BIOMARGIN

PUBLIC WORKSHOP

Methodological aspects of biomarker discovery and validation: BIOMARGIN as a case study

BioPark Paris, Tuesday 21st Feb 2017

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Synopsis

The discovery, confirmation and validation of candidate biomarkers of disease conditions, treatment efficacy or toxicity may require a number of experimental and clinical steps to achieve sufficient evidence to become transferable to the clinics, and to convince physicians to incorporate them in their practice. In this workshop, we will mostly address the methodological bottlenecks, or in contrast differentiating designs, that could be identified in different research programs on biomarkers, taking biomarkers of allograft lesions in transplantation as an example.

Programme

10h10	**Registration** **Welcome Coffee**
10h20	Welcome by Pierre Marquet, scientific coordinator of the BioMargin Consortium
10h30 - 11h40 Session I – Methodology of clinical studies and levels of evidence of biomarkers *(Chair: Marie Essig)*	
10h30 – 10h50	Wilfried Gwinner, University of Hanover, Germany *Challenges in the assessment of the transplant*
10h50 – 11h20	Petra Reinke, Charité, Germany *Methodological and clinical aspects/issues of BIODRIM*
11h20 – 11h40	Pierre Marquet, Inserm, France *BioMargin Design and expected evidence of the outcomes*
11h40 - 13h00 Session II – Nucleic acids as biomarkers: technical aspects and constraints *(Chair: Joost Schanstra)*	
11h40 – 12h10	Ekkehard Schütz, Chronix Biomedical, Germany *Methodological considerations about cell-free DNA as a source of biomarkers*
12h10 – 12h30	Maarten Naesens, KULeuven, Belgium *Profiling and validation of mRNA biomarkers in blood and biopsies*
12h30 – 12h50	Dany Anglicheau, Hôpital Necker, France *Methodological issues of miRNA profiling and validation of miRNA biomarkers in different matrices*
12h50 - 14h00 Lunch	
14H00 - 17H00 Session III – Mass spectrometry for proteomic and metabolomics biomarkers: methodology and clinical transfer *(Chair: Maarten Naesens)*	
14h00 – 14h30	Vladimír Havlíček, Academy of Sciences of the Czech Republic, Czech Republic *From MSI to REIMS: meet interesting abbreviations in clinical mass spectrometry*
14h30 – 15h00	Anna Baud, UCL IRIS, UK *Protein biomarker discovery for translation to the clinic*
15h20 – 15h40	Inge Mertens, VITO, Belgium *Mass spectrometry based clinical proteomics as a tool for liquid biopsy biomarker discovery*
15h40 – 16h00	Jochen Metzger, MOS, Germany *Capillary electrophoresis-mass spectrometry for clinical diagnosis – A commercialization model based on proprietary methodology rather than a patented test*
16h00 - 16h30	**Coffee Break**
16h30 – 17h00	Short oral presentations: *Endogenous metabolic profiling as a fundament in personalized theranostics* Torbjörn lundstedt, AcureOmics, Sweden *The SWATH mass spectrometry technology as applied to urine peptidomics* François- Ludovic Sauvage, Inserm, France
17H00	**End of meeting**

Lectures

Methodological and clinical aspects/issues of BIO-drIM

Petra Reinke
Charité, Germany

The central focus of the BIO-drIM project is the implementation of biomarker-driven strategies for personalizing immunosuppression (IS), with the aim of:
1. improving the long-term outcome in solid organ transplant patients,
2. decreasing adverse effects (graft toxicity, diabetes, cardiovascular events, opportunistic and community acquired infections, bone loss, and malignancies),
3. optimising the costs-benefit-ratio of chronic IS.

