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HEALS

HEALS

Health and Environment-wide Associations based on Large population Surveys

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http://www.heals-eu.eu/

D5.2 Established Standard Operating Protocols and workflows for application of omics on human cohort data

WP5 Omics, epigenetics and confirmatory in vitro analyses

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1 Scope of the report

The report outlines the activities performed in WP5 omics within HEALS towards the development of protocols and workflows for omics analysis in human exposome studies

During the first year of the project main activities involved the inventory of the available omics technology protocols from the different HEALS partners.

Secondly, some activities for specific omics protocols involved the optimization of sample preparation requirements (adductomics and untargeted metabolomics). This also included the selection of the appropriate HEALS SNP platform to understand the contribution of genetic heterogeneity in relation to environmental exposure and health outcome.

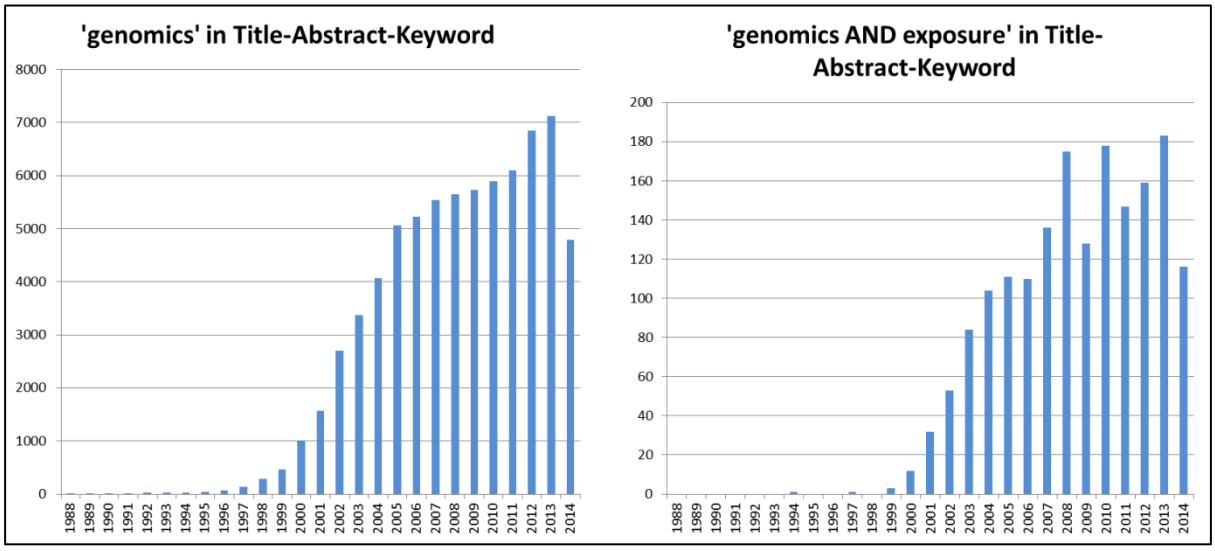
Thirdly, the activities involved the making of an inventory for existing molecular epidemiological studies available within the HEALS project, to be used as pilot cases for the application of omics technologies in exposome studies. This was discussed together with WP4 at the Ljubljana workshop in May 2014.

Finally, the activities involved the contribution of WP5 to advice on sample collection compatible with future omics analysis, for EXHES, the HEALS exposome twin study.

The report includes an overview of available omics technologies including SOPs for selected technologies.

2 Introduction

With the advent of omics technologies it has become possible to measure all the biological and chemical constituents of a cell or tissue. Bioinformatics analysis on the emerging data allows for better understanding of biological mechanisms or for categorization of samples or subjects into different classes, e.g. diseased versus non-diseased, exposed versus non-exposed. During the turn of the century Cy-dye based spotted oligonucleotide microarray analysis and 2D gel based MALDI-TOF proteome analysis were examples of main stream omics technologies. Gradually, this has evolved to more advanced technologies including e.g. DNA and RNA sequencing, phosphoproteome profiling, lipidomics, whole genome DNA methylation analysis. The application of omics has landed in numerous studies, including studies to understand the effect of external stressors on the internal exposome. A search in Scopus on the term ‘genomics’ in Title-Abstract-Keyword showed that until 2013 the number of papers increase until ~7000 in 2013, with the largest increase in the first 5 years of the 21th century. Around 1/40 of the total number of these papers also involves the term ‘exposure’ in Title-Abstract-Keyword, and in terms of yearly increase a similar pattern is observed.



