [BASELINE][FOLLOW-UP]

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, please indicate with a check how often you felt or thought a certain way.

	Never	Almost never	Sometimes	Fairly often	Very often
In the last month, how often have you felt that you were unable to control the important things in your life?	❍	❍	❍	❍	❍
In the last month, how often have you felt confident about your ability to handle your personal problems?	❍	❍	❍	❍	❍
In the last month, how often have you felt that things were going your way?	❍	❍	❍	❍	❍

3. UCLA Loneliness Scale-short version [BASELINE][FOLLOW-UP]

The next questions are about how you feel about different aspects of your life. For each one, tell how often you feel that way.

	Hardly ever or never	Some of the time	Often
How often do you feel that you lack companionship?	❍	❍	❍
How often do you feel left out?	❍	❍	❍
How often do you feel isolated from others?	❍	❍	❍

4. Brief Resilience Scale (BRS) [BASELINE][FOLLOW-UP]

Please indicate the extent to which you agree with each of the following statements by using the following scale:

	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
I tend to bounce back quickly after hard times	❍	❍	❍	❍	❍
I have a hard time making it through stressful events	❍	❍	❍	❍	❍
It does not take me long to recover from a stressful event	❍	❍	❍	❍	❍
It is hard for me to snap back when something bad happens	❍	❍	❍	❍	❍
I usually come through difficult times with little trouble	❍	❍	❍	❍	❍
I tend to take a long time to get over set-backs in my life	❍	❍	❍	❍	❍

5. Center for Epidemiologic Studies Depression Scale (CES-D Scale) [BASELINE][FOLLOW-UP]

Below is a list of the ways you might have felt or behaved. Please tell me how often you have felt this way during the past week.
During the last week …

	Rarely or None of the time (less than 1 day)	Some or a Little of the time (1 - 2 days)	Occasionally or a moderate amount of time (3 - 4 days)	Most or All of the time (5 - 7 days)
I was bothered by things that usually don't bother me	❍	❍	❍	❍
I did not feel like eating; my appetite was poor	❍	❍	❍	❍
I felt that I could not shake off the blues even with help from my family or friends	❍	❍	❍	❍
I felt that I was just as good as other people	❍	❍	❍	❍
I had trouble keeping my mind on what I was doing	❍	❍	❍	❍
I felt depressed	❍	❍	❍	❍
I felt that everything I did was an effort	❍	❍	❍	❍
I felt hopeful about the future	❍	❍	❍	❍
I thought my life had been a failure	❍	❍	❍	❍
I felt fearful	❍	❍	❍	❍
My sleep was restless	❍	❍	❍	❍
I was happy	❍	❍	❍	❍
I talked less than usual	❍	❍	❍	❍
I felt lonely	❍	❍	❍	❍
People were unfriendly	❍	❍	❍	❍
I enjoyed life	❍	❍	❍	❍
I had crying spells	❍	❍	❍	❍
I felt sad	❍	❍	❍	❍
I felt that people dislike me	❍	❍	❍	❍
I could not get "going"	❍	❍	❍	❍

ANNEX 3 – Questionnaire for monitoring of adverse events to COVID-19 vaccination

A) Demographics – Anamnesis

Age: _____

Sex: □ M □ F

Special population: □ HIV □ Cancer □ Solid organ transplant □ Haematological stem cell transplant

If solid organ transplant, please specify: □ Liver □ Kidney □ Heart □ Lung

Drug/food allergies: □ Y □ N If yes, please specify:_____

Previous serious adverse events to vaccination: □ Y □ N

Underlying diseases:

□ Renal □ Cardiovascular □ Asthma □ Diabetes mellitus □ Anaemia/Blood disorders □ Seizure/Epilepsy

Other nervous system disease: □ Y □ N If yes, please specify:_____

Concomitant medications:_____

Blood transfusion in the last year: □ Y □ N

Treatment with immunoglobulin or antivirals in the last year: □ Y □ N

Other relevant conditions: _____

B) Adverse event report

First dose (dd/mm/yy): _____

Second dose (dd/mm/yy): _____

Clinical events after the first dose: □ Y □ N

Clinical events after the first dose: □ Y □ N

Headache: □ Y □ N

Asthenia: □ Y □ N

Fever (≥38°C): □ Y □ N

Nausea: □ Y □ N

Vomiting: □ Y □ N

Lymphadenopathy: □ Y □ N

Tachycardia: □ Y □ N

Flushing: □ Y □ N

Syncope: □ Y □ N

Pruritus, erythema or pain in injection site: □ Y □ N

Generalised urticaria: □ Y □ N

Serious allergic events (anaphylaxis, angioedema): □ Y □ N

COVID infection after vaccination: □ Y □ N

Use of paracetamol or non-steroidal anti-inflammatory drugs: □ Y □ N

Requirement for use of other drugs: □ Y □ N If Yes, please specify: _____

Demyelinating disorders: □ Y □ N

Encephalitis/Encephalopathy: □ Y □ N

Peripheral neuropathy: □ Y □ N

Muscular weakness and/or paralysis: □ Y □ N

Visual impairment (including optic neuritis): □ Y □ N

Walking disturbance: □ Y □ N

Blood disorders (anaemia, thrombocytopenia, leukopenia): □ Y □ N

Seizures/Epilepsy: □ Y □ N

Weakness and/or paralysis of facial muscle: □ Y □ N

Vasculitis: □ Y □ N

Renal disorders: □ Y □ N

Liver function disorders: □ Y □ N

Cardiac disorders: □ Y □ N

Other adverse events: □ Y □ N If Yes, please specify: _____

If yes (complete for each reported event): day of onset (dd/mm/yy):_____

hospitalization/emergency department admission/general practitioner visit: □ Y □ N

Description of the event (laboratory data/administered drugs/other relevant information):

_____

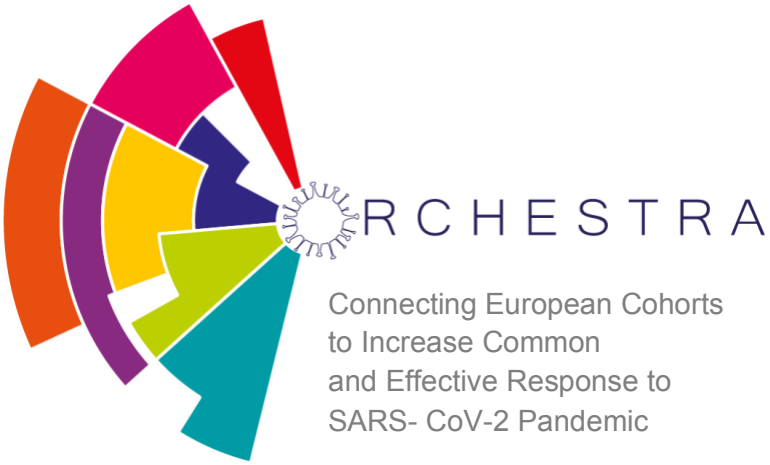
_____

_____

_____

Recovery: □ Y □ N

ANNEX 4 – WP6 Deliverable 6.1_Mapping of retrospective samples and development of standardized protocols for prospective sampling