Challenges in the assessment of the transplant

Wilfried Gwinner
University of Hanover, Germany

Renal allograft survival has only slightly improved in recent decades. The reasons for graft loss are manifold and often multifactorial in the individual patient. Major diagnostic challenges in the continuous monitoring after transplantation are the anticipation and timely diagnosis of smoldering injuries and acute damage in the allograft. Evaluation of the immunological status and clinical complications during the posttransplant course can help identify patients at risk. Monitoring of the renal function can indicate allograft deterioration but is not informative of the underlying causes and nature of tissue damage. An allograft biopsy is currently the gold standard for establishing a specific diagnosis. However, biopsies are not suitable for close control of the allograft status. Issues of representativeness and reproducibility limit their value. Moreover, distinct lesions localized in the tubulointerstitium, the microvascular system and in larger arteries may not always be pathognomonic for T cell- and antibody-mediated rejection, as suggested by previous studies on the molecular level

BioMargin Design and expected evidence of the outcomes

Pierre Marquet, on behalf of the BIOMARGIN consortium
INSERM U850, Univ. Limoges, CHU Limoges, France

BIOMARGIN aims to discover, select and validate: (1) blood and/or urine biomarkers at different omics levels of renal allograft lesions; and (2) early predictors of chronic graft dysfunction and ultimately graft loss. Another goal is to provide clinicians with tools to obtain such information in a timely manner. Finally, the most pertinent biomarkers will be transferred to the clinics.

Indeed, biomarker translation to the clinics first requires identifying and thoroughly validating candidate biomarkers for clearly defined disease entities. This implies a multistage program, from biomarker discovery to validation and qualification applying the highest possible quality controls at all clinical and analytical steps. Multicenter investigations in large cohorts should further evaluate the predictive, diagnostic and prognostic performance of biomarkers, identify relevant confounding factors and most importantly, estimate the generalizability of the proposed biomarker models.

Secondly, the clinical utility of innovative biomarkers should be demonstrated in target populations, including description of thresholds and confounders. This requires proof of the utility of the candidate biomarker at the individual level through both large observational cohort studies and ideally prospective interventional trials.

Methodological considerations about cell-free DNA as a source of biomarkers

Ekkehard Schütz,
Chronix Biomedical, Germany

Cell-free DNA (cfDNA) biomarkers are increasingly used in medical laboratory diagnostics. Almost all approaches rely on the detection and/or quantification of cfDNA that differs from the inherited germ-line DNA of the patient. There are three major field of use, which are: non-invasive prenatal testing (NIPT), cancer and transplantation, where such differences are usually present and can be used as blood biomarkers biomarkers. The increasing use is linked to the improvement of technologies in molecular diagnostics, which has in particular benefited from improvements in next-generation sequencing (NGS) and the availability of robust digital PCR systems. These technologies are used to overcome the issues that are faced in cfDNA based biomarker analytics, based on their biologic nature: cfDNA is only present in trace amounts in the circulation (plasma) at concentrations of usually below 4,000 genomic copies/mL. cfDNA is highly fragmented with a major length of only ~170 base pairs. cfDNA consists of a major fraction of genomic DNA released by circulating nuclear cells (white blood cells). The challenge therefore is to detect e.g. single nucleotide differences in a low fraction of trace amounts of highly fragmented DNA. To ensure this, the entire process, beginning with blood draw needs to be optimized and standardized for cfDNA. Any used detection method has its strength and weaknesses (e.g. ligation-based vs PCR based), which needs to be considered in assay design to generate a reproducible result. This talk will give an overview of different technologic and methodologic approaches to overcome these caveats for robust and precise diagnostic testing.

Session II

Profiling and validation of mRNA biomarkers in blood and biopsies

Maarten Naesens
KULeuven, Belgium

Biomarkers are the cornerstone of personalized medicine. In this presentation, the necessity to implement biomarkers in clinical transplantation will be discussed, and the recent literature on this topic will be reviewed.
More specifically, the opportunities and hurdles of mRNA expression analyses in blood and kidney transplant biopsies will be covered. Technical advancements will be integrated with recent examples from European and US centres. Finally, we will present the mRNA expression data obtained in the FP7 BIOMARGIN project, and put these data into a broader perspective.

Methodological issues of miRNA profiling and validation of miRNA biomarkers in different matrices

Dany Anglicheau
Hôpital Necker, France

Because the noncoding microRNAs (miRNAs) regulate the expression of a vast array of genes, their expression level has been investigated intensively in many physiological and pathological processes. Within the BIOMARGIN project, miRNA profiling was investigated in renal tissue, in whole blood and in urine samples using different technologies in order to define miRNA-based biomarkers of renal allograft injuries. In this presentation, we will (i) discuss several technological challenges of miRNA profiling including the strengths and weaknesses of different platforms and the challenge of miRNA normalization, (ii) provide a short overview of the available literature in miRNA biomarkers in renal transplantation, (iii) provide preliminary results of miRNA profiling in the BIOMARGIN project.

