Executive summary:

Both immune and nonimmune mechanisms contribute to the progression of chronic histological damage and scarring of the kidney graft in transplanted patients. These injuries jeopardize graft function and long-term graft survival. It is essential that the transplant community develops reliable and noninvasive approaches to diagnose early these lesions, or even predict which patients are most likely to develop graft failure, so that patient care be adapted accordingly.

The practical objectives of BIOMARGIN were to discover, select and validate: (i) blood and/or urine biomarkers of renal allograft lesions, i.e. body molecules with good diagnostic performance of two different types of rejection process, or of tissue fibrosis; (ii) predictive biomarkers of chronic graft dysfunction and ultimately graft loss, less invasive than graft biopsy and with improved predictive values of long-term outcome; (iii) biomarkers within the graft, to help pathologists to read biopsies; and (iv) provide clinicians with such information in a timely manner.

To reach these goals, the BIOMARGIN consortium employed an ambitious strategy encompassing: (i) a succession of clinical studies in 4 hospitals in three European countries for the discovery, confirmation and validation of biomarkers in which transplanted patients provided blood, urine and biopsy samples; (ii) a large exploration of nucleic acids, proteins, lipids and smaller molecules in this samples using state of the art analytical technologies; and (iii) cutting-edge statistics.

In a first step, 9 different laboratories from 4 countries compared samples from 134 patients with normal biopsy (controls), antibody-mediated rejection (ABMR), T-cell mediated rejection (TCMR) or Interstitial Fibrosis / Tubular Atrophy (IFTA) and identified a wealth of molecules with concentration differences between groups. A second, identical study in 138 other kidney transplant recipients helped select a shorter list of confirmed candidates, the diagnostic performance of which was then tested in a larger, representative group of patients (n= 458). Combinations of nucleic acids (messenger-RNA - mRNA - and micro-RNA - miR -) in blood or in the biopsy, or small molecules (called metabolites) in blood, as well as combinations of peptides or proteins in urine yielded very good to excellent diagnostic performance with respect to ABMR and TCMR, while no proper signature could be validated for IFTA. In addition, in the biopsy: a 5-miR signature discriminated ABMR from all other diagnoses and a 10-miR signature identified ABMR from normal biopsies, both with an excellent performance; and >500 mRNAs robustly diagnosed ABMR and pointed towards two rather unexpected causes, namely innate immunity and NK cell activation.

This wealth of positive results should translate into different clinical and commercial strategies, after all these biomarkers have been patented and published.
The predictive performance of these biomarker signatures, and potentially others, will be tested on a huge number of blood and urine samples (about 2900 of each) collected from 637 adult and 38 pediatric patients followed-up prospectively in the BIOMARGIN European Cohort Study.

The BIOMARGIN consortium co-organized a symposium entitled "The coolest clinical trials in transplantation sponsored by EU" at the European Society of Transplantation (ESOT) 2017 meeting in Barcelona, Spain in September 2017. BIOMARGIN was presented in front of 120 people along with other EU projects related to organ transplantation. Questions were numerous and the audience showed a real interest for BIOMARGIN. This was followed by the presentation of BIOMARGIN results through 7 oral or poster presentations by different partners.
Summary description of project context and objectives:

For patients with end-stage renal disease, kidney transplantation has become the preferred treatment, providing better life expectancy and quality than prolonged dialysis, in both adults and children. Kidney allograft survival in the first transplant year has improved in the past decades to more than 95%, mainly driven by better peritransplant management and development of new immunosuppressive drugs. Also, the frequency of acute rejection which is a major risk factor for the development of chronic allograft nephropathy and subsequent graft loss, has markedly decreased by the immunosuppressive treatment. However, these improvements have hardly been translated into improved long-term outcomes, which is still only 65-70% after 10 years. Both immune mechanisms (T cell mediated and antibody-mediated rejection, de novo or recurrent glomerular disease, etc.) and nonimmune mechanisms (nephrotoxicity of calcineurin inhibitors, accelerated cellular 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 develops 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 acute and chronic graft injury (and failure), so that appropriate interventions can be instituted as early as possible (before this becomes clinically apparent).