WP6 Deliverable 6.1

Mapping of retrospective samples and development of standardised protocols for prospective sampling

UANTWERPEN

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Project Classification

Project Acronym:	ORCHESTRA
Project Title:	Connecting European Cohorts to Increase Common and Effective Response to SARS- CoV-2 Pandemic
Coordinator:	UNIVR
Grant Agreement Number:	101016167
Funding Scheme:	Horizon 2020
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Duration:	36 months
Website:	www.orchestra-cohort.eu
Email:	info@orchestra.eu

Document Classification

WP No:	WP6
Deliverable No:	D6.1
Title:	Standardised protocol and manual on collection and storage of samples
Lead Beneficiary:	UANTWERPEN
Other Involved Beneficiaries:	UNIBO, INSERM, HMGU
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Dissemination Level:	Confidential
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Author(s):	Surbhi Malhotra-Kumar, Matilda Berkell

History of Changes

Version	Date	Created/Modified by
1		Surbhi Malhotra-Kumar, Matilda Berkell

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Executive summary

WP Context

The objective of WP6 in the ORCHESTRA project is to study human (epi)genetic, immunological, microbial, and viral features to identify markers of disease severity and to study the long-term impact of SARS-CoV-2 infection as well as the effect of immunization and characteristics underlying breakthrough infections post vaccination. Samples will be included retrospectively as well as prospectively within the study to study severe COVID-19 infection in hospitalized and outpatients during the acute disease stage as well as study long-term effects of infection and immunization on the host immune response.

Purpose of the document

The purpose of the WP6 protocols is to provide an overview of the biosamples to be collected within the prospective ORCHESTRA study, at which timepoints, and which analyses these samples are to be subjected to.

Content of the document

The WP6 protocol outlines the objectives of the biological studies foreseen within the project, the methodology to be utilized to meet these goals, which patient populations that are to be analyzed, and how these samples are to be handled and stored.

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Target of the document

The target audience of the document are the clinical WPs 2-5 within the ORCHESTRA study in which prospective sample collection is conducted.

Dissemination level

Confidential, only for members of the consortium (including the Commission Services).

Core content

The WP6 prospective protocol document is available in Appendix 1.

References

N/A

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ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Appendix 1

PROSPECTIVE SAMPLE COLLECTION AND MANAGEMENT DOCUMENT

WP6 – Biobanking, genomics, and viral-host interactions

University of Antwerp (UANTWERPEN) in collaboration with all WP6 partners

Version dated 11-05-2021

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Thank you for your participation in the ORCHESTRA study. The purpose of this document is to provide you with additional information and instructions on the collection and processing of samples for WP6 within the prospective tier of ORCHESTRA. This document is a guideline for research staff including (sub-) investigators, research nurses, and laboratory staff. The clinical protocol takes precedence over this document. If there is a discrepancy between the sample collection and management document and the clinical protocol, the ORCHESTRA clinical protocol should be followed.

This document outlines how samples should be handled after patient inclusion. Specifically, it describes the time points desired for biosampling.

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Project Classification

Project Acronym:	ORCHESTRA
Project Title:	Connecting European Cohorts to Increase Common and Effective Response to SARS- CoV-2 Pandemic
Coordinator:	UNIVR
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Funding Scheme:	Horizon 2020
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Website:	www.orchestra-cohort.eu
Email:	info@orchestra.eu

Document Classification

Title:	PROSPECTIVE SAMPLE COLLECTION AND MANAGEMENT DOCUMENT
Lead Beneficiary:	UANTWERPEN
Other Involved Beneficiaries:	INSERM, APHP, UNIBO, HMGU
PIs, Participating centres	Surbhi-Malhotra-Kumar, UANTWERPEN Samir Kumar-Singh, UANTWERPEN Patrizia Brigidi, UNIBO Valerio Carelli, UNIBO Benoit Visseaux, INSERM/APHP Laurent Abel, INSERM Anette Peters, HMGU
Authors:	Surbhi Malhotra-Kumar, Samir Kumar-Singh, Matilda Berkell, Patrizia Brigidi, Benoit Visseaux

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2.0		

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ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

LIST OF ABBREVIATIONS

APHP	Assistance Publique – Hôpitaux de Paris
COVID-19	Coronavirus Disease 19
DMSO	Dimethyl Sulfoxide
eCRF	Electronic Clinical Research Form
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HMGU	Helmholtz Zentrum München
IFN	Interferon
INSERM	Institut National de la Santé et de la Recherche Médicale
LMM	Laboratory of Medical Microbiology
MSD	MesoScale Discovery
NGS	Next Generation Sequencing
NP	Nasopharyngeal
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
UANTWERPEN	University of Antwerp
UNIBO	University of Bologna
UTM	Universal Transport Medium
VOC	Variant of Concern
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

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1. INTRODUCTION TO ORCHESTRA

ORCHESTRA is a three-year international research project aimed at tackling the coronavirus pandemic, led by the University of Verona and involving 26 partners (extending to a wider network of 37 partners) from 15 countries: Argentina, Belgium, Brazil, Congo, France, Gabon, Germany, India, Italy, Luxemburg, Netherlands, Romania, Slovakia, Spain, Venezuela. The project is divided into a retrospective and a prospective tier in which clinical data and biosamples are analysed in order to

- develop evidence-based recommendations for effective prevention, protection and optimized treatment of COVID-19 patients (including long-term sequelae) with a special focus on 'at risk' populations, including healthcare workers and fragile individuals
- assess impact of environmental factors, socio-economic determinants, lifestyle and confinement measures on the spread of COVID-19
- provide knowledge on the efficacy of vaccines against SARS-CoV-2
- provide a model for responsiveness for future pandemic outbreaks.

2. OBJECTIVES AND BRIEF DESCRIPTION OF SUBSTUDIES WITHIN WP6

Several different sample types will be analysed within the ORCHESTRA study for the purposes of identifying human and viral genetic markers indicative of disease severity as well as to study immune responses over time as a result of infection and immunization. Specifically, samples will be collected from patients developing COVID-19 (including breakthrough and reinfection) in order to study both short- and long-term effects of infection on host immunity, respiratory and intestinal microbiome dynamics as well as host and viral genetic determinants underlying infection. Additionally, samples will be collected from vaccinated fragile populations as well as vaccinated healthcare workers in order to study effects of vaccination on host immunity and respiratory and intestinal microbiome dynamics.