From MSI to REIMS: meet interesting abbreviations in clinical mass spectrometry

Vladimír Havlíček

Academy of Sciences of the Czech Republic, Czech Republik

In this presentation the quest for disease biomarkers will be illustrated on a diverse set of analytical technologies. Molecular signatures of aspergillosis, standing here for an infectious disease, will be illustrated by high price liquid chromatography (HPLC) combined with Fourier transform ion cyclotron resonance mass spectrometry. Dereplication of fungal siderophores from HPLC datasets and also of some other important secondary metabolites was addressed by our in-house tool called CycloBranch. Aspergillosis caused by a filamentous fungus *Aspergillus fumigatus* was also probed by multimodal imaging. The pathogen was visualized by positron emission tomography, optical and scanning electron microscopy with energy dispersive X-ray spectroscopy and namely by means of mass spectrometry imaging (MSI).. The tissue aging process on human eye lenses will be demonstrated. Whereas the total lipid content in this fragile tissue decreased with age, MSI revealed subtle lipid composition changes and indicated the formation of lipid degradation products. Metabolic and inherited disorder tracking will be illustrated on Fabry disease. MALDI MSI indicated the distribution of various globosylceramides in murine renal coronal and transversal sections. The talk will be concluded with Rapid Evaporation Ionization Mass Spectrometry (REIMS). Particular attention will be paid to subcutaneous and metastatic melanomas in murine skin and lungs, respectively. MSI will be shown as a promising approach for biomarker discovery, whereas the REIMS as an excellent tissue classification tool.

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Protein biomarker discovery for translation to the clinic

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Despite great recent advances in medicine, currently there are no effective treatments for many metabolic or neurodegenerative diseases. Finding new biomarkers for identifying, stratifying and monitoring new treatments of these disorders are important in the development of future novel therapies. To diagnose patients early and more accurately, biomarkers need to be disease-specific, quantitative, reproducible and ideally non-invasive.

Biomarker discovery using deep-proteomic profiling analysis can identify panels of potential candidate biomarkers that can be multiplexed into high-throughput targeted multiple reaction monitoring mass-spectrometry assays (MRM-MS) for verification. Candidate biomarkers are evaluated on larger cohorts of samples, and those that are reliably confirmed are retained during the development of the assay. To address the clinical needs, the final assay should be specific, sensitive, reproducible, high-throughput and cost-effective.

We will describe the streamline approach we take from initial discovery experiments to biomarker validation for clinical translation. We will also discuss the importance of sample selection, experimental design and the major bottlenecks of discovery and targeted proteomic approaches. Studies from our laboratory using targeted proteomics will be presented and include newly described markers for mucopolysaccharidoses stratification in urine, dementia from cerebrospinal fluid and a high throughput alternative targeted proteomic assay for iPS cell pluripotency

Mass spectrometry based clinical proteomics as a tool for liquid biopsy biomarker discovery

Inge Mertens
VITO, Belgium

Today, a clinical diagnosis is often based on the examination of a tissue biopsy by a pathologist. The procedure to collect the tissue is very invasive and unpleasant. Moreover, the diagnosis largely depends on the expertise and training of the pathologists. In the future, we believe that this typical workflow could be optimized by using liquid biopsies, that are less invasive to collect and also allow better follow up of the patients. To do that, we need sensitive and selective biomarkers to detect the pathology of interest.

In this presentation, we will give an overview of ongoing research projects that have the goal to identify biomarkers for several pathologies (renal allograft rejection, bladder cancer, colorectal cancer) using mass spectrometry based clinical proteomics. We will highlight several aspects of good practice in setting up studies and show that a multidisciplinary team is required to obtain high quality results.

Capillary electrophoresis-mass spectrometry for clinical diagnosis – A commercialization model based on proprietary methodology rather than a patented test

Jochen Metzger[1], Petra Zürbig[1], Bill Mullen[2], Harald Mischak[1,2]

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Biomarker research, for the development of clinical tests, faces several major challenges. One such challenge is providing a clear segregation of disease features from variables stemming from biological differences, such as age, sex, exercise, diet and circadian rhythm. The fact that a disease is often multifactorial in its molecular origin and single disease features are shared between different diseases make it a significant challenge to distinguish one disease unequivocally from all other clinical conditions. Under these circumstances, a combination of biomarkers to produce a molecular signature of a disease is a potentially more effective and powerful strategy than a single biomarker diagnostic test.