The practical objectives of BIOMARGIN were to:

Discover, select and validate:

(1) blood and/or urine biomarkers, at different omics levels (transcriptomics, proteomics and metabolomics), of renal allograft lesions, with good diagnostic performance as compared to biopsy histological analysis (‘Gold standard’);

(2) mechanism- based classifiers of graft lesions, including intragraft mRNA or miRNA as well as lipid, peptide and protein localization within the graft, to help histological interpretation of the biopsy; indeed, as it entails transcriptomics analyses in the graft and a systems biology approach, BIOMARGIN will also help understand the mechanisms involved in the various kidney allograft injury processes. This, combined with mass spectrometry imaging of the graft should offer pathologists new molecular targets for the analysis of renal graft biopsy and may improve, in the process, the “gold standard” diagnosis of graft lesions and injury processes.

(3) predictive biomarkers of chronic graft dysfunction and ultimately graft loss, less invasive than graft biopsy and with improved prediction of long-term outcome.

Provide clinicians with tools (analytical techniques, interpretation algorithms, a dedicated website) to obtain such information in a timely manner.

Promote these innovations towards scientific societies and patient associations

Set-up a research environment for further biomarker research in transplantation by providing:

- a database of all biomarker candidates in renal transplantation, either issued from BIOMARGIN or previously discovered by BIOMARGIN partners and other groups (“2BIO-DB”)
- a BIOMARGIN network-biobank of urine and plasma samples from kidney transplant recipients (“BiBUP”)
The BIOMARGIN consortium had planned to employ an ambitious strategy encompassing:

- An extensive clinical study scheme entailing two retrospective, one trans-sectional and one prospective study for the discovery, confirmation and validation of biomarkers (figure 1)
- Highly standardized sample collection, processing and storage (figure 2)
- An indisputable gold standard (blind centralized biopsy reading by a college of three experts)
- Several complementary –omics approaches
  - Urine: messenger RNAs, micro-RNAs, peptides & proteins, lipids, metabolites
  - Blood: messenger RNAs, micro-RNAs, metabolites
  - Graft biopsy: messenger RNAs, micro-RNAs, mass spectrometry imaging of lipids and proteins
- State of the art analytical technologies for the untargeted discovery and identification of biomarker candidates (first 2 steps), and then for their quantitative measurement.
- Sophisticated statistics adapting and combining multivariate statistical methods and systems biology modelling

Figure 1: Succession of clinical studies planned to discover, select and validate biomarkers of kidney graft injuries. (Ab: antibody-mediated)
Urine in an 100 mL cup

Leucocyturia and hematuria detection (dipstick) in the local labs BEFORE freezing

Warning:
• In cases with significant leucocyturia, a bacterial culture should be performed and results recorded

Figure 2: Flow-chart of urine sample collection, processing, storage and shipment, shown as example

In the discovery and selection phases (first two clinical steps, each comparing four groups of renal allograft recipients with distinct histological-clinical conditions: normal biopsy, T-cell mediated rejection, antibody-mediated rejection and IFTA without rejection or inflammation), we planned to use:

- Untargeted microarray analysis of mRNA and miRNA expression in blood and tissue and of miRNA expression in urine; candidate-gene approach of mRNA expression in urine
- Three different high-resolution mass spectrometry technique in parallel for urine proteomics and peptidomics investigations, to address potential problems of discrepant results with different types of liquid-phase separation and mass spectrometry principles and systems used, as previously reported in the literature.
- Optimized, untargeted, high-resolution LC-MS/MS for lipids in plasma and urine
- A combination of LC-MS, GC-MS (and NMR if necessary) for plasma and urine metabolites.
- MALDI-TOF/TOF and TOF-SIMS imaging for mass spectrometry imaging of the graft

For the validation phases (a trans-sectional study and an ambispective cohort of kidney transplant patients) we planned to set up and validate robust, easily transferable reference techniques aimed to be implemented in clinical laboratories for routine analysis of biomarkers:

- Targeted RT-PCR with robust, validated and transferable primers and probes
- LC-MS/MS in the SRM mode and CE-MS for the targeted analysis of peptides and proteins
- Transferable (robust, validated), targeted, quantitative mass spectrometry techniques for lipids and metabolites in plasma and urine
**Description of the main S/T results/foreground**

**Period 1 (12 months):**

For the first two, case-control clinical sub-studies of 3xBIOS², 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 inferior reliability of analytical results. Then, after discussion among the consortium, a standard operating procedure 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 later on.