Samples collected within the framework of the ORCHESTRA study will in many cases be subjected to more than one type of analysis. To provide an overview of research questions of interest within the project, a brief summary of each analysis can be found below.

2.1 CHARACTERISATION OF SARS-COV-2 VIRAL VARIANTS (TASK 6.2)

This task will target two main points among selected patient populations: [i] to characterise the viral variants and to identify variants of concern (VOCs) by whole genome sequencing (WGS) in COVID-19 patients and in vaccinated individuals with breakthrough infections, and [ii] to study potential

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mutation selection in populations presenting with long viral replication or receiving immunoglobulin therapies, and [iii] to study the viral replication (viral load and excretion duration).

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2.2 CHARACTERISATION OF SEROLOGICAL MARKERS OF SARS-COV-2 INFECTION (TASK 6.3)

This task will characterize the host antibody responses with quantitative serology (anti-S and anti-N) on Abbott, Roche, MesoScale Discovery (MSD), or similar platforms as well as (pseudo-) seroneutralisation assays. These assays will be performed on populations with various degrees of COVID-19 severity and vaccinated individuals, also with breakthrough infections.

2.3 CHARACTERISATION OF CELLULAR IMMUNITY FOR SARS-COV-2 INFECTION (TASK 6.4)

Vaccinated and non-vaccinated COVID-19 patients with varying degrees of disease severity and SARS-CoV-2-positive non-symptomatic individuals will be studied for the balance and the phenotypes of T and B cells as a function of disease course and severity as well as response to vaccination. This task will chiefly employ flow cytometric analyses with CD45, CD3, CD4, CD19, FOXP3, Ki-67, CD38 markers, viability and IFNγ release assays.

2.4 CYTOKINOME ANALYSIS (TASK 6.5)

Vaccinated and non-vaccinated COVID-19 patients with varying degrees of disease severity, SARS-CoV-2-positive non-symptomatic individuals and vaccinated individuals will be studied on MSD panels, Luminex panels and on select ELISAs. As an outcome, panels of cytokine markers predicting disease severity, mortality, breakthrough infections, and long term sequalae will be generated.

2.5 NEXT GENERATION SEQUENCING (NGS) OF COVID-19 COHORTS (TASK 6.6)

In-depth human genetic analysis will be conducted using WGS or whole exome sequencing (WES) followed by functional analyses of the most promising variants. Additionally, serum samples will be utilized for detection of auto-antibodies against type I interferons (IFNs).

2.6 EPIGENOME-WIDE ANALYSES (TASK 6.7)

Genome-wide methylation analyses of COVID-19-positive patients in addition to a small number of control patients will enable differentiation of inherited and acquired genomic regulatory features through COVID-19 infection, which result in severe disease or an efficient clearing of infection through immune responses.

2.7 INTESTINAL MICROBIOME PROFILING (TASK 6.8)

This task will profile compositional and functional structures of the microbiome from faecal samples by NGS approaches, in order to elucidate the role of the intestinal microbiome in the susceptibility, progression and severity of COVID-19 infection.

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2.8 RESPIRATORY MICROBIOME DYNAMICS (TASK 6.9)

We will investigate differences in the respiratory microbiome composition by combining meta-transcriptomic and metagenomic sequencing to analyse both RNA and DNA viruses and the bacterial and fungal fractions. This will firstly elucidate the role of commensal flora and of co-infecting respiratory pathogens in influencing COVID-19 disease severity. Secondly, long-term carriage and impact of SARS-CoV-2 on the respiratory microbiome will be assessed on longitudinally collected prospective samples (6-12 months post-recovery and new infections).

3. SAMPLE TIMEPOINTS

An overview of sample types to be collected at each time point can be found in Table 1 below. No collection material will be provided within this study, but recommended materials to be used for each collection can be found in the section entitled “Detailed sample collection and storage instructions”.

Table 1. Overview of required biosamples associated analysis within WP6. Task numbers correspond to tasks described in the Description of Work, Amendment 1, Annex 1.

Sample type	Task	Analysis
NP swab	Task 6.2	Viral variant sequencing
	Task 6.9	Respiratory microbiome analysis
Serum	Task 6.3	Serology
	Task 6.6	Assessment of auto-antibodies against type I IFNs
EDTA plasma	Task 6.5	Cytokinome analysis
Heparin blood (PBMCs)	Task 6.4	Cellular immunity characterization
EDTA whole blood	Task 6.6	Human WGS or WES
	Task 6.7	Epigenomics
Stool sample (faeces or rectal swab)	Task 6.8	Intestinal microbiome analysis

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3.1 SAMPLING TIMEPOINTS FOR COVID-19 PATIENTS (INCLUDING REINFECTIONS AND BREAKTHROUGH INFECTIONS IN VACCINATED INDIVIDUALS)

3.1.1 Sample collection and management

Sampling is required to be performed on the day of diagnosis and at following timepoints as shown in Table 2. Patient inclusion will primarily be based on availability of informed consent for the outlined tasks as well as availability of multiple samples per patient. Informed consent forms should clearly request permission to perform human genetic and epigenetic analyses, without which these analyses cannot be undertaken.

Table 2. Overview sampling and data collection in ORCHESTRA cohorts in COVID-19 patients including long-term sequelae.

	D0[1]	3 months[1] ±1 month	6 months[1] ±1 month	12 months[1] ±1 month	18 months[1] ±2 month	Objective
NP swab	x	x[2,3]	x[2,3]	x[2,3]	x[2]	Viral variant and metagenomic sequencing
2 x 2 mL serum tube (serum)	x	x	x	x	x	Immune - serology and type I IFNs autoantbodies
4 mL EDTA blood tube (plasma)	x	x[4]	x[4]	x[4]	x[2]	Immune - cytokine and chemokine
1 (if possible 2) 9 mL heparin tube (PBMCs)	x	x[4]	x[4]	x[4]	x[2]	Immune - cellular
2 x 2 mL EDTA tube (whole blood)	x	x	x	x	x	Genetic and epigenetic analyses
Stool sample (faeces or rectal swab)	x		x		x	Metagenomic sequencing

1. Day 0: first positive SARS-CoV-2 PCR test. Follow-up of 3, 6, 12, and 18 months start from Day 0.
2. Reassessed only if outside the normal ranges at the previous assessment or if clinically indicated.
3. At least one of the three timepoints (month 3, month 6, month 12) is required to perform metagenomic analyses.
4. At least one of the three timepoints (month 3, month 6, month 12) is required.