Following this combinatorial approach, capillary electrophoresis coupled to mass spectrometry (CE-MS) was developed in order to gain an analytical platform for multidimensional analysis of peptides within the mass range 0.8 to 20 kilodalton. Based on large numbers of individual CE-MS peptide profiles, mostly in urine, but also in other bio-fluids, followed by their group-wise statistical comparison, disease-specific peptide marker patterns were established in a standardized proteomic workflow. This mathematical process was applied to various renal and non-renal diseases using support vector machine learning algorithms. By optimizing sample preparation protocols, CE-MS analysis and proteomic data processing methods, CE-MS has progressed over the last ten years to a versatile methodology for clinical diagnostic tests based on such peptide marker patterns, with at least 20, but up to as many as 300 peptide components. An example of this was demonstrated in large clinical trials and technical reports and was certified recently in a letter of support given by the US Food and Drug Administration for the prognostic chronic kidney disease marker pattern CKD273.

Molecular marker patterns contain to some extent redundant information thereby increasing robustness in their classification accuracy and several different marker patterns can be defined and employed resulting in similar test characteristics. This makes patent claims on biomarker patterns almost impossible. Therefore the economic strategy for developing and implementing this innovative technology must be focused on the CE-MS technology as a modular analyzing system together with proprietary proteomic data processing algorithms for centralized prognostic/diagnostic test applications.

Abstracts for Short oral Presentation

Endogenous metabolic profiling as a fundament in personalized theranostics

Torbjörn Lundstedt[1,2,7], Katrin Lundstedt-Enkel[1,3], Kate Bennett[1], Claire Russell[4], Rebeca Martín-Jiménez[4], Michelangelo Campanella[4], Sara Mole[5], Julia Petschnigg[5], Thomas Moritz[1,6], and Johan Trygg[1,7]

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Metabolomics has grown into an established tool in research for;
A. Diagnosis, i.e. classification.
B. Identification of biomarkers in relation to e.g. diseases.
C. Dynamic studies when identifying effects from, for instance, medical treatment, changes in life style, environmental or genetic changes.

In this presentation the use of metabolomics as a tool in drug discovery and diagnostics will be highlighted. In the first part the differences in biochemical profiles between healthy volunteers and persons with the diagnosis rheumatoid arthritis (RA) are discussed and identification of involved biochemical pathways for understanding the underlying factors of the disease are presented.
In the next part a comparison to different animal models is made, in order to identify the most relevant animal model for describing the disease in humans. The animal models are used for evaluation of novel treatments.
In the last part, an example from the BATCure project will be presented for a CLN3 disease yeast model, comparing the *btn-1* mutant *vs.* wild type. In addition, from the zebrafish (*Danio rerio*) CLN2 disease model, we compared tpp1-/- with the metabolic profile of wild type. Results will be presented and discussed in relation to metabolic profiles and biochemical pathways and how these findings can help us to identify novel methods of treatments.

Abstract 2

The SWATH mass spectrometry technology as applied to urine peptidomics

François Ludovic-Sauvage

Inserm U850 , France

François-Ludovic Sauvage1, Marie Essig1,3 and Pierre Marquet1,2

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Background: In renal transplantation, the discovery of early urine biomarkers of graft lesions would be useful to help physicians to improve patient care and minimize the use of invasive graft biopsies. For untargeted proteomic analysis, papers have recently reported the SWATHTM MS (AB-Sciex) technology (1), a data-independent acquisition mode, with fixed or variable m/z windows, allowing the simultaneous identification and quantitation of large numbers of compounds. The urine peptidome, made up of the peptides naturally present in urine, excreted or shed by the kidney, or resulting from natural enzymatic hydrolysis of urine proteins, may be an interesting source of such biomarkers. We aimed at building an MS library of human natural urinary peptides and applied the SWATHTM technique in FP7 BIOMARGIN.