In parallel, all the actions needed to conduct the clinical trial 3xBIOS² were finalized:
- Regulatory and ethical application and approval of the case-control & trans-sectional and cohort studies
- Central reading of the first set of preselected biopsy slides
- 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
- Anonymization and shipment of the first set of samples from the 4 biobanks to the 8 labs involved

The analytical tasks performed for step 1 of 3xBIOS2 study ("training set") for biomarker discovery were 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 was begun
- 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 had begun (10 samples disqualified after quality check)
- Untargeted analyses of peptides in urine were 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.
- Untargeted analyses of proteins in urine: half of the proteomic analyses were performed by INSERM #1a, using NanoLC and MALDI-TOF/TOF. VITO #7 had begun analyses by NanoLC-ESI-HRMS.
- Untargeted analyses of lipids in urine: after method comparison, optimization and validation, UNIVERSITE PARIS DESCARTES #11 analyzed all samples and detected and annotated all molecules 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 was ongoing. Due to missing the initial time-slot, analyses were postponed.
- 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 samples with high quality 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 were 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 has been populated with biomarkers from the literature by the consortium and implemented as an online resource by INSERM #1a.

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 we wrote the 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 Clinical Research File for BECS study was finalized in July 2014 and its electronic CRF was in preparation.

Period 2 (18 months):

The actions made with respect to the BIOMARGIN clinical studies were:

- Regulatory and ethical application of the case-control & trans-sectional (3xBIOS2) and cohort (BECs) studies
- Central reading of the second and third sets of biopsy slides
- Anonymization and shipment of the second and third sets of samples from the 4 biobanks to the 8 labs involved
- Development of an electronic Clinical Research File (eCRF) for the steps 2 and 3 of 3xBIOS2
- Preselection and final selection (after central reading by expert pathologists) of 138 sample triplets (urine, blood, biopsy core) collected in the standardized conditions at step 2 of 3xBIOS2
- Shipment of 458 unselected sample triplets (urine, blood, biopsy core) collected in the standardized conditions at step 3 of 3xBIOS2 (with central reading by expert pathologists in parallel)
- Quality monitoring of the samples and of the clinical research files
- Sample shipment to the 8 laboratories on months 26-27 for step 2 samples and on months 33-35 for step 3 samples
• BECS regulatory approval was obtained in France, Germany and Belgium between 23 July 2014 and 8 July 2015.
• At the end of this second study period, 574 adult patients, but only 15 pediatric patients had been enrolled. The time-period of children enrolment was prolonged, as well sample and clinical data collection in all patients over the third study period.

The analytical tasks performed for step 2 of 3xBIOS2 study (“selection set”) for biomarker discovery were as follows:

• Urine protein and creatinine concentrations were measured in all samples by INSERM #1a
• mRNA and microRNA profiling of blood samples: Partner #1c extracted the 138 Paxgene® blood tubes selected, verified RNA quality and concentration but analyses were only scheduled for Month 40. 458 Paxgene® tubes from the trans-sectional study (step 3) were received later on, RNAs purified and quality assessed as described above.
• mRNA and microRNA profiling of urine samples: same as above.
• Untargeted analyses of peptides in urine: Since the evaluation of statistical data of the Training Set (3xBIOS 2 step I) was lagging behind, it was decided that Part 1a (INSERM Limoges) and 7 (VITO) use also untargeted shotgun peptidomics/proteomics instead of switching to their targeted MRM techniques for this second step. Therefore, untargeted analysis of the Selection Set was accomplished by all WP4 partners.
• Untargeted analyses of lipids in urine: Likewise, because step 2 samples were received before the statistical results of step 1 it was decided to carry on using untargeted lipidomics analysis on this second series of samples. LC-MS analyses of the 137 urinary extracts were performed in one batch.
• Untargeted analyses of metabolic biomarkers in plasma and urine was performed by partner 12 on urine and plasma samples from the selection set (step 2). As the most discriminative metabolic differences in step 1 were found using GCMS, it was decided that GCMS should be used to analyze plasma and urine samples from the selection set (step 2). No clear Biomarker candidates were found in urine, while in plasma a number of pathways were identified.
• messenger RNA and microRNA profiling of biopsy samples: Partner #6 extracted all Allprotect® tubes that were selected for step 2 (N=137). After extraction, RNA quality and concentration were evaluated using the Nanodrop technology. All samples were ready by month 36 for the quantification of biopsy mRNA and miRNA biomarker candidates, scheduled for month 40. The 319 Allprotect® samples from the trans-sectional study (Step3) were received by Partner #6 between months 33 and 35. RNAs were purified and their quality was assessed as described.
• Mass-spectrometry imaging of renal allograft biopsies: During this second reporting period, 10 human kidney biopsies (one control sample, two normal transplant kidneys and 7 rejection samples) were evaluated using ToF-SIMS (Time of Flight-Secondary Ion Mass Spectrometry) and MALDI-TOF/TOF (Matrix Assisted Laser Desorption Ionization- Time of Flight) in parallel. The first provides the localization of elements and lipids, and the second of lipids, peptides and proteins. The data showed interesting preliminary findings.