Level I	Assessments of Level I are mandatory
Level II	Customized according to the feasibility of each cohort

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3.2 SAMPLING TIMEPOINTS FOR VACCINATED INDIVIDUALS

3.2.1 Sample collection and management

Vaccinated individuals in WP4 and WP5 will be sampled according to the time points outlined in Table 3. In case of breakthrough infections post vaccination, samples will be collected as outlined in Table 2. Vaccination is (mostly) performed in two doses. Collection is to be performed prior to administration of dose 1 and dose 2, and 3, 6, and 12 months after the first dose (Table 3). Patient inclusion will primarily be based on availability of informed consent for the outlined tasks as well as availability of multiple samples per patient. Informed consent forms should clearly request permission to perform human genetic and epigenetic analyses, without which these analyses cannot be undertaken.

Table 3. Overview sampling and data collection in ORCHESTRA cohorts for vaccinated individuals.

	1st dose	2nd dose[1]	3 months[2] (± 1 month)	6 months[2] (± 2 months)	12 months[2] (± 3 months)	Objective
NP swab	x[3]	x[3]	x[4]	x[4]	x[4]	Viral variant and metagenomic sequencing
2 x 2 mL serum tube (serum)	x	x	x	x	x	Immune - serology and type I IFNs autoantbodies
4 mL EDTA blood tube (plasma)	x	x[5]	x[5]	x[5]	x[5]	Immune - cytokine and chemokine
1 (if possible 2) 9 mL heparin tube (PBMCs)	x	x[5]	x[5]	x[5]	x[5]	Immune - cellular
2 x 2 mL EDTA tube (whole blood)	x		x	x	x	Genetic and epigenetic analyses
Stool sample (faeces or rectal swab)	x		x			Metagenomic sequencing

1. The assessment at 2nd dose is mandatory in patients who will receive such dose within 8-12 weeks after first dose (current AstraZeneca vaccination schedule).
2. 3, 6, and 12 months counted from 1st dose.
3. At least one timepoint at 1st or 2nd dose is required.
4. At least one of the three timepoints (month 3, month 6, month 12) is required.
5. At least one of the four timepoints (2nd dose, month 3, month 6, month 12) is required.

Level I	Assessments of Level I are mandatory
Level II	Customized according to the feasibility of each cohort

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4. DETAILED SAMPLE COLLECTION AND STORAGE INSTRUCTIONS

4.1 NP SWAB COLLECTION

4.1.1 Recommended materials

Swabs

- NP FLOQSwabs – Regular flocked swab (Copan Italia, Cat. No. 503CS01)

Storage media (in order of preference) – use 1mL storage media per swab

- DNA/RNAShield (Zymo Research, Cat. No. R1100-50 / R1100-250)
- TRIzol (Invitrogen, Thermofisher Scientific, Cat. No. 15596026)
- UTM Tubes – 12x80 mm tube size prefilled with 1 mL UTM (COPAN Italia, Cat. No. 350C)
- RNALater (Thermofisher Scientific, Cat. No. AM7021)

Swab-medium combinations (in order of preference)

- DNA/RNA Shield collection tube with swab – 12x80 mm screwcap vial pre-filled with 1 mL DNA/RNA Shield (Zymo Research, Cat. No. R1107)
- NP UTM flocked Swabs – Regular NP FLOQSwab (Sterile) with tube (12x80 mm) prefilled with 1 mL UTM (COPAN Italia, Cat. No. 360C)
- Sigma Virocult – Liquid viral transport media (1 to 2 mL) and regular flocked swab (Cat. MW951S or MW951S2ML)

Due to the large number of available materials, we cannot provide an exhaustive list of acceptable swab-medium combinations. We have presented some of the most common and well accepted solutions. If another is used for collection, it should contain a universal viral transport medium and not only be adapted to a single PCR technology (such as those provided by several RT-PCR manufacturers) with a transport medium volume ranging from 1 to 2 mL, and using a flocked swab.

4.1.2 Sample collection

1. Label the tube containing the storage medium as instructed in the section entitled “Labelling instructions”.
2. Register the sample collection in the Requisition Form displayed in Appendix X.
3. Collect the sample according to your routine practice protocol.
4. Immediately after collection, place the swab into the tube containing storage medium. Ensure that the entire swab is immersed in medium.
5. Break the swab at the scored line as instructed by the manufacturer.

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6. Using the aid of the cap, push that swab in the tube and close the cap tightly
7. Transfer the sample to the Local Laboratory.

4.1.3 Storage conditions

- At arrival in the Local Laboratory, store the tube containing storage medium and the swab in designated boxes in the freezer at -70°C or below until shipment.
- In case you do not have immediate access to a -70°C freezer, store them at -20°C and transfer them to a -70°C as soon as possible and within 2 days. Keep them at -70°C until shipment is arranged.

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4.2 SERUM SAMPLE COLLECTION

4.2.1 Recommended materials

- 2 mL BD Vacutainer Serum tube (e.g., BD #368492)
- 2 mL Cryovial (e.g., Simport # T309-2A)
- Disposable plastic pipettes (2.5, 5 mL size)

4.2.2 Sample collection

1. Label the Serum tubes as instructed in the section entitled "Labelling instructions".
2. Register the sample collection in the Requisition Form displayed in Appendix X.
3. Draw the patient's blood into two Serum tubes (2 mL).
4. Slowly and gently invert the tubes 180° and back 5-6 times.
5. Transfer as soon as possible (within one hour) to the Local Laboratory.

4.2.3 Sample processing

6. Before centrifugation, allow blood to clot thoroughly for 60 minutes.
7. Label the tubes as instructed in the section entitled "Labelling instructions".
8. Centrifuge the sample at 1300 g for 10 min at 20°C WITH THE BRAKE ON.
9. Transfer approx. 1 mL supernatant from each tube into separate cryovials using sterile disposable pipette taking care to not disturb the buffy coat.

4.2.4 Storage conditions

- After processing, store the cryovials as soon as possible in your freezer at -70°C or below until shipment.
- In case you do not have immediate access to a -70°C freezer, store them at -20°C and transfer them to a -70°C as soon as possible and within 2 days. Keep them at -70°C until shipment is arranged.

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4.3 EDTA PLASMA ISOLATION

In this section, two protocols for EDTA plasma isolation have been described. Depending on the laboratory protocol utilized, material required for isolation may vary.

4.3.1 Recommended materials

Common materials

- 4 mL BD Vacutainer K2E (EDTA) (e.g., BD Cat. No. 368861)
- 3 mL vial (e.g., Simport Cat. No. T309-3A)
- Disposable plastic pipettes (2.5, 5 mL size)

EDTA Plasma Protocol 1: Single-spin EDTA plasma isolation

- No additional material is required.