Methods: After solid-phase extraction (2) of urine samples from kidney transplant recipients, native urine peptides were: (i) identified by means of a 2-hour long nano-LC-ESI-Q-TOF mass spectrometry method using the classical, data-dependent acquisition mode and a combination of proteomics search engines, to build up a dedicated MS library; and then (ii) retrieved in library and quantified based on the areas of the chromatographic peaks obtained using the same method, except that acquisition was in the SWATH mode with 60 SWATH windows of variable width.

Results: 258 urine samples from kidney transplant recipients with a normal (n=80), antibody mediated rejection (ABMR) (n=65), T-cell mediated rejection (TCMR) (n=67) or interstitial fibrosis/tubular atrophy (IFTA) (n=100) were analyzed, leading to the identification of 514 different naturally occurring peptides issued from 38 proteins. The SWATH library thus constituted was found to be efficient for the analysis of further patient samples from the BIOMARGIN trans-sectional study (step 3).

Conclusions: We developed a nano-LC-ESI-Q-TOF mass spectrometry technique using the SWATH acquisition mode, and a dedicated MS library of 514 peptides issued from 38 different proteins, for the accurate identification and quantification of naturally occurring peptides in the urine samples of patients enrolled in FP7 BIOMARGIN. A small number of these peptides seem to be able to discriminate between groups of graft lesions.

(1) Schubert OT, Gillet LC, Collins BC, et al. Nat Protoc. 2015;10:426-441.

(2) Sauvage FL, Gastinel LN, Marquet P. J Chromatogr A. 2012;1259:139-147.

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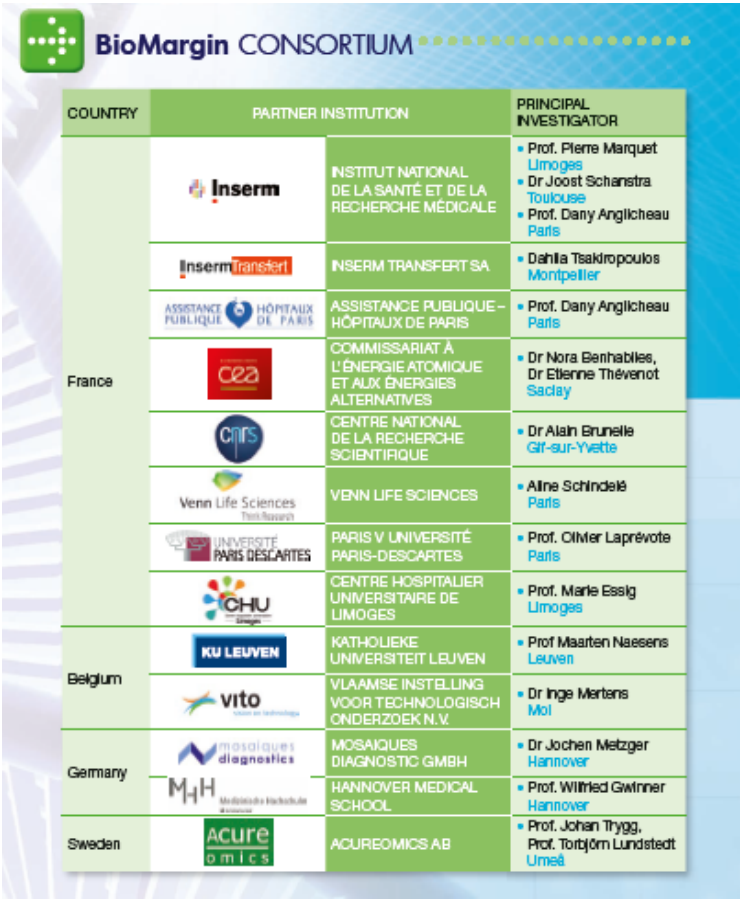
The BioMargin consortium is coordinated by the French National Institute of Medical Research (INSERM – Prof Pierre Marquet) and brings together 13 complementary European partners, including three small and medium enterprises, five academic laboratories, and four University Hospitals, and one technology transfer/ management company from four European Member States (France, Belgium, Germany, and Sweden). The partners are highly complementary and the consortium combines all the skills from clinical nephrology, clinical trials, histology, -omics, statistics and mathematical modelling, regulatory and ethical expertise in clinical setting.



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