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Project context and objectives:

For patients with end-stage renal disease, transplantation has become the preferred treatment, providing better survival than prolonged dialysis, in both adults and children. In renal allograft recipients, graft survival at one year post-transplantation has improved to more than 95%. However, at 10 years, it is only approximately 65-70%, with no major improvement with regards to the previous decade. Also, although acute rejection (AR) is a major risk factor for the development of chronic allograft nephropathy leading to graft loss, decreases in the incidence of AR in recent years have not translated into improved long-term outcomes. Both immune mechanisms (Tcell mediated and antibody-mediated rejection, de novo or recurrent glomerular disease, etc.) and nonimmune mechanisms (nephrotoxicity of calcineurin inhibitors, accelerated aging, epithelial mesenchymal transition, etc.) contribute to the progression of chronic histological damage and scarring of renal allografts. These injury processes jeopardize graft function and long-term graft survival. As only a subset of patients develop chronic injury and because at present physicians do not have the ability to reverse chronic fibrotic kidney damage, it is essential that the transplant community develops reliable and noninvasive approaches to predict which patients are most likely to develop graft failure, so that appropriate interventions can be instituted before this becomes clinically apparent.

The practical objectives of BIOMARGIN are to:

- Discover, select and validate: (1) blood and/or urine biomarkers, **at different omics levels**, of renal allograft lesions, with good diagnostic performance as compared to biopsy histological analysis ('Gold standard'); (2) mechanism-based classifiers of graft lesions, including intra-graft mRNA or miRNA as well as lipid, peptide and protein localization within the graft, to help histological interpretation of the biopsy; and (3) early biomarkers of chronic graft dysfunction and ultimately graft loss, less invasive than graft biopsy and with improved predictive values of longterm outcome.
- Provide clinicians with tools (analytical techniques, interpretation algorithms, a dedicated website) to obtain such information in a timely manner, and promote these innovations towards scientific societies and patient associations.
- Set-up a research environment for further biomarker research in transplantation.

The BIOMARGIN consortium employs an innovative strategy tackling several complementary –omics and mass spectrometry imaging approaches. The biomarker candidates will be selected and integrated using molecular biology, computational biology, statistics and disease progression models.

Description of the work performed and main results:

The objectives of the first 18-months period were mainly to:

- (1) Collect, distribute and analyze triplets of blood, urine and kidney biopsy samples from ca. 120 renal transplant patients, with normal biopsy, or histological signs of T-cell mediated rejection (TCMR), Antibody-mediated rejection (AbMR) or Interstitial Fibrosis/Tubular Atrophy (IFTA).
- (2) Write the protocol, patient information and consent forms of the BIOMARGIN European Cohort Study (BECS), translate them into the different languages of the Partners' member state, submit them to the national authorities and national/regional/local ethics committee (as appropriate) and obtain the signatures of the transplant centers members of the consortium to participate in this study.

The plan was to select banked urine, blood and biopsy samples collected as usual practice in the 4 Biobanks participating in the project (APHP Necker #3, KU Leuven #6, MHH #9 and CHU Limoges #13). However, when reviewing the results of the first exchange of test samples for the purpose of quality control, an unexpected disparity of sample collection and preparation conditions was discovered, resulting in a risk for the reliability of analytical results. Then, after a long discussion among the consortium, a SOP was written to harmonize sample collection, preparation and storage. As a consequence, sample collection started late leading to an overall delay of 10 months which has impacted all the work-packages.

In parallel, all the activities planned by CARDINAL SYSTEMS #10 for the clinical trial 3×BIOS² were finalized:

- Regulatory and ethical application of the case-control & trans-sectional and cohort studies
- Central reading of the first set of preselected biopsy slides
- Anonymization and shipment of the first set of samples from the 4 biobanks to the 8 labs involved
- Development of an electronic Clinical Research File (eCRF)
- Preselection and final selection (after central reading by expert pathologists) of 134 sample triplets (urine, blood, biopsy core) collected in the standardized conditions
- Quality monitoring of the samples and of the clinical research files
- Sample shipment to the 8 laboratories on May 13, 2014