EDTA Plasma Protocol 2: Double-spin EDTA plasma isolation

- 3 mL BD Vacutainer EST Tubes (e.g., BD Cat. No. 362725)

4.3.2 Sample collection

1. Label the K2E (EDTA) tube as instructed in the section entitled “Labelling instructions”.
2. Register the sample collection in the Requisition Form displayed in Appendix X.
3. Draw the patient’s blood into the EDTA tube (4 mL).
4. Slowly and gently invert the tube 180° and back 8-10 times.
5. Transfer as soon as possible (within one hour) to the Local Laboratory.

4.3.3 Sample processing

EDTA Plasma Protocol 1: Single-spin EDTA plasma isolation

6. The samples should be processed within 120 minutes.
7. Label the 3 mL vial as instructed in the section entitled “Labelling instructions”.
8. Centrifuge the sample at 1300 g for 10 min at 20°C WITH THE BRAKE ON. This will give three layers: (from top to bottom) plasma, leucocytes (buffy coat), and erythrocytes.
9. Transfer approx. 2 mL of plasma into the 3 mL vial using sterile disposable pipette taking care to not disturb the buffy coat.

EDTA Plasma Protocol 2: Double-spin EDTA plasma isolation

6. The samples should be processed within 120 minutes.
7. Label the 3 mL vial as instructed in the section entitled “Labelling instructions”.
8. Register the sample collection in the Requisition Form displayed in Appendix X.

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9. Centrifugation I: Centrifuge the sample at 1500 g for 15 min at 20°C WITH THE BRAKE ON. This will give three layers: (from top to bottom) plasma, leucocytes (buffy coat), and erythrocytes.
10. Collection of supernatant I: Transfer the plasma in a 3 mL centrifugation tube (e.g. 3 mL BD Vacutainer EST Tube) using sterile disposable pipette taking care to not disturb the buffy coat.
11. Centrifugation II: Centrifugation at 2000 g for 15 min at 20°C WITH THE BRAKE ON to remove all potentially remaining cells.
12. Collection of supernatant II: Transfer approx. 2 mL of plasma into the 3 mL vial using sterile disposable pipette taking care to not disturb the buffy coat.

4.3.4 Storage conditions

- After processing, store the cryovial as soon as possible in your freezer at -70°C or below until shipment.
- In case you do not have immediate access to a -70°C freezer, store it at -20°C and transfer it to a -70°C as soon as possible and within 2 days. Keep them at -70°C until shipment is arranged.

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4.4 HEPARIN PLASMA COLLECTION AND PBMC ISOLATION

In this section, three protocols for PBMC isolation have been described (Table 4). Depending on the laboratory protocol utilized, material required for isolation may vary. PBMC Protocol 1 and PBMC Protocol 2 are slightly more expensive but are time saving and easier to perform. These protocols employ specialized tubes that provide a clear separation, especially PBMC Protocol 1. PBMC Protocol 3 is a conventional protocol of PBMC isolation based on Ficoll-Paque as a separating medium, no specialized tubes are needed here.

Table 4 also provides an approximate cost per sample by each of these procedures. These prices include the cost of tubes and separation media. Please note that these calculations are based on prices offered to UANTWERPEN in Belgium (Table 5). Sites are advised to get the quotations by the local distributors.

Table 4. Common PBMC isolation procedures.

	PBMC isolation procedures		Cost per sample		
			Low volume	Bulk	Comment
1	SepMate tube / Ficoll-Paque PLUS	Protocol 1	8.83 €	5.07 €	Sepmate utilizes 15 mL separation medium (can process 4 mL to 17 mL blood)
2	LeucoSep tube / Lymphoprep medium	Protocol 2	7.94 €	4.59 €	LeucoSep utilizes 15 mL separation medium (can process 4 mL to 17 mL blood)
3	Falcon 50 mL tube / Ficoll-Paque PLUS	Protocol 3	5.93 €	3.84 €	Ficoll-Paque Plus separation utilizes 15 mL separation medium (can process 4 mL to 17 mL blood)
4	LeucoSep tube with pre-filled Lymphoprep	optional	7.20 €	7.20 €	LeucoSep tubes are pre-filled with Lymphoprep
5	BD CPT (tube with pre-filled Ficoll-Paque)	optional	15.11 €	15.11 €	CPT tubes are pre-filled with Ficoll-Paque

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Table 5. Cost of different centrifuge tubes and separation media.

Product name	Supplier	Catalogue number	Cost	Unit price (EUR) per mL/tube
Media				
Ficoll-Paque PLUS	Cytiva	17144003 (6x100mL)	6 x 100 mL – 225EUR	0.38
Ficoll-Paque PLUS	Cytiva	17144002 (6x500mL)	6 x 500 mL - 706EUR	0.24
Lymphoprep	StemCell	07801 (250mL)	250 mL - 100 EUR	0.40
Lymphoprep	StemCell	07851 (500mL)	500 mL - 156 EUR	0.31
Lymphoprep	StemCell	07811 (4x250mL)	4x250 mL - 227 EUR	0.23
Lymphoprep	StemCell	07861 (6x500mL)	6x500 mL - 531 EUR	0.18
Centrifuge tubes				
Falcon 50 mL tubes	Greiner	227261	500 tubes - 51,40 EUR	0.10
LeucoSep	Greiner	227289 (non-sterile)	300 tubes - 520,25EUR	1.73
LeucoSep	Greiner	227290 (sterile)	300 tubes - 430,00EUR	1.43
SepMate	StemCell	85450 (100 tubes)	100 tubes - 310EUR	3.10
SepMate	StemCell	85460 (500 tubes)	500 tubes - 882EUR	1.76
Centrifuge tubes prefilled with separation medium				
BD CPT (pre-filled Ficoll-Paque)	BD	362753	60 tubes - 894EUR	14.90
LeucoSep (pre-filled Lymphoprep)	Greiner	227288 (prefilled)	25 tubes - 174,96EUR	7.00

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4.4.1 Recommended materials

Common materials

- 9 mL Lithium Heparin Tube (e.g., Greiner #455084)
- Phosphate Buffered Saline (PBS) (e.g., Lonza #17-516F)
- Fetal Bovine Serum (FBS), heat inactivated (e.g., Sigma #F7524)
- Falcon tubes, 50 mL (e.g., Greiner #227261)
- Dimethyl Sulfoxide (DMSO) (e.g., Sigma #D2650)
- Cryo freezing tubes (e.g., Greiner #126263)
- Cryo freezing container (e.g., Nalgene #5100-0001)
- Pipette tips, 5-1000 µL
- Disposable plastic pipettes (5, 10, 25, and 50 mL size)

PBMC Protocol 1: PBMC isolation with SepMate tube/ Ficoll-Paque Plus or Lymphoprep