Statistical analyses:

• CEA (Part 4) received the step 1 datasets between July 2014 (M17) and February 2015 (M24) and then performed statistical analyses using the first version of the pipeline mainly developed during the first phase of the project. The first results presented during the annual meeting in Hannover (March 2015) and the discussion with the clinicians showed that it would be better to take into account a mixed phenotype definition of the samples for the
statistical analysis, instead of the “single diagnosis category” since a significant proportion of samples exhibited mixed phenotypes.

- Therefore, a second version of the pipeline was developed and the statistical results for each dataset presented during the statistical analysis Workshop held in Paris in October 2015. Since then, Partner 4 received eight step2 datasets, the statistical analyses were carried out with the same pipeline and comparisons were made between the relevant variables of step1 and step2 in order to generate a restricted list of biomarkers.

❖ Period 3 (18 months):

Clinical studies:

- 3xBIOS2 step 3 (trans-sectional study): the results of blind centralized histological reading of graft biopsies by expert pathologists (after adjudication of discrepant results by consensus between the experts) was reported to the Partners on month 46; the clinical data was cleaned and the clinical database sent to all partners on month 47; the biopsy slides were returned to the clinical sites at months 47-48; monitoring visits for the 3xBIOS2 study step 3 were performed.

- BECS: A specific Investigator Study File (ISF) for the BECS study was prepared and provided by Partner #10, following regulatory approvals, to each participating site (adult and pediatric teams). More adult patients have been enrolled than planned and recruitment was stopped, while recruitment of paediatric patients was prolonged to try and reach the target of 50 individuals. A new version of the CRF and eCRF with minor modifications was provided by Partner #10.

- As per month 48, 2313 sets of blood and urine sample doublets and at least 1000 biopsy samples had already been collected, but dispatching of these samples had not yet begun.

Analytical tasks:

- Based on the results obtained at the first 3 steps, it was decided that the laboratory Partners involved in BECS (step 4) would be: Inserm Limoges #1a, Inserm Paris #3, KU Leuven #6, MOS #8 and possibly AcureOmics #12 and VITO #7 if they wish.

- Messenger RNA and microRNA profiling in blood samples: miRNA biomarker candidates (restricted list) were determined in 458 blood samples by Partner#1c and data were subsequently submitted to partner #4 (CEA) for further data analysis on Month 48. Messenger RNA profiling was assessed in the same samples by Partner #6.

- Urine mRNA analysis by Partner #1c: after RNA purification from 485 urine samples, the samples were pre-amplified using a new pre-amplification protocol and 24 RNAs (including additional mRNA candidates identified from other platforms) were quantified by qPCR in all of them.

- Messenger RNA and microRNA profiling of biopsy samples: the quantification of biopsy mRNA and miRNA biomarker candidates from the extensive list defined in WP7 was performed in samples from the Selection set (step2). Allprotect® samples (N=319) from 3xBIOS2 step3, so-called “trans-sectional study” were sent to partner #6 between Month 33 and Month 35. RNAs were purified and their quality was assessed as described. Measurement of miRNA and mRNA expression had not yet begun at the end of this period.

- Proteins and peptides in urine samples: Partners #1a, #7 and #8 completed the analyses of the selection set (3xBIOS2, step 2) and provided absolute or relative concentrations for the proteins and peptides of the extensive lists of biomarker candidates. During this third period, the WP4 partners improved their technological platforms (i.e. partner 1a by establishing a Q-TOF SWATH method for peptidomic analysis), or their raw proteome data processing
workflow (i.e. partner 7 by introducing the SWISS algorithm to fill up the gaps in the protein data matrix). The decision made at the beginning of the second reporting period that partners 1a and 7 should use untargeted shotgun proteomics/peptidomics again instead of switching to a targeted MRM technique turned out to be the right choice, since it was observed that the disease groups in this sample set were somewhat different from the respective disease groups of step1 with respect to their proteomic/peptidomic composition. Subsequently, all WP4 partners analyzed the proteins and peptides of the restricted list in the trans-sectional study (step3) samples.

