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1. EXECUTIVE SUMMARY

Molecular in vitro diagnostics have enabled a significant progress in medicine. Further progress is expected by new technologies analysing nucleic acids, proteins, and metabolites signatures (profiles) in various human tissues and body fluids. This includes new applications for Personalized Medicine. However, the profiles of these molecules can change drastically during sample collection, transport, storage, and sample processing thus making a reliable diagnostic or biomedical research unreliable or even impossible as the subsequent analytical assay will not determine the bioanalyte profile as it was in the patient but an artificial profile generated after sample collection. A major impediment to progress in the hunt for biomarkers is therefore the lack of standardization in how specimens are collected (G. Poste. Nature 2011, Vol 469, 156-157). Also, pre-analytical errors in sample collection and handling account for 60-70% of all problems occurring in laboratory diagnostics (Lippi el al.. Clin Chem Lab Med. 2011 Jul; 49(7)).

Further progress is limited due to the lack of guidelines standardizing pre-analytical workflows and due to still missing new and improved sample preservation and handling technologies. The European project SPIDIA (www.spidia.eu), a 4.5 years large-scale integrating project, aims to close this gap by providing guidelines, quality assurance schemes and innovative pre-analytical technologies and tools. SPIDIA's results are of importance for diagnostics including patients, hospitals, doctors' offices, and clinical diagnostic laboratories as well as for biomedical and clinical research in the pharmaceutical, biotech and diagnostic industry, for government funded research programs and biobanking.

SPIDIA was organized around three main activities. Each of these was associated with major goals.

The first activity intends to lead to pan-European quality assurance schemes and guidelines for the pre-analytical phase of in vitro diagnostics. These guidelines will be based on evidence gathered during ring trials and other studies performed in order to identify and improve problematic steps in pre-analytical workflows and procedures.

For human blood samples, SPIDIA planned and executed pan-European ring trials for three different analytes: genomic DNA from whole blood, circulating cell-free DNA from blood and plasma as well as cellular RNA from whole blood. For each analyte, two consecutive ring trials were executed and evaluated. About 320 laboratories from 29 different European countries participated in each of the two ring trials series. The findings about pre-analytical impact factors on blood samples qualities derived from these different ring trials are the basis for the development of new CEN Technical Specification documents for the standardisation of the pre-analytical phase for molecular in vitro diagnostics in the European Union. These documents are currently under development by a dedicated working group within the CEN / Technical Committee 140 ("In-vitro diagnostic medical devices").

Time course studies on metabolome profile changes during pre-analytical workflows for processing human body fluids generated evidence for writing another CEN Technical Specification document on how to handle samples for metablome profile and compounds analysis.

For the standardization and validation of pre-analytical tissues collection and handling, more than 5.000 malignant and non-malignant tissue samples were collected and analysed during the project, using different tissue stabilization technologies including formalin fixation and a novel tissue fixation and stabilization technology developed within SPIDIA. The impact of pre-analytical variables like tissue fixation and ischemia times on the quality of histomorphology as well as on the quality of biomolecules like DNA, RNA, proteins and metabolites were investigated. These studies indicated that the novel tissue fixation and stabilization technology showed promising advantages compared to the classical formalin fixation with regard to the detection and analysis of proteins, phospho-proteins and nucleic acids. In addition, three cancer morphology ring trials involving 69 renowned pathologists from Europe and the US were executed, using breast, colon and prostate cancer samples in order to evaluate the applicability of the new SPIDIA tissue stabilization technology for routine pathology in comparison to the gold standard formalin. As for blood samples, all findings about pre-analytical impact factors on tissue samples qualities will be a basis for the development of new CEN Technical Specification documents.

Another important goal was the identification of sample quality biomarkers for selected sample types. These were intended to either allow the evaluation of pre-analytical workflows if these lead to good quality samples or to allow a judgement if a sample of unknown pre-analytical workflow history can be used for a dedicated analysis. Fourteen blood RNA quality biomarkers were discovered and validated. For tissue samples, the effects of warm and cold ischemia on the RNA transcript level let to the discovery of 28 candidate biomarkers for tissue quality. Also stable and unstable proteins/ phospho-proteins during cold ischemia were identified. In both ischemia types, 16 metabolites were found which could serve as profiling markers for the sample history.

The second SPIDIA activity was dedicated to the discovery, development and integration of breakthrough new technologies and tools that improve weak steps and links in pre-analytical workflows of in vitro diagnostics.

As mentioned above, a completely new crosslinking-free tissue fixation and stabilization technology was developed and evaluated within SPIDIA. The new technology showed superior performance in stabilizing biomolecules like DNA, RNA, proteins, and phospho-proteins while leaving the morphology of the same tissue sample also intact. The new system was thus compatible with immunohistochemistry, classical pathological analysis and molecular analyses. The technology is meanwhile also successfully used by other large government funded research programs.

Another screening and development program was executed to develop new stabilization solutions which preserve the white blood cells morphologies in human whole blood as well as their cellular bioanalyte profiles during the entire pre-analytical workflow. Some of these new solutions showed very promising proof-of-principle results.

SPIDIA also worked on the integration of pre-analytical and analytical workflows. An automated integrated pre-analytical workflow was developed for processing stabilized blood samples in order to isolated cellular RNA including miRNA. This was directly linked to a new automated RT-qPCR assay setup on the same platform. This resulted in a standardized complete sample-to-result workflow. Other successfully developed sample-to-result workflows address new RNA expression based Alzheimer's Disease and cancer diagnostics.

Human plasma samples can serve as minimal invasive sample materials for diagnostic purposes. It is known that they contain cell-free circulating nucleic acids (ccfNA) from different origins, like tumours or a foetus in the case of pregnancy. An unsolved problem is the preservation of these ccfNA profiles during pre-analytical workflows. SPIDIA therefore developed a new stabilization technology that stabilizes ccfNA profiles in blood and in plasma generated therefrom. In addition, a new manual and automated technology for the enrichment and purification of ccfNA from larger plasma volumes was developed which can also process samples stabilized by the new preservation technology. This is of high importance as the concentration of specific ccfNA molecules in blood / plasma is low.

Moreover SPIDIA looked into the collection of swab samples for molecular analyses and improved the release of DNA and the pathogen inactivation for enabling higher safety and sensitivity.

The third SPIDIA activity focused on management, ethics, spreading of excellence and dissemination.

The operational SPIDIA management organisation was installed since the beginning of the project. Strategic, management and workshops meetings were taking place every six months to monitor the project and work progress and to define the project strategies. They also hosted members of the Scientific Advisory Board (SAB) and the Project Ethic Committee (PEC).

The main tasks of this SPIDIA activity were to develop an internal and external training program and to disseminate SPIDIA's results to the scientific community and general public. During the project 27 different training activities have been performed including internal and public workshops, seminars, and round-table discussions. The SPIDIA webpage (www.spidia.eu) was established and has been continuously updated with

information about results from the SPIDIA project. In addition, new sections include meetings and events with SPIDIA participation, publications, news and press, and links to related organisations. Today more than 800 people have registered for the newsletter. In total six newsletters have been published.

The SPIDIA consortium has been very active in disseminating the results. More than 60 oral or poster presentations have been presented at international conferences and meetings. Also, 11 scientific papers have so far been published in international peer-review journals. Three scientific papers have been accepted for publication. A significant number of additional articles are in preparation or have been submitted for publication. In addition, articles about the SPIDIA project have been published in non-specialized media. A live webinar event has been broadcasted in September 2012. The intellectual property committee of SPIDIA successfully monitored all activities for dissemination and reported the generation of intellectual property to the SPIDIA Executive Management Board. For constant awareness, the PEC organized workshops on every meeting discussing the latest ethics views including discussions on biobanking.

Under the roof of the US National Cancer Institute (NCI) there are two additional large research and standardisation programs for improving sample qualities running, the Biospecimen Research Network (BRN, http://biospecimens.cancer.gov/researchnetwork) and the Cancer Human Biobank (caHUB, http://cahub.cancer.gov). SPIDIA is successfully collaborating with the NCI on these programs.

A new descriptive model for achieving a comprehensive and complete international pre-analytical workflow standardisation was proposed by the BRN. The model defines each pre-analytical workflow in three dimensions: i) sample type (e.g. blood), ii) bio-analyte in this sample type to be analysed (e.g. cellular RNA), iii) the final assay test technology used (e.g. qRT-PCR). In the model these three dimensions represent one “ice cube”. There are many different of such three dimensional individual pre-analytical workflows to be standardised – many “ice cubes” which form a final “ice cube tray”. The entire model is therefore called the “Ice-Cube-Tray Model. SPIDIA’s research and standardisation program is built on the same model principles. In 2011 both programs therefore agreed to promote and use this model and to cooperate on its basis.

SPIDIA further established additional collaborations with the Christian-Doppler-Laboratory grant project at the Medical University of Graz on pre-analytical workflow standardization, with the m4 Top Cluster in Munich on the development of a new sample-to-result multimodality diagnostic for Barrett Syndrome, with BBMRI Sweden on tissue related pre-analytical workflow topics and with various other organizations on pre-analytical workflow standardisation topics.

Although SPIDIA’s funding period has ended, several activities will continue beyond this stage. These include the development of CEN Technical Specification documents for the European Union, important dissemination activities as well as the continued cooperation with the US NCI and international organizations as the US Clinical Laboratory and Laboratory Standards Institute (CLSI) or other larger grant projects.

As a conclusion we can state that SPIDIA has achieved all its major goals. The high importance of international pre-analytical workflow standardization including the generation of international standards documents and the development of new sample technologies is much more recognized internationally than prior to the project. The consortium would nevertheless like to express that there is still research and standardization work needed for completing the whole task of international pre-analytical workflow standardization. This includes additional pre-analytical workflows which were not part of SPIDIA’s program but also the final achievement of full ISO and EN international standards as the highest level of standardisation once the entire field has matured. The large US initiatives BRN and caHUB will consequently continue. Additional EU / EC activities would be highly beneficial for maintaining Europe's interests and its leading position within this field including resulting economic benefits.

2. DESCRIPTION OF THE PROJECT CONTEXT AND OBJECTIVES

SPIDIA was organized around three major activities. These are used here to structure this chapter.

2.1 Activity 1 – Evidence-based, international guidelines and quality-assurance schemes

Within SPIDIA's work package (WP) 1.1 current tissue preservation technologies (Formalin Fixed and Paraffin embedded tissue – FFPE and CRYO preserved tissue) were intensively assessed for stabilising nucleic acids, proteins, metabolites and tissue's antigenicity. Critical parameters for tissue samples qualities were identified and evaluated. These results served as a baseline data set for the evaluation of a novel tissue fixation and stabilisation solution developed within SPIDIA. This novel technology was developed by SPIDIA's industrial partners and is called PAXgene Tissue. The comprehensive technology evaluation addressed its applicability in routine medical service including the preservation of morphology (evaluated by international ring trials), antigenicity and biomolecules (nucleic acids, proteins, metabolites) in comparison with current tissue preservation techniques (FFPE, CRYO).
Finally, these comprehensive tissue studies on critical aspects of the pre-analytical phase provide a profound baseline data set for the pre-analytical workflow standardisation activities at the European Committee for Standardization (CEN) within WP1.4. This includes the preparation of CEN Technical Specification documents for national roll out in EU countries and their assessment by the CEN Technical Committee 140.
Conclusively, all main objectives of WP1.1 were achieved by a great collaborative effort between the SPIDIA partners of WP1.1 and other tissue-related WPs.

The main goal of WP 1.2 was the development and evaluation of External Quality Assurance (EQA) Schemes for pre-analytical workflows of human blood specimens. For this evaluation three different pre-analytical workflows were analysed: blood samples for genomic DNA analysis (SPIDIA-DNA), blood samples for cellular RNA analysis (SPIDIA-RNA), blood samples and plasma generated therefrom for circulating cell-free DNA analysis (SPIDIA-DNAplas). Two consecutive pan-European ring trials were planned and executed for each of these applications. The first one allowed the participating laboratories to use their own procedures. The second ring trials protocols asked the laboratories to follow SPIDIA's improved workflows. In total more than 300 laboratory applications were intended to be collected for participating in the first and in the second ring trial. The participating laboratories were planned to be located all over Europe. The ring trials including participants' data collection were supposed to be organized via by the web site of the European Federation of Clinical Chemistry and Laboratory Medicine EFCCLM with support via the SPIDIA web page.

The implementation of the two ring trials series included the development of ring trial protocols, participants recruitment and qualification, blood and plasma sample collection / generation, sample shipment to the participating laboratories, development of data collection forms, statistical analysis of data generated by the participants or by SPIDIA laboratories when analysing nucleic acids isolated by the participants, generation of specific reports for each participating laboratory, and the definition and optimization of the specific blood DNA, ccfDNA and RNA quality parameters for improving the results.
The nucleic acids isolated by the participating laboratories were shipped back to SPIDIA and were subsequently analysed by a panel of parameters: nucleic acid quantity (DNA, RNA, cfDNA), purity (DNA, RNA), integrity (DNA, ccfDNA, RNA), presence of PCR interferences (DNA, ccfDNA, RNA) and performance in dedicated validated RT-qPCR gene expression anylsis (RNA). Due to pre-analytical workflow improvements implemented in the second round of ring trials, the overall performance on the participating laboratories improved significantly compared to the first trials. This clearly demonstrated that improved and standardised pre-analytical workflows can improve the analytical test results.

SPIDIA also looked into the stability of proteins in blood and plasma samples. Two time courses studies aimed to investigate the protein profile stability in blood specimens during pre-analytical workflows. Immunological or mass-spectrometry methods were used to identify stable and unstable protein species during these studies.

The SPIDIA Blood-DNA, -RNA and –DNAplas ring trials results as well as the blood protein studies results provide evidence for developing CEN Technical Specification documents for standardising pre-analytical workflows in European countries (see below).

The aim of the work WP1.3 was to characterize potential fluctuations of various bioanalyte levels in tissue and blood samples during the pre-analytical phase for identifying sample quality biomarkers. WP1.3. identified changes of proteins, phosphoproteins, mRNA, and metabolite levels in human and animal tissues exposed to known or experimentally controlled pre-analytical conditions. Keeping cold ischemia times short or the samples on ice prior to stabilization significantly reduced this effect. Importantly, although extensive inter-individual variability at all levels of analyses was seen, this work package identified several bioanalytes that did not vary during the pre-analytical phase from tissue collection until preservation and, therefore, may be considered as stable reference markers for biomarker development programs. This work package successfully designed, used, and validated assays for proteins, phosphoproteins, mRNAs, and metabolites for monitoring pre-analytical variations in tissues. For blood, in total 14 RNA quality biomarkers were identified and validated for monitoring transcript changes affected by pre-analytical variations. In addition, four of these biomarkers were applied as sample quality parameters for the second SPIDIA-RNA ring trial.

The goal of work package 1.4 was to use the results of the collaborative research carried out under SPIDIA as a basis for standardisation activities at the CEN Technical Committee (CEN/TC) 140, "*In vitro diagnostic medical devices"*. The CEN/TC 140 plenary meeting 2012 accepted a New Work Item Proposal (NWIP) presented by SPIDIA representatives for developing CEN Technical Specification documents on pre-analytical workflows for selected types of samples. A dedicated working group within the CEN/TC 140 is currently working on drafting these documents. SPIDIA representatives are active members in this working group. Drafting these CEN Technical Specifications could not start earlier than February 2013 as SPIDIA's research data, which was gained up until March, have to be considered. Taking into account also the finalization of documents and the approval process, it is anticipated that the first CEN Technical Specifications will be finalized for national roll out in EU countries by Q3 or Q4 2014. This standardisation work will therefore continue beyond SPIDIA's funding period end.
In addition SPIDIA's results are intended to be used for updating a relevant US Clinical and Laboratory Standards Institute (CLSI) guideline. This is expected to happen during the course of 2014.

2.2 Activity 2 – Research leading to pre-analytical in vitro diagnostic tools that eliminate human error

The second activity was dedicated to the development of new technologies and tools to improve weak steps and links in the pre-analytical sample handling. Special emphasis was laid on the discovery and development of novel stabilization technologies for tissues, blood, and non-invasive samples, such as swab samples or plasma samples for pre-natal and cancer analysis and on the development of integrated complete sample-to-result workflows.

