Foundation for Polish Science

TEAM TECH PROGRAMME

LAUREATE ANNUAL RESEARCH PROGRESS REPORT

Project title:	**Mass Spectrometry of Biopharmaceuticals - improved methodologies for qualitative, quantitative and structural characterization of drugs, proteinaceous drug targets and diagnostic molecules.**		
Laureate:	**Prof. Michał Dadlez**		
Reporting period:	From 01.11.2017 to 30.09.2018.	**Period no.**	**1**
Agreement No.:	TEAM TECH CORE FACILITY/2016-2/2	from 01.11.2017 to 31.10.2020	

NOTE: Information provided within a progress report shall consider only the said project within a particular reporting period.

1. INFORMATION ON THE RESEARCH PROGRESS

1.1. Achieved deliverables and milestones (up to 1 page A4)

List up to 3 (three) the most important research achievements within a past reporting period:

1. Development of a new hydrogen-deuterium data analysis tool HaDeX, available as a web-server *(http://mslab-ibb.pl/shiny/HaDeX/).*

2. Establishment of in-house DIA protocol QExactive Classic mass spectrometer, positively verified in multilaboratory testing programme: “Evaluation of Data-Independent Acquisition (DIA) for Protein Quantification in Academic and Core Facility Settings”. New protocol for protein extraction from urine samples resulting in 3-fold increase in the number of identified proteins.

3. Including two new panels of LMW compounds (14 fatty acids, aminoacids and metabolites) into the portfolio of the Laboratory offer for external users.

Provide a brief and concise summary of the above mentioned achievements, indicating also impact of such deliverables/milestones on the overall project progress:

Deliverables mentioned fulfill in part the aims of relevant tasks planned in the project at the present stage.

a. Project challenges and risk assessment (up to 1 page A4)

List up to 3 (three) the most important project and research challenges experienced within a given reporting period supplemented with a brief and concise information on the actions taken:

- Difficulties met do not exceed the routine level of scientific conduct and are of technical or merit-based nature.
- […]
- […]

Provide a brief and concise information on the obstacles in the project implementation - research and managerial – that are likely to occur within a forthcoming reporting period and your strategy to respond and mitigate:

[…] No obstacles reported.

b. Socio-economic impact of the project and its tech-transfer potential (up to 1 page A4)

Provide information on how the results achieved during the project implementation influence the key scientific, technological and socio-economic challenges of the modern world.

Procedures being worked out aim at improvement of a spectrum of analyses provided by the laboratory for biomedical sciences community but also for direct use in medicine.

Indicate any actions taken in the past reporting period that may help facilitate a tech-transfer, including collaboration with identified economic partners.

Proteogenomic approaches directed in the frame of the project to detection of non-canonical peptide sequences will be used in a common project with a company “Kucharczyk – techniki elektroforetyczne”.

Established in the lab procedures for drug and metabolites monitoring are directly transferred to application for medical purposes.

c. Detailed description of the research activities (up to 6 pages A4)

This section shall provide a thorough information on the progress of the research programme in the past reporting period. In case of consortium, sections shall be sub-divided between two members of the consortium.

In the second reporting period the implementation of the new analytical procedures was continued as planned. In the frame of all four tasks of the project numerous procedures were designed, tested using pilot sample sets and validated according to appropriate procedures. SOPs were worked out when necessary.

For global proteomics procedures:

a) optimization of protein extraction from a patient's urine samples was carried out. With a combination of FASP 10kDa filters, LysC/Trypsin digestion, followed with HLB desalting step, achieved significant improvement in protein identification. Comparing to 956 in the publication in 2014 (Mucha et al.) to 2510 after the use of the described method, as tested on 156 sample set. 3 more proteins distinguishing control samples from each of 3 nephropathies under study were found. The results are prepared for filing a patent application.

