Project Final Report

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4.1 Final publishable summary report

Executive Summary

Currently, clinical diagnosis and monitoring of urinary bladder cancer (UBC) relies on invasive, highly costly cystoscopy. Additionally, due to high recurrence rates, UBC treatment is associated with frequent follow-up of patients, making it one of the most costly types of cancer in terms of management cost. The TransBioBC project was based on the identification of novel biomarkers (BM) for UBC detection in urine and powerful technological platforms (capillary electrophoresis mass spectrometry; CE-MS and micro-capillary ELISA; mELISA), as extensive foreground work of the involved partners in the framework of FP7 EU projects DECanBio and GENINCA. The objective of TransBioBC was to translate this foreground into valuable products and to specifically employ the involved technologies (CE-MS and micro-ELISA), represented by the participating SMEs (MOS, DS), for the development of non-invasive urine tests for routine monitoring of UBC. The experimental workflow included:

a) Assuring platform and assay analytical performance according to regulatory guidelines,
b) Analysis of well characterized clinical samples from existing sufficiently powered UBC cohorts in a two-phase approach (phase I: targeting definition of optimized BM combinations for the specific contexts of use; and phase II: validation of the latter in existing prospectively collected urine samples);
c) Development of database platforms integrating biomarker and clinical information;
d) Thorough analysis of the biomarker added value;
e) Development of a prototype kit for quantification in urine of the optimal UBC biomarker (s);
f) Biomarker multi-stakeholder dissemination and exploitation plans.

Tested biomarkers included peptide biomarker profiles defined by CE-MS (Theodorescu et al., *Lancet Oncol* 7, 230, 2006), as well as proteins associated with UBC based on high resolution mass spectrometry analysis of urine, including SPARC (Secreted Protein Acidic and Rich in Cysteine), profilin 1, histone 2B (Frantzi et al., *J Proteome Res*. 12, 3969 2013, Zoidakis et al, *Mol Cell Proteomics*;11(4):M111.009449, 2012). Following collection of proper ethics approvals, extensive analytical performance analysis indicated interference of ELISA assays by urine matrix for the vast majority of tested biomarkers, with the main exceptions of SPARC (ELISA; *PLOS One*, 11(2):e0149471, 2016) and vitamin D binding protein (VDBP; micro-ELISA). Further quantification of SPARC (BRFAA) in the prospectively collected ISBLaC study (CNIO) (n=488), supported the marker discriminatory potential for mainly invasive UBC and an association with tumor relapse. Nevertheless, hematuria was found as a cofounder for biomarker measurements. Similarly, results from micro-ELISA also provided discrimination for UBC, nevertheless at sub-optimal diagnostic rates. Based on these technical difficulties encountered with ELISA, mass spectrometry based assays (multiple reaction monitoring-MRM) were developed for the quantification of these UBC protein biomarkers. MRM assays were found to exhibit superior performance in comparison to ELISA, being impacted to a lower extent by hematuria, nevertheless measurements of SPARC, NMP22 (Nuclear Matrix Protein 22), profilin1 and VASP (Vasodilator-Stimulated Phosphoprotein) in the ISBLaC cohort provided overall lower diagnostic accuracies in comparison to the CE-MS peptides.

In parallel, 1433 urine samples from various clinical centres were analysed using CE-MS in Phase I, and 712 in Phase II. The former included a discovery and test set to refine previously described biomarkers for specific use in primary diagnosis and detection of tumor relapse. Support vector machine models based on 116 (primary diagnosis) and 106 (tumor relapse) peptides provided AUC of 0.87 (n=270, test set) and 0.75 (n=211, test set), respectively. The peptide biomarkers outperformed cytology, providing complementary information to the latter (combination of both tests resulted in an AUC of 0.90; Frantzi et al., *Clin Cancer Res*. 22, 4077, 2016).