For tissue samples, there is currently no pre-analytical collection, stabilisation and storage technology available, which can be used for both, classical histopathological examination and molecular analytical methods from the same piece of tissue. Methods for tissue fixation currently used in traditional histology are of limited use for molecular analysis. Fixatives that contain formaldehyde cross-link and modify biomolecules. Such modifications lead to biomolecule degradation and inhibition in analytical downstream applications.
In WP2.1 a new tissue fixation and stabilization technology was developed for simultaneous preservation of morphology and biomolecules consisting of a fixative and a stabilization reagent. Within WP2.4 the task was to develop collection containers to integrate the new fixation and stabilization technology and to evaluate it for use in routine diagnostic workflows. In summary, the main objectives were:

- Discover compositions of stabilising compounds protecting both tissue morphology and biomolecular targets, including DNA, RNA and proteins.

- Provide a proof of principle that tissues fixed and stabilized with the new tissue reagents are compatible with immunohistochemistry, classical pathological analysis and can be used for isolation of high qualtiy nucleic acids as well as proteins.
- Perform workflow analysis, develop guidelines for usage of the new technology in routine pathology.
- Create new containers for a pre-analytical system, which can be used for tissue collection, transport and storage and to integrate the stabilisation technologies discovered within WP2.1.
- Test and adapt the tissue stabilization chemistry for fixation of fine needle aspirates and show in a proof of principle its applicability.

Work package 2.3 dealt with the optimization of pre-analytical workflows for IHC based diagnostics in pathology laboratories. Histology, immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) are procedures based on subjective assessment of stained tissue by individual pathologists. The pathologists are trained for years to recognize the different features of the tissues slide. Therefore, it is of outermost importance that a new fixative will give acceptable features and staining pattern compared to the golden standard, formalin fixed paraffin embedded tissues (FFPE). A core objective of WP 2.3 was to test and thereafter optimize staining protocols for H&E, IHC and ISH technologies for the PAXgene Tissue fixative.

In clinical practice today, tissue sample pre-treatment, processes, and protocols for IHC and FISH/CISH can vary widely between different laboratories. For several assays optimization was therefore performed independently by SPIDIA partners in their own laboratories thereby testing if different procedures could be used.

SPIDIA also looked into the discovery of new stabilization technologies for blood samples (WP2.2). Evolving new molecular diagnostics require new pre-analytical tools and procedures in order to ensure valid results. For example, cellular transcript profiles can change over time after a blood sample is taken from a patient and thus do not reflect the situation in the patient but an artificial profile generated by sample handling, storage and shipment. This is can be avoided if the sample material is stabilised appropriately. Technical solutions available today allow the stabilisation of gene transcript levels in blood but lyse all cells. This allows the analysis of whole blood RNA gene expression profiles but does not allow the isolation / enrichment of white blood cells or circulating tumor or impacted organ cells. In contrast, solutions that keep cells intact are not capable to stabilize the transcriptome in these cells. This currently limits progress in cancer diagnostics and personalized medicine projects. The objective of WP 2.2 was to fill this gap by a three step approach:

1. Discover stabilising compounds / compound mixtures that protect both, the morphology of white blood and other circulating cells as well as cellular biomolecules,
2. External proof-of-principle evaluation of the best stabilisation candidate by applying a broader set of downstream technologies
3. Introduction of the best candidate stabilization solution into a prototype blood collection tube and its performance testing.

The WP 2.5 was dedicated to the improvement of non or minimally invasive sample collection technologies, like swabs and plasma samples. Many types of swabs are already available for all kinds of sample materials but there is a need to have dedicated swabs for nucleic acid based analyses, which stabilize the analyte, prevent bacterial overgrowth after sample collection and allow an optimized release of this biomolecular target during sample analysis. Additionally, current swab collection systems are not certified as pathogen inactivating. One objective of this WP was the discovery of an optimised collection and stabilisation system for swab samples for use in DNA based diagnostic applications, as well as the discovery of pathogen inactivating technologies for swab collection, transport and storage.
Human plasma and serum samples can serve as minimal invasive sample materials for diagnostic purposes, as it is known, that they contain cell-free circulating nucleic acids (ccfNA) from different origins, like tumours or a foetus in the case of pregnancy. This DNA can be used for new diagnostic tests. Another WP 2.5 objective was therefore the development of a stabilization technology that prevents the dilution (“contamination”) of plasma cell-free DNA with release genomic DNA from blood cells as well as ccfNAs degradation after blood collection.

At the start of SPIDIA, there was no sample-to-result system for RNA gene expression analysis from stabilized human whole blood available that integrates all pre-analytical and analytical steps. There were automated systems available for the extraction of RNA from these samples, but all systems were limited to the stage where the pure RNA is isolated. Another disadvantage of available automated systems was the lack of flexibility to process different samples numbers at a time. Therefore, the aim of WP 2.6 was to develop a robust automated system that integrates the pre-analytical steps and the analytical assay. Furthermore this system should be have been able to efficiently isolate all RNA species (including mRNAs and miRNAs)

WP2.7 had been dedicated to the development of additional validated sample-to-result workflows. The results of other work packages were combined for achieving this goal. WP2.7 included the evaluation of these complete workflows for molecular diagnosis of two case pathologies, namely Alzheimer's disease and colorectal cancer. These belong to the most common diseases that affect patients in western countries. The implementation of optimized pre-analytical workflows processing stabilized human blood, were seen as a key element for enabling high sensitivity and correct analytical test results for early diagnosis. Colorectal cancer was further characterized via metabolomics analysis of metastatic patients' serum.

2.3 Activity 3 – Management, ethics and spreading of excellence

The main tasks of WP3.1 had been to develop an internal and external training program and to disseminate the results of SPIDIA to the scientific community and the general public. During the project time 27 different training activities have been performed including three major workshops summarizing the main results from SPIDIA. The SPIDIA webpage (www.spidia.eu) has since then been continuously updated with information about SPIDIA results as well as meeting and events with SPIDIA participation, publications, and links to related organisations. On the webpage it has also been possible to sign up for the SPIDIA newsletter. Today more than 800 people have registered for the newsletter. Six newsletters have been published. The SPIDIA consortium has contributed more than 60 oral or poster presentations to international conferences and meetings. Also, 11 papers have been published in international peer-review journals. In addition, three scientific papers have been accepted for publication. Several articles are currently in preparation or have been submitted for publication

The role of the management team, composed by the project coordinator (QIAGEN) and the project management partner (NOVAMEN) was to ensure that consortium activities are performed in accordance to the EC expectations in terms of content and timescale.
A manual of management was released during the third month of the project and set out the main decision-making rules and the main procedures regarding the running of the project.
Partners have nominated a committee to deal with all the Industrial and Intellectual Property aspects related to the SPIDIA project. The aims of this committee were i) to identify the knowledge that could require protection, commercial development or dissemination, ii) to guide the consortium in choosing the best measures to secure its intellectual and industrial property and iii) to define procedures for handling the industrial property rights.
The dissemination and the exploitation of the project's knowledge are described in a Plan for the use and dissemination of foreground and planned to be delivered at the end of the project.

Within WP3.3, the SPIDIA PEC (Project Ethics Committee) was formed. The main objectives were to ensure that: i) all experiments performed in the project complied with national and European rules and all new ethical issues raised by SPIDIA research were properly addressed, ii) it was constantly ensured that the 3R's in relation to animal work are observed. Therefore, documents and letters of intent were asked from the participants involved in research with human biological materials. The matters were discussed at every SPIDIA meeting where direct questions were dealt with. Certain topics were further deepened in ethics workshops for either SPIDIA consortium or the general public. In addition, there was attention for transparency resulting in an explanation on how SPIDIA worked with human and animal materials on the SPIDIA web site. Dealing with the subject in this way made all the SPIDIA participants very aware of the ethical side, which goes far beyond the regulatory side, of working with animal and human materials. SPIDIA's external advisors Ruth Chadwick and Anne Cambon Thompson accompanied the consortium with great support during the entire course of the project.

3. DESCRIPTION OF THE MAIN SCIENTIFIC AND TECHNICAL RESULTS

3.1 WP1.1 – Validation of tissue-related technologies and documentation for standardization activity

Molecular characterization of human cancer samples requires the analysis of multiple parameters ranging from classical histopathological features to an increasingly broader spectrum of molecular biomarkers, e.g. for Personalized Medicine. The morphological characterization is routinely based on the analysis of Formalin Fixed and Paraffin Embedded (FFPE) tissues but it is known that formalin fixation impairs molecular analyses. Therefore molecular analytical tests typically require frozen tissue samples. For the assessment of current and novel tissue preservation techniques as well as critical aspects of tissue sample quality, a major tissue sample collection was performed at SPIDIA's partners MUG, EMC and TUM which resulted in more than 5000 differently processed malignant and non-malignant tissue samples collected for SPIDIA. Additionally, dedicated tissue collections were performed to evaluate critical pre-analytical variables such as ischemia, and for preparation of the SPIDIA cancer morphology ring trials.

Current state-of-the-art techniques for tissue preservation (FFPE; snap-freezing) were evaluated as well as the impact of several pre-analytical variables on tissue sample quality and subsequent molecular analyses. Results demonstrated that established methods for RNA quality control (e.g. 28S:18S ratio, RIN value) were well-suited for frozen tissue but do not readily correlate with e.g. RT-PCR amplification efficiency of RNA isolated from FFPE tissues (Fig. 1). For these samples we recommend a more detailed analysis, like a RT-qPCR assay based on different amplicon lengths. We developed such an assay, to estimate the suitability of isolated RNA for downstream analytical applications. Furthermore, we could demonstrate that results of RT-qPCR were sensitive to degradation of RNA introduced by pre-analytical procedures such as time or type of fixation and storage conditions (Fig. 2).

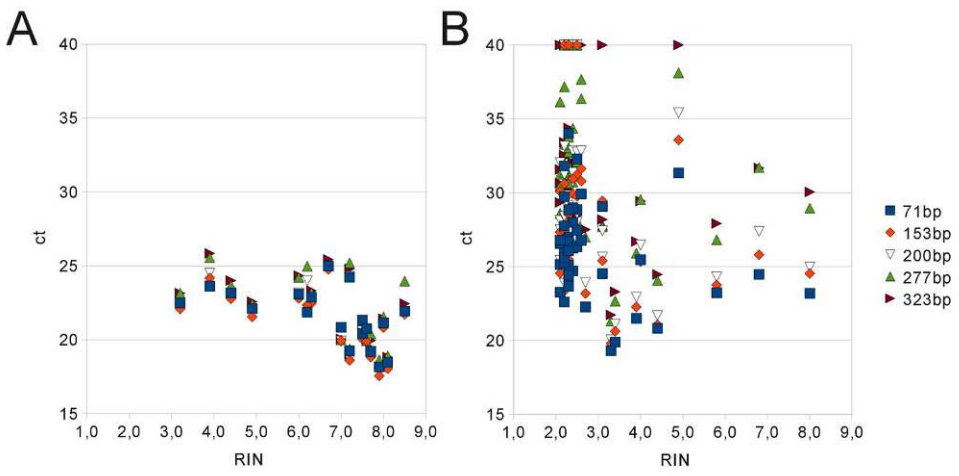


***Fig. 1**: **Comparison of RIN values and RT-qPCR results of different FFPE and cryopreserved samples.** RIN and ct-values generated by RT-qPCR of RNA extracted from 65 different samples of 15 patients are shown.*

*Corresponding aliquots of different tissue types were **(A)** cryopreserved or **(B)** fixed in formalin and embedded in paraffin. The majority of cryopreserved samples had RIN values above 5 while the majority of FFPE samples showed low RIN values between 2 and 3. In CRYO samples the Pearson correlation factor between RIN and ct values was -0.5 to -0.6, in FFPE samples the correlation factor was -0.25 to -0.28 depending on amplicon length. The difference in ct values obtained from CRYO or FFPE samples was statistically significant (p<0.01). Viertler, Kashofer et al, (PLoS ONE, accepted for publication).*

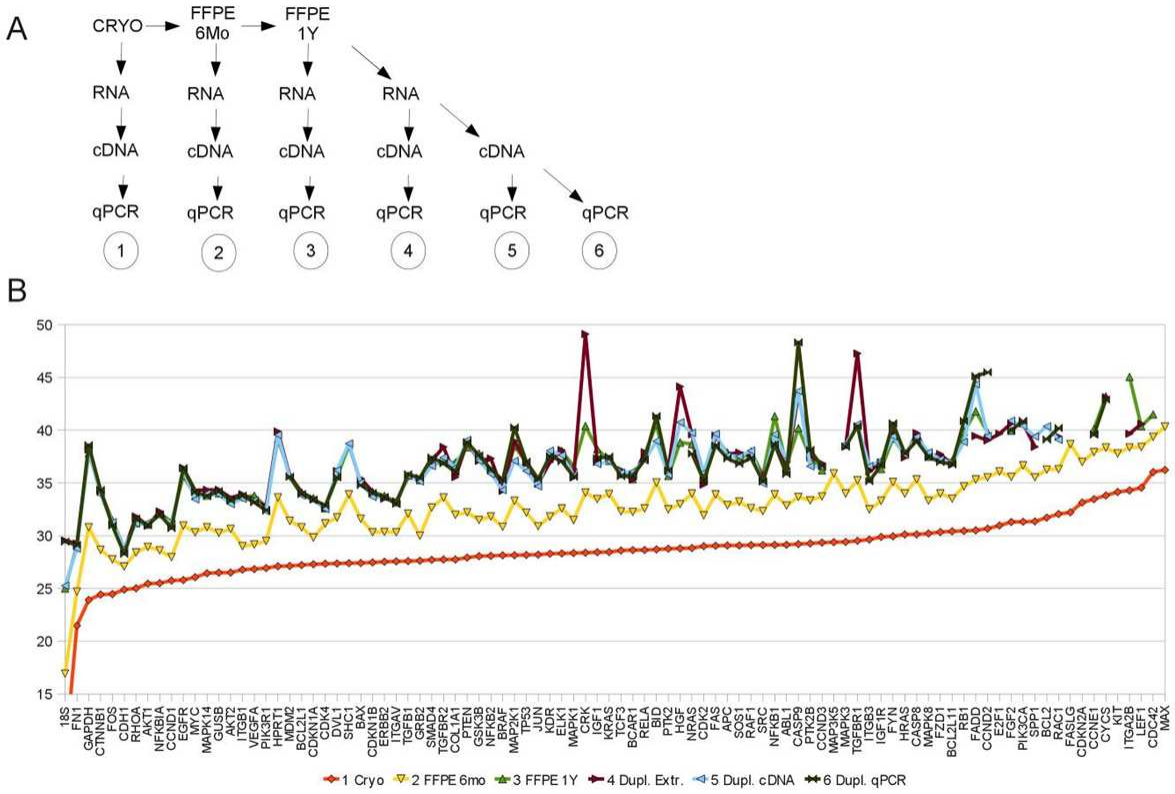


***Fig. 2**: **Fixation and storage introduces major gene-to-gene variations in RT-qPCR efficiency.** Human liver samples were cryopreserved or fixed in formalin and paraffin embedded. **(A)** RNA was extracted from samples at different time points including technical replicates. **(B)** Comparison of RT-qPCR data for 92 genes from cryopreserved and FFPE human liver samples revealed an average difference of the ct values ranging from 4 cycles (6 months) to 8 cycles (1 year) increasing with storage time at room temperature. Extraction from the FFPE sample which had been stored for one year was done in duplicates. cDNA generation was performed in duplicates from the same RNA, and qRT-PCR was performed twice from the same cDNA. Data was generated with the TaqMan "Human Molecular Mechanisms of Cancer" assay, individual ct values are shown. Viertler, Kashofer et al (PLoS ONE, accepted for publication).*

Additionally, we could show effects of pre-analytical parameters on protein yield and quality. We also optimized protein extraction protocols for FFPE and cryo-preserved samples. Furthermore, SOPs for NMR sample preparation and spectral acquisition for metabolomic studies were established. The effect on the metabolome by pre-analytical variables such as ischemia times, sample storage and preservation techniques was investigated. A detailed study on the effects of intraoperative warm and postoperative cold ischemia was performed on the level of RNA, proteins and metabolites and was reported in detail within the tissue biomarker studies in WP1.3 and 2.7. An example of the effect of ischemia on the NMR profiles is shown in Fig. 3.