b) the data-independent acquisition method for QExactive Classic mass spectrometer was implemented in the frame of the international study "Evaluation of Data-Independent Acquisition (DIA) for Protein Quantification in Academic and Core Facility Settings" of The Association of Biomolecular Resource Facilities Proteomics Research Group. The method is mainly used on high-end spectrometers, so adapting it to slightly older equipment present in the laboratory was a challenge requiring a good theoretical preparation and in-depth knowledge of the parameters of the HPLC and the mass spectrometer. As part of optimization, various settings were tested, including: resolution, AGC target (automatic gain control; maximum number of ions collected for each cycle before fragmentation and detection), maximum injection time (maximum period in which ions are collected), number of m/z windows, window width, windows overlap and total acquisition time. The parameters depend on each other and on other system values such as the resolution of the column used in HPLC, which affects the average chromatography peak width. In the course of the optimization the method has been developed for HPLC M-class coupled with QExactive mass spectrometer capable of producing data for the greatest amount of peptides while still maintaining good resolution and lowest CV between successive measurements. Within the tests of Proteomics Research Group of the Association of Biomolecular Resource Facilities nine samples derived from the human HeLa cell line with four spiked-in proteins in unknown concentration were measured using the DIA method described above. Data were also collected in standard DDA (data-dependent acquisition) mode to create a spectral library for targeted DIA analysis. The resulting files were sent to the study organizers and analyzed in-house using two programs: commercial Spectronaut (Biognosys) and free platform Skyline. From 63 laboratories that took part in the survey only two including ours used the older model of QExactive. Despite the hardware limitations, we were able to correctly determine ratio for 3 out of 4 spiked-in proteins with a CV around 10% between and within study groups, which is better than for most participants using much newer equipment. Although this results are satisfactory, it is necessary to perform further experiments on various types of samples to achieve sufficient experience to carry out this type of analysis for cooperating laboratories and commercial clients.

For targeted proteomics in collaboration with a team from Dr. Zbigniew Szewczuk's lab at the Faculty of Chemistry, University of Wrocław, we develop new MS technology for analysis of trace amounts of peptides by use of ionization enhancers based on 2,4,6-triphenylpyridinium salts (TPP) in MRM/PRM peptide detection. In the last reporting period tests were performed on the TPP compound and its effectiveness by analyzing 69 peptides for 45 proteins that are known cancer markers. The estimated potential of the salt to increase the sensitivity of the analytical method was determined for each target. An increase of up to 100-fold was observed for certain important cancer markers, such as

CEA5. We have also observed a decrease in solubility for many peptide targets in result of derivatisation by TPP salts and are currently working on resolving this issue.

Also, to increase the detection of low level proteins such as phosphoproteins and cancer markers in MRM/PRM analysis in limited biological samples we have been developing a high-pH reversed-phase fractionation 2D-Micro-Nano-LC method with 96-well-plate microliter fraction collection using a PAL System robot. Such devices are not commercially yet available and we are developing one in-house. In this reporting period we have been solving challenges due to the high-pH degradation of capillaries and testing alternative buffer systems that are more stable.

With respect to task on mAbs characterisation native MS procedures have been optimised using Synapt G2 mass spectrometer and appropriate SOP prepared for large molecule masses analyses. Commercially available therapeutic mAbs (Herceptin and Pertuzumab) were used for testing. Results show that both glycosylation patterns, structure-dependent charge envelope shape, and oligomerisation status of mAbs can be precisely characterised and that the spectra are of very good quality. Library of spectra of therapeutic mAbs built in the frame of the project can be used to benchmark the other mAbs preparations.

For HDex protocols in MAbs analysis of 53 different digesting conditions for Pertuzumab and Rituximab was carried out. Conditions included different amounts of protein per one MS run, exchanging original antibodies' buffer to PBS, stop buffer volume and composition, disulphide bridges reduction conditions (reducing agents, concentration, time, temperature), digesting enzymes immobilized on columns (pepsin, pepsin with protease XIII, nepenthesin I, nepenthesin II), digesting column flow rates, effect of freezing after quench. Especially a new protease Nepentesin-2 (nep-2) proved useful, and was tested using Synapt G2HDMS for improved peptide coverage using Pertuzumab, Rituximab, Herceptin as test therapeutic antibodies. The sequence coverage was much better than for pepsin, and for heavy chain of Herceptin it was 92.5%, excluding peptide which is known to be glycosylated and 97.5 % for light chain, which by pepsin cleavage was covered in only ca 70%. Full mapping of hydrogen-deuterium exchange patterns was performed for mAb samples with nep-2 cleavage. The deuteration uptake was analyzed in DynamX 3.0 program for 5 time points of exchange. The results initiate the library of benchmark exchange patterns of a canonic therapeutic mAb, and can be used as "HDex-MS pattern" for deuterium uptake for Herceptin biosimilars (e.g Herzuma, Kanjiti) and other mAbs biosimilars.

For targeted analysis of mAbs quantitation in body fluids 28 specific peptides were selected from bioinformatics analysis and their synthetic isotope-labeled peptide standards were obtained to be used for the quantitative measurements by MRM/PRM methods of three therapeutic monoclonal antibodies: Trastuzumab, Rituximab, and Pertuzumab. MRM method development was began for the three antibodies.