- Lipidomics and metabolomics analysis in blood and urine samples: Partner 11 designed a reference LC-HRMS technique for the quantification of 80 putative lipid biomarkers and analysed the 454 samples of the “trans-sectional study”. Partner 12 implemented a method for the simultaneous quantification of 26 amino-acids in plasma. In addition to this, quantitative methods were developed for endogenous metabolites that might be used as rejection biomarkers. Furthermore, a draft priority application for a patent was filed.

**Statistical analyses:**

- At the end of the 2nd period, Partner #4 had received 8 step-2 datasets from Partners #1a, #7, #8, #11 and #12. These datasets had been obtained from untargeted analyzes which means that the same set of variables was available in both step 1 and step 2 data. The first strategy was to apply the statistical pipeline developed during step 1 on step 2 datasets, in order to identify the variables that were selected in both steps as a restricted list of biomarkers. This strategy that corresponds to the intersection of results from step 1 and step 2 was used as a first approach.

- Partner #4 also received step-2 datasets corresponding to targeted analyses, where the variables measured were the best identified during step 1. The strategy presented in the case of untargeted data is obviously not adapted for targeted analyses, so that different approaches were successfully developed. At this stage, the main objective was to test the restricted list of biomarkers on step 2 datasets, but also to identify relevant groups of variables as biomarkers signatures.

**Period 4 (12 months):**

The European Commission granted the BIOMARGIN programme a one-year prolongation, as per their decision in August 2016, corresponding to this fourth period.

**Clinical studies:**

- **Data entry in the e-CRF:** As of 28 February 2018, 637 adult and 38 paediatric patients had been enrolled in BECS, representing approx. 2900 visits for adult patients and 150 visits for paediatric patients. Data entry was mostly performed directly by the clinical investigators, but at the end of the project (28Feb2018), not all clinical data had been entered in the eCRF yet. In addition, the consortium decided to pursue patient follow-up beyond February 2018 until all have reached 5 years post-transplantation, using other sources of budget. Consequently, all clinical sites will carry-on filling-in patient data at each new protocol visit, and Necker Sites 3 and 10 committed to catch up with their
backlog of visits. The OpenClinical e-CRF system and SAS database were transferred from Partner #10 to Partner #13 (CHU Limoges, official sponsor of BECS) in March 2018.

- **Data Cleaning:** A first round of data cleaning has been done at the time of Data entry in the eCRF by automatic edit checks. A second round was performed after data had been transferred to the database using SAS edit checks. Query management was completed for patients declared in the e-CRF by 28 Feb 2018. Altogether, approx. 8600 queries were emitted and solved.

- **Monitoring:** Between September 2017 and February 2018, Partner #10 (Venn Life Sciences) monitored each of the four clinical study sites (Partners #3, 6, 9 and 13) between 3 (Partner #13) and 9 (Partner #3) times, depending on the number of patients included into the study and the status of data entry in the e-CRF. The last monitoring visit at each site corresponded to an administrative close-out with respect to Partner #10 involvement. All the Informed Consent Forms signed by the patients and investigators in the investigating centres in France (but not in Belgium and Germany because it was not required by national regulations) were recovered by a CRA and sent to the French Coordinator.

- **Sample dispatching:** By February 2018, 2891 sample doublets/triplets had been collected in the four clinical sites. It had been decided previously by the consortium that the biopsy samples would not be analysed at this stage. Consequently, only urine and blood samples were dispatched to the 4 laboratories (Partners 1a, 1c, 6 and 9) still involved in biomarker validation at this stage. A total of approx. 15 000 tubes were dispatched in three shipments between June 2017 and February 2018.

**Analytical tasks:**

a. Complementary analyses in samples from the trans-sectional study

- Partner#1b (INSERM Toulouse) determined the concentrations of 4 selected miRNAs in step-3 urine samples. Although detected, these miRNAs showed disappointing statistical results and could not be validated.

- Partners #1a, 7 and 8 completed their evaluation of the diagnostic performance of their selected urine peptides and proteins in urine using their previously established “reference technique”. They obtained nearly the same very good performance using the established biomarker sets. Therefore, the decision was made that each would use its own proteomic/peptidomic reference technology to develop it further beyond the project end for best operability in a laboratory setting.

