Standard Operating Procedure

Blood Sampling
WP5 Biology, HARMONIC

Version: 1.0

Date of validity: (First patient inclusion)
Valid until: (Last patient inclusion)

Compiled by:	Approved by:	Accepted by:
Date:	Date:	Date:
Signature:	Signature:	Signature:

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1. Objectives

The purpose of this Standard Operating Procedure (SOP) related to Biology WP is to ensure the procedure of blood sampling for patients included in this WP.

This SOP is specific to WP5 and not include the procedures of the blood samples for the specific markers in WP2 (ex. BNP, NT-Pro-BNP, IGF-1, GH, LH etc). A separate SOPs for tasks of WP2 are available describing the procedures for specific WP2 markers.

2. Contact related to this SOP

If there is any question or information needed, please contact:

Responsible	Email
Siamak Haghdoost	siamak.haghdoost@su.se
Maria Grazia Andreassi	andreas@ifc.cnr.it
Nadia Haddy	Nadia.haddy@gustaveroussy.fr

3. Applicable documents

WP5 Study Protocol, Version 26/11/2019 — V.2

4. Responsibilities/Partners

Partners	**Clinicians and researchers**
Gustave Roussy (France)	Dr. Brice Fresneau Dr. Stephanie Bolle Dr. Valentine Martin Dr. Nadia Haddy
Centre Régional François Baclesse	Dr. Charlotte Demoore Dr. Juliette Thariat
Institute of Clinical Physiology-National Research Council (IFC-CNR, Italy)	Dr. Jonica Campolo Dr. Maria Grazia Andreassi
DCPT, Aarhus University Hospital (Denmark)	Dr. Yasmin Lassen Dr. Sonja Karabegovic
KU Leuven (Belgium)	Dr. Gilles Defraene Dr. Karin Haustermans

5 Inclusion and exclusion criteria

Radiotherapy cohort **WP2**	**Interventional cardiology cohort**
Inclusion criteria	
✓ Age at diagnosis =< 21 years ✓ Informed consent of parent/guardian as well as child/patient ✓ Patients treated for : **brain tumours (except malignant gliomas); head and neck tumours (e.g. rhabdomyosarcomas and nasopharyngeal carcinoma); Hodgkin's lymphoma** ✓ Patients receiving Pulmonary and chest radiation for: **Ewing sarcoma; other chest sarcomas; Lung metastasis of Wilms and Ewing tumours, and other tumours** ✓ Patients receiving Craniospinal radiation therapy for: **Medulloblastoma or other tumours**	✓ Age of patients: 5-22 years ✓ Patients with congenital heart disease ✓ Informed consent of parent/guardian as well as child/patient
Exclusion criteria	
Chromosomal abnormalities and/or genetic syndromes Absence of informed consent	

6 Timeline for blood and saliva sampling (3 times)

Inclusion **T0**
Before irradiation

T1
After irradiation, up to 3 months

T2
1 year after irradiation

Each time. 3 tubes with blood and 1 tube with saliva to be collected

BD Vacutainer CPT Mononuclear Cell Preparation Tube - Sodium Citrate) (4 ml)

Standard plastic tube without any preservative

1 EDTA tube (~3-5 ml)

Serum tube without anti-coagulant (~3-5 ml)

As shown above, blood and saliva samples will be collected at 3 time points:

T0: Before start of radiotherapy (WP2)/interventional cardiology (WP3)

T1 After radiotherapy up to three months (WP2) or day of finishing radiotherapy/interventional cardiology (WP3)

T2 One year after finishing radiotherapy (WP2)/interventional cardiology (WP3)

7 Samples collection

7.1 Before sampling

7.1.1 Materials needed to be prepared

a) Tubes

Types of tubes	**Number of tubes**	**Reference**
EDTA (3-5ml)	1 per patient per time	
CPT Tube - Sodium Citrate (4 ml)	1 per patient per time	https://www.bdbiosciences.com/us/applications/blood-collection/cell-biomarker-preservation/bd-vacutainerreg-cpttrade-mononuclear-cell-preparation-tube---sodium-citrate/p/362760
Clot activator serum separation tube (tube without anti-coagulant)	1 per patient per time	
Standard 10 or 15 ml plastic tube for collecting saliva	1 per patient per time	

a) Other materials

Type	**Quantity needed**	**Reference**
Sterile 2 ml tubes with screw cap	Packages of 1000 tubes	https://www.sigmaaldrich.com/catalog/product/aldrich/br780763?lang=en&region=SE
Sterile Phosphate Buffered Saline PBS	500ML	https://www.sigmaaldrich.com/catalog/product/sigma/d8662?lang=en&region=S
Ice to keep sample at 0 to 4°C		

b) Equipment

Freezer (-80°C)

Centrifuge adapted to different tubes

7.1.2 Patient information and sample labelling

Patient Information: Local patient ID label is to be attached to the patient's medical record and matched with the name of the actual patient, before biosampling.