Within the subproject focusing on HDex data analysis procedures the improved HDex data and results visualization package was worked out. A novel approach for error assessment in deuteration comparisons in protein regions was worked out and included into the package, by implementation of a multi-state variability test (MSVT) which offers a statistical assessment of regional variability. A user can compare relative deuteration between multiple states in a specific regions of the interest (ROI).

MSVT relies on information coming from all peptides which at least partially cover the specified ROI. It means that instead of per-peptide results, we take into account a whole regional variability, which leads to the increase of sensitivity. The application - HaDeX is available as a web-server (http://mslab-ibb.pl/shiny/HaDeX/). The application saves time-consuming data processing work, ensures reproducibility of the results, orders and simplifies the whole process and produces publication-quality visualizations. Transparency is provided by full documentation available online. HaDeX is a part of an R package with tools for analyzing the data for bioinformaticians. Current work assumes further development of HaDeX in discussions with experimentalists to make sure it covers all the needed aspects of providing understandable results.

Within Task 4 new procedures for quantitation of low molecular compounds were established and tested as planned. The development of tissue homogenization procedures was finalised. Procedures were tested on a set of tissue sample within the frame of a project carried out in collaboration with prof. Ufnal from Warsaw Medical Unversity. Processing samples from 5 mg up to 1 g of soft tissue is possible, bone homogenisation was not tested yet. In the next stage of method development the compatibility of different homogenates with Competition Rapid Equilibrium Dialysis (RED) will be tested.
Implementation of plasma fatty acid panel was continued and in large part finalised. At present Laboratory can offer the analyses of a panel of 14 fatty acids. Stearic and palmitic acid had to be omitted by now. The panel will be further expanded to include oleic and oleopalmitic acid and used in NAWA (National Agency for Academic Exchange) project titled "Health-promoting importance of human milk donation". The methods for amino acids and their metabolites quantitation are still under development. PITC (phenyl isocyanate) and PhNCO (phenyl isocyanate) derivatisation methods were tested and discarded, while finally the commercially available Waters kit (AccQ-Tag -with modifications made by us for use with our LC/MS) and dansyl chloride derivative was successfully implemented. Thus aa quantitation panel enriched the offer of the Laboratory. LC/MSMS method for L-carnitine determination in plasma and tissue homogenates (human skeletal muscle) was established for total and free fraction of L-carnitine. Also, we started work on new procedures of small molecule quantitation in microsomal and S9 hepatic fraction.

1. PROJECT PROMOTION AND DISSEMINATION OF THE RESULTS

Project achievements are in a preliminary stage. Nevertheless several posters were presented on scientific conferences.

2. PARTNERSHIP IN THE PROJECT

d. Description of collaboration with foreign research partner(s)

This section shall provide a brief and concise information on the nature of collaboration with foreign research partner.

A direct end-user of the developed technologies such as the ionization enhancer (TPP) in MRM/PRM peptide detection, and the 2D-Micro-Nano-LC fractionation system are Dr. Mark Basik an oncologist, and collaborating researcher from the Lady Davis Institute of Medical Research (Associate Professor of Surgery and Oncology, McGill University). Such technologies are invaluable for the detection of low level proteins such as phosphoproteins and cancer markers in limited biological samples such as biopsies often encountered in Dr. Basik's research.

HDex exchange mapping: prof. Harald Herrmann, DKFZ Heidelberg, Germany, prof. Boris Negrutskii IMBG, Kiev, Ukraine in the frame common projects on a variety of protein systems for testing the new procedures.

e. Description of collaboration with local research partner(s)

This section shall provide a brief and concise information on the nature of collaboration with local research partner.

Tissues homogenization procedures were tested on a set of tissue sample within the frame of a project carried out in collaboration with prof. Ufnal from Warsaw Medical Unversity. Amino acid quantitation methods were used in a common project with dr hab. Robert Olek (Gdansk University of Physical Education and Sport). Common publications in preparation.

For targeted proteomics we are working together in collaboration with a team from Prof. Zbigniew Szewczuk's lab at the Faculty of Chemistry, University of Wrocław, to develop new MS technology for analysis of trace amounts of peptides. We are developing the use of ionization enhancers based on 2,4,6-triphenylpyridinium salts (TPP) in MRM/PRM peptide detection.

HDex exchange mapping: prof. Marszałek, prof. Pokrzywa in the frame common projects on a variety of protein systems.