- Ficoll-Paque Plus (e.g., Cytiva #GE17-1440-02). Alternatively, Lymphoprep (StemCell #1114547) can also be used
- SepMate tubes (e.g., StemCell #15440)

PBMC Protocol 2: PBMC isolation with Leucosep / Lymphoprep

- RPMI-1640 medium with L-glutamine and Hepes 25 mM (e.g., Lonza #12-115F)
- L-glutamine (e.g., Lonza #BE17-605E)
- Gentamycin (e.g., Sigma #G1397)
- Penicillin-Streptomycin (e.g., Sigma #DE17-602E)
- Lymphoprep (e.g., Lymphoprep #1114547)
- Leucosep tubes (e.g., Greiner #227290)
- Trypan blue stain (0,4%) (e.g., Gibco #15250-061)

PBMC Protocol 3: Conventional Ficoll-Paque PBMC isolation protocol

- Ficoll-Paque Plus (e.g., Cytiva #GE17-1440-02)

4.4.2 Sample collection

1. Label the Heparin tube as instructed in the section entitled “Labelling instructions”.
2. Register the sample collection in the Requisition Form displayed in Appendix X.

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3. Draw the patient's blood into one or two Heparin tubes (9 ml).
4. Gently invert the tube 180° and back 5-6 times.
5. Transfer as soon as possible (within one hour) to the Local Laboratory.

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4.4.3 Sample processing

PBMC Protocol 1: PBMC isolation with SepMate tube/ Ficoll-Paque Plus or Lymphoprep

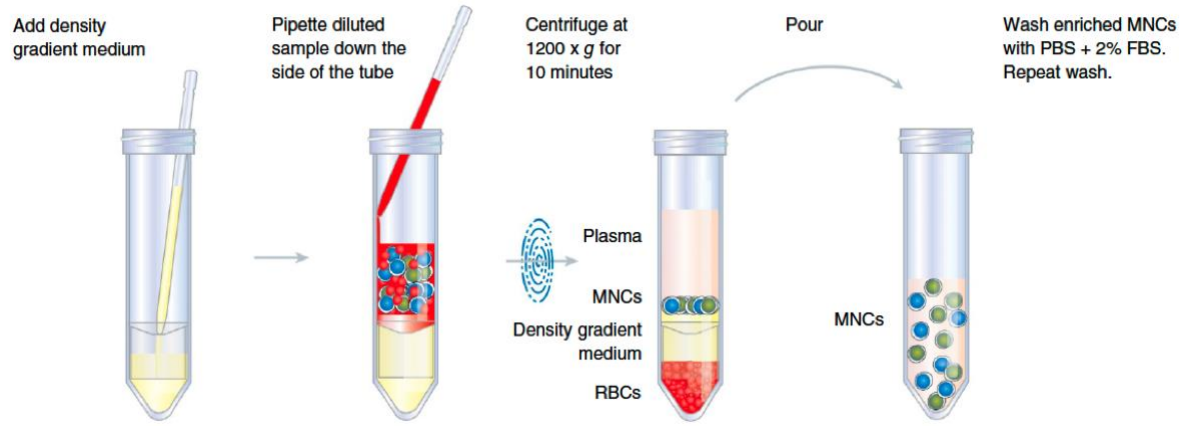

Figure 1. PBMC isolation procedure using SepMate tubes.

Buffer and other reagents preparation

1. Warm up PBS + 2% FBS at 37°C.
2. Ficoll-Paque or Lymphoprep (stored at ambient temperature of 18-20°C).
3. Heat inactivated (56°C – 30 min) FBS and heat inactivated FBS-DMSO 20% at 4°C.
4. Keep cryo freezing container (with isopropanol 100%) at 4°C. Replace isopropanol after 5 freezings.

PBMC Isolation (day of scheduled study visit)

5. Label each tube as instructed in the section entitled "Labelling instructions".
6. Add 15 mL density gradient medium to the SepMate tube by carefully pipetting it through the central hole of the SepMate insert. The top of the density gradient medium will be above the insert.
7. Spin tubes at 200 x g for 1 minute at room temperature.
8. Dilute 8-9 mL of blood sample with an equal volume of PBS + 2% FBS. Mix gently (up to 16 mL of blood with equal volume of PBS + 2% FBS can be loaded on one tube)
9. Keeping the SepMate tube vertical, add the diluted sample by pipetting it down the side of the tube. The sample will mix with the density gradient medium above the insert.

   NOTE: The sample can be poured down the side of the tube. Take care not to pour the diluted sample directly through the central hole.
10. Centrifuge at 1200 x *g* for 10 minutes at room temperature, WITH BRAKE ON.

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11. Pour off the top layer, which contains the enriched PBMCs, into a new tube. Do not hold the SepMate tube in the inverted position for longer than 2 seconds.

    NOTE: Some red blood cells (RBCs) may be present on the surface of the SepMate insert after centrifugation. These RBCs will not affect performance.
12. Wash enriched PBMCs by adding PBS + 2% FBS to top off the 50 mL tubes. Cap the tubes and mix by gently inverting tubes several times.
13. Spin tubes at 300 x *g* for 8 minutes at room temperature WITH BRAKE ON.
14. Dump supernatant into a waste in one smooth motion being careful not to disturb the cell pellet.
15. Repeat the wash of enriched PBMCs by adding PBS + 2% FBS to top off the 50 mL tubes. Cap the tubes and mix by gently inverting tubes several times.
16. Spin tubes at 300 x *g* for 8 minutes at room temperature WITH BRAKE ON.
17. Dump supernatant into a waste in one smooth motion being careful not to disturb the cell pellet.

To speed up the procedure, PBMCs are not counted before freezing. Instead, one cryotube of 1 mL is prepared per 8-9 mL of blood.

(ONE TO TWO 9 ML TUBES ARE RECOMMENDED FOR EACH SAMPLE)

18. Resuspend the pellet in multiples of 0.5ml FBS (previously kept at 4°C) for every 8-9 mL of processed blood volume.
19. Label cryo-tubes as instructed in the section entitled “Labelling instructions”.
20. Transfer 0.5 mL of the resuspended PBMCs to the labelled cryo-tubes containing 0.5 ml FBS/DMSO 20% (previously kept at 4°C). The final storage solution is therefore FBS/DMSO 10%.
21. Close and invert cryo-tubes and transfer to cryo-freezing container (previously kept at 4°C).