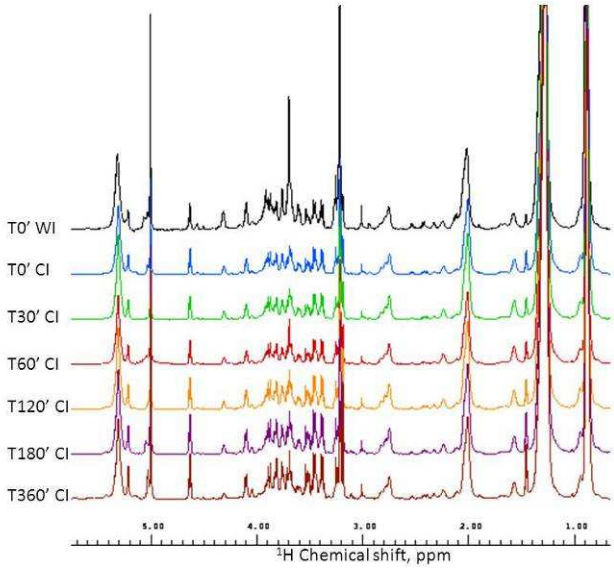


***Fig. 3*: HR-MAS ^{1}H NMR spectra of human liver samples from the same donor, exposed at different ischemia times and types.** *Spectra have been acquired at 277 K and 600 MHz, using 4 kHz of MAX spin rate. WI = warm ischemia, CI = cold ischemia.*

In conclusion, pre-analytical parameters had a major impact on molecular tissue diagnostics and assay results. Consequently, the quality of tissue-based molecular analyses can only be defined in the context of the pre-analytical procedures. Hence, the results obtained by these studies also served as a baseline data set for the evaluation of novel tissue-related technologies.

We also evaluated a new tissue fixation and stabilisation technology (PAXgene Tissue), developed by SPIDIA´s industrial partners, for preservation of biomolecules, morphology and antigenicity in comparison with current state of the art techniques (FFPE and cryo-preservation). The preservation of biomolecules was evaluated by a multitude of state-of-the-art techniques for quality control and in several analytical downstream applications.

An excellent preservation of RNA integrity, RNA profiles (Fig. 4), microRNA, high-molecular-mass DNA, proteins as well as phospho-proteins (Fig. 5) and antigenicity by the new SPIDIA PAXgene Tissue technology was demonstrated by our studies. This opens the opportunity for the analysis of a broad spectrum of biomolecules from the same tissue samples collected for histopathological diagnosis. This minimizes the risk that molecular analyses negatively interfere with routine healthcare and guarantees that molecular data can be directly compared with histopathologically characterized lesions.

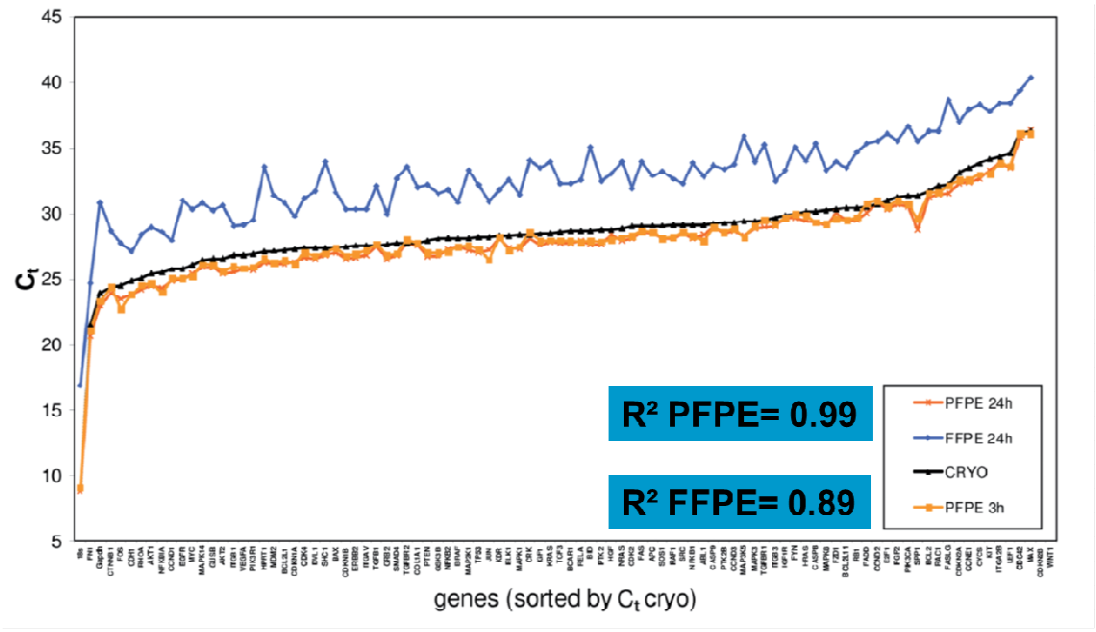


Fig. 4: Strong correlation of gene signature from PFPE and snap-frozen tissue, lower amplification efficiency and major gene-to-gene variations from FFPE tissue. *Gene expression analysis of 92 cancer pathway-associated and 4 endogenous control genes (18S, GAPDH, GUSB, HPRT1) was performed. Gene signature from PFPE, FFPE, and snap-frozen (CRYO) human liver analyzed by RT-qPCR on predefined TaqMan array "Human Molecular Mechanisms of Cancer" plate. Genes are sorted by increasing Ct value using the frozen sample as reference. Viertler et al, J Mol Diagn, 2012.*

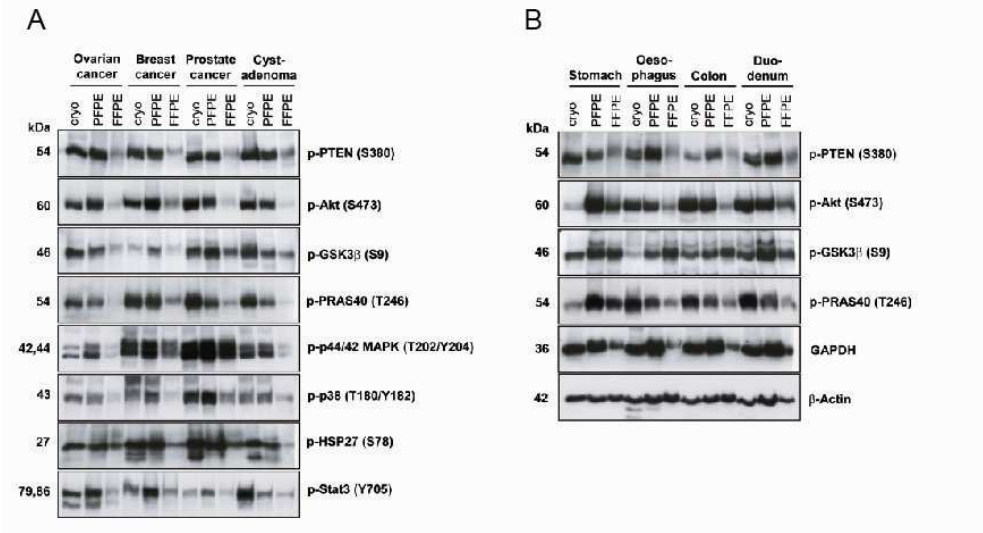


Fig. 5: The PAXgene Tissue System preserves phospho-proteins in human clinical tissue specimens. (A) *Three human malignant (ovarian, breast and prostate cancer), one pre-malignant (cystadenoma), and* ***(B)*** *four non-malignant (stomach, oesophagus, colon, duodenum) tissue specimens were each divided into three samples and either cryopreserved (cryo), fixed and stabilized in the PAXgene Tissue reagents and paraffin-embedded (PFPE) or fixed in formalin and paraffin-embedded (FFPE). Proteins were extracted with optimized protocols and 15 µg protein of each was separated by SDS-PAGE. Western blot analysis was performed using indicated antibodies. Gündisch et al., PLOS ONE, 2013*

Critical parameters for applying this new stabilization technology to routine medical service such as the impact of tissue samples storage conditions on nucleic acids, retraction artefacts in certain tumor samples or the reliability of IHC results were defined. The impact on morphology and suitability for routine diagnostics were evaluated in detail by a morphology scoring and by three cancer morphology ring trials (breast, colon, prostate) involving 69 renowned pathologists from Europe and the US. First results indicated the suitability of the new PAXgene Fixed Paraffin Embedded (PFPE) tissues for histopathological diagnosis, as demonstrated for the grading of breast and colon cancer.

The versatility of the novel tissue stabilisation system provides new opportunities for a series of application scenarios, e.g. in the context of clinical trials, biomarker discovery programs or molecular analysis of lesions where a collection of snap-frozen material is impossible for logistic, medical or ethical reasons.

The results of these comprehensive studies in collaboration of all tissue related WPs were published in six manuscripts in peer-reviewed journals. Additional publications are currently in review or in preparation. Furthermore, all milestones and deliverables of WP1.1 were achieved. Public international workshops were organized in Graz, Austria 2012 as well as in Hilden, Germany 2013. Several international collaborations e.g. with the National Cancer Institute in the US were established.

Critical aspects for standardisation of the pre-analytical phase have been identified and investigated in detail within WP1.1 and other tissue-related WPs. The available results provide a solid baseline data set for the standardisation activity at CEN and the development of new CEN Technical Specification documents. The SPIDIA studies initiated and supported this standardisation activity and led to a first best practice document which serves also as a baseline for further development into CEN Technical Specification documents. As this is a complex, multi-year process involving national and international standardisation bodies, these activities were not finalized by the end of the SPIDIA project, but will be actively continued by representatives of the SPIDIA consortium within the relevant CEN Technical Committee 140 Working group 3.

3.2 WP1.2- Evidence-based Quality Guidelines for the pre-analytical phase of Blood Samples

The WP 1.2 results presented here have partly been published in two scientific papers in peer-reviewed journals. Additional publications summarizing other results described below are currently in preparation.

Molecular *in vitro* diagnostics will play an important role in future health care practice. This includes gene expression profiling promising to provide insight into normal biological and pathological processes and to enable individualized therapies as well as therapy monitoring. In this field, significant improvements of analytical downstream assays and data analysis (analytical process) have been made during the last years. In contrast, the influence of the pre-analytical steps, such as sample collection, stabilization, transport, storage, and processing has been highly underestimated. The SPIDIA WP 1.2 was focused on the standardisation of these pre-analytical workflows for blood samples and on the identification of critical steps in these pre-analytical workflows that need further improvement. These goals were achieved by the development and implementation of a panel of Pan-European External Quality Assurance schemes (EQAs) / ring trials specifically designated for genomic DNA analysis from blood (SPIDIA-DNA), cell-free circulating DNA analysis from plasma (SPIDIA-DNAplas) and cellular RNA analysis from blood (SPIDIA-RNA). In addition, time course studies for investigating the stability of proteins in blood / plasma were performed.

The results obtained from the pan-European ring trials were expected to serve a basis for writing evidence-based guidelines (**Fig.6)**.

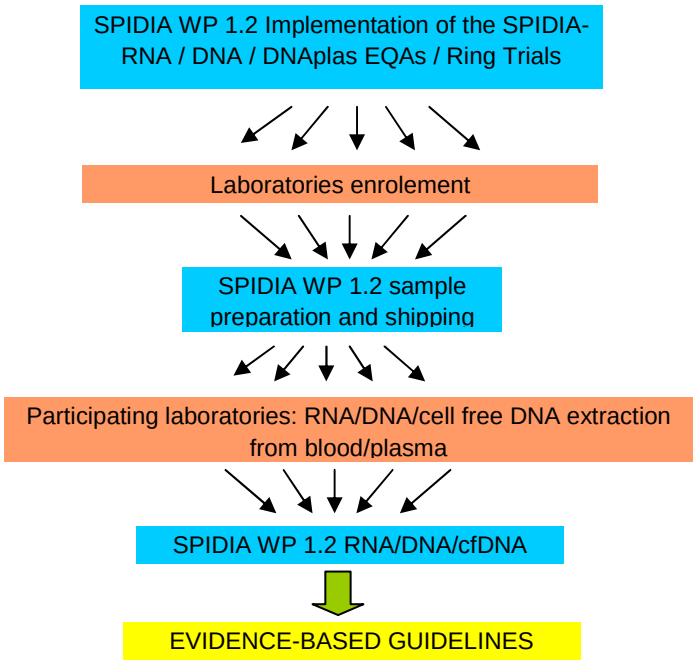


***Fig. 6**: SPIDIA-DNA/RNA/DNAplas workflow*

***Table 1:** Number of expected (planned), accepted and free applications of the SPIDIA 1st and 2nd Ring Trials*

SPIDIA	Expected		Accepted		Free	
	1st ring trials	2nd ring trials	1st ring trials	2nd ring trials	1st ring trials	2nd ring trials
DNA	200	200	131	127	69	73
DNAplas	60	60	67	66	-7*	-6*
RNA	160	160	124	122	36	38
Total	420	420	322	315	98	105

* *The number of DNAplas registrations exceeded that planned.*

***Table 2**: Percentage re-entry samples according the DNA, DNAplas and RNA shipped kit 1st ring trials and 2nd ring trials.*

DNA				**DNAplas**				**RNA**			
1st ring trials		2nd ring trials		1st ring trials		2nd ring trials		1st ring trials		2nd ring trials	
Sent	*Returned*	*Sent*	*Returned*	*Sent*	*Returned*	*Sent*	*Returned*	*Sent*	*Returned*	*Sent*	*Returned*
131*	116	127*	119	67*	61	61*	56	102*	93	119*	109
Return rate: 88.5%		Return rate: 94%		Return rate: 91%		Return rate: 92%		Return rate: 92%		Return rate: 92%	

* as of some laboratories renounced to participate in the Ring Trials, this number represents the effective kit shipped to the participants

The participants recruitment as well as the data collection from these participating laboratories were organized via a dedicate webpage of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). Tables 1 and 2 describe the number of participating laboratories for the individual rings trials 1 and 2.

The participating laboratories received homogenous samples from SPIDIA's member UNIFI. They sent their isolated nucleic acids back to SPIDIA. In SPIDIA's laboratories at different partner sites these isolated nucleic acids were analysed in depth by various analytical downstream tests for judging their qualities. Based on statistical parameters, the performance of each laboratory was classified for each analytical performance parameter into one of the three categories "in control", "warning" or "out of control". Based on the results obtained from the first ring trials, several improvements were incorporated into the second ring trial series (preparation of samples, handling of samples at the participants' laboratories as storage times and conditions, and data analysis). Comparison of the laboratories' performances between the two series of Blood RNA and of Blood DNA ring trial runs showed significant improvements in the second ring trial: the number of the parameters that were in control increased and the parameters that were in the warning category or out of control category decreased (Figure 7 and 8). This result clearly demonstrates, that the measures, that were taken by SPIDIA improved the pre-analytical workflows for blood samples collected for RNA or DNA analysis significantly.