The analytical tasks performed for step 1 of 3xBIOS² study ("training set") for biomarker discovery are as follows:

- Urine protein and creatinine concentrations were measured in all samples by INSERM #1a
- Untargeted microRNA profiling in blood was completed by INSERM #1c.
- The extracted RNA samples were subsequently sent by INSERM #1c to KU Leuven #6 and mRNA expression analysis is on-going
- Untargeted analyses of microRNA in urine: 119 samples underwent microarray analysis by INSERM #1b (11 were disqualified, as they did not meet the QC criteria). All raw data and QC reports were sent to CEA #4 for statistical analysis
- Untargeted analyses of mRNA in 120 urine samples by INSERM #1c are still on-going (10 samples disqualified after quality check)
- Untargeted analyses of peptides in urine was successfully performed in all samples except one by MOSAIQUES DIAGNOSTICS GmbH #8 using CE-MS, and in 48 samples by INSERM #1a using MALDI-TOF/TOF. The rest of the analyses, as well as all analyses by NanoLC-ESI-HRMS by VITO #7, are on-going.

- Untargeted analyses of proteins in urine: half of the proteomic analyses were performed by INSERM #1a, using NanoLC and MALDI-TOF/TOF.
- Untargeted analyses of lipids in urine: after method comparison, optimization and validation, UNIVERSITE PARIS DESCARTES #11 analyzed all samples and detected, annotated using an on-line database, and semi-quantified many lipids of different categories in a large range of concentrations.
- Untargeted analyses of metabolic biomarkers in plasma and urine: 3 different GCMS methods were developed. The development of scripts for MS data preprocessing is on-going. Due to missing the initial time-slot, analyses have been postponed to Nov. and Dec. 2014.
- Untargeted analysis of mRNA and miRNA gene expression in biopsy samples: KU Leuven #6 extracted 131 biopsy samples and evaluated RNA quality and concentration. mRNA of 118 good-quality samples was amplified and hybridized onto microarrays. Microarray gene expression profiles were then calculated and normalized. At month 18, the extracted RNA samples were subsequently sent for miRNA expression analysis to APHP Necker #3.
- Mass-spectrometry imaging of renal allograft biopsies: specialized staff training was delivered and reproducible and efficient methods developed and validated, for sample preparation and analysis by MALDI-TOF (lipids, proteins) and TOF-SIMS (lipids).

Data integration and disease prediction modelling:

- The "Prototype "2BIO-DB" biomarker database for data and metadata collection" was built by CEA #4 and sent to INSERM #1a at M12. The database is currently being filled with 120 miRNA biomarkers from the literature by APHP Necker #3. Proteins and mRNAs will follow shortly. In parallel, the database is currently being implemented as an online resource by INSERM #1a. 2BIO-DB will now be appended up to M48 with the new biomarkers discovered during the project.
- A genetic data mining algorithm was applied on a dataset previously published by MOSAIQUES DIAGNOSTICS GMBH #8. At M17, CEA #4 received the first Biomargin datasets, of miRNA and peptidomics in urine. Preliminary univariate and multivariate analyses of both datasets have been performed.

Longitudinal evaluation of the diagnostic and prognostic performance of selected biomarkers:

- The Biomargin ambispective European Cohort Study (BECS) protocol was discussed with, and agreed upon, by all the clinical Partners and presented at the Consortium annual meeting in March 2014 to have a feedback from the other Partners and the Advisory Board. In parallel were written information and consent forms for adult patients, for parents/legal guardians of children and for the children, of different age groups, themselves. Each form was translated into the languages of the clinical Partners, who further corrected them.
- Finally, the protocol and associated consent and information forms were submitted for regulatory approval in France, Germany and Belgium between May and August 2014.
- The enrolment of patients in the BECS study will start after regulatory approvals. However, BECS includes a retrospective part for patients with samples already banked as part of usual clinical care. The pre-screening since month 6 of BIOMARGIN identified approx. 50 such patients who can already be included as soon as approvals are obtained.
- The Clinical Research File for BECS study was finalized in July 2014. The electronic CRF is planned to be released in October 2014.