New urine protein extraction protocol will be invaluable for a nephropathy diagnostics project carried out in collaboration with prof. prof. Leszek Pączek, Krzysztof Mucha, Department of Immunology, Transplant Medicine and Internal Diseases, Medical University of Warsaw, Poland

3. IMPACT OF THE PROGRAMME ON THE PI's CAREER

Provide information and necessary feedback on how is this programme affecting your scientific and professional development.

Project enables transfer of research into new analytical procedures made available for a wider scientific community, but also provides new potential diagnostic tools of importance to medicine. Therefore it enables to disseminate the developments in a science oriented environment into the society. It ensures a broader perspective for the activity of the Mass Spectrometry Lab, I have a privilege to direct.

4. DEVELOPMENT OF RESEARCH TEAM MEMBERS

f. Information on the supervision of PhD students, including additional mentors

Since the aim of the project is to increase the human potential of the R&D sector, provide necessary information on the scientific and professional supervision of PhD students participating in the projects. It is crucial to provide information on mandatory supervision of PhD students by external mentors.

All students are supervised on a daily basis by the PI and postdocs in direct contact and consultations. Lab meetings of are held on a weekly basis. External mentors for PhD students are in contact with PhD students: Prof. J. Pierzynowski and prof. H. Herrmann, they actively participate in common projects in which both PhD students are involved. However, the formal acceptance of prof. Herrmann has not been obtained yet.

g. Supervision of research staff

Since the aim of the project is to increase the human potential of the R&D sector, provide necessary information on the scientific and professional supervision of research team members.

All students and lab members are supervised on a daily basis by the PI and postdocs in direct contact and consultations. Lab meetings of are held on a weekly basis.

5. IMPLEMENTATION OF THE PROJECT ACCORDING TO THE PLANNED SCHEDULE

YES X NO ☐

In case an actual implementation of the project differs from the accepted schedule, please do provide the rationale behind it:

No deviation from schedule.

6. ADDITIONAL INFORMATION

Provide – if necessary – all other important information relevant to the project implementation.

[…]

7. PI's WORKLOAD

I, the undersigned, hereby state that:

X☐ My total commitment to projects financed from EU Structural Funds, ESF and other activities financed from other sources, including the beneficiary's and other entity's funds, did not exceed 276 hours in any month from the reporting period.

☐ My total commitment to projects financed from EU Structural Funds, ESF and other activities financed from other sources, including the beneficiary's and other entity's funds, exceed 276 hours in the below indicated months from the reporting period*:

Year/Month	Number of hours of the total commitment per month
Dec. 2017- Mar.2018	220
Apr.2018-Sep.2018	200

8. TEAM MEMBERS WORKLOAD:

X☐ According to the team member's statements in no case the total commitment to projects financed from EU Structural Funds, ESF and other activities financed from other sources, including the beneficiary's and other entity's funds, did not exceed 276 hours in any month from the reporting period.

☐ According to the team member's statements in the following cases the total commitment to projects financed from EU Structural Funds, ESF and other activities financed from other sources, including the beneficiary's and other entity's funds, exceeded 276 hours in the below mentioned months from the reporting period*:

Name/Surname	Year/Month	Number of hours of the total commitment per month

* In case of exceeding 276 hours monthly of the total commitment to any projects of an employee/stipendee, his/her remuneration/stipend from the Project funds will constitute an ineligible expenditure for each month in which 276 hours have been exceeded.

9. Appendixes to the merit-based annual progress report in the electronic version:

- Project realization indicators (on-line data base: *Progress reports* tab/ *progress report*/*Indicators*)
- Scientific Achievements of the Laureate and Team Members (on-line data base: *Progress reports* tab/ Publications/Patents/Academic degrees and titles/Awards and grants),
- List of conferences, scientific exchanges and business meetings (doc. file uploaded to on-line data base: *Progress reports* tab/*Progress report/Attachments*)
- Progress reports of team members – if applicable - (doc. file uploaded to on-line data base: *Progress reports* tab/*Progress report/Attachments*)

Note: The Laurate's Annual Research Progress Report should be uploaded to the Foundation's database (in the *Progress reports tab/Progress report/Attachments tab*) in two formats: as a '.doc' file and its signed, scanned version as a '.pdf' file.

I, the undersigned, hereby confirm that the information contained in the merit, periodic report (both electronic and paper version) are true. I am aware of the legal consequences of giving untrue information in a legally significant situation, as stated in article 271 of the Penal Code.

Date:……

Laureate Signature……