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PBMC Protocol 2: PBMC isolation with Leucosep / Lymphoprep

Buffer and other reagents preparation

1. RPMI complete media (supplemented with 10% fetal bovine serum, 1% L-glutamine, 0.1% gentamicin and 1% Pen/Strep).
2. Warm up RPMI and RPMI complete media at 37°C.
3. Lymphoprep (stored at ambient temperature of 18-20°C).
4. Heat inactivated (56°C – 30 min) FBS and heat inactivated FBS-DMSO 20% at 4°C.
5. Keep cryo freezing container (with isopropanol 100%) at 4°C. Replace isopropanol after 5 freezings.

PBMC Isolation (day of scheduled study visit)

6. For each sample, pipet 15 mL of lymphoprep into a 50 mL leucosep tube. Prepare appropriate number of tubes per sample (max 35 ml of diluted blood per leucosep tube).
7. Label each tube as instructed in the section entitled “Labelling instructions”.
8. Spin tubes at 200 x g for 1 minute at room temperature.
9. Add blood into a leucosep tube and fill till 50 mL with warm RPMI 1640 complete media. Mix gently.
10. Spin tubes at 880 x g, for 20 minutes at room temperature WITHOUT BRAKE.
11. After centrifugation, the tubes will contain 4 layers (from top to bottom): 1) a plasma layer, 2) a cloudy interface layer of PBMCs containing white blood cells: lymphocytes + monocytes, 3) lymphoprep layer, 4) RBC layer with the granulocyte layer on top.
12. Collect the ring (PBMC layer) and place cloudy interface layer into a new 50 mL centrifuge tube, combining cells from the various tubes of each individual participant sample.
13. Add RPMI to top off the 50 mL tubes containing the PBMCs. Cap the tubes and mix by gently inverting tubes several times.
14. Spin tubes at 690 x g, for 10 minutes at room temperature WITH BRAKE ON.
15. Dump supernatant into a waste in one smooth motion being careful not to disturb the cell pellet.
16. Add 1 mL RPMI to tube and resuspend the cell pellet by gently swirling the tubes.
17. Add RPMI to top off the 50 mL tubes containing the PBMCs. Cap the tubes and mix by gently inverting tubes several times.
18. Spin tubes at 480 x g, for 10 minutes at room temperature WITH BRAKE ON.
19. Dump supernatant into a waste in one smooth motion being careful not to disturb the cell pellet.
20. Add 1 mL RPMI to tube and resuspend the cell pellet by gently swirling the tubes.

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21. Add RPMI to top off the 50 mL tubes containing the PBMCs. Cap the tubes and mix by gently inverting tubes several times.
22. Spin tubes at 260 x g, for 10 minutes at room temperature WITH BRAKE ON.
23. Dump supernatant into a waste in one smooth motion being careful not to disturb the cell pellet.

To speed up the procedure, PBMCs are not counted before freezing. Instead, one cryotube of 1 mL is prepared per 8-9 mL of blood.

(ONE TO TWO 9 ML TUBES ARE RECOMMENDED FOR EACH SAMPLE)

24. Resuspend the pellet in multiples of 0.5 ml FBS (previously kept at 4°C) for every 8-9 mL of processed blood volume.
25. Label cryo-tubes as instructed in the section entitled “Labelling instructions”.
26. Transfer 0.5 mL of the resuspended PBMCs to the labelled cryo-tubes containing 0.5 ml FBS/DMSO 20% (previously kept at 4°C). The final storage solution is therefore FBS/DMSO 10%.
27. Close and invert cryo-tubes and transfer to cryo-freezing container (previously kept at 4°C).

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PBMC Protocol 3: Conventional Ficoll-Paque PBMC isolation protocol

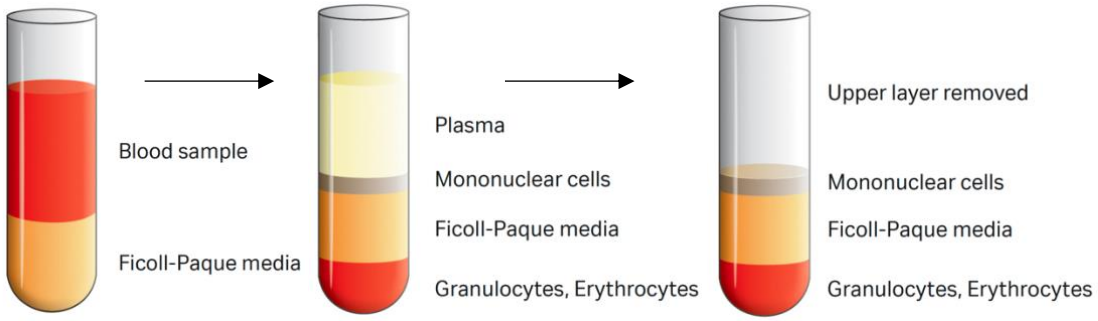

Figure 2. Liquid phases at different PBMC isolation stages.

Buffer and other reagents preparation

1. Warm up PBS at 37°C.
2. Ficoll-Paque Plus (stored at ambient temperature of 18-20°C).
3. Heat inactivated (56°C – 30 min) FBS and heat inactivated FBS-DMSO 20% at 4°C.
4. Keep cryo freezing container (with isopropanol 100%) at 4°C. Replace isopropanol after 5 freezings.

PBMC Isolation (day of scheduled study visit)

5. Dilute the blood 1:1 in PBS.
6. Mix the blood and PBS by inverting the tube several times or by drawing the mixture in and out of a pipette.
7. Invert the bottle of Ficoll-Paque Plus several times to ensure proper mixing.
8. Add Ficoll-Paque Plus (15 mL) to a new 50mL centrifuge tube.
9. Carefully layer the diluted blood sample onto the Ficoll-Paque Plus.

   IMPORTANT: When layering the sample do not mix the Ficoll-Paque Plus and the blood sample.
10. Centrifuge at 400 g for 30-40 min at 18-20°C with WITHOUT BRAKE.
11. Remove the upper layer containing plasma and platelets using a sterile Pasteur pipette, leaving the mononuclear cell layer undisturbed at the interface. The upper layer, which contains the plasma, may be discarded or saved for later use.
12. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean 50 mL centrifuge tube. It is critical to remove all the material at the interface but in a minimum volume.
13. Estimate the volume of the transferred mononuclear cells. Add at least 3 volumes (~ 6 mL) of PBS to the PBMCs in the centrifuge tube.

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14. Suspended the cells by gently drawing them in and out of a Pasteur pipette.
15. Centrifuge at 400-500 g for 10-15 min at 18-20°C WITH BRAKE ON.
16. Discard the supernatant.
17. Suspend the lymphocytes in 6-8 mL of PBS by gently drawing them in and out of a Pasteur pipette.
18. Centrifuge at 400-500 g for 10-15 min at 18-20°C WITH BRAKE ON
19. Discard the supernatant.

To speed up the procedure, PBMCs are not counted before freezing. Instead, one cryotube of 1 mL is prepared per 8-9 mL of blood.