Further validation of these optimal classifiers was performed in a phase II study involving blinded analysis by CE-MS of a total of 712 samples from prospectively collected samples – (Radboud University Nijmegen (Netherlands) and University of Barcelona (Spain). Based on the results, a prototype kit for the measurement of the CE-MS UBC biomarkers was developed including detailed manuals for use and distributed to collaborating clinical centers. In parallel, besides the highly ranked publication in Clinical Cancer Research, results were disseminated to the scientific community via presentations to relevant meetings as well as by contacting patient groups, and by generating and distributing TransBioBC flyers. Contacts with pharma companies were established, currently addressing potential use of the TransBioBC markers in clinical trials. Health economic calculations were performed suggesting the cost-effectiveness of the biomarkers in UBC management.

TransBioBC enabled the establishment of a novel non-invasive approach for UBC detection especially during UBC surveillance with anticipated positive impact on UBC management. In addition, acquired know-how (on MRM assays, data management) open new avenues in biomarker research for UBC and other diseases alike.

Summary description of the project context and the main objectives

Urinary bladder cancer (UBC) ranks second in incidence and mortality among cancers of the genitourinary tract and accounts for more than 350,000 new cases worldwide per year. It is staged into three main groups of distinct clinical presentations with different prognosis and primary treatment: (i) non-muscle invasive (NMIBC) restricted to the urothelium (Tis, Ta) or up to the muscularis mucosa (T1), muscle invasive (MIBC, T2, T3 or T4), and metastatic disease (N+/M+) (Dinney et al., *Cancer Cell* 6, 111, 2004). NMIBC has very high recurrence rates of 50% - 70% and a 15% overall progression rate to higher grade and/or muscle invasive stages. Progression to T2 tumors varies from 6% to 25% in Ta and from 27% to 48% in T1 tumors of all grades. The problem of diagnostic under-staging in Ta or T1 tumors range from 35% to 62% (Madersbacher *et al., J Clin. Oncol.* 21, 690 ,2003).

Currently the standard for UBC initial diagnosis and surveillance is cystoscopy, and the histopathologic analysis of biopsy specimens obtained during this procedure. While the specificity of this approach is close to 100%, the sensitivity is modest particularly for low grade tumors (Lee et al. *BJU. Int.* 102, 1228 ,2008). Of the non-invasive methods, urinary cytology is being applied in adjunct to the cystoscopic evaluation, even though it is of low sensitivity, particularly in the detection of low grade disease, as well as prone to inter and intra-observer variabilities (Howlader et al., *J. Natl. Cancer Inst.* 101, 533, 2009). It is general practice to monitor patients after initial diagnosis, typically by cystoscopy every 6-12 months to facilitate early diagnosis of tumor recurrence. However, this approach does not allow any prognosis of recurrence and/or progression, initiating an adjustment of therapy. In addition, associated cost is high while patient compliance during surveillance is moderate to low, due to the highly unpleasant procedure, frequently resulting in delayed detection of tumor recurrence and progression (Chamie *et al., Cancer* 117, 5392, 2011). More accurate, optimally non-invasive tests are thus needed for UBC surveillance, which consists (Lotan *et al., Urol. Oncol.* 28, 441, 2010) and ismajor area of application due to the high disease prevalence (Frantzi etal. *Curr. Opin. Urol.* 22, 390, 2012). Specific clinical needs are the identification of biomarkers, which could replace cystoscopy especially for low risk patents, allow early detection and/or scoring of disease aggressiveness, and prognosticate recurrence and/or progression and guide adjustment of treatment accordingly.
In addition, the current prognosticators, e.g. tumor grade, stage, size, and multifocality, do not accurately reflect clinical outcome (Sexton *et al., Cancer Control* 17, 256, 2010). Several potential biomarkers associated with various disease phenotypes, which could be potential of use during disease surveillance, have been described (Bryan et al., *BJU. Int*. 105, 608, 2010; Lintula &. Hotakainen, *Expert. Opin. Biol. Ther*. 10, 1169, 2010). Selected markers (NMP22 Bladder Check, UroVysion FISH assay, ImmunoCyt, BTA Stat and Trak) have been approved by the FDA for application in UBC surveillance and/or initial screening of high risk populations. However, their added value and application in disease surveillance are questionable, due to low specificity (Lotan *et al., Urol. Oncol.* 28, 441, 2010). Additionally, conflicting results on their utility (or absence thereof) are reported. For example, in the recent prospective longitudinal UroScreen study, the NMP22 assay was evaluated and found to be of low overall utility in early disease detection in UBC screening of a high-risk population, and significant interference of the measurements by haematuria and infection could be demonstrated (Huber *et al., BJU. Int.* 110, 699, 2012). In addition, in a recent study including a surveillance cohort of 270 patients who had undergone cystectomy, the FISH assay targeting the detection of chromosomal alterations commonly detected in UBC was evaluated in the detection of disease recurrence in the face of urinary diversion: similar positive and negative predictive values to urinary cytology were obtained, but no added value of the marker test in this occasion could be suggested (Fernandez *et al., Urol. Oncol.*, 2011). Hence, none of these assays are routinely used in UBC management. These facts demonstrate the urgent clinical need for the development of new, non-invasive approaches for early detection of recurrence and/or progression Such BMs would have **significant impact o**n: **(a)** disease management, as they would reduce the number of necessary cystoscopies and allow evidence-based guidance of therapeutic interventions (Stockle *et al., J. Urol.* 153, 47, 1995), increase patient compliance and