Amongst but not excluded to these, studies are reported using peripheral or cord blood lymphocytes gene expression profiling to detect effects due to: air pollution (Ahangari et al. 2013); diesel exhaust (Pettit et al. 2012); arsenic exposure (Andrew et al. 2008), (Argos et al. 2006), (Wu et al. 2003); Nickel (Arita et al. 2013), (Bonin et al. 2011), lead (Kasperczyk et al. 2012); lead in relation to autism (Tian et

al. 2011); mercury (Stamova et al. 2011); Cadmium (Dakeshita et al. 2009); metal fumes (Wang et al. 2004; Wang et al. 2008); alcohol (Beech et al. 2012), (Kupfer et al. 2013); acetaminophen (Bushel et al. 2007); perinatal exposure to antiretroviral therapy (Cote et al. 2008); Benzene (Bi et al. 2010; Forrest et al. 2005); smoking (Buttner et al. 2007; Charlesworth et al. 2010); smoking in twins (van Leeuwen et al. 2007); endocrine disruptors in relation to fertility (Caserta et al. 2013); TCDD (McHale et al. 2007); in utero exposure to carcinogens (Hochstenbach et al. 2012); exposure to allergens (Kam et al. 2012); 50 Hz electromagnetic field (Kirschenlohr et al. 2012); age and shift work (Ando et al. 2010), (Reszka et al. 2013); sleep/wake patterns (James et al. 2007); chronic fatigue syndrome (Kaushik et al. 2005), and chronic psychological stress (Kawai et al. 2007), (Rokutan et al. 2005). These studies are clear examples of using omics to detect and understand the possible biological consequences of individual stressors, which in some instances could also be associated to health status. Instead, HEALS aims at the development of a platform for integrated analysis of a multitude of these stressors, using a multitude of omics technologies, internal exposure data e.g. via biomarker data or PBPK modelling, jointly with additional parameters such as geographical localization, external exposure data from personalized and environmental sensor and modelling data.

Therefore, there is a need to develop *multiple* protocols, standardized workflows to apply omics in exposome studies towards analytical exposure biology concepts. The outcome of these development of protocols within HEALS, also in relation to existing and planned studies is described in the present report.

3 Omics technologies available for HEALS

Within HEALS, several omics and biomarker protocols have been identified in the first year that are available or are being developed for HEALS studies. The overview is provided in Table 1.

Table 1. Overview of available omics technologies in HEALS. Partner is indicated between brackets.
DNA damage and repair assays:
8-oxoguanine by LC-MS/MS, (ISS).
N7-methylguanine,(UM)[1]
O6-alkylguanines,(UM)[1]
Comet assay, (ISS).
DNA repair functional assays, (ISS).
Gamma-H2AX analysis, (ISS).
DNA repair capacity. Quantitation of ATase activity levels (transfer of 3H methylated substrate DNA to alkyl transferase protein), (UM)
DNA repair capacity: quantitation of total Atase protein in cell/tissue extracts by ELISA , (UM)
Metabolomics and proteomics:
Metabolomics (non targeted approach for small molecules) and other targeted contaminant analysis NMR spectroscopy, (U)HPLC - High Resolution MS , (U)HPLC - MS/MS, (FERA)[2]
LC-MS/MS triple quadrupole based amino acid analysis performed on cells, plasma and breastmilk (the latter to be developed), (UPD).
GC-MS/MS (triple quadrupole) organic acid analysis performed on cells, urine, breast milk (the latter to be developed), (UPD).
Metabolomics_Untargeted HR-MS, (AUTH).
Metabolomics_Targeted HILIC-MS/MS, (AUTH).
Metabolomics_Untargeted GC-MS analysis using TOF or Quadrupole MS, (AUTH).
Metabolomics_NMR Varian 500 MHz, (AUTH).
Proteomics employing various technologies: 1 Orbitrap Velos – ETD (Thermo); 1 QTOF (Global,