(ONE TO TWO 9 ML TUBES ARE RECOMMENDED FOR EACH SAMPLE)

20. Resuspend the pellet in multiples of 0.5mL FBS (previously kept at 4°C) for every 8-9 mL of processed blood volume.
21. Label cryo-tubes.
22. Transfer 0.5 mL of the resuspended PBMCs to the labelled cryo-tubes containing 0.5 mL FBS/DMSO 20% (previously kept at 4°C). The final storage solution is therefore FBS/DMSO 10%.
23. Close and invert cryo-tubes and transfer to cryo-freezing container (previously kept at 4°C).

4.4.4 Storage conditions

- Transfer the cryo-freezing container to -70°C or below.
- After 1-3 days, transfer cryo-tubes to liquid N_2 or store at -70°C until shipment is arranged.

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4.5 WHOLE BLOOD COLLECTION

4.5.1 Recommended materials

- 2 mL BD Vacutainer K2E (EDTA) (e.g., BD Cat. No. 368841)

4.5.2 Sample collection

1. Label two EDTA tubes as instructed in the section entitled "Labelling instructions".
2. Register the sample collection in the Requisition Form displayed in Appendix X.
3. Draw the patient's blood into two EDTA tubes (2 mL).
4. Slowly and gently invert the tubes 180° and back 8-10 times.

4.5.3 Sample processing

5. Snap freeze the EDTA tubes containing whole blood samples in liquid nitrogen or freeze them at -20 °C or -80 °C.

4.5.4 Storage conditions

- Store the cryovial as soon as possible in your freezer at -70°C or below until shipment.
- In case you do not have immediate access to a -70°C freezer, store them at -20°C and transfer them to a -70°C as soon as possible and within 2 days. Keep them at -70°C until shipment is arranged.

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4.6 STOOL SAMPLE COLLECTION

4.6.1 Recommended materials

- Faecal sampling TUBE (e.g APTACA #2688)
- RNALater solution (e.g, Thermo Fisher Scientific #AM7021 / #AM7024 (500 and 100mL respectively) or Sigma-Aldrich #R0901)
- Freezer for storage (ideally -80°C, also -20°C can be temporarily used)

4.6.2 Sample collection

1. Label the collection tube as instructed in the section entitled "Labelling instructions".
2. Collect of faecal matter according to your routine practice protocol.
3. Transfer the sample to the laboratory

   NOTE: In case the sample cannot be transferred to the laboratory immediately, the sample can be stored at +4°C up to 24 h in a proper container (RNase- DNase- free, sterile tube e.g.,Thermo Fisher Scientific #AM12501) or directly in the faecal sampling tube.

4.6.3 Sample processing

4. Label transfer tube as described in Section 5.
5. Transfer 1-2 g faecal matter to the faecal sampling tube.
6. Add 5-10mL of RNALater solution (all the specimen must be covered) and gently mix by inverting the tube few times.

   NOTE: In case RNALater is unavailable, direct freezing at -70°C without adding any carrier fluid is also acceptable.

4.6.4 Storage conditions

- At arrival in the lab, store the tube containing faecal matter (and storage medium where applicable) in the freezer at -70°C or below until shipment.
- In case you do not have access to a -70°C freezer, samples can be stored temporarily at -20°C for 2-3 weeks.
- If needed, samples can be stored +4°C for up to 24 h immediately after collection before transfer to the Local Laboratory.

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4.7 RECTAL SWAB SAMPLE COLLECTION

4.7.1 Recommended materials

- Fecal SWAB (e.g. APTACA CliniSwab #2160/SG or #2170/SG).
  NOTE: if you choose other brands pay attention the swab comes without any carrier fluid or surface treatment (dry swab)
- RNALater solution (e.g, Thermo Fisher Scientific #AM7021 / #AM7024 (500 and 100mL respectively) or Sigma-Aldrich #R0901)
- Freezer for storage (ideally -80°C, also -20°C can be temporarily used)

4.7.2 Sample collection

1. Label transfer tube as instructed in the section entitled “Labelling instructions”.
2. Remove the swab from the tube (do not touch the swab tip, always hold the shaft applicator above the marked breakpoint)
3. Insert the flocked swab through the rectal sphincter 1-1.5 inches (2.5-4cm) and gently rotate it between your fingers
4. Remove the swab and examine to make sure there is faecal material visible on the tip of the swab
5. Transfer the swab into the tube. Holding the end of the swab shaft, place the marked breaking point against the rim of the tube and bend it to break at the marked breakpoint. Discard the broken upper part and tighten the screw cap.
6. Transfer the sample to the laboratory

   NOTE: In case the sample cannot be transferred to the laboratory immediately, the sample can be stored at +4°C up to 24 h

4.7.3 Sample processing

7. Add 2-10mL of RNALater solution, the sufficient volume to fully submerge the swab tip, which may vary according to the chosen brand/model of the swab.

   NOTE: In case RNALater is unavailable, direct freezing at -70°C without adding any carrier fluid is also acceptable.

4.7.4 Storage conditions

- At arrival in the lab, store the tube containing the swab (and storage medium where applicable) in the freezer at -70°C or below until shipment.
- In case you do not have access to a -70°C freezer, samples can be stored temporarily at -20°C for 2-3 weeks.

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- If needed, samples can be stored +4°C for up to 24 h immediately after collection before transfer to the Local Laboratory.

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5. STORAGE INSTRUCTIONS AND DESTINATION SITES

Table 6. Overview of samples to be shipped per time point per patient and their shipping conditions.

Sample type	Number of tubes/samples	Storage temp. (°C)	Shipping temp. (°C)	Destination site
NP swab	1	-70°C or below	Dry ice	INSERM UANTWERPEN
Serum	2	Short-term at -20°C, long-term at -70°C or below	Dry ice	INSERM UANTWERPEN
EDTA plasma	1	Short-term at -20°C, long-term at -70°C or below	Dry ice	UANTWERPEN
PBMC	1 or 2	-70°C or below, or liquid nitrogen	Dry ice	UANTWERPEN
2 mL EDTA whole blood	2	-20°C or -70°C or below	Dry ice	INSERM UNIBO HMGU
Stool or rectal swab	1	+4°C (up to 24 h), long-term at -70°C or below	Dry ice	UNIBO

6. CONTACT DETAILS

6.1 QUESTIONS CONCERNING PROTOCOLS FOR BIOLOGICAL SAMPLING

NP swabs

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PBMC

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Whole blood

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Valerio Carelli (UNIBO); email: valerio.carelli@unibo.it

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Stool – Rectal swabs

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6.2 COORDINATING LABORATORY FOR SAMPLE SHIPMENTS

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