ultimately decrease cost of care (Lotan *et al.*, *Urol. Oncol.* 28, 441, 2010). **(b**) decrease the cost of clinical trials on new therapeutic agents by allowing stratification of patients at inclusion, reducing thus the number of patients at inclusion and as surrogate predicting endpoints, allowing assessment of outcome at earlier points. To implement these BM in routine clinical practice and clinical trials, a well conducted study is required that clearly demonstrates the value of these BM, ideally both from a clinical, as well as from a health economical point-of-view.

The TransBioBC proposal was based on the extensive foreground work of the involved partners in the framework of FP7 EU projects DECanBio and GENINCA. In the context of FP7 DECanBio Partners BRFAA, CNIO and DS collaborated in establishing the clinical resources, identifying BM with potential use in early detection of UBC recurrence and progression in urine and establishing immunoassay platforms for their measurement. Notably, the association of several of these BM with UBC were verified in initial independent studies conducted within DECanBio (Zoidakis *et al.*, *Mol. Cell Proteomics* 11, M111 ,2012). In parallel, in the context of GENINCA, partner MOS successfully established the application of capillary electrophoresis mass spectrometry (CE-MS) and the concept of BM signatures enabling proteome analysis for the non-invasive early detection and prediction of progression of cancer, i.e. gastrointestinal tumors (Lankisch *et al.*, *Hepatology* 53, 875 ,2011). Collaborations of MOS with BRFAA have demonstrated the applicability of CE-MS in the detection of BC biomarkers in urine (Schiffer et al., *Clin. Cancer Res*. 15, 4935, 2009; Theodorescu et al., *Lancet Oncol* 7, 230, 2006). Within DECanBio, this collaboration was expanded where the DECanBio developed fractionation strategies in combination to CE-MS allowed for the detection of various additional, novel potential biomarkers including fragments of histone H2B, and the transcription factor NIF-1 (Frantzi M. et al. *J Proteome Res*. 12(9), 3969-79, 2013). **TransBioBC aimed at translating this FP7 foreground work toward actual BM implementation in BC diagnostics** (see **Figure 1** below).