- Specifically, Partner #1a (INSERM Limoges) generated: a combination of 5 urinary peptides showing a very good diagnostic performance with respect to ABMR (AUCROC =0.77); (ii) a combination of 6 peptides yielding a very good diagnostic performance with respect to TCMR (AUCROC=0.77); (iii) a combination of 5 urinary proteins with a very good diagnostic performance with respect to ABMR (AUCROC=0.80); and (IV) a list of 4 proteins with excellent diagnostic performance for TCMR (AUCROC=0.85).

- Partner #7 (VITO) analysed 12 peptides from 6 different proteins in a subset of 185 urine samples and found a sensitivity of almost 80% and a specificity of about 70%. The TOP 20 most relevant proteins (so not only the 6 proteins of the validated signature) were included in a patent application that was filed in December 2017 (P117691EP00). The tryptic peptides that will be used as internal standards for targeted analysis were synthesized. The preparation of a manuscript is ongoing. As a follow up to BIOMARGIN, VITO will develop, together with other partners (also outside the BIOMARGIN
consortium), an antibody-based assay that will be tested and validated in a clinical setting. The performance of this test will be compared to the targeted mass spectrometry data as reference.

- Partner #8 selected a 206 urinary peptide marker pattern of T-Cell mediated rejection (TCMR) with an AUC of 0.79 (95% CI range: 0.74-0.83, P<0.0001) in the ROC analysis, and sensitivity and specificity values of 76.5 and 73.6 %. Interference of the TCMR model was observed in patients with ABMR (47% specificity, N=16) and combined ABMR and IFTA phenotype (44% specificity, N=17), which either can be attributed to deficits of the model to appropriately differentiate the TCMR and ABMR phenotypes or to the fact that ABMR is most often associated with concomitant TCMR features. Good discrimination was obtained for patients with normal biopsy results (77% specificity, N=196), IFTA (71% specificity, N=83), glomerulonephritis (86% specificity, N=7) and polyomavirus nephropathy (73% specificity, N=11). Part#8 also validated their final ABMR model containing 146 urinary peptides, obtaining an AUC of 0.76 (95% CI range: 0.71-0.80, P<0.0001) in the ROC analysis and sensitivity and specificity values of 80.0 and 64.7 %. Interference of the ABMR model was observed in patients with glomerulonephritis (33% specificity, N=15), IFTA (52% specificity, N=73) and polyomavirus nephropathy (62% specificity, N=13). Good discrimination was obtained for patients with normal biopsy results (70% specificity, N=196) and TCMR (87% specificity, N=15).

- In addition, Partner#1c (INSERM Paris) determined CXCL9 and CXCL10 proteins in urine by ELISA and confirmed them as good markers of ABMR with an AUC of 0.832 and 0.851 against normal, respectively.

- Partner #11 set up their reference LC-HRMS technique for the quantification of 80 lipids selected as candidate biomarkers. They determined these lipids in the 454 urine samples from the “trans-sectional study” and the results were analysed by Partner #4 (CEA) to select a short list of the most discriminant compounds. At the end of BIOMARGIN, the diagnostic value of 22 lipid compounds and combinations thereof (4 to 6 lipids) was being evaluated.

- Partner #12 validated a gas chromatography mass spectrometry (GCMS) method for the absolute quantitation of 6 plasma metabolites as biomarker candidates. Both this method and a LC-MS/MS technique for 26 amino acids were used to quantitate a panel of metabolic biomarker candidates in the 456 plasma samples from the trans-sectional study. Additional metabolite candidates were also measured at the Clinical Chemistry Laboratory at Umeå University. Multivariate and univariate statistical analyses were performed in order to determine whether differences could be observed in the levels of candidate markers between grafts showing signs of rejection and grafts with normal biopsy. In summary, a higher number of metabolites were observed at altered levels in the ABMR rejection group with much fewer changes found in the TCMR group compared to normal. At the end of BIOMARGIN, CEA was performing additional statistical analyses. A priority application towards a patent is envisaged.