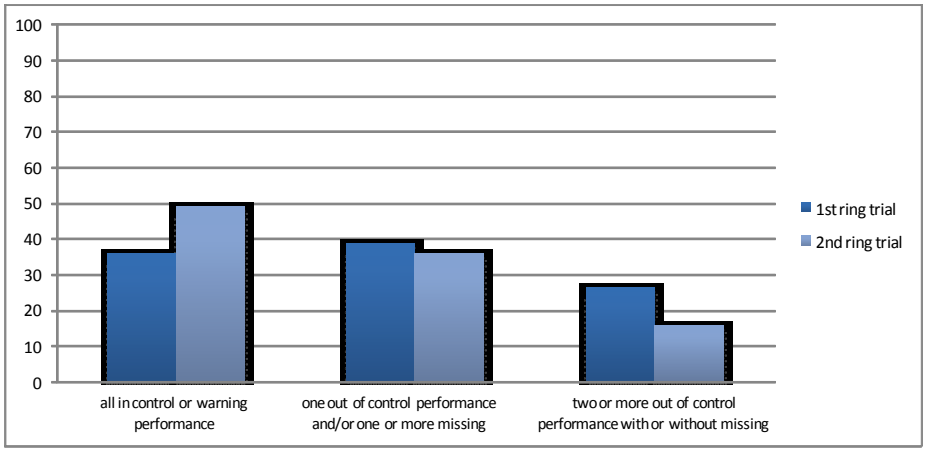


***Fig. 7**: SPIDIA-DNAs laboratories performances (manuscript for publication in preparation)*

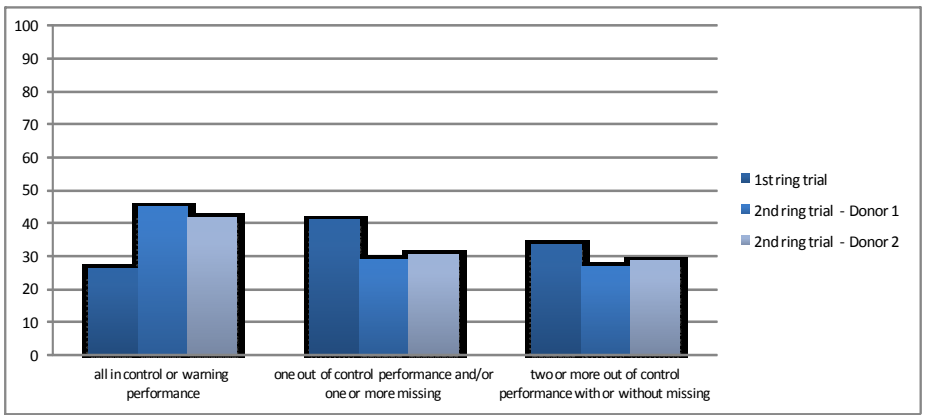


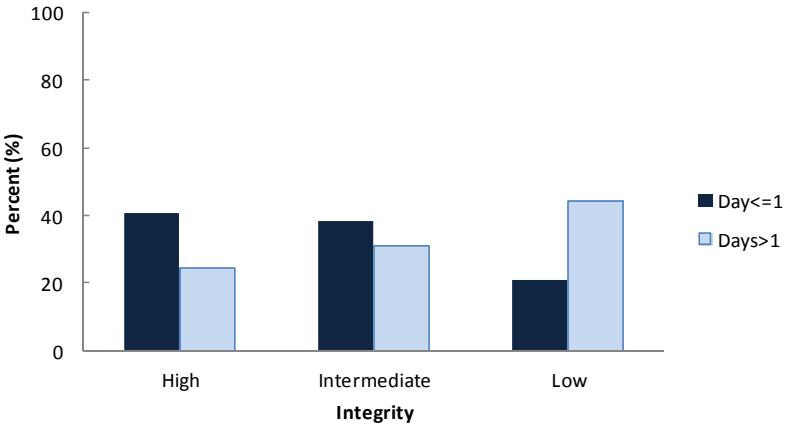
***Fig. 8:** SPIDIA-RNAs performances (manuscript for publication in preparation)*

SPIDIA-DNA (Blood DNA) RING TRIALS

SPIDIA's partner UNIFI sent cooled aliquots from a fresh blood donation as fast as possible to all participating laboratories in Europe. These were asked to store the samples and to extract genomic DNA according to their own routine procedures, to measure the DNA purity and quantity and to send back the extracted DNA to UNIFI. In addition, each laboratory was asked to file their sample storage and processing details on the dedicated EFLM website.

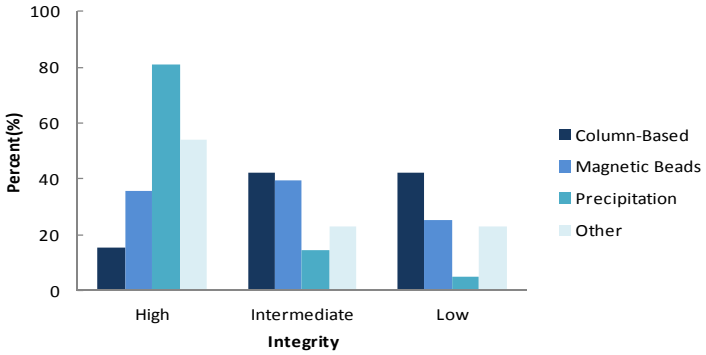
The DNA was isolated by the participants and sent back to SPIDIA. It was analysed in different SPIDIA laboratories by quality parameters such as purity and total yield (by spectrophotometric measurements and by qPCR single-gene quantification), integrity (by Pulse Field Gel Electrophoresis)), and the presence of matrix interferences (by Kineret analysis)). Furthermore the influence of the genomic DNA length on PCR assays of different amplicon lengths was investigated. Each participating laboratory received a detailed report containing its results. All data were also statistically analysed by SPIDIA's partner INT in order to identify the most critical pre-analytical variables that can influence the sample quality parameters.

The second SPIDIA Blood-DNA ring trial scheme was improved in terms of the blood collection procedure, the preparation of ring trial samples and the conditions for shipping blood samples to the participating laboratories (box guaranteeing +4℃ for 48h). In addition the participants were asked not to follow their own internal procedures as during the first ring trial but to follow SPIDA's protocols (sample storage at 4℃, storage time limited, DNA isolation within 3 days). Also, the methodology to evaluate the High Molecular weight DNA integrity in SPIDIA laboratories was changed to an "ad hoc algorithm" combining the "by eye" evaluation of Pulse Field Gel Electrophoresis by a panel of experts and ImageJ software data analysis.
The ring trial results demonstrate that pre-analytical variables such as blood samples storage time prior to DNA extraction and the DNA extraction method can influence quality parameters as DNA integrity (Figures 9 and 10).



	Day ≤ 1	Days > 1	***Total***
High Integrity	39 (40.6 %)	15 (24.6 %)	*54*
Intermediate Integrity	37 (38.6 %)	19 (31.1 %)	*56*
Low Integrity	20(20.8 %)	27 (44.3 %)	*47*
Total	*96*	*61*	*157*

Fig. 9: *Contingency table and histogram for DNA Integrity according to the Time interval blood sample arrival-extraction (manuscript for publication in preparation)*



	Column-Based	Magnetic Beads	Precipitation	Other	***Total***
High Integrity	11 (15.1 %)	17 (35.4 %)	17 (81.0 %)	7 (53.8 %)	*52*
Intermediate Integrity	31 (42.5 %)	19 (39.6 %)	3 (14.3 %)	3 (23.1 %)	*56*
Low Integrity	31(42.5 %)	12 (25.0 %)	1 (0.48 %)	3 (23.1 %)	*47*
Total	*73*	*48*	*21*	*13*	*155*

Fig. 10: *Contingency table and histogram for the DNA integrity according to the Extraction Method (manuscript for publication in preparation)*

SPIDIA-DNAplas (plasma cell-free circulating DNA): 1st and 2nd RING TRIAL

As for the other ring trials, SPIDIA's partner UNIFI prepared the samples. Plasma sample generated from whole blood were sent to the participating European laboratories. For the first ring trial these were asked to handle the samples and to extract cell-free circulating DNA following their own routine procedures. The participants were asked to send the isolated ccfDNA back to UNIFI. Each laboratory was also asked to file their sample storage and processing details on a dedicated EFLM website. SPIDIA laboratories analysed the patricipants' ccfDNA samples by quality parameters such as total ccfDNA yield (by qPCR absolute quantification of a single copy gene), integrity (by Isohelix kit + Agilent electrophoresis). Each participating laboratory finally received a detailed report describing its results.
All data were also analysed on an overall level in order to identify the most critical pre-analytical variables that can influence the sample quality parameters.

Based on the analysis of ring trial 1, the second ring trial was improved in terms of the blood collection procedure, the preparation of the ring trial plasma samples and the conditions for shipping blood samples to the participating laboratories (boxes guaranteeing +4℃ for 48h). In addition, the participants were asked not to follow their own internal procedures as for the first ring trial but to apply SPIDA's protocol (sample storage at 4℃, DNA isolation within 3 days).

The DNAplas ring trial demonstrated that the extraction procedure can influence the ccfDNA yield (Figure 11).

It should be noted that SPIDIA demonstrated in another work package that the release of genomic DNA from blood cells during pre-analytical workflows is a critical artefact which can change the overall ccfDNA profile thus potentially influencing analytical test results. As the SPIDIA ring trial was based on plasma samples which were promptly generated after blood collection, this artefact did not occur and could thus not be monitored.

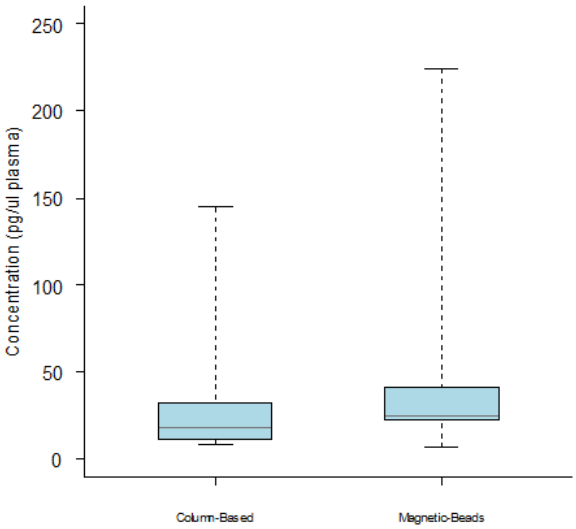


Fig. 11: *Descriptive statistics and box-plot for the RNaseP quantification by qPCR according to the Extraction Method in plasma DNA (p<0.023) (manuscript for publication in preparation)*

SPIDIA-RNA (Blood Cellular RNA): 1st and 2d RING TRIAL

SPIDIA's partner UNIFI prepared the blood samples and sent them to the participating laboratories. For the first ring trial blood was distributed either in K_2EDTA blood collection tubes or into cellular RNA stabilizer containing PAXgene Blood RNA tubes, depending on the participants' requests. The cellular RNA had to be extracted from these blood samples at two different time points following the participating laboratories' own internal routine procedures (day of arrival and 24 h thereafter). The two isolated RNA samples were shipped back to UNIFI. Each laboratory was also asked to file their sample storage and processing details on a dedicated EFLM website.
The isolated RNA samples sent back to UNIFI were analysed by quality parameters such as purity and total yield (by spectrophotometric measurements), integrity (by Agilent technology-RIN evaluation, UNIFI), RT-qPCR assays for GAPDH, IL1 beta, IL8 and c-fos trancsripts, and the presence of interference substances by Kineret analysis. After statistical analysis each participating laboratory received a detailed report summarizing its results.

Based on the data and experiences obtained from the first ring trial, the second SPIDIA Blood-RNA ring trial scheme was changed in terms of the blood collection procedure and preparation of samples for the participants. In contrast to the first one, blood was not pooled for generating a large volume of homogenous blood prior to filling the smaller volumes into the blood collection tubes for shipment to the participants. This change was implemented in order to avoid gene expression profile artefacts (e.g. levelling out transcript level differences between individual sample donations). Instead two different donors' blood was distributed to individual blood collection tubes (K_2EDTA tubes, PAXgene Blood RNA tubes). In addition shipping boxes were used which guaranteed shipments of +4℃ for 48 h. Also, the panel of analytical downstream assays / quality parameters was broadened by adding new blood RNA sample quality biomarkers developed within WP1.3 (RT-qPCR assays) and an assay to evaluate the DNA contamination in RNA samples. After statistical analysis, the second ring trial data indicated several pre-analytical variables which had an influence on the analytical downstream test results. Amongst these was the type of blood collection tube (containing stabilization reagent or not) and the storage temperature if a non-stabilizing EDTA tube was used. The statistical data also showed that the DNase treatment (Fig 12) and the extraction procedures (Fig 13) can be pre-analytical variables influencing analytical test results. These later findings have to be interpreted carefully as participants who requested to use the IVD PAXgene Blood RNA tube had to use the linked PAXgene Blood RNA Kit (optimized and integrated in vitro

diagnostic system) whereas the participants who requested to use the EDTA Blood Collection tube were free to use their own cellular RNA isolation kits or laboratory procedure.

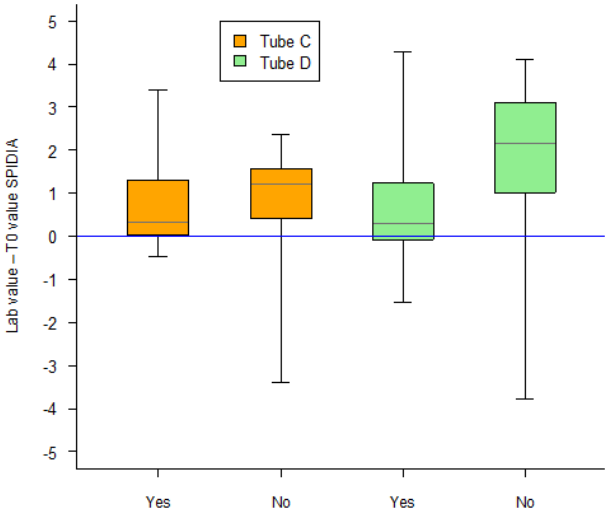


Fig. 12: *Relationship between RT-qPCR analysis data (IL8 mRNA), DNase Treatment and time blood storage (p<0.001, for tube 72h. Tube C: RNA isolated at the day of sample arrival, Tube D: RNA isolated 24 h after sample arrival. (manuscript for publication in preparation)*

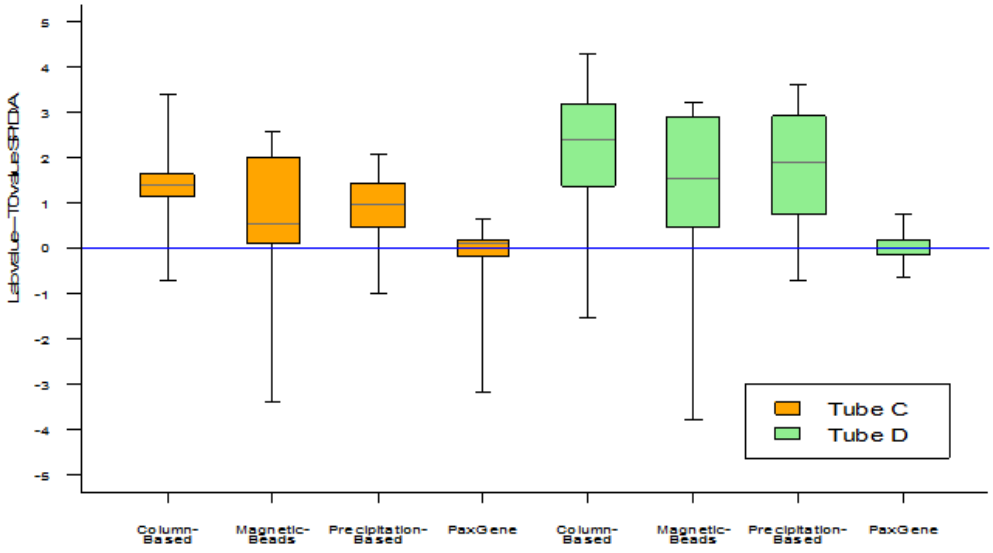


Fig. 13*: Relationship between Gene expression (IL8) and Extraction Method (p< 0.001 for Tube C and D) (manuscript for publication in preparation)*

In order to explore the association between RNA integrity and gene expression revealed by the first ring trial data, we dichotomized the continuous variable RIN values according to the cut-off value of 5. The box plots in Figure 14, obtained by analysing the GAPDH mRNA quantification by RT-qPCR, are representatives of data indicating a lower GAPDH mRNA quantification when RNA with RIN values of lower than 5 were analysed.