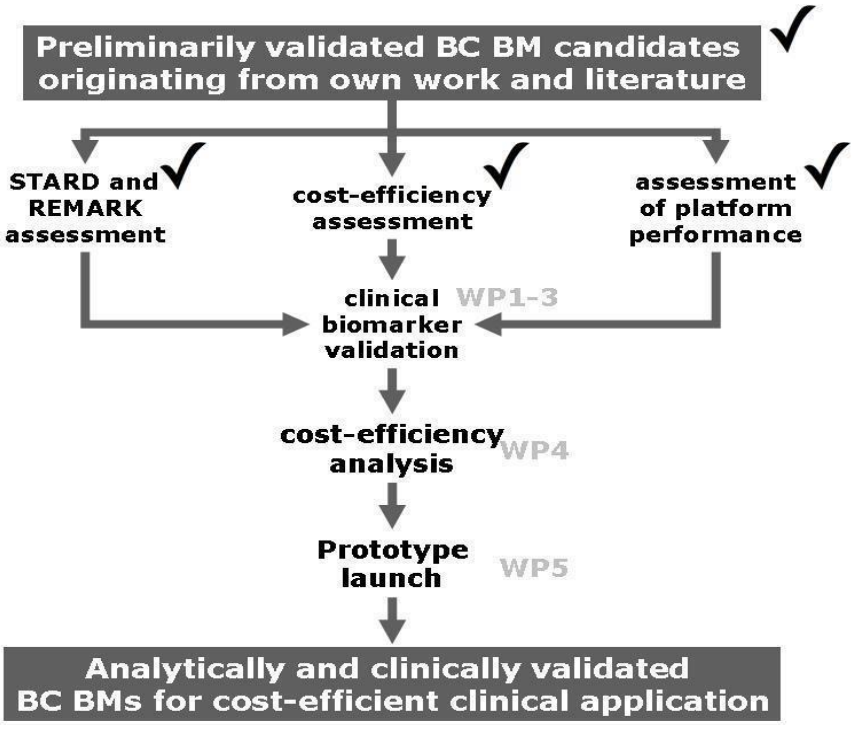

Figure 1. Primary objective of the TransBioBC approach: translating potential biomarkers for UBC into a product of value in patient management

Specific objectives included

1) Platform optimization for the detection of the proposed biomarkers in clinical routine settings.
2) Clinical validation of urinary BM.
3) Development and validation of classifiers based on BM combinations for the early detection of BC recurrence and/or progression.
4) Added value of these BM combinations/classifier in predicting recurrence and/or progression.
5) Health economic evaluation for cost-efficient BM applications.
6) Exploitation of the BM combinations/ classifier for clinical use in patient management and as "business-to-business" product for usage in clinical trials of both scientific institutions as well as pharmaceutical industry.

Collectively, TransBioBC targeted proteomic BM implementation in UBC diagnostics by further validation of discovered promising BM and BM combinations and by optimizing the involved highly innovative technologies (CE-MS and micro-ELISA) for clinical routine applications. **The project built on the novel concept that a combination of BM will result in superior accuracy compared to individual biomarkers (**Schiffer *et al.*, *Clin. Cancer Res.* 15, 4935, 2009) **and recruited innovative platforms to realize this notion,** providing thus a clear competitive advantage over the existing single marker methodological approaches. Even more, it proposed to use these state-of the art platforms (MS- and ELISA-based) in concert to combine their strengths and achieve optimal accuracy rates. This concept of application of BM panels /classifiers has been proposed in science for several years, but was not yet implemented in clinical application; TransBioBC provided the opportunity to bring it forward in a strongly implementation-oriented approach.

Description of the main S&T results/foreground

General Workplan:

The TransBioBC Project focused on the establishment of biomarker (BM) classifiers for the detection of urothelial bladder cancer (UBC). Building upon biomarker candidates and state-of the art platforms and resources developed within European projects, TransBioBC targeted the establishment of clinically useful assays for further implementation in UBC clinical management and clinical trials.

The TransBioBC workplan was structured into 5 highly interrelated WPs targeting respectively:

WP1: Coordination of clinical studies and centres,

WP2: BM measurements in urine,

WP3: Statistical assessment of added value of the BMs and developing appropriate multi-marker classifiers

WP4: Industrial applications including evaluation of cost effectiveness and marketing dissemination,

WP5: Prototype Launch,

WP6: Management of the consortium and general dissemination.