Expected final results and their potential impact and use

The final goal is to provide renal transplant clinicians with innovative biomarkers enabling closer, more accurate, more predictive and/or less invasive monitoring of renal transplant patients than serum creatinine or graft biopsies. Such currently unavailable biomarkers will likely improve patient care and will have several positive impacts.

- Diagnostic tools:

The whole purpose of BIOMARGIN is to develop new biomarkers that will enable a closer monitoring of the graft in order to detect acute or chronic injuries earlier, which will translate into a more rapid intervention and hopefully better long-term outcome.

- Improve treatment outcome for transplanted patient:

Early and specific diagnosis of immunological or non-immunological allograft injuries is a major prerequisite for a successful intervention. The earlier therapy can be started, the greater the chances are to stop, or even reverse, the injury process and prevent irreversible scarring of the renal tissue. Based on this, we expect to better conserve renal tissue and function, thus prolonging allograft survival, which is currently limited to approximately 12 years on average. This is particularly important for pediatric patients, who are expected to need several transplantations during their lifetime to avoid prolonged dependence on dialysis.

- Better understanding of the mode of action of existing or potential treatments:

So far, the pathophysiology of progressive loss of allograft function has been poorly understood. There are only a few clearly defined allograft injuries, such as acute T-cell mediated rejection and acute/chronic antibody-mediated rejection, but the underlying causes of graft loss appear to be much more diverse. The use of different 'omics' technologies in BIOMARGIN holds the promise of delineating specific molecules and pathways in these processes, of immunological or non-immunological origin, which can serve to define therapeutic targets.

Moreover, biomarkers may bring a new understanding of drug effects on the renal intra-cellular pathways and on biomarker levels that should help transplant clinicians select the best therapeutic option in a given situation and monitor its effects, using biomarkers. In addition, by unravelling signalling pathways involved in graft lesions such as fibrosis, BIOMARGIN should also open new paths for therapeutic interventions.

- Impact on graft outcome, patient survival and quality of life:

Owing to closer monitoring of the graft and faster reaction regarding the adaptation of individual patient treatment, the rates of renal graft function deterioration and of graft loss should be reduced. At the present time, the therapeutic arsenal does not allow for the reversal of chronic antibody-mediated rejection or allograft fibrosis, for instance. This is the reason why biomarkers with a strong predictive value would be important, as the most efficient therapeutic measures nowadays are preventive in nature. The general condition of the patients will improve, resulting in a better quality of life for both the patient and his/her entourage. Also, prolonged graft survival should translate into prolonged patient survival, as patients on dialysis have a shorter life expectancy than transplanted patients. Less kidney allograft recipients will come back on the waiting list for kidney transplantation each year, and consequently this will increase the total number of patients being transplanted and shorten the time patients spend on the waiting list, which in turn will also increase their life expectancy, as recently demonstrated.

In Europe, 50,000 to 100,000 patients have end-stage renal failure. Compared to dialysis treatment, for most patients kidney transplantation is better suited to regain health, quality of life, and the ability to pursue an individual and self-sufficient lifestyle. Approximately 18,000 kidney transplantations are performed annually in Europe. However, this figure is far exceeded by the number of patients on the waiting list for renal transplantation. Improving the success rate of transplantation by prolonging the allograft survival would contribute to have less second or third renal transplantations, hence more new patients transplanted, instead of being dependent on dialysis.

- Socio-Economical Impact:

The BIOMARGIN non-invasive biomarkers will reduce the need for, and the costs related to, graft biopsies. Extended graft survival will also result in less patients returning to dialysis, the cost of which is clearly much higher than the cost of transplant patient medical care, especially years after

transplantation. Also, shortening the time patients spend on a waiting list is cost saving. Finally, patients with a functioning graft are much more likely to be able work and to earn their living.

A second important economic impact expected is for the European industry, especially the SME sector, through the commercialization of key-in-hand techniques for biomarker analyses and interpretation.

Finally, the involvement of patient associations (through the external advisory board and active communication actions planned) will help spread the results of this research quicker and to a wider community.

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