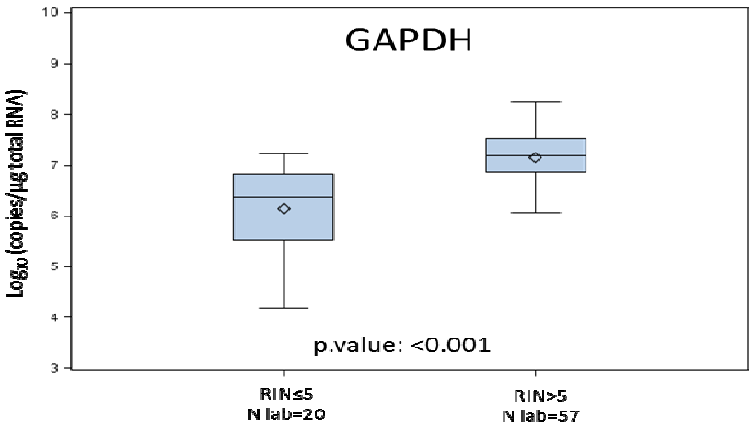


Fig. 14: *Relationship between RIN values and the gene expression quantification by RT-qPRC (according to the RIN values categorized on the basis of the cut-off of 5) (manuscript for publication in preparation)*

The SPIDIA WP 1.3 identified, developed and validated a set of blood RNA biomarkers suitable for monitoring pre-analytical workflows (RT-qPCR assays). These markers showed in all individual samples of a 60 donor cohort either an increase or decrease of the relevant transcripts during pre-analytical workflows when non-stabilising blood collection tubes were used. These assays therefore allow by comparing the specific transcript levels at time 0 and other time points during pre-analytical workflows if artificial RNA profile changes have occurred during pre-analytical workflows. When such non-stabilizing EDTA blood samples were used by the second ring trial participants such artificial changes in transcript levels indeed occurred (Figures 15 and 16).

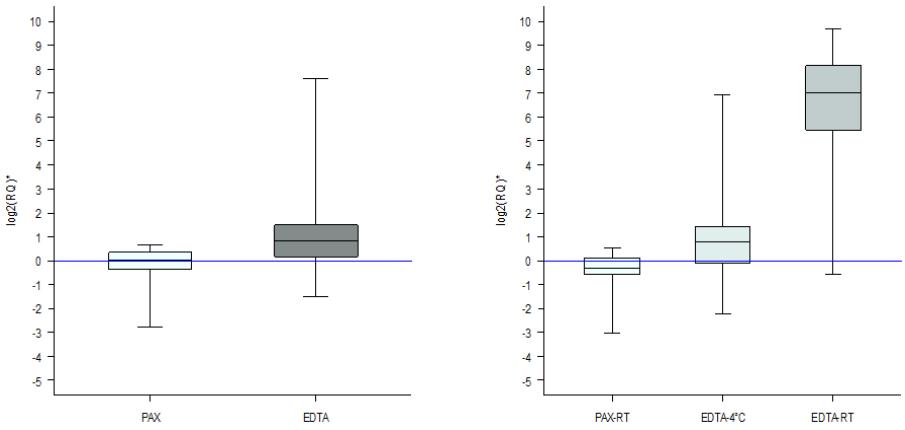


Fig. 15*: Relationship between blood collection tube and gene expression (up-regulation) (p<0.001 for PAXgene Blood RNA tube vs EDTA blood collection tube) and relationship among blood collection tube-temperature blood storage and gene expression (up-regulation) (p<0.001 for PAXgene room temperature (RT) vs EDTA -4℃ vs EDTA room temperatur e (RT)). Left: Tube C - RNA isolated at the day of sample arrival after +4 ℃ shipment. Right: Tube D – RNA isolated 2 4 h later). Shown is the relative quantification normalized to time 0 when blood collection at UNIFI took place prior to shipment. (manuscript for publication in preparation)*

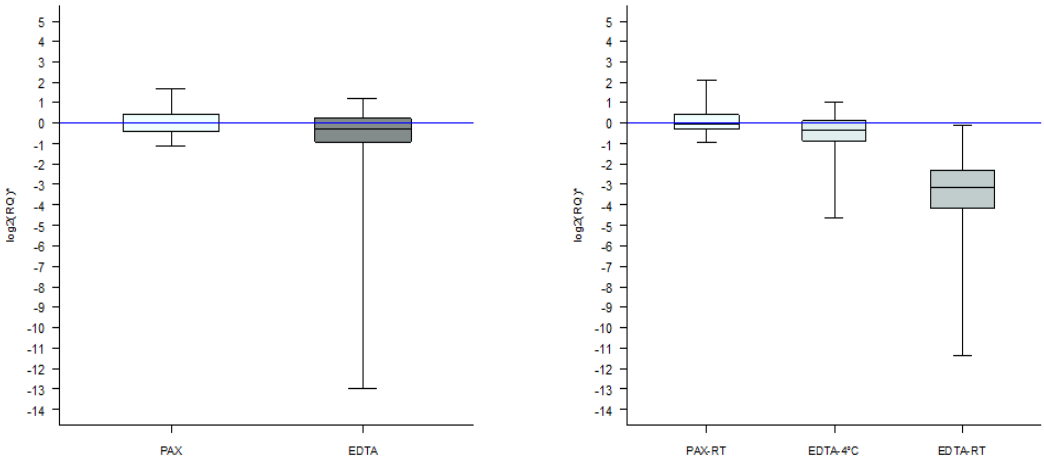


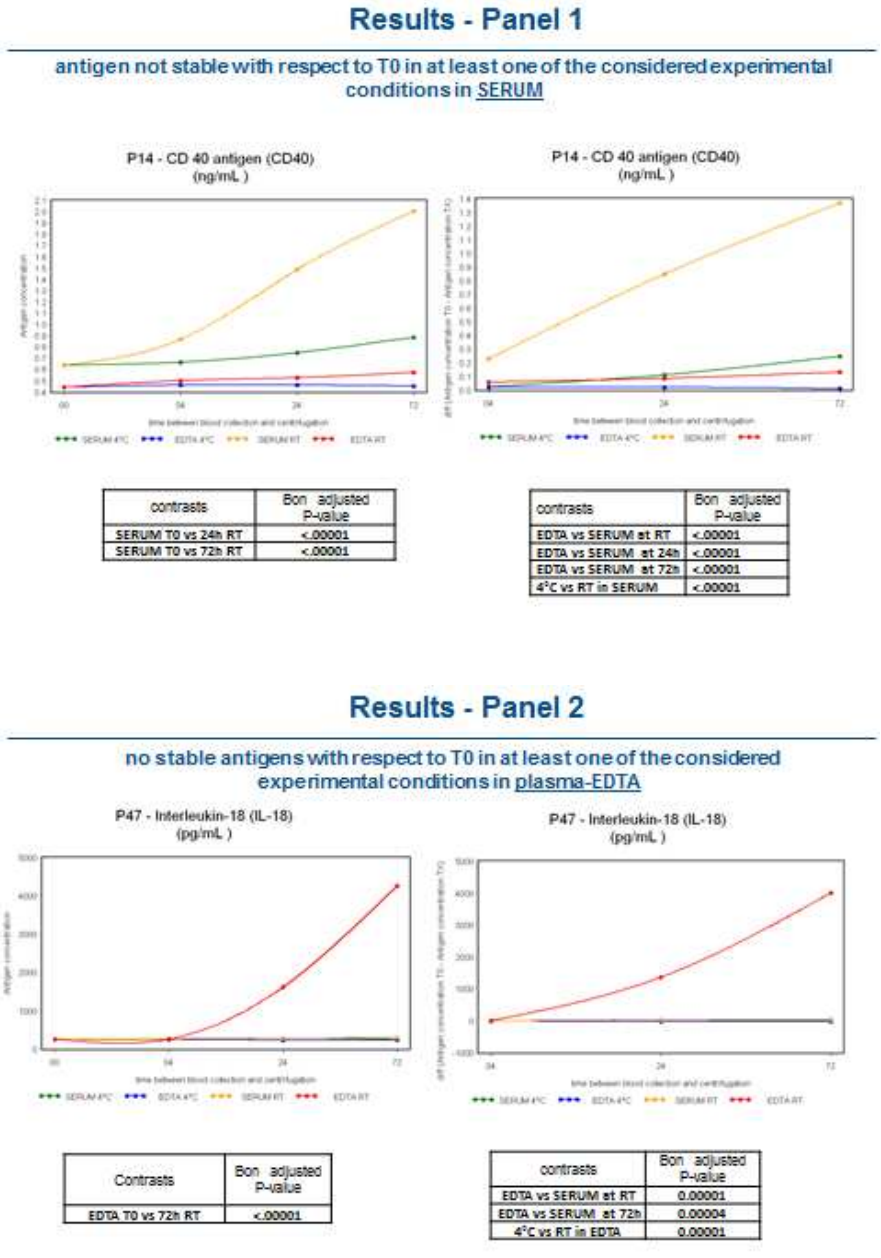
Fig. 16: *Relationship between blood collection tube and gene expression (down-regulation) (p<0.001 for PAXgene Blood RNA tube vs EDTA blood collection tube) and relationship among blood collection tube-temperature blood storage and gene expression (down-regulation) (p<0.001 for PAXgene room temperature (RT) vs EDTA -4℃ vs EDTA room tempera ture (RT). Left: Tube C - RNA isolated at the day of sample arrival after +4 ℃ shipment. Right: Tube D – RNA isol ated 24 h later). Shown is the relative quantification normalized to time 0 when blood collection at UNIFI took place prior to shipment. (manuscript for publication in preparation)*

SPIDIA-PROT (Blood Protein):

The blood protein profile stability was tested by two time courses experiments in order to identify specific protein species changes which might be used as blood protein quality markers for monitoring pre-analytical workflows. Pre-analytical variables such as blood collection tubes (with/without anticoagulant, serum and plasma samples) as well as blood, serum, and plasma storage time and storage temperature were analysed. The blood proteins were analysed by three methodologies: multiplex immunoassay (a panel of 88 antigens was analysed by the company Rules Based Medicine), TAILS (Terminal Amino Isotopic Labeling of Substrates, by the Medical University of Graz) and 2DIGE-Mass Spectrometry (UNIFI, Medical University of Graz).

The multiplex immunoassays analysis showed that 20 out of 88 antigens were undetectable or missing, 41 were not significantly influenced by the pre-analytical phase and 27 antigens were significantly influenced by at least one pre-analytical parameters (Figure 17: Panel 1, Panel 2, Panel 3 and Panel 4).

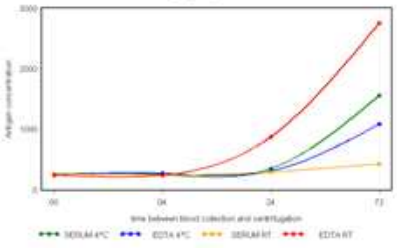
Fig 17: *Protein changes depending on collection tubes, blood derivative (plasma/serum), time and temperature blood storage (Panel 1 - Panel 2 - Panel 3 and Panel 4) (unpublished data)*



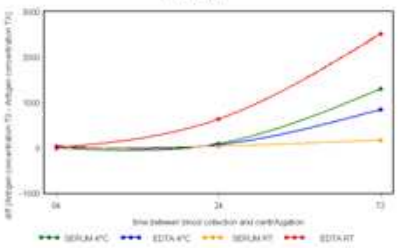
Results - Panel 3

no stable antigens with respect to T0 in at least one of the considered experimental conditions in both plasma-EDTA and SERUM

P46 - Interleukin-16 (IL-16) (pg/mL)



P46 - Interleukin-16 (IL-16) (pg/mL)



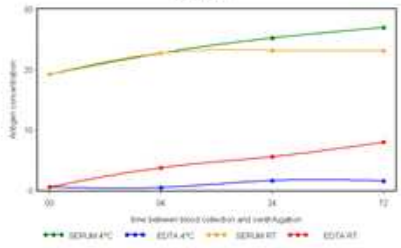
Contrasts	Bon adjusted P-value
EDTA T0 vs 72h RT	<.00001
SERUM T0 vs 72h 4°C	0.01205

contrasts	Bon adjusted P-value
EDTA vs SERUM at RT	0.00152
EDTA vs SERUM at 72h	0.02601
4°C vs RT in EDTA	0.04254

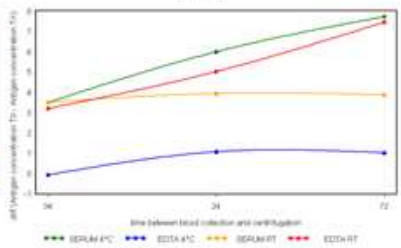
Results - Panel 4

antigens with different expression in the two sample types (SERUM vs plasmaEDTA) at T0

P9 - Brain-Derived Neurotrophic Factor (BDNF) (ng/mL)



P9 - Brain-Derived Neurotrophic Factor (BDNF) (ng/mL)



Contrasts	Bon adjusted P-value
EDTA T0 vs SERUM T0	<.00001

contrasts	Bon adjusted P-value
EDTA vs SERUM at 4°C	0.02697

TAILS identified 6 peptides as potential blood protein quality markers since they altered quantitatively by time or temperature (up-regulated: two antigens of Complement C4-A and 1 antigens of Interleukin-10, down-regulated antigens of: Probable histone-lysine N-methyltransferase, Zinc finger FYVE domain-containing protein 26 and Paired immunoglobulin-like type 2 receptor beta).

2D-DIGE + Mass spectrometry identified several proteins applying the threshold of “two fold protein changing”
The comparison of the results obtained bythe multiplex immunoassay approach (RBM), by the 2D-DIGE + mass-spectrometry or by the TAILS analysis has shown a complex situation that can be summarized as follows:
1. The three systems revealed the contemporaneous presence of a sub-set of proteins that remains stable in serum or plasma in different storage and time interval conditions.
2. Other blood proteins, on the contrary, show significant changes in all the three analytical approaches used in this study.

3. The multiplex immunoassay approach measures mainly blood proteins of lower concentration, whereas the 2D-DIGE/mass spectrometry focuses on proteins of higher concentrations.
4. Consequently, only a limited number of specific protein level changes were identified by both methods(n= 6) (Figure 18).
5. The three different approaches used by SPIDIA are more complementary than confirmatory and they should be used in order to cover the entire range of blood proteins in studies addressing the role of the pre-analytical phase.

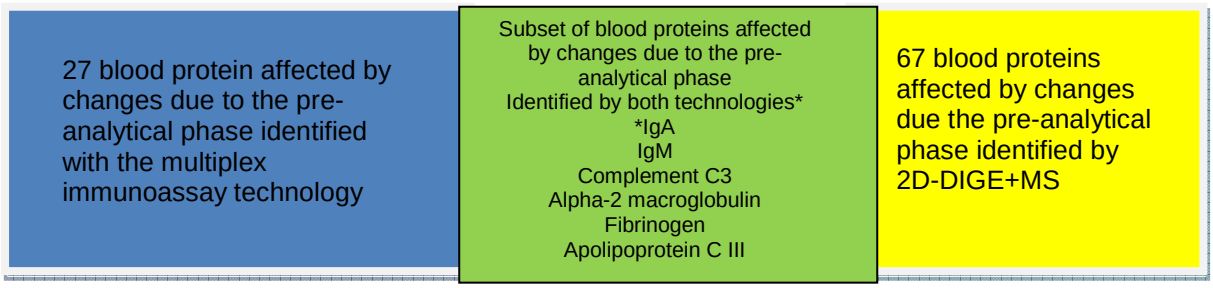


Fig. 18: *Matching and merging of blood changing protein by the immunoassay and 2D-DIGE+MS methodologi*es (manuscript for publication in preparation)

3.3 WP1.3 - Identification and Validation of biomarkers to monitor changes in clinical sample materials

Accurate determination of the quantity and quality of biomarkers in benign as well as in malignant tissues is crucial for making effective treatment decisions. Unfortunately, the lack of standardization in the collection and storage of clinical tissue specimens may create artificial modifications in tissues subjected to varying pre-analytical handling parameters. Unlike administration of pre-surgery medication or anaesthesia protocols, the factors of time and temperature between surgical resection and preservation of a tissue specimen are among the most important pre-analytical factors that could lend themselves to standardization. The number of studies which investigate the impact of warm and cold ischemia on molecular profiles is limited and the results are often contradictory. In addition, most studies used only a few samples and one or two methods to assess the potential variability of bioanalytes in tissue samples during the pre-analytical phase.

This work package has addressed a comprehensive analysis of mRNA, proteins, phosphoproteins, and metabolites in a set of homogeneous clinical and animal tissue samples exposed to known or procedural controlled pre-analytical conditions. We applied a variety of methods, such as Affymetrix gene arrays, RT-qPCR assays, reverse phase protein arrays, Western blot, mass spectrometry, and HR-MAS 1H- NMR spectroscopy to get a broad overview of the molecular changes during warm and cold ischemia. In addition, the work was subdivided to several European labs with special expertise in molecular analysis of tissue samples.