The workplan was designed according to the general principles of regulatory agencies for biomarker validation, in an organized application –oriented format (Summarized in **Figure 2** below:)

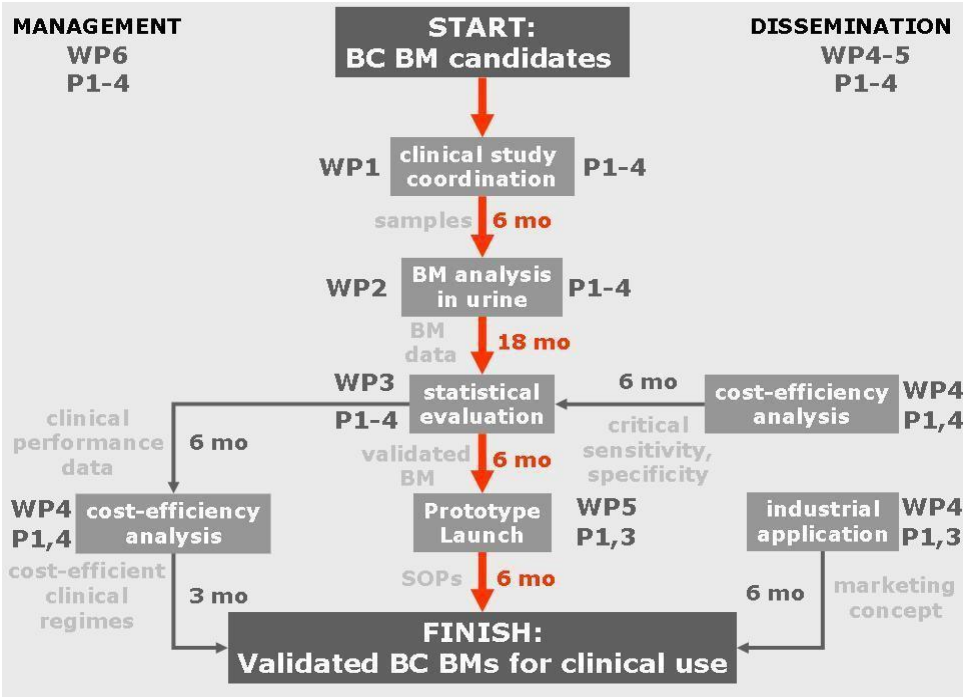

Figure 2. Project evaluation and review technique (PERT) diagram. PERT method was applied to analyze the involved tasks in completing the project. Events are depicted as grey boxes and activities as arrows. For each activity time needed to complete the task and deliverable are given. The longest continuous pathway taken from the initial event to the terminal event defines the critical path and is highlighted in red. This path determines the total 36 months required for the project. Efficient project management will especially review the progress in this path, and ensure avoiding delays in reaching of the terminal event; P: partner number P1 BRFAA, P2: CNIO; P3: MOS; P4: DS

In brief, the main components of the TransBioBC workplan included:

(a) Proposed BC biomarkers:
1) Urinary peptides. Using CE-MS, a panel of urinary peptides associated with UBC was identified and verified (Theodorescu et al., Lancet Oncol 7, 230, 2006, and Schiffer et al., *Clin. Cancer Res*. 15, 4935, 2009). These included peptides derived from collagen, fibrinogen, apolipoprotein A1, Metastasis-suppressor KiSS-1 and other proteins. The combination of a specific selection of these peptides in a high-dimensional Support vector machine (SVM)-based classifier allowed detection of BC with 100% sensitivity and 86% specificity in a blinded multi-institutional cohort (n=180). In addition, in the form of nomogram with tumor grade, urinary BMs could predict muscle invasiveness with 88% accuracy rates. These results demonstrated an association of the CE-MS profile with BC; forming the basis of further investigation of these peptides in patient surveillance in the context of TransBioBC.
Protein markers: In parallel, studies conducted within DECanBio demonstrated BC-association of the various proteins including profilin 1, myeloblastin and aminopeptidase N, histone 2B, matricellular protein SPARC, NIF-1, following application of comprehensive proteomics techniques in urine. Results were confirmed in independent sets of samples and in multiple occassions differential expression of the proteins in UBC tissue was further suggested (Zoidakis et al., *Mol. Cell Proteomics* 11, M111.009449, 2012, and Frantzi et al., *J Proteome Res*. 12, 3969, 2013). Additional marker to be tested was survivin, a promising urinary marker of potentially high specificity according to recent studies (Guey et al., *Eur. Urol*. 57, 283, 2010; Court et al., *Proteomics* 11, 1160, 2011), nevertheless not yet adequately characterized for the specific contexts of use of detection of recurrence.
(b) Platforms for BM measurements:
1) Capillary electrophoresis mass spectrometry (CE-MS):
SME partner MOS has developed and validated CE-MS to a stage ready for clinical routine applications. Performance parameters of the CE-MS platform have been extensively assessed through a series of studies (Mischak Vlahou, Ioannidis, *Clin. Biochem.*, 2012) and were accepted by the regulatory agencies (EMA and FDA).
2) Mirco-ELISA platform: DS has developed biosensor chips platform dedicated to immunoassay analysis, which emanated from the combination of microfluidics and electrochemistry technologies. With incubation times adapted to assay kinetics, beads and reagents are greatly pre-concentrated to yield results in the ng/ml to pg/ml range depending on the sample matrix and on the specificity of the capture and detection antibodies.
c) Available prospective clinical cohorts: The Integrated Study on Bladder Cancer (ISBlaC; CNIO), including series of prospective patients both incident and prevalent, with a suspicion of UBC at time of inclusion.
d) Study design: Clinical validation of the selected BMs followed a **2-phase strategy**: (1) Evaluation of the added value of BMs following a large-scale case/control design and establishment of an optimized BM combination for early detection of tumor relapse, and (2) Clinical validation of the optimized urinary BM combination in unselected, prospectively followed-up cohort, providing the necessary high quality clinical evidence needed for TransBioBC's ambitious exploitation objectives.