Micromass/Waters) ; 1 4700 MALDI-TOF/TOF (ABI); HPLC: 1 2D LC system nanoAcquity (Waters) 1 2D LC system nanoEksigent (Eksigent) 1 Ettan system (GE) 2-DE system (GE)", (CERETOX)
Transcriptomics, DNA methylation, SNP and miRNA profiling
Transcriptomics (Affymetrix) on cell cultures (microarray experiments followed by RT PCR for validation), (UPD).
transcriptome via RNA seq, (SXS).
DNA cytosine methylation, (SXS).
DNA cytosine methylation, (ISS).
miRNA expression_Agilent array, (CNR).
mRNA expression profiling_Agilent SurePrint G3 Whole Human Genome 4x44K Microarray, (CNR).
SNP genotyping via Axiom® Biobank Genotyping Arrays, (SXS).[3]
SNP genotyping via custom design Fluidigm Biomark 96.96 Dynamic Array, (SXS). [3]
[1]Pilots are being performed by UM to define the best sample preparation requirements [2] Metabolomics pilots are performed on spare samples send by JSI to FERA, to define the best sample and analytical requirements. Further resampled blood from PHIME is available, analysis expected fall 2015 [3]the final selection of the SNP genotyping platform is described in paragraph 6.1. and applied in a pilot to the existing Repro-PL samples

An extensive overview of the available technologies and sample requirements is provided in the excel file available in the Addendum and supplementary data file 'matrix omics_V1_RS040414_MASTER_password protected.xlsx'.

Further, the available SOPs and workflows from the majority of these technologies are attached in the Addendum to D5.2 Established Standard Operating Protocols and workflows for application of omics on human cohort data to this report (File 'Addendum to D5.2_V1.pdf'). This is a working document and can be updated during the course of the HEALS project.

For the two omics technologies metabolomics and adductomics, piloting is ongoing to define the best sample preparation requirements. These are described in paragraph 5.

4 Sample preparation requirements for untargeted analysis

Metabolomics

For best results, the dietary diversity in the human population must be minimized. All subjects should fast overnight or refrain from food before collection of urine or blood samples. A brief description of consumed food should be included in any report. One approach that is currently used in clinical metabolomics studies is to make all subjects fast overnight and void the bladder first thing in the morning. The subject should then drink 500mL of water and avoid any other fluid or foodstuff for at least an hour, before voiding the bladder once again.

This is considered to be the metabolically 'clean' sample; accepting that we know there are starvation components (ketone bodies, etc.) that are beginning to appear. The presence of starvation components increases greatly if starved for an additional 4 hours. For clinical studies that involve patients with the misfortunate of severe disease, it may be considered undue burden, or unhealthy, to request dietary restriction for minimization of potential diet-related influences on metabolomic profiles. Another approach is to recruit best controls based on life-style factors following the collection of such information for the patient population. For all clinical studies, independent of dietary restriction, it is desirable to recruit for control populations that provide best controls based on gender, age, and ethnic origin.

For effective metabolomics analysis it is crucial that all samples are handled in a certain way as to not compromise the quality and accuracy of data acquired. A number of critical sample preparation instructions, including brief analytical procedures are noted below for a number of matrices.

Blood (including cord blood)

Alcohol swabs should not be used just prior to the collection of blood. If possible at least 1 mL of whole blood should be taken and added to a glass heparin plasma collection tube (e.g. BD Vacutainer, Becton, Dickinson and Company, New Jersey, USA). Volume and time of sampling should be documented. If possible at blood sampling site, the sample should be spun at 2000 - 3000 x g at 4°C for 15 minutes and the plasma supernatant removed. The plasma should be aliquoted into 3 x 1 mL Eppendorf (Eppendorf AG, Germany) tubes before freezing at -80°C. The plasma sample should then

remain frozen at all times and transported with a reputable courier to the respective laboratory (either FERA, York, UK or AUTH, Thessaloniki, Greece) for metabolomics analysis.

Once at the laboratory (FERA) the plasma sample will be thawed immediately prior to analysis before centrifugation at 21, 000 x g for 10 minutes at 4°C. Supernatant (200 µL) is mixed with 800 µl of chilled (4°C) methanol thoroughly vortexed and re-centrifuged at 21, 000 x g for 10 minutes at 4°C. Supernatant (500 µl) is removed and 500 µl of de-ionised water (18.2 MΩ) added before analysis by Liquid chromatography – High Resolution Mass Spectrometry (LC-HRMS). A pooled QC aliquot is produced by taking 20 µl of each of the sample extracts before mixing comprehensively on a vortex shaker for 10 seconds. This is continuously injected for the first 15 analyses to condition the LC column and subsequantly analysed every 6 injections for QC purposes. Samples are analysed in a random order (pre-determined from www.random.org) and analysed typically in batches of 25-30.