The aim of this work package was to characterize possible fluctuations of various bioanalyte levels in tissue samples during the pre-analytical phase with special emphasis on warm and cold ischemia times as basis for SPIDIA's standardization activities (see WP1.4). WP1.3. identified changes of protein, phosphoprotein, mRNA, and metabolite levels in human and animal tissues exposed to known or experimentally controlled pre-analytical conditions. Keeping cold ischemia times short or the samples on ice prior to stabilization significantly reduced

this effect. Importantly, although extensive inter-individual variability at all levels of analyses was seen, this work package identified several bioanalytes that did not vary during the pre-analytical phase from tissue collection until preservation. These may therefore be considered as stable reference markers for biomarker development programs.

In addition, this WP aimed to develop biomarkers for monitoring changes in clinical blood samples during the pre-analytical phase, i.e. after primary blood sample collection. qPCR based RNA biomarkers for the analysis of pre-analytical variation in blood samples (EDTA and PAXgene blood) were identified, extensively validated in several patient cohorts, and made available to the SPIDIA blood RNA ring trials.

3.4 WP1.4 - Dissemination of Research Results to the Standardisation Community

In 2009 and 2010, SPIDIA representatives presented project updates to the CEN/Technical Committee 140 (In-vitro diagnostic medical devices). In 2011 a New Work Item Proposal (NWIP) for a CEN Standard was prepared by SPIDIA members within the CEN/TC 140 Working Group (WG) 3. The 2011 the CEN/TC plenary meeting could not agree on this proposal yet. The committee asked for modifications and recommended not to go directly to the highest standardization level of a CEN Standard as pre-analytical workflow standardizations have not scientifically matured yet. The CEN/TC 140 2012 plenary meeting accepted a revised NWIP for a CEN Technical Specification.

Within the CEN/TC 140 Working Group (WG) 3 it was decided to develop several CEN Technical Specification documents, addressing the different sample types, (Blood, Plasma, Tissues) and analytes (DNA, RNA, proteins). A separate document will be drafted for Metabolites. This standardization work at CEN will continue after the end of SPIDIA's funding period. It is expected that CEN Technical Specifications will be available for EU national countries roll out by Q3 or Q4 2014.

In addition to CEN as a SPIDIA partner and SPIDIA's collaboration partner US National Cancer Institute, the SPIDIA consortium has established another collaboration with the US Clinical Laboratory Standards Institute (CLSI). An update for a CLSI guideline addressing pre-analytical workflow standardization will take SPIDIA's results and work at CEN into account. Thus SPIDIA is connected with major guideline generating bodies in the EU and in the US.

SPIDIA and its collaboration partners have created a strong basis for a first wave of international pre-analytical workflow standardization documents. Additional international research programs are still needed for standardizing also other workflows not covered by SPIDIA or the US initiatives. These are also needed for achieving a scientific mature status for developing the now upcoming CEN Technical Specifications to full international standards in several years (ISO, CEN EN etc.). At the CEN/TC 140 Plenary Meeting in October 2012 in Berlin several EU country representatives and stakeholders therefore emphasized that continued work in standardizing pre-analytical workflows on an international level will be of high importance. Keeping Europe in its leading driving position is important. International final goal should be to have documents standardizing all relevant diagnostic pre-analytical workflows in a three-dimensional manner (sample type & bioanalyte type & assay technology: Ice Cube Tray Model). This still means an additional significant international effort but would finally lead to full international standards (ISO, EU EN Standard Documents).

Although SPIDIA has ended, the large US initiatives working on this topic will continue during the upcoming years (NIH/NCI: BRN, caHUB). SPIDIA has successfully co-operated with this US programs. From the US view a continued active co-operation with SPIDIA would be much appreciated. New EU / EC projects on pre-analytical workflow standardization would therefore be very beneficial based on the broad expertise generated within SPIDIA for keeping Europe's interests including economic benefits.

3.5 WP2.1 - Stabilisation technologies for in vitro diagnostics that enable classical and molecular stabilisation in one tissue sample

Formalin, a 4% aqueous formaldehyde solution, is the most widely used fixative for the preservation of tissue morphology and examination by histological or immunohistochemical techniques. Formalin has been used for this purpose with only a few minor changes since its introduction in the late 19th century. Although its mechanism of action is still not fully understood, formalin fixes tissue by chemical modification of biomolecules, forming crosslinks between proteins and nucleic acids. Due to the harsh conditions necessary to break the protein-nucleic acid crosslinks and reverse or partially reduce chemical modifications, biomolecules isolated from Formalin Fixed Paraffin Embedded (FFPE) tissue are of limited use for molecular analysis. Snap-freezing of tissues in liquid nitrogen and storage at low temperatures, widely accepted to be the best way to preserve proteins and nucleic acids in tissues, can be inappropriate for a clinical environment because of intracellular ice formation and consequent morphological changes in the tissue. Also, the use of liquid nitrogen in clinics is restricted. During the last few years, a number of fixatives have been proposed as alternatives to formalin, but none of these reagents has gained broader acceptance. SPIDIA has therefore developed a new tissue fixation and stabilization technology allowing both, the classical pathological tests as well as molecular testing.

Initially, a model system using rat tissue to screen for tissue fixation reagents was established. Using this model system, QIAGEN under supervision of PreAnalytiX developed the fixation and stabilization reagent solutions for simultaneous preservation of morphology and biomolecules, including RNA, miRNA, DNA and proteins in solid tissue samples. The new technology is called PAXgene Tissue. The system consists of a fixation reagent and a stabilization reagent. An optimized workflow was developed for this system to standardize fixation, stabilisation and processing of tissue for optimal preservation of histomorphology and biomolecules.

The applicability of the new technology for histomorphology, RNA, DNA and IHC analyses as well as classical pathological tissue staining methods was demonstrated by SIDIA partners by a high number of human tissue samples. By large morphology evaluation studies involving three SPIDIA pathology departments (MUG, EMC and TUM) it could be demonstrated that in PAXgene Tissue fixed, stabilised and paraffin-embedded (PFPE) human tissue samples the morphology and antigenicity are preserved equally or better in most tissue types compared to FFPE tissue. The samples can be analysed by conventional histochemical or immunohistochemical staining. In addition it could be shown by different SPIDIA groups (QIAGEN, ImmunID, Aros, MUG, and TUM) that Bio-molecules purified from PFPE tissue are of high molecular weight and integrity. Non-degraded and immune-reactive proteins can be recovered from PFPE tissue suitable for Western blotting. DNA can be used for demanding applications like long-range and multiplex PCR. Expression profiles of RNA and non-coding small RNAs can be quantified with real time PCR arrays. They show high concordance to the expression profiles from snap frozen samples, generated from the same tumor. SPIDIA's member CRIMMP demonstrated that metabolome analysis from PAXgene tissue treated samples on HR-MAS NMR is difficult due to dominating signals by the fixation reagent itself.

For the development of an Fine Needle Aspirate (FNA) stabilization technology, a model system was established by QIAGEN consisting of human tumor cells from cell cultures mixed with whole blood or plasma and tiny pieces of rat tissue to simulate fine needle aspiration specimens. Using this model system, QIAGEN tested if the most favourable compositions developed for stabilisation of solid tissue can be used for fine needle aspiration fixation. Since all existing fixatives and compositions including the PAXgene Tissue fixative did not stabilize cell morphology in biological fluids, a new screening program was initiated to modify the compositions of the PAXgene Tissue reagents. Finally a reagent composition, a modified PAXgene Tissue fixative, called FNA-Fixative, was identified which fulfilled the criteria for preservation of cells in a biological fluid.

In a proof of principle investigation under supervision of AROS the new FNA-Fixative was tested for preservation of fine needle aspirations from human non-small cell lung carcinoma in liquid based cytology and cell-block preparations. Results indicated that the new FNA-Fixtive can be used as cytological fixative for morphological diagnosis.

3.6 WP2.2 - Stabilisation technologies for in vitro diagnostics that enable classical and molecular stabilisation in one blood sample

The aim of this work package was to develop a whole blood stabilization solution that stabilizes DNA and RNA in the white blood cells but also keeps the morphology of these cells intact instead of lysing them as current state of the art technologies like the PAXgene Blood RNA technology do. QIAGEN screened a total of 112 chemicals in 350 different combinations of formulations and final concentrations of substances when added to the blood samples. The screening program identified multiple chemicals that were capable to either stabilise transcripts in blood while lysing the cells or kept the cells intact while not stabilising the transcripts. Such chemicals typically showed a chemical dosage effects in the way that higher concentrations of chemicals often led to improvements with regard to transcript stabilisation, while the integrity of cells changed for the worse.

An additional screening program led to the discovery of another solution that seemed to have sufficient stabilization properties but left the white blood cells intact as shown by flow cytometry and FACS sorting techniques. This solution was successfully tested by SPIDIA partners in different analytical test applications thus demonstrating proof-of- principle for a new blood stabilization technology.

3.7 WP2.3 - Linking the pre-analytical workflow in the pathology laboratory from fixation to reporting to improve cancer diagnostics

In order to create a unique encrypted tissue identification system for pathology laboratories, a system has been developed that enables the personnel to identify and trace the samples going through the different processing steps from acquisition of the sample to a possible diagnosis. The sample has a unique code and can be scanned during the procedures to make sure mistakes are minimized and the work flow is running smoothly. Cassettes to use for tissue processing have been thoroughly tested. A flap free label candidate was chosen but unfortunately very close to validation this label failed some essential tests. The recommended label was the flap-label that has been through a complete validation.

A second objective of this work package was the valuation of stabilised tissues in histology and immunohistochemistry. The aim was to investigate the effect of the new fixation and stabilisation technique developed by SPIDIA (PAXgene Tissue System) on tissue morphology and antigenicitity. To fully evaluate the effect of the new fixation technique, the golden standard, formalin fixed paraffin embedded (FFPE) tissue was used as a reference. Both, morphology and antigenicity were preserved though alteration of standard procedures is in different cases needed. More than 30 antibodies were tested. After this screening phase no antibodies were found that did not work at all with the new fixative even though the appearance is in some cases slightly different. In the regard of CISH and FISH some hurdles need to be overcome to obtain similar results to formalin.

The procedures of haematoxylin and eosin-staining, immunohistochemistry and in situ hybridization are used in anatomic pathology to support the diagnosis of disease. Achievements have been directed to optimize procedures for these processes on PAXgene Fixed Paraffin Embedded (PFPE) tissue in order to get comparable results to FFPE.

In general, protocols developed for haematoxylin & eosin-staining (H&E), immunohistochemistry and in situ hybridization on formalin fixed tissue need to be optimized prior to applying them on PAXgene fixed tissue. Pre-treatment was found to be the parameter with largest influence on the result. In general it is recommended to use milder conditions in all processes for PAXgene Tissue fixation than is used for formalin fixation.

PAXgene fixed tissue shows differences in morphology in H&E staining. A new fixative will however always be a matter of habit since tissue will have a different appearance than the golden reference. In immunohistochemistry most of the tested antibodies resulted in acceptable staining on PAXgene fixed tissue. However, for about 20 % of the antibodies staining was weaker compared to formalin. This may lead to false negatives. Further optimization is therefore needed to reach optimal appearance for these remaining antibodies. For CISH (DNA

and mRNA) and FISH (DNA) staining, the morphology and also signal strength is an issue still be worked on since the procedures can be very harsh on the tissue with both, enzymatic pre-treatment and boiling in buffer. Experiments for achieving improvements demonstrated that for optimising the protocols, pre-treatments should be left out.

3.8 WP2.4 - Investigation of integrated sample collection / stabilisation / transport technologies for tissues

QIAGEN under supervision of PreAnalytiX has developed a two reagent system (see WP2.1) consisting of a tissue fixative and a stabilization reagent for simultaneous preservation of morphology and biomolecules including RNA, DNA, small RNAs, and proteins in tissue specimen.

Different design concepts for prototype container devices, in which the two reagent fixation/stabilization system could be integrated, were moulded. From the most favoured design concept was selected and produced ("Two Chamber one Closure Concept", Figure 19). A standard protocol for tissue fixation and stabilization by using the container device was established.

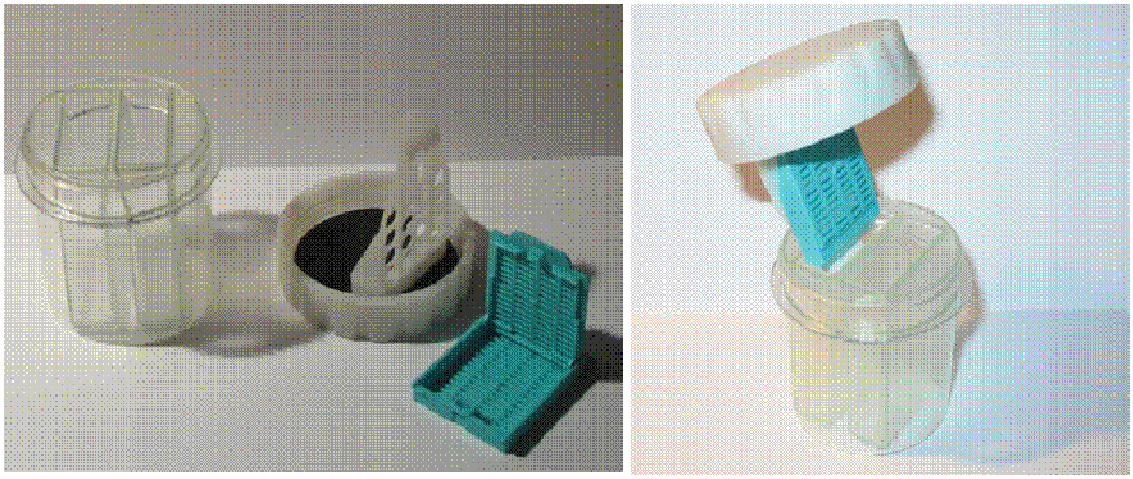


***Fig. 19**: Prototype container for the "Two chamber one closure concept"*
Left hand side: Container concept with 2 chambers, the lid contains a holder for a standard histocassette. Each of the 2 chambers holds one of the two stabilization components developed within WP 2.1
Right hand side: assembled tissue container

Upon discussions with routine pathologists, it became clear, that the container concept could be suitable as a standardized format for fixation and stabilization of biopsies. But as the size of resected tissue is too heterogeneous and unpredictable before surgery, a more flexible format to suite all needs within the workflow of a routine pathology laboratory was needed. Therefore an alternative workflow for fixation of larger pieces of tissue as well as for integration of the Stabilizer reagent into tissue processing was developed. A large container filled with 50 ml fixative solution was able to cover either four tissue cassettes with smaller tissue samples or one larger sample with up to 20 x 20 x 20 mm in size. In addition, the Stabilizer was provided in larger quantities as a concentrate in 500 ml bottles filled with 125 ml stabilizer-concentrate. The concentrate can be reconstituted by adding 350 ml ethanol. Fixative can be replaced by the stabilizer for transport and storage until processing or the stabilizer can be integrated into the processing protocol i.e. by filling the first position of an automated processor with reconstituted stabilizer.

In a first step QIAGEN tested the different containers with rat tissue. This showed comparable performance to the bulk reagent used within WP 2.1 with regard to RNA stabilization and quality as well as to preservation of histomorphology. In a next step SPIDIA's partners QIAGEN and MUG tested both sample collection containers for use in routine clinical settings including biopsies or larger pieces of tissue of normal or malignant tissue types. Tests comprised the whole workflow from the operation theatre to the downstream analytical steps including, fixation, transport, storage, processing, histological staining and purification of biomolecules. Comparison studies were performed with corresponding tissue samples fixed with formalin, the new PAXgene Tissue containers or snap frozen in liquid nitrogen. Morphology preservation in histological stains and immunohistochemistry, integrity of nucleic acids and performance in downstream applications like PCR, RT-PCR (including PCR arrays), multiplex- and long range PCR were evaluated and compared.

Tissue collection with the new container was easy to handle, even for staff not especially trained in biobanking or pathology. The tissue containers fulfilled all requirements for easy collection, tissue fixation and stabilisation, temporary storage and safe transport. All samples shipped from distinct collection sites arrived at the pathology labs in well condition.

Morphology was preserved in PAXgene fixed and paraffin embedded (PFPE) tissue collected with both containers, similar or indistinguishable from FFPE tissue. Nucleic acids isolated from the PFPE samples showed higher integrity compared to corresponding FFPE samples and performed as good as nucleic acids purified from snap frozen samples. Sections of PFPE tissue could be used for immunohistochemistry staining and achieved equivalent results compared to sections of the corresponding FFPE tissue, after having implemented some optimization of staining protocols.