Main Results:
Ethics Requirements (WP1):

A high number of urine samples (approximately 2100) was analysed in the context of TransBioBC. Besides samples from the ISBLaC study, existing case-control series from additional clinical centers in Greece and Germany were employed to increase power of phase I study in defining optimal biomarker combinations. In addition, in support of the phase II prospective validation, clinical samples from the running prospective studies on UBC in Radboud University Nijmegen (Netherlands) and University of Barcelona (Spain) were employed. In

all cases ethics approvals from local ethics committees were received allowing sample use in the context of TransBioBC and submitted to the EU in line to respective requirements.

Data Management System (WP1)**.**

The initial plan in TranBioBC was to integrate existing phenotyping (TURB, pathology, biochemical data, information on co-morbidities at baseline and during follow up) and newly collected data using the EPIQuest platform established at CNIO. CNIO initially adapted the EPIQuest tool to accommodate TransBioBC data requirements. However, unforeseen informatics problems were encountered posing difficulties in offering access to TransBioBC partners while ensuring privacy and confidentiality of the data. Despite several attempts, the problem was not solved. Initial data sharing among partners was conducted through encrypted excel files. The problem was finally solved by implementing two approaches: REDcap, a secure, web-based application developed and owned by Vanderbilt University, designed to support electronic data capture for research studies (CNIO). Moreover, a data management system developed by MOS "de novo" based on the needs of the TransBioBC Project. The Database structure was developed using MySQL programming source codes, while the interface was built primarily using PHP programming language. The databases are accessible via https://olimpia.bioinfo.cnio.es/ and http://transbiobc.mosaiques.de/ for authorized users. Capabilities offered include:

a) Clinical (as allowed by ethics restrictions), demographical and proteomics data are displayed, which the authorized user can search and filter online.
b) Data, or selected sub-cohorts can be exported in an automatic way.
c) To not jeopardize the integrity of the database and preserve security, inclusion of new data can only be conducted by the Administrator.

As an example, the TransBioBC-REDCap database contains information from a total of 1607 subjects classified as cases (confirmed bladder cancer patients) or controls (non-confirmed cancer patients) and further categorized in incident (newly diagnosed) or prevalent patients according to different time points. Biomarker measurements as conducted within TransBioBC are recorded along with detailed clinicopathological and follow-up information as available. Patients included in ISBlaC (N=589) have been followed-up at a yearly basis. In addition, information on 349 newly recruited patients (ISBlaC2) with respective clinical and pathological data is included and continuously updated. A MasterFile describing all data registered in REDCap is available to the Consortium members. Similarly, the TransbioBC database (http://transbiobc.mosaiques.de/) contains information on 2145 subjects which also will be updated with additional information as soon as it becomes available after the end of the project.