LC conditions: The LC column to be used is an ACE 3Q 150 x 3 mm, 3 µm (Advanced Chromatography Technologies, Aberdeen, UK) or equivalent. Mobile phases are 0.1% formic acid in water (mobile phase A, MPA) and 0.1% formic acid in acetonitrile (mobile phase B, MPB). Gradient applied is 100% MPA for 5 minutes before increasing to 100% MPB over 15 minutes. This is held for 10 minutes before reverting back to 100% MPA and held for 2 minutes. Injection volume is 10 µl, flow rate is 0.4 mL/min and column temperature set to 25°C.

MS conditions: The MS to be used is a Thermo Exactive (Thermo Fisher Scientific, MA, USA) set at 50,000 resolution FWHM @ 200 m/z with an acquisition speed of 2 Hz, or an equivalent high resolution MS (e.g. ToF MS). Analysis will be undertaken in both positive and negative ionisation mode (separate experiments).

Data analysis: All raw data will be aligned against a QC data file before mass features are selected using either Progensis QI software (Nonlinear Dynamics, Newcastle, UK) or the open-source software XCMS (Scripps, CA, USA). Both software packages also offer data reduction techniques to de-isotope / de-adduct features selected in all data files before the option of sample differentiation by multivariate and univariate statistical analysis. If necessary, data features (includes m/z's, retention times and peak intensity, often peak area) will be normalized (using a script in R) using the QC data to correct for any within or between batch variation.

Once a satisfactory list of features are produced, tentative identification based on accurate mass only will be applied using in-house or online databases (e.g. Human Metabolome Database). Confirmation

of metabolite identity will be by either NMR or MS/MS^n comparing spectra against an analytical standard.

Urine

If possible at least 1 mL of urine should be taken into a sterile container, aliquoted into 3 x Eppendorf tubes and immediately frozen before transportation. Volume and time of sampling should be documented. If possible at sampling site, the addition of 1 % sodium azide should be undertaken in at least one of the urine aliquots.

Once at the laboratory (Fera) the urine sample will be thawed immediately prior to analysis before centrifugation at 21000 x g for 10 minutes at 4°C. Supernatant (200 µL) is mixed with 400 µL of chilled (4°C) methanol: water (v/v 1:1) and analysed by Liquid chromatography – High Resolution Mass Spectrometry (LC-HRMS).

QC procedures, LC-MS conditions and data analysis are as described for blood samples above.

Tissue (From Want et, al. (2013)):

To obtain useful global metabolic profiles, sampling must be performed as rapidly as possible. Tissues should be collected on to ice to minimize further metabolic activity, and the samples should be snap-frozen using liquid nitrogen and stored at the lowest available temperature (a minimum of −20 °C, preferably at −80 °C) in order to avoid any further loss of unstable metabolites. Although some studies have been conducted (Huang et al. 2011), the stability of metabolite profiles for tissue samples in storage is unclear at present.

If possible, at least 500 mg of tissue should be collected and aliquoted into 3 x Eppendorf tubes. If the tissue samples are large, it is good practice to cut them into small pieces for freezing and storage as small pieces of tissue will freeze more rapidly.

The person taking the sample should record any possible contaminant signals resulting from the collection of the tissue samples, e.g., from anesthetic, surgical instrument cleaning solutions or tube-related contaminants.

Once at the laboratory (Fera) the tissue sample will be thawed immediately prior to analysis before homogenisation in the presence of chilled extraction solvent (methanol: water (v/v 1:1)). The amount of sample for analysis and method of homogenisation is tissue specific. For example, 50 mg of cord /

placenta tissue (a “worst case” tissue matrix due to its rubbery consistency) is homogenised using a bead beater and 2.3 mm diameter silica \ zirconia beads (Thistle Scientific, Glasgow, UK) in the presence of liquid nitrogen to keep the extract cool. The tissue is extracted with 1.5 ml of chilled methanol: water (v/v 1:1) which is then centrifuged at 21,000 g for 5 minutes at 4°C. Supernatant (1.2 ml) is dried in a Savant vacuum concentrator (Fisher Scientific, Loughborough, UK) and reconstituted in 600 µL methanol: water (v/v 1:1) before analysis by LC-HRMS.

QC procedures and data analysis are as described for blood samples above.

Breast Milk (including colostrum)

If possible, at least 3 mL of breast milk should be taken into a sterile container, aliquoted into 3 x 1 mL Eppendorf tubes and immediately frozen before transportation. Volume and time of sampling should be documented.