- Partner #1c (INSERM Paris) determined 38 miRNA biomarker candidates of the restricted list and 7 control miRNAs in 312 biopsy samples from the trans-sectional study. Multivariate analyses by Partner #4 showed that a 5-miR signature discriminated ABMR from all other diagnoses with an AUC of 0.87, and a 10-miR signature identified ABMR from normal biopsies with an AUC of 0.91. Moreover, another 7-miR signature distinguished rejection cases (ABMR and TCMR) from normal biopsies, with an AUC of 0.89. Partner#1c determined 48 miRNA biomarker candidates of the restricted list in 453 blood samples from the trans-sectional study. Final analysis by Partner #4 identified the best combination of biomarkers for the diagnosis of ABMR among all other diagnoses,
for which a 3-miR signature gives an AUC of 0.71. Finally, a second pre-amplification protocol was performed and 7 additional RNAs were quantified by qPCR in 458 urine samples from the trans-sectional study. The best ABMR signature combines 7 RNAs with an AUC of 0.74 versus all other diagnoses, and 5 RNAs diagnose ABMR versus normal cases with an AUC of 0.69.

- To further prioritize on the best biopsy mRNA signatures, Partner #6 employed additional micro-arrays for global gene expression analysis on Step 2 and Step 3 biopsy samples with sufficient quality RNA. Expression data were normalized and outliers identified. The numerous genes (N=503 unique genes) previously reported to be differentially expressed are highly robust and collinear, and the diagnostic performance of each of these 503 transcripts is high. Therefore, Partner #6 currently investigates more global gene expression alterations in the gene expression profiles, to allow better phenotypic characterization of these cases. Subsequent pathway enrichment analysis on the unselected significant genes identified “Allograft rejection” as the top pathway, confirming many genes involved in innate immunity and NK cell activation. These data will be further used in the service platform to other partners and for further diagnostic refinement of the phenotypic grouping of the patients included in the Biomargin study.

b. Analyses of samples from the BECS prospective study

- Biopsy samples were collected as part of BECS, but it was decided by the consortium, owing to time and money constraint, not to analyze them in this fourth period.
- More than 2500 blood samples and >2500 urine samples from the BECS study were distributed to the Partners involved in urine analyses.
- Partner#1c extracted and purified mRNAs from these samples. Analyses will be performed beyond the timeframe of BIOMARGIN.
- The preparation of these samples for peptide or protein analyses (Partners 1a, 7 and 8) was still ongoing on 28 February 2018. The analyses will be completed beyond the end of BIOMARGIN, as decided during the last consortium meeting in February 2018.

Data integration and disease prediction modelling:

- Partner #4 (CEA) received 9 datasets from the trans-sectional study in order to estimate the performance of the selected biomarkers or biomarker signatures. By 28 February 2018, they had analyzed seven datasets. The two remaining datasets will have to be analyzed in a particular way since the values have been obtained using quantitative methods, as opposed to untargeted semi-quantitative analysis in the previous steps.
- Histological reading of the biopsies identified mixed phenotypes such as “ABMR+TCMR”, “ABMR+IFTA”, “TCMR+IFTA”, etc. Partner #4 trained logistic regression models, SPLS-DA models and SVM with polynomials kernel with pure and mixed phenotypes. In each configuration of validation, they estimated model performance using the AUC of the ROC curve.
- As already mentioned above, very good to excellent diagnostic performance was found for several biomarker signatures (in blood, biopsy or urine) with regards to ABMR (mostly), TCMR and rejection as a whole, while hardly any sensitive and specific biomarker of IFTA was found.
• The task devoted to Disease progression modelling could not be addressed before 28 February 2018, since the BECS biomarker results were not available and the BECS clinical database was still incomplete. These objectives will be reached beyond the end of the project, since the patients have been included, the samples collected, the biomarker signatures to validate identified and the clinical database to assess the outcome of the patients will soon be complete, cleaned and frozen.

• Accordingly, the “mechanistic model of renal disease progression and prognostic dynamic tool to assist the clinicians” and the “BIPART website, online platform to assist clinicians in decision making” could not be delivered in time but will be beyond the BIOMARGIN time-frame.

Dissemination and exploitation

• BIOMARGIN has co-organized and participated in a symposium entitled “The coolest clinical trials in transplantation sponsored by EU” at the ESOT 2017 meeting in Barcelona in September 2017. Both the coordinator and Maarten Naesens (one of BIOMARGIN members; KUL) presented the project in front of 120 transplant experts along with other EU projects related to organ transplantation. There was a lively and very interested discussion regarding the BIOMARGIN approach. This was followed by the presentation of detailed BIOMARGIN results through 7 oral or poster presentations by different partners.