Markers measured by micro-ELISA (WP2)

A. Development of a reference assay (WP2): The micro-ELISA is a biosensor chips platform dedicated to immunoassay analysis, developed by DS and emanating from the combination of microfluidics and electrochemistry technologies. At the start of the project, DS offered two types of electrochemical chips, the microwell (immuDrop) and the microchannel based sensors (immuSpeed), both applicable to immunoassays. The development of a reference assay, (the TSH- Thyroid Stimulating Hormone- assay), allowed to assess the performance of the two approaches. The ImmuDrop system was found to require larger amount of reagents and longer detection times before stabilization of the signal, in comparison to the ImmuSpeed system. In addition, the

ImmuDrop-chips cannot be regenerated which is the case for the ImmuChips. Thus, the ImmuSpeed system was chosen for further UBC assay development.

B. Establishment of micro-ELISA assays for BM and transposition to urine (WP2)

As aforementioned, several biomarker candidates were considered (APN, SPARC, NIF, H2B, survivin, profilin 1 and myeloblastin) in TransBioBC. Efforts were undertaken to develop immunoassays, however, mainly due to antibody specificity issues, only one assay could be developed, targeting the matricellular protein SPARC. When tested in multiple urine samples, high matrix effects were observed. Other BMs were therefore investigated following discussions among partners and a literature search (Frantzi et al., Nat. Rev. Urol 12, 317, 2015) resulting in the development of 3 assays quantifying ApoE (Apolipoprotein E), VDBP and TS-1(Thrombospondin 1), respectively. High analytical performance was obtained in all cases, with limits of detection in the picomolar range and CVs in reproducibility assays below 15%. In urine, these assays demonstrated to give linear results with good recovery percentages within the 75-125% acceptance range. In addition, the results were highly correlated with commercial ELISA.

C. micro-ELISA assays for analysis of urinary BM in prospective cohort (WP2, 3)

The successfully developed assays were therefore further used initially for the analysis of a case/control (n=110) and the ISBLaC prospective cohort. Unfortunately, the received diagnostic rates were sub-optimal for a clinical test (AUCs in the range of 0.60) hence further development of the assay to a clinical test was not pursued. Despite this negative result for the specific markers, a significant S&T foreground for DS was the demonstration that the immuSpeed platform could be used in clinical routine. Indeed, TransBioBC provided the opportunity for the first time to use the platform for large scale sample analysis in a clinical perspective. From this project a robust workflow could be established, from the sample preparation to the statistical analysis of the results in a systematic and accurate way.

Markers measured by ELISA

Characterization of classical ELISA for selected BM (WP2)

A total of 11 commercially available ELISA tests for the proposed markers (survivin-two assays, SLIT-2, NIF-1-two assays, H2B-two assays, profilin 1-three assays, SPARC, Proteinase 3-PR3) were tested in depth by standard curve analysis, intra-assay reproducibility, linearity and spiking experiments. Aminopeptidase N was replaced by SLIT-2 (Slit Homolog-2; Frantzi et al, *J Proteome Res*. 2013; 12(9):3969-79) due to low discriminatory power of the marker in initial reproducibility experiments involving analysis of a total of 80 samples. Assays were performed according to regulatory requirements (FDA, Draft Guidance for Industry: bioanalytical method validation 2001), using similarly existing samples approved for use in proteomics investigations. In brief, all assays were performed in multiple replicates and/or days, as applicable, a wide range of marker amounts was tested (low, medium, high marker concentrations) and, in all cases, a threshold of 15% CV was applied, as criteria of acceptance. Only 3 assays (for SPARC, survivin and SLIT-2) passed the accuracy thresholds and were found applicable for further application in marker quantification. These results collectively reflect the difficulties in developing urine-based ELISA assays of sufficient analytical performance for clinical application, presumably attributed to the urine matrix itself and/or presence of markers in various isoforms (Chatziharalambous et al, *PLOS One*, 11(2):e0149471, 2016).