3.9 WP2.5 - Improvement of non- or minimally invasive sampling techniques

One scope of WP 2.5 was the discovery of improved collection systems for swab samples for use in DNA-based diagnostic applications. Two swab types have been discovered which fulfill the quantitative release of nucleic acids in different ways: One swab has a specific fibric structure for optimal sample release, the other swab material self-disintegrates thereby releasing the sample material into the buffer. The search for a buffer system providing nucleic acid stabilisation resulted in the selection of a specific chaotropic buffer composition which has been shown to stabilise genomic and viral DNA for up to 4 weeks at RT and 37℃. In addition, viral RNA could be stabilised for up to 4 weeks at room temperature and up to 2 weeks at 37℃. Bacteria inactivating p roperties of the buffer could been shown by specific studies with gram positive and negative bacteria. In addition, virus inactivating properties of the buffer are assumed due to the similarity to the QIAGEN lysis buffer AVL, which has been shown to be virus inactivating by Blow et al. (J Virol Methods. 2004 Aug;119(2):195-8.). The overall performance of the systems has been examined in detail in comparison to state-of-the-art swab collection technologies, by using human, bacterial, and viral material.

In summary, the results demonstrated multiple advantages of the developed systems over state-of-the-art systems in terms of efficient nucleic acids release, stabilization of DNA and RNA, and pathogen inactivating properties.

The second scope of this workpackage was to develop new stabilization technologies for cell-free circulating DNA (ccfDNA) in blood samples. The analysis of ccfDNA profiles in plasma generated from the primary whole blood sample opens attractive new diagnostics. During the course of the SPIDIA project pre-natal diagnostics assays analyzing fetal ccfDNA in the maternal blood were developed by several companies and introduced into the market. A second promising diagnostic application is cancer diagnostics and therapy monitoring. Others as organ disease diagnostics may follow. For all these new promising diagnostics it is key to preserve the ccfDNA and ccfRNA profiles. The most critical factor seems to be the release of genomic DNA from white blood cells starting after blood collection and progressing during the pre-analytical workflow. SPIDIA therefore developed a new stabilization technology for ccfNA profiles in human whole blood. The technology is novel and stabilizes

human blood samples for up to six days at room temperature. The technology was tested with blood from healthy donors as well as blood from pregnant women and cancer patients by three SPIDIA partners. The new technology significantly reduced the release of DNA from white blood cells and therefore prevented a dilution of the ccfNA target profiles during blood sample transport and storage, which is an important prerequisite for reliable prenatal or cancer test procedures.

The second challenge for ccfDNA and ccfRNA based diagnostics is the sensitivity needed. The ccfNA targets are usually low concentrated in blood. Processing larger blood volumes of several milliliters for the automated isolation of ccfDNA or ccfRNA but concentrating the eluted nucleic acids in a small volume is needed. Therefore SPIDIA also developed a new automated isolation and concentration technology for the enrichment of ccfNA from large volumes of plasma.

Both new technologies can be combined in one pre-analytical workflow. This enables the complete integration and standardization of all pre-analytical steps from whole blood collection, stabilization, transport, storage, plasma generation and isolation of ccfDNA for analytical downstream testing.

3.10 WP2.6 - Integration of pre-analytical and analytical steps into the workflow of processing blood samples

Standardized complete sample-to-result diagnostic workflows should be the final goal for achieving optimal analytical test results. Within SPIDIA such workflows were developed for cellular RNA target testing.

Based on the existing automation platform QIAsymphony from QIAGEN, a complete new workflow was developed for the automated isolation and analysis of cellular RNA from stabilized whole blood. As the PAXgene Blood RNA Tubes are the only in vitro diagnostic product in the market, they were used for blood collection to build this workflow.

After intensive screening a new dedicated cellular RNA isolation chemistry was developed. This enables the efficient isolation of all types of RNA (small RNA species as miRNA and larger RNA species as transcripts) from stabilized whole blood collected in PAXgene Blood RNA tubes. The RNA isolated with this protocol was of high purity and integrity. The new chemistry was used to design and optimize a dedicated script and dedicated reagents for the QIAsymphony platform. The final workflow allowed to process 72 blood samples within one run with a hands-on time of about 30 minutes and robot runtime of 5h and 45 minutes. The new system was also challenged by processing difficult blood samples. Its robustness against interference sample compounds that normally influence preparation methods could be proofed. The system is highly flexible in a way that all numbers of samples from one to 72 can be processed in a single run. With a maximum throughput of 144 samples per day the needs of small to midsize laboratories (medium throughput) can be addressed.

After the development of the RNA extraction protocol was finalized, a qRT-PCR setup protocol on a QIAsymphony module was developed that directly uses the eluted RNA from the blood samples. The link of the two protocols resulted in a streamlined automated workflow, which covered the whole process starting with the blood sample collection and stabilization and ending with the ready to use qRT-PCR plate. No significant differences compared to manual reaction set ups was detected in the final evaluation made by SPIDIA's partners AROS, BTU and DiaGenic by running own analytical assays targeting different mRNAs as well as miRNAs.

3.11 WP2.7 - Test of new pre-analytical tools and guidelines in biomarker discovery programs

The development of pre-analytical tools and procedures and the development of diagnostic assays are intimately connected: better pre-analytical procedures that minimize alterations in the original molecular profiles of samples are of fundamental importance to unravel the presence of a disease signature during downstream analyses. SPIDIA's WP2.7 has been devoted to the evaluation of procedures to be implemented in routine clinical activities and at the definition of best practice procedures for analytical settings. Using optimized procedures, the ability to extract a disease signature has been evaluated for two case studies, selected among the most common diseases that affect patients in western countries, namely Alzheimer's disease (AD) and colorectal cancer.

As far as AD is concerned, a new algorithm and optimized assay conditions have been implemented to make an automated platform suitable for DiaGenic's new ADtect® assay. The analysis of a large set of samples (140) collected at SPIDIA's member UNIFI using the new algorithm to distinguish AD from other forms of dementia has been performed. The data are intended to be published after CE-IVD marking of the new analytical test. The new AD gene expression signature was combined with an optimized pre-analytical workflow processing stabilized whole blood in PAXgene Blood RNA tubes thus creating a complete sample-to-result workflow.

NMR metabolomic analysis of serum generated from metastatic colorectal cancer patients' blood allowed the discovery of a strong signature of the disease. The analysis of this signature may offer an independent tool to predict overall survival (Bertini et al. Cancer Res; 2011, 72(1); 356–64) (Figure 20).

Attempts to identify colorectal cancer specific wrongly regulated miRNAs levels that could predict overall survival also provided promising results: high expression of miR-345 and low expression of miR-31 in whole blood were proposed as prognostic biomarkers of short overall survival and miR-345 was also a predictor for treatment response in patients with metastatic CRC treated with 3rd line therapy with cetuximab and irinotecan. These data will be ready for publication after validations with further statistical analysis. Also these new assays were combined with optimized pre-analytical workflows, again resulting in standardized complete sample-to-result workflows.

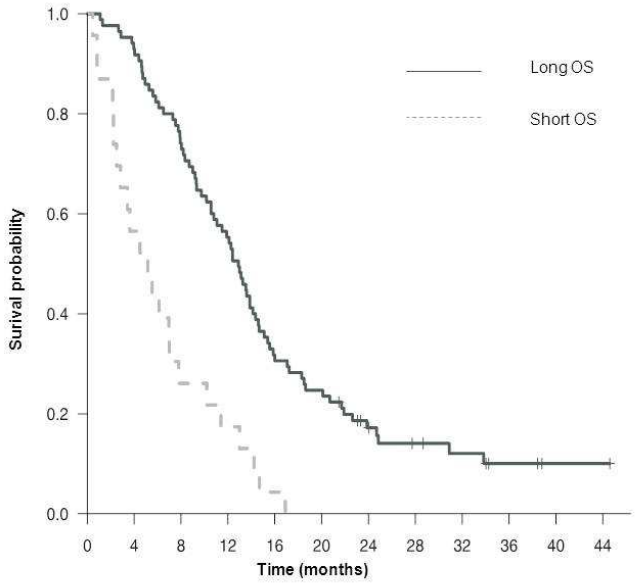


Fig. 20: *Metabolomics of metastatic colorectal cancer patients. Kaplan-Meier curves showing survival probability based on ^{1}H-NMR profiling predictive model (P = 1.33 x 10^{-6}). OS, overall survival. (Bertini et al. Cancer Res; 2011, 72(1); 356–64)*

Metabolomics have revealed to be a very powerful tool to monitor the effect of pre-analytical treatments in urine, serum and plasma, i.e. the most commonly used bioanalytes in metabolomics studies (Bernini et al. J. Biomol. NMR; 2011, 49(3-4):231-43). Indeed, some of the most abundant metabolites change significantly their concentration as an effect of residual enzymatic activity and redox reactions due to exposure to aerobic conditions. Similar results have been obtained for tissues, where, in addition to the above effects, we have been able to identify molecular markers of apoptotic processes. A systematic study of the effect of warm and cold ischemia on human liver tissues performed on samples provided by MUG and EMC has been reported in a manuscript presently submitted for publication.

3.12 WP3.1 – Training, dissemination and exploitation of results

The main tasks of WP3.1 have been to develop an internal and external training program and to disseminate the results of SPIDIA to the scientific community and the general public. During the project time a total of 27 different training activities have been performed including workshops, seminars, round-table discussions and practical workshops. During the first period the majority of the training events were for the SPIDIA partners to improve the outcome of the project. These included training events for financial and administrative reporting, ethics, and sample transportation. During the remaining time of the project the majority of the training events have been open to an outside audience and especially during the last two years there have been three major workshops focusing directly to an audience outside SPIDIA. The first was a workshop on the outcome of the first ring-trials performed in SPIDIA that was held in conjunction with the qPCR Symposium 2011 in Prague. The second was a public workshop held at the Medical University of Graz in 2012 and the third was a public workshop held in Hilden in conjunction with the final SPIDIA consortium meeting in 2013. All three events attracted a high international attendance and were very well received.

The SPIDIA webpage (www.spidia.eu) was established during the first year of the project and has since then been continuously updated with information about results from the SPIDIA project. In addition, the webpage has evolved with new sections about for example meeting and events with SPIDIA participation, publications, news and press, and links to related organisations. On the webpage it has also been possible to sign up for the SPIDIA newsletter. Today more than 800 people have registered for the newsletter and a total of six newsletters have been published.

The SPIDIA consortium has been very active in disseminating the results and more than 60 oral or poster presentations has been performed at international conferences and meetings. Also, 11 papers have so far been published in international peer-review journals. Three additional scientific papers are accepted for publication. A significant number of articles are in preparation or have been submitted for publication. In addition, articles about the SPIDIA project have been published on at least seven occasions in non-specialized media. A live webinar event has been broadcast in September 2012.

SPIDIA has also established a significant number of collaborations with other important international initiatives and projects for disseminating and exploiting results (see 4. “Potential Impacts” and other chapters of this report).

3.13 WP3.2 - Strategic, operational and IPR/Knowledge Management

The operational management organisation was installed since the beginning of the project.
Strategic, management and workshops meetings were taking place every six months to monitor the execution of the project and the work progress and to define the project strategies. They also host members of the Scientific Advisory Board (SAB) and the Project Ethic Committee (PEC).
In addition to these steering committee meetings, regularly conference calls were organised on the WP level to ensure effective communication and coordination of the scientific work.

Work progress and results were reported to the European Commission by the consortium after a first period of 18 months, after a second period of 36 months and at the end of the project (at month 54).
The protection, management and transfer of IPR appeared to be an important and strategic issue for a successful implementation of SPIDIA project, as well as for open opportunities for commercial exploitation. This element included the sharing of knowledge. To comply with the rules of participation in FP7 programs, a database was created in the first months of the project to define the background information each beneficiary wanted to make accessible to other participants and the background they prefer to exclude from SPIDIA project.

After Spidia became an operative running project, an Intellectual Property Committee (IIPC) was set up in order to monitor and approve all dissemination activities and also to deal with all Industrial and Intellectual Property Rights issues according to the Consortium Agreement. An IPR manager or Chairman of the IIPC committee was appointed in October 2008.

The Intellectual Property Committee (IIPC) was in charge of providing advice on request on:

- the evaluation of SPIDIA-results before they are disseminated
- the determination of IPR ownership,
- the management of joint ownership,
- the granting of Access Rights,
- the freedom to operate and patentability,
- the choice between patents or other protections.

Rules and protocols were established during the first 12 months of the project to guarantee appropriate Intellectual Property Rights protection.

During the whole duration of the project, 76 requests for dissemination were submitted and evaluated by the IIPC (46 requests within the first 36 months and 30 requests during the second period of the project): 15 publications, 42 poster presentations and 19 talks. In total 10 notifications of generation of intellectual property were reported to the SPIDIA Executive Management Board,
No critical issues, like disagreement with regard to the ownership of intellectual property rights or critical data for dissemination were observed. No results were disseminated without prior approval from the IIPC.

3.14 WP3.3 - Project Ethics

The Project's Ethical Committee (PEC) played an essential role during the entire course of the SPIDIA project. The exchange of human material and use of animal materials for health care research purposes obligated the SPIDIA project organisation to take care of regulatory issues and ethical issues involved.

The way sample exchange was set-up could be an example to other European projects working with human and animal materials for medical research.

SPIDIA PEC workshops on ethics for working with human materials in medical research were performed.

A SPIDIA PEC website for transparency in SPIDIA research with human and animal materials was established.

4. POTENTIAL IMPACTS

4.1 Introduction

Molecular in vitro diagnostics have enabled a significant progress in medicine. Further breakthrough progress is expected by new technologies analysing signatures (profiles) of nucleic acids, proteins, and metabolites in human tissues and body fluids. This includes the upcoming high potential field of Personalized Medicine. However, the profiles of these molecules can change drastically during collection, preservation, transport, storage, and sample processing thus making a reliable diagnostic or biomedical research unreliable or even impossible because the subsequent analytical assay will not determine the profile in the patient but an artificial profile generated after sample collection. Therefore further progress is limited due to the lack of guidelines in sample collection, handling, stabilization, storage, and processing of clinical samples and due to still missing new and improved sample preservation and handling technologies.

Lippi G. et al. reported that pre-analytical errors in sample collection and handling account for 60-70% of all problems occurring in laboratory diagnostics (Lippi el al; Preanalytical quality improvement: from dream to reality. Clin Chem Lab Med. 2011 Jul; 49(7):1113-26. Epub 2011 Apr 25). These pre-analytical errors cause costs of about 350.000 € / year in an average German hospital (Frost & Sullivan 2011, commercial study). The new evidence based standardization documents based on SPIDIA's results will enable to reduce this error rate for the selected molecular diagnostic applications. A reduced error rate will lead to better patient diagnosis and treatments as well as reduced health care costs. It will also enable related cost savings in the healthcare system including hospitals. As the same workflows are used for research and development, the documents will also enable to develop more reliable biomarker and diagnostic tests.

Biomarker discovery and development programs might have failed in the past due to lack of high quality samples. G. Poste stated that a major impediment to progress in the hunt for biomarkers is the lack of standardization in how specimens are collected. Unless specimens are taken from people who are matched for as many variables as possible, all the subsequent steps in efforts to correlate biomarkers with people's conditions and responses to treatments are compromised. Too many researchers rely on whatever specimens they can obtain conveniently from local institutions (G. Poste. Bring on the biomarkers. Nature 2011, Vol 469, 156-157).

Improving and standardizing pre-analytical workflows is therefore one of the major decisive leverage effects for improving the health care system by more reliable diagnostics and resulting patients treatments but also for enabling higher research success rates in the pharmaceutical, biotech, and diagnostic industry as well as in government funded research and biobanking. Combining such standardized pre-analytical workflows with analytical tests will create complete standardized and validated sample-to-result diagnostic workflows.

SPIDIA has worked on this so important improvement of sample qualities for 4.5 years. The consortium has achieved all its major goals. These achievements for the standardization and improvement of generic pre-analytical tools and procedures are of interest for diagnostics including hospitals, doctors' offices, clinical diagnostic laboratories but also for biomedical research in the pharmaceutical, biotech and diagnostic industry, government funded research, and biobanking.