• A plan to raise awareness about BIOMARGIN and its results was discussed at the final consortium meeting. The consortium members expressed a real interest in carrying on presenting the results in conferences under the “BIOMARGIN” label.

• A Web conference was organized on 5th February 2018 to discuss the BIOMARGIN Publication strategy with WP leaders. A couple of weeks later, a session dedicated to intellectual property (including discussion on patent and publication strategy) was organized at the final Consortium meeting with all members of the consortium. Several patents and scientific articles are expected within months, and more should come when the final results of the BECS study will be available.
**Potential impact**

The BIOMARGIN research programme has resulted in several blood, biopsy or urine biomarker signatures of kidney graft rejection, with very good to excellent diagnostic performance. The predictive (or very early diagnostic) performance of these signatures is still being tested in the BIOMARGIN European Cohort Study, which will be carried on for another 3 years owing to complementary grants.

**- Clinical Impact:**

These signatures will enable a closer non-invasive monitoring of the graft in order to detect acute or chronic injuries earlier, which should enable renal transplant clinicians to improve patient care, resulting in several positive impacts. Indeed, 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.

Still, to elicit or facilitate the clinical transfer of our biomarkers, a few last steps need to be completed:

- Patenting all these biomarkers and/or combinations thereof (in addition to the two priority applications already filed)
- Publishing them in specialized international journals to raise awareness among the clinical transplant community
- Transferring the biomarker analytical techniques developed to several clinical sites outside the BIOMARGIN consortium and/or developing simpler, certified tests
- As well as, ideally (although not compulsory), setting-up a randomized clinical trial comparing biomarker-driven patient care to the standard of care

In addition, the untargeted transcriptomics analysis of graft biopsies has identified specific pathways involved in graft antibody-mediated rejection, previously overlooked or unknown, namely genes involved in innate immunity and NK cell activation, which might open up new therapeutic avenues. This confirms our initial hypothesis that the underlying causes of graft loss cannot be reduced to “T-cell mediated” or “antibody-mediated”. Further investigations will be made on other graft lesions, such as fibrosis, and BIOMARGIN might open other innovative paths for therapeutic interventions.

Owing to closer monitoring of the graft and faster 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 are 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 be in need for re-listing 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 BIOMARGIN biomarkers and their interpretation.

Address of the project public website: http://www.biomargin.eu
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<th>COUNTRY</th>
<th>PARTNER INSTITUTION</th>
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<tbody>
<tr>
<td>France</td>
<td>Institut National de la Santé et de la Recherche Médicale</td>
<td>Prof. Pierre Marquet, Limoges, Dr Joost Schanstra, Toulouse, Prof. Dany Anglicheau, Paris</td>
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<td></td>
<td>Inserm Transfert SA</td>
<td>Dahlia Tsakiropoulos, Montpellier</td>
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<td></td>
<td>Assistance Publique – Hôpitaux de Paris</td>
<td>Prof. Dany Anglicheau, Paris</td>
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<tr>
<td></td>
<td>Commissariat à l’Energie Atomique et aux Energies Alternatives</td>
<td>Dr Nora Benhabiles, Dr Etienne Thévenot, Saclay</td>
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<tr>
<td></td>
<td>Centre National de la Recherche Scientifique</td>
<td>Dr Alain Brunelle, Gif-sur-Yvette</td>
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<td></td>
<td>Venn Life Systems</td>
<td>Dr Marylin Labart, Paris</td>
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<td>Paris V Université Paris-Descartes</td>
<td>Prof. Oliver Laprévote, Paris</td>
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<td></td>
<td>Centre hospitalier universitaire de Limoges</td>
<td>Prof. Marie Essig, Limoges</td>
</tr>
<tr>
<td>Belgium</td>
<td>Katholieke Universiteit Leuven</td>
<td>Prof. Maarten Naessens, Leuven</td>
</tr>
<tr>
<td></td>
<td>Vlaamse Instelling voor Technologisch Onderzoek N.V.</td>
<td>Dr Inge Mertens, Mol</td>
</tr>
<tr>
<td>Germany</td>
<td>Mosaiques Diagnostic GmbH</td>
<td>Dr Jochen Mezger, Hannover</td>
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<td>Medizinische Hochschule Hannover</td>
<td>Prof. Wilfried Gwinner, Hannover</td>
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<td>Sweden</td>
<td>Acureomics AB</td>
<td>Prof. Johan Trygg, Prof. Torbjorn Lundstedt, Umeå</td>
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