Once at the laboratory (FERA) the milk sample will be freeze-dried immediately for a minimum of 48 hours and stored in an air-tight sterile container at room temperature. Assuming 13% solid content of breast milk, approximately 450mg of solid material should be recovered. For analysis, 50 mg of dry sample is extracted with 1.5 mL chilled methanol: water (v/v 1:1) by shaking for 30 minutes on a Disrupter Genie (VWR, Lutterworth, UK) before centrifugation at 21,000 g for 5 minutes at 4°C. Supernatant (500 µl) is diluted 4 fold with methanol: water (v/v 1:1) before shaking and analysis by LC-HRMS.

QC procedures and data analysis are as described for blood samples above.

<u>Adductomics</u>

DNA sample preparation and enzymic digestion protocols are likely to be key factors in the reproducibility of any adductome analysis. Preliminary studies using MS-MS analysis using two different published DNA digestion protocols (Balbo et al Anal Chem 2014,86,1744-52; Wild et al, Carcinogenesis 1983, 4,1605-9) revealed important differences in the types and levels of different base adducts in calf thymus DNA that had been modified with a methylating agent, temozolomide. This has been ascribed to the presence of a contaminating enzyme that can remove certain types of adducts (Schumacher et al, Anal Biochem 2013,434,4-11).

Together with FERA and UM, complementary liquid chromatography - mass spectrometry methodologies have been evaluated (LC-MS/MS and LC-HRMS) in order to increase detection and confidence in identification capabilities of a wide range of DNA adducts.

5 Implementation of omics workflows and protocols to (1) existing studies within HEALS, (2) EXHES protocol development and Conclusions

As the inventory of available omics technologies was completed and largely the SOPs for all of these have been established, next steps were to define the possible implementation into HEALS exposome studies. This was done for selected existing HEALS studies (6.1), as well as the EXHES protocol (6.2).

5.1 Existing HEALS studies

During the Ljubljana workshop in May 2014, (associated with Deliverable D5.1), it was identified that the Repro-PL and Phime study were candidate studies, for application of omics.

Repro-PL

Repro-PL is a Polish mother-birth cohort (Polanska et al. 2009), (Polanska et al. 2011) involving the recruitment of 1300 mother-child pairs. Research visits are/were scheduled 3 times during pregnancy, at birth, and postnatal for a subset of the subjects. Each visit involved the collection of biomaterials (saliva, urine, hair, maternal blood and cord blood). In addition, 6 weeks post partum breast milk was collected. The study aims at the understanding of environmental factors in relation to health outcome in the children. Emphasis is on the exposure to metals, polycyclic aromatic hydrocarbons and environmental tobacco smoke in relation to small-for-gestational age, and preterm delivery. Since the start of the study, several biomarker data have been collected: a.o. plasma glutatione peroxidase activity, erythrocyte glutatione peroxidase activity (GPx1), Erythrocyte superoxide dismutase activity, TBARS (thiobarbituric acid reactive substances, selenium, zinc, copper, DNA damage (single strand breaks and oxidative damage, vitamin A, vitamin E, β-carotene, ceruloplasmin activity, genetic polymorphisms of selected genes, urinary polycyclic hydrocarbons, urinary phthalates, lead, mercury, cotinine, 1-hydroxypyrene. To improve the understanding of the internal exposome in the context of exposome studies (endotype), a start will be made with the application of omics in Repro-PL. The following biomaterials are available and have been shipped from HEALS partner NIOM to the corresponding HEALS partners (AUTH and SXS) for analysis:

- Urine samples stored at – stored at - 20oC (n=1500)
- Buffy coat – stored at -20oC (n=1000)

- Plasma (HepLi) – (~1 ml) stored at - 70oC (n=700)

So far, metabolomics analysis will be performed on **plasma and/or urine** using metabolomics technologies available at Thessaloniki.

- Metabolomics_Untargeted HR-MS, (AUTH).
- Metabolomics_Targeted HILIC-MS/MS, (AUTH).
- Metabolomics_Untargeted GC-MS analysis using TOF or Quadrupole MS, (AUTH).
- Metabolomics_NMR Varian 500 MHz, (AUTH).

Denis, George please provide details, has a selection been made for a specific platform, or will you run the Repro-PL samples on all four platforms. Plasma heap Li, or will it be NMR urine? Would you please be so kind to specify?