4.2 New evidence-based, international guidelines and quality-assurance schemes

SPIDIA's large pan-European ring trials on collecting, processing and analyzing blood samples (DNA, RNA, cell-free circulating DNA) have generated broad scientific evidence on the importance to standardize pre-analytical workflows for these applications. These data as well as other SPIDIA data (see various chapters of this report) and experiences from other international initiatives will be used for developing EU and US international documents for standardizing and harmonizing pre-analytical diagnostic workflows. The steps addressed include patient's sample collection in hospitals and doctors' offices, shipment and storage of samples as well as

processing of samples in clinical diagnostic laboratories. Writing the standardization documents at the European Committee for Standardization CEN is ongoing with leading support by several SPIDIA representatives. SPIDIA's work will lead to a first series of CEN Technical Specification documents for national roll out in European countries. These will be the first of this kind and will address selective important sample types and bioanalytes.

SPIDIA's results are also intended to be used for updating a specific guideline of the US Clinical Laboratory Standards Institute (CLSI).

Developing and implementing the SPIDIA blood ring trials was a great effort and important achievement (recruitment of participants, high volume sample collection and aliquotting, sample shipment and storage, data collection from participants, analysis of isolated nucleic acids, statistical analysis, ring trial reports for participants). These schemes can serve as a basis for future proficiency testing on these molecular applications.

Pre-analytical workflows can lead to artificial changes in blood RNA profiles after sample collection from the patient / donor. Therefore new control tools are needed for verifying pre-analytical workflows established at individual institutions if they secure blood samples of sufficient quality. Via large screening programs, SPIDIA discovered and validated new cellular blood RNA quality biomarkers. These are intended to control, assess and validate pre-analytical workflows if they sufficiently secure high quality blood samples. More detailed results will be published via a peer-reviewed scientific paper.

A similar program was performed for identifying new ample quality biomarker candidates for monitoring pre-analytical workflows for tissue RNA gene expression, protein & phospho-protein analysis, and metabolites. Results will be published via peer-reviewed scientific papers. Within the SPIDIA project a limited number of tissues (organs) could be analyzed. More studies including other tissue types are needed for further analyzing if some of the marker candidates can further developed to fully validated general quality markers

Within the comprehensive SPIDIA tissue work, we also investigated critical parameters for tissue sample quality and evaluated a novel tissue stabilisation procedure developed by SPIDIA's industrial partners for its applicability in routine medical service and for preservation of morphology, antigenicity and biomolecules in comparison with current tissue preservation techniques (FFPE, CRYO). These studies also provided a profound baseline data set for the standardisation activities at CEN and the development of new European guidelines. Also for tissue related diagnosis and research we assume that this work will improve molecular diagnostics of patient samples and facilitate biomarker development in clinical trials, and thereby lead to better diagnostics and targeted therapies, save health care costs and substantially contribute to medical research in general and further technology development. Furthermore, international collaborations e.g. with the National Cancer Institute in the US were established. The broad expertise generated within the SPIDIA consortium should be used for follow-up projects and project calls to continue with this highly valuable work and to develop additional new tools, technologies and guidelines in this broad and complex field.

The measurement of small-molecule metabolites has been an integral part of clinical practice and clinical chemistry for more than one century. What distinguishes the emerging technology "metabolomics" from clinical chemistry is that the former has the ability to measure not just one or two compounds at a time, but tens to hundreds of them. So, metabolomics extends the ensemble of measurable compounds in a standard experiment, providing a more comprehensive picture of the individual metabolism: the metabolic profile. It has been shown in the literature (and in WP2.7 of this project) how metabolic profiles can be useful for diagnosis or prognosis where a single biomarker does not provide adequate discrimination. The use of metabolomics in biomarker discovery and the concept of metabolic profiles are gaining more and more importance at the level of research activities but are not yet implemented at the level of clinical practice or clinical chemistry.
Although metabolomics has been shown to have the potential to be applied in the clinic, it has not yet entered in the routine due to a number of obstacles: metabolomics analysis cannot be performed with existing technology that is commonplace in most hospital/clinical chemistry environments; more extensive validation of biomarkers discovered by metabolomics is needed; the concept of metabolic profiling as a more powerful tool than search of

few target metabolites has to be accepted by the common mentality. Adherence to strict pre-analytical procedures to preserve the original metabolome, as proposed on the basis of the results obtained in SPIDIA, will be crucial in studies aimed at biomarker discoveries and is expected to help their validation reducing the noise and errors introduced by different preanalytics. Use of the appropriate procedures and knowledge of the metabolites that are most commonly affected by preclinical treatments is also of fundamental importance for the application of metabolic biomarkers in drug development studies in Pharma. Additionally, the metabolomic activities performed within SPIDIA have clearly shown their potential impact in biobanking. Quality control and assessment of the collection, handling and storage procedures implemented at any biobanks can be validated via metabolomics as a sensitive and rapid way to record changes of the molecular profiles in biofluids and tissues and can help implementing existing standard operating procedures. In particular, metabolomics may become an efficient means to fill the void due to the present limited availability of data on the “shelf-life time” of samples stored in biobanks.

4.3 New pre-analytical tools for molecular in vitro diagnostics and biomedical research

Within the second activity of SPIDIA, several new breaking technologies were developed which will be the basis for new product developments. These new technologies enable strongly improved sample qualities by new pre-analytical workflows thus enabling improved diagnostic test results and patient treatment. In particular PreAnalytiX aims to commercialize the new SPIDIA PAXgene Tissue technology developed within WP 2.1 and 2.4 for enabling to perform the classical histopathology as well as the increasing molecular pathology applications from the same tissue / tumor sample. These new opportunities in diagnostic will improve the characterization of e.g. cancer samples with molecular methods. Cancer patients will benefit from that. The products will have the potential for a worldwide use in healthcare and research as well as in the biobanking arena, which gives them also a high potential for an economic success and the generation of workplaces in the industry.

Under the lead of PreAnalytiX, SPIDIA started to collaborate within a dedicated Munich m4 Top Cluster grant project. This project intends to validate new molecular biomarkers for a new future Barrett Syndrome diagnostics (endoscopic samples). This would lead to multimodality diagnostics on Barrett Syndrome endoscopic samples (morphology, immuno-histochemistry, nucleic acids). The traditional formalin based fixation (FFPE) could not be used due to negative effects on the nucleic acid based new biomarker assays. QIAGEN and PreAnalytiX were therefore contacted for a co-operating on using the new SPIDIA PAXgene tissue technology within this m4 project. After initial evaluation, the new SPIDIA technology including the new tissue collection containers will now be used within this m4 Top Cluster grant project for the collection of biopsies from up to 2000 patients with Barrett Syndrome. In parallel, samples will also be collected for processing via formalin fixation. Performance of SPIDIA PAXgene Tissue processing and paraffin embedding vs. formalin fixation and paraffin embedding will be compared (morphology staining, IHC, molecular analysis). Results will be used for establishing a future complete sample-to-result workflow for Barrett Syndrome diagnostics and for validating the new nucleic acid based biomarkers. This project will continue at least until end of 2015.

PreAnalytiX and QIAGEN initiated to collaborate with the large-scale US GTEx Consortium on implementing workflows based on the new SPIDIA PAXgene Tissue technology. The Genotype-Tissue Expression Project (GTEx) is a collaborative pilot initiative of the US National Institutes of Health (NIH) that is studying how inherited variations in genes relate to common diseases. GTEx tissue collection is managed by The National Cancer Institute's cancer Human Biobank (caHUB) in conjunction with tissue source sites across the country (http://commonfund.nih.gov/GTEx). The success of GTEx depends on the ability to successfully procure and store high quality biospecimens for analysis. SPIDIA is collaborating with the US NIH / NCI as described in this report on the BRN and caHUB programs. The GTEx consortium has evaluated all available technologies including the new SPIDIA PAXgene Tissue technology and has finally decided to use the new SPIDIA technology for all tissue collections within the project. This has led to published SOPs for tissue collection and processing based on PAXgene Tissue (http://biospecimens.cancer.gov/resources/sops/default.asp). GTEx’s very positive evaluation of SPIDIA’s new PAXgene Tissue technology confirms the positive results obtained for

this new technology within the SPIDIA consortium including international morphology ring trials and the CD-Laboratory grant project in Graz, Austria (see below). The GTEx project is planned to continue for the next years.

During the development and evaluation of the new SPIDIA PAXgene tissue fixation and stabilization technology some important aspects came up which could not be deeply analysed as these were not part of the SPIDIA program (e.g. pathogen inactivation, pathogen detection, some analysis on a molecular level as ultra-structures and suitability for high safety class laboratories etc.). SPIDIA's partners QIAGEN and the Medial University of Graz (MUG) therefore established a co-work between the Christian-Doppler (CD)-Laboratory grant project in Graz and SPIDIA. Within this CD laboratory grant project these open aspects were investigated. Several important results could be obtained thus complementing the SPIDIA results. The CD-Laboratory project will continue for the next four years.

SPIDIA developed also a new and first technology which enables current classical Fine Needle Aspirates (FNAs) analysis as staining, immunohistochemistry etc. as well as molecular analysis (RNA, DNA, etc.) with high sensitivity and reliability from the same sample. PreAnalytiX intends to further develop this technology and to integrate it into collection devices for Fine Needle Aspirates (FNAs) collection & preservation, for transport, and storage thus enabling the parallel analysis of cytology and molecular biomolecules form the same sample. Based on current plans, a commercialization of such a new system is expected by 2015 / 2016.

Also the new stabilization technology developments within WP 2.5 for swab samples and for cell-free circulating DNA (ccfDNA) from plasma samples are breakthrough achievements. Especially the new ccfDNA profile preservation and ccfDNA enrichment technology for blood and plasma samples has a huge potential for a broad application in the diagnostic and research fields (pre-natal testing, cancer and organs disease diagnostics). The prenatal diagnostic as well as the cancer diagnostic are moving to free circulating DNA that can be detected in the plasma. The collection of a blood sample and the generation of plasma therefrom are much less invasive than an amniocentesis or the collection of a biopsy from a tissue. Because of this easy sample collection and the high information content of plasma samples, interest in the scientific community and also the diagnostic and pharma industry in ccfDNA from plasma has greatly increased during the last years. Many companies work on assays for pre-natal and cancer diagnostics, but the pre-analytic sample handling is a bottleneck so far. The plasma stabilization technology, developed within SPIDIA could close that gap and serve as a general pre-analytic tool for pre-natal and cancer diagnostic tests based on ccfDNA, when this technology gets integrated into a blood collection tube.

SPIDIA has developed several sample-to-result workflows within WPs 2.6 and 2.7 which cover all steps from blood sample collection, stabilization, transport, storage, processing, and the analytical downstream test. One of these workflows includes the fully automated isolation of cellular RNA including miRNA from stabilized whole blood (PAXgene Blood RNA tubes) on QIAGENs QIAsymphony platform. Because the extraction procedure is fully automated, it reduces the risk of human errors or sample mix-up and therefore increases the safety and reliability of the test results, generated from these samples. QIAGEN intends to further develop this workflow into a dedicated system for in vitro diagnostic use (IVD) in the near future.

4.4 Future view on international pre-analytical workflow standardization

SPIDIA was the largest EU initiative on analyzing and standardizing pre-analytical workflows for improving clinical samples qualities. There are two large US programs also targeting pre-analytic workflow research and standardization: i) Biospecimen Research Network (BRN), ii) Cancer Human Biobank (caHUB). Both are under the responsibility of the US National Cancer Institute (NCI). SPIDIA and the NCI have decided in 2010 to start a formal collaboration for exchanging results and standardization efforts. It is the first time that the NCI has entered such a collaboration with an EU initiative. The collaboration will continue wherever possible, e.g. joint workshops, exchange of results etc. although on a limited base as the SPIDIA project has ended.

The US BRN program has brought the pre-analytical workflow standardization requirements into a descriptive model. Following this model will enable to achieve a comprehensive final status of international pre-analytical workflow standardization for diagnostics, pharma/biotech research, biobanking, and government funded research programs. The model defines each pre-analytical workflow in three dimensions: i) sample type (e.g. blood), ii) bio-analyte in this sample type to be analysed (e.g. cellular RNA), iii) the final assay test technology used (e.g. qRT-PCR). In the model these three dimensions represent one “ice cube”. There are many different of such three dimensional individual pre-analytical workflows to be standardized – many “ice cubes” which form a final “ice cube tray”. The entire model is therefore called the “Ice-Cube-Tray Model”. Once all work has been done for filling each important ice cube, each research program or each standardization activity can pick its relevant pre-analytical workflow (= ice cubes) which will contain all relevant research information and standardization documents. In 2011 this model was presented to SPIDIA by the BRN and caHUB management team as SPIDIA’s program was also built on the same model principles. Both initiatives agreed to work together for bringing the ice cube model to reality. This transatlantic collaboration between the US NCI / BRN & caHUB programs and the EU SPIDIA initiative was publically announced by the BRN Director Prof. Dr. Carolyn Compton in her closing speech at the 2011 Annual BRN Symposium in Bethesda (US). This was the start of a formal collaboration between the EU SPIDIA and the US programs.

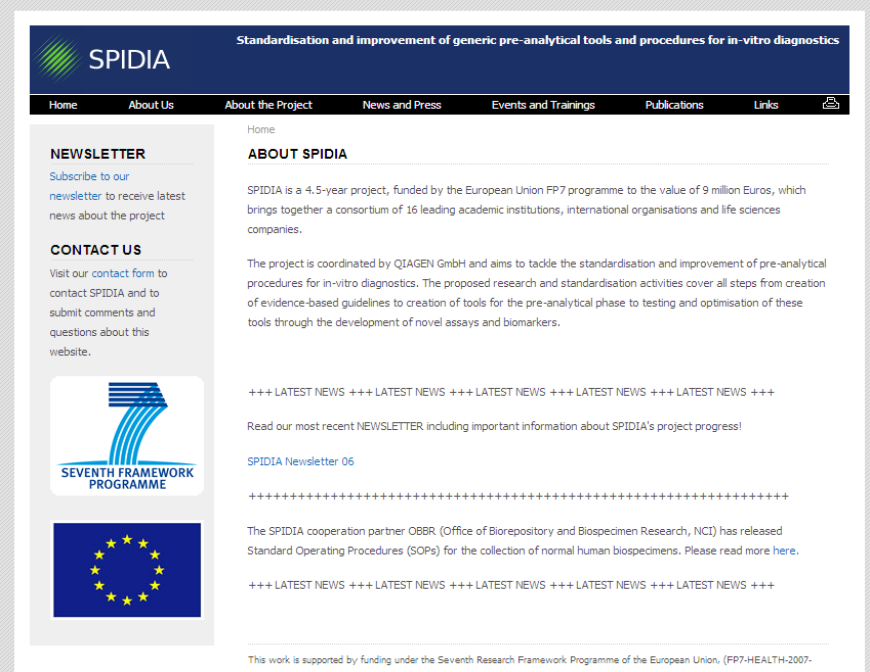
First important key “ice cubes” will be filled by SPIDIA and also by both US NCI initiatives. For SPIDIA, this will include the upcoming CEN Technical Specifications which are planned to be ready for roll out in the EU member states by the end of 2014. Since there is the large number of pre-analytical workflow combinations, additional international efforts by larger research programs as EU SPIDIA, US BRN, and US caHUB will still be needed to standardize these remaining pre-analytical workflows. Progress in diagnostics, therapies and research for the benefit of patients will still be limited if suited sample qualities are not secured for all applications. International final goal should be to fill all relevant “ice cubes” and to achieve full international EN or ISO Standards.

The US initiatives working on this topic will therefore continue during the upcoming years (NIH/NCI: BRN, caHUB). SPIDIA has successfully co-operated with this US programs. SPIDIA has enabled great international progress but the consortium’s contribution in the future will be limited as the project has ended. New EU / EC projects on pre-analytical workflow research and standardization based on the broad expertise generated within SPIDIA would therefore be very beneficial for maintaining Europe's strategic interests and competitiveness in this key field. Being silent would lead to a loss of Europe’s leading position and influence.

5. CONTACTS AND ADDRESSES

5.1 Project website

www.spidia.eu



5.2 Project logo

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