Sample Information: Available samples ID number plus specify the time points (T0; T1 and T2). If possible use corresponding barcode attached to the tube.

7.2 Sample Processing

Knowledge regarding blood sampling is required.

At each time point, blood should be collected in 3 tubes as follows: one vacutainer containing EDTA K2 (~ 4 ml), one clot activator serum separation tube (a tube without anticoagulants) (~ 4 ml) and in one BD Vacutainer® CPT™ tube for isolation of lymphocytes (~ 4 ml).

7.3 Post Sampling (technics and storage)

All blood tubes should be centrifuged within **2 hours of blood collection for best results**.

Steps by type of the tube

CPTblood tube* (to be analysed within 2 hours)	Clot activator serum separation tube	**EDTA tube**
Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times	Coagulation 30-40 min at RT	Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times
Blood centrifugation (1700 x g-force* 20min at RT)	Blood centrifugation (400 x g-force 15min at RT)	Blood centrifugation (350 x g-force 15min) Seperate plasma and the rest of sample
Isolation of PBMC cell pellet	Serum (aliquots of ~ 1 ml) in 2-3 small tubes**	Save the plasma (3-4 aliquots of ~ 0.5 ml) preferable at 4°C
Washing cell pellet Discard supernatant without disturbing PBMC cell pellet	**Store part of the clot in 1 small tube** at -80°C**	Keep the rest of the blood (blood cells) in 2 aliquots in small tubes**
Please store all the materials (cell pellet; serum; plasma; blood cells) in sterile 2 ml tubes with screw cap		
Store the dry pellet cells at -80°C	**Store both of them at -80°C**	**Store both of them at -80°C**

*Check detailed protocol for conversion of g-force to RPM in the Annex

** Sterile 2 ml tubes with screw cap

Saliva samples, it will be important to require that patients do not eat 30 minutes before giving a saliva sample. Prior to saliva sampling, patients have to wash the mouth or drink water.

Collect appoximately 4-5 ml saliva in a standard clean plastic tubes (10 or 15 ml adapted to the centrifuge) without any preservatives and divide the saliva in to the 2 or 3 small tubes with screw cap, almost 2 ml in each. The samples have to be transferred to -20 freezer within 5-10.

8 Samples packaging and transfer

Blood and saliva samples will be sent from hospitals to Sweden SU, in boxes containing **dried ice** to keep the samples (blood, serum, plasma) frozen during the transport. Transport is done by an authorized delivery company to the SU, Sweden and from there the samples are distributed to the other partners in Europe for analyses. The samples should be send to SU, Sweden 2 times during the project: month 33 and month 46.

9. Annex

Detailed protocol of Isolation of PBMC using Cell Preparation Tubes (CPT):

This link could be helpful: www.youtube.com/watch?v=5Z25H8JLtDk

The BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (CPT) is a single tube system for the collection of whole blood and the separation of mononuclear cells. Isolation of PBMC in these tubes occurred according to the manufacturer's instructions:

Steps:

1. Collect blood into CPT using venipuncture technique. Note: Blood tubes should be centrifuged within **2 hours of blood collection for best results**.

2. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.

3. Centrifuge CPT tubes at 1700 × g-force for 20 min *(PS: you should convert g-force (or also called RCF) to RPM for your particular centrifuge, please check provided link for coverting g-force to RPM)**** at room temperature. Note: Do not centrifuge CPT over 2000 g-force, as it may cause tube breakage.

4. After centrifugation, carefully open the CPT into a biological safety cabinet II. Using a pipette (ex. Pasteur pipette), gently collect the mononuclear cells, which can be found in the layer just under the plasma.

5. Transfer cells to a 10 mL (or 15 mL) conical standard tube. Avoid vigorous pipetting that would disintegrate the gel plug itself.

6. Add 3 mL PBS (Dulbecco's Phosphate Buffered Saline) to wash cells. Mix cells by inverting tube 3 to 5 times.

7. Centrifuge at 400 × g-force for 8 min. Discard supernatant without disturbing cell pellet.

8. Resuspend cell pellet by gently tapping tube with index finger.

9. Add 3 ml PBS again and mix cells by inverting tube 3 to 5 times.

10. Split the volume in to 2 small tubes (sterile 2 ml tubes with screw cap)

11. Centrifuge at 400 × g-force for 5 min. Discard supernatant without disturbing cell pellet.

12. The dry PBMC pellet cells should be stored at -80ºC.

Store samples at -80ºC until transfer to the SU in dry ice

*For coverting g-force to RPM:https://www.sigmaaldrich.com/technical-documents/articles/biology/g-force-calculator.html