In addition, from the buffy coats, transcriptomics analysis will be performed using Illumina etcetera Denis could you please add, details not known to me

Finally, from the same buffy coats, genomic DNA will be isolated and subjected to SNP profiling. Within WP5, discussions have been taken place on selection of a relevant HEALS platform for SNP profiling. Initially, two platforms were considered for implementation into HEALS exposome platform: [1] the Fluidigm Biomark 96.96 Dynamic Array and [2] Affymextrix Axiom® Biobank Genotyping Arrays. Considering the Fluidigm Biomark 96.96 Dynamic Array, for this targeted platform, it is possible to design a dedicated set of SNPs of interest. Using this approach, it was estimated that approximately 2.000 – 3.000 SNPs could be analyzed in 2,500 individuals, within the context of the HEALS budget. An initial effort was made to retrieve list of relevant SNPs for implementation on the Fluidigm platform from WP13-W17: Allergy and asthma - link with particulate matter (PM) and biologicals; neurodevelopmental and neurodegenerative disorders - link with metals/metalloids; obesity and childhood diabetes - link with endocrine disruptors. This has yielded a list up to a few hundreds of relevant candidate genes for neurodevelopment/metals and organic pollutants. However, during this initial process, it also became evident that the exact genetic localization of the polymorphism was very often not indicated (lack of reference SNP ID number). Thus, it was anticipated that the time needed for the final the selection of most relevant SNPs for inclusion on the array would be tremendous. For one gene of interest, for example involved in the phase I metabolic activation of xenobiotics, in fact

multiple polymorphisms may exist e.g. in the transcriptional regulatory or active domain. The appropriate bioinformatics retrieval, analysis and selection was estimated to be very time consuming.

Therefore, it was concluded to resort to the second platform proposed: Affymextrix Axiom® Biobank Genotyping Arrays. Axiom® Biobank Genotyping Arrays, is a ~whole genome SNP platform with GWAS markers selected from Axiom® Genomic Database of SNPs and indels.

The purpose of the SNP profiling pilot to Repro-PL is not aiming for a complete genome wide association study, linking specific individual SNPs strongly to health endpoints (e.g. preterm delivery), as the total number of subjects will be too low, in particular for less frequent SNPs. Instead, it is believed that the combined data from transcriptome, metabolome and SNP analysis contribute to the understanding of the endotype, the internal mechanistic constitution, underlying the external phenotype.

PHIME

As described in the final report (Skerfving 2011), on page 5, regarding the FP6 funded PHIME project:

'The overall aim was to improve assessment of the public-health impact of toxic and essential metals via food, addressing the complexity of exposures, interactions (e.g. with nutrition), and risk groups (women, children, the elderly and individuals with genetic susceptibility). PHIME also addressed mechanisms for uptake of metals in plants, and thus into the human food chain'

As part of this project, the PHIME cohort was started (Slovenia and Croatia, n = 675, which was extended to additional populations along the adriatic coast line) in which the relation between maternal mercury intake (as a consequence of fish consumption) and fetal and childhood development was studied. In these studies, a range of biosamples have been collected including hair, urine, breast milk, cord tissue and cord blood as well as data on neuropsychological and psychological development in the offspring.

Original cord and cord blood samples were unfortunately stored at -20°C long term and were therefore unfit for metabolomics analysis for HEALS due to possible metabolite degradation However, a number of these samples have still been delivered to Fera and have been invaluable for method development / optimisation (see blood and tissue method details in section 5 of this report).

Between 20 and 30 additional blood samples will also be re-sampled (predicted spring 2015) from the original Phime Mothers. These samples will be aliquoted, frozen and stored at -80°C before transportation to FERA who will carry out the analysis described in section 5 of this report.

5.2 EXHES protocol development

WP5 helped out with the design of the EXHES protocol, in particular to ensure the correct sampling and storage of samples for future omics analyses. As the exact nature of the omics technologies applied to the EXHES study is at the moment not defined in, and partially depend on the first results from the omics pilots in Repro-PL and PHIME, exact advice is difficult to give at the moment. This has resulted in a general advisory comments:

- Keep samples after collection as cold as possible e.g. on ice
- Freeze samples as quickly as possible, preferably in liquid nitrogen
- Providing minimum amounts of biosample required for the various omics analyses potentially foreseen
- Record timing between collection of sample and ultimate storage in -80 oC.
- Advice to store samples at -80 oC at least for metabolome and transcriptome analysis
- Consider the use of serum for metabolome analysis (instead of plasma)
- If plasma is collected use heparin as anticoagulant
- In addition, contributions were made specifically to SOP_Plasma_Urine_for metabolomics.docx

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