Implementation of the ELISA assays in the prospective cohort (WP2-WP3)

The diagnostic potential of these markers using the optimized assays was subsequently tested using urine samples from UBC patients and controls in a step-wise fashion. For SPARC, 196 samples were initially analyzed. Along the same lines, for SLIT-2, samples corresponding to controls (n= 52), Ta (n=49), T1 (n=36) and $T2^+$ (n=29) and for Survivin, 35 (controls), 35 (Ta), 35 (T1) and 27 ($T2^+$) samples were assayed. Nevertheless, a very high number of samples negative for survivin was observed and a very high number of positive controls with high inter-individual variabilities were obtained for SLIT-2, which resulted in excluding these two markers from further analysis. SPARC however showed better performance rates involving a gradual increase from controls to low risk and high risk disease, and as such was also further tested in the CNIO ISBlaC cohort (n=586). The later analysis supported a significant difference between the SPARC levels in T0 (controls) and the T1, T2+ groups and also between Ta and the T1, T2+ groups ($p<0.05$) as well as between the high-risk samples compared to controls ($p<0.05$) in both incident (primary) as well as prevalent (under surveillance) cases. However, in the case of low risk disease no significant changes in the protein levels compared to controls were observed. In addition, the false positive rate of the SPARC ELISA assay was approximately two and three-fold greater among hematuric patients with incident and recurrent UBC, respectively. As a result, the sensitivity of the ELISA assay was found to be 3.6 fold greater in incident UBC patients with hematuria, and 4.2 fold greater among recurrent UBC patients with hematuria, as compared to their respective non-hematuric counterparts. Hence, while SPARC was found to be associated with UBC, nevertheless its diagnostic performance appears to be insufficient for clinical implementation. The study findings on SPARC have been compiled as an Original Article submitted for publication.

Development of mass spectrometry assays for biomarker quantification (WP2)

Based on the poor performance of ELISA assays, validation of the protein markers through an alternative MS-based platform was pursued (BRFAA). The latter corresponds to the multiple reaction monitoring (MRM) approach, allowing for protein quantification based on specific peptides (per protein). In the context of TransBioBC, MRM assays were developed for a series of protein markers and their analytical performance was evaluated. The specificity of the assays was ensured in all cases by using peptide standards labelled by heavy isotopes (^{15}N, ^{13}C), corresponding to the selected biomarker peptides. The peptide standards have the same chromatographic properties (elution time) than the respective endogenous (urine) peptides, therefore providing a means to confirm signal specificity. Assays for SPARC, profilin 1, SLT-2, VASP and the classical UBC marker NMP-22 were established, followed by the measurement of the respective markers initially in a case control study (for profilin 1, SLT2 and SPARC; n=97) as a proof of principle and subsequently in the ISBLaC cohort. Even though discrimination for UBC could be observed, the received accuracy rates were inferior to the ones received by the CE-MS peptides (results on all markers are summarized below).

Diagnostic potential of biomarkers (WP3)

Overall the design used to assess the diagnostic performance of the biomarkers was a cross-sectional study with confirmed urothelial bladder cancer (UBC) patients as cases and individuals with UBC suspicion or benign urological conditions as controls. The study follows a discovery-replication approach for a diagnostic test. The outcomes of interest were diagnosis of a primary UBC (incident) and of a relapse of a previous UBC (prevalent). Sensitivity (Se, 95%CI), specificity (Sp, 95%CI), positive predictive value (PPV, 95%CI), negative predictive

value (NPV, 95%CI), and the area under the ROC curve (AUC-ROC, 95%CI) have been estimated. Association analyses were performed using unadjusted and age- and gender-adjusted logistic regression. Potential confounding effects of urine parameters (creatinine, protein, and blood) have been taken into account following gender- and age-adjustments. The analyses were stratified according to the tumour characteristics: low-risk (LR, TaG1/2) and high-risk (HR, TaG3 and T1) non-muscle invasive bladder cancer (NMIBC), and muscle invasive bladder cancer (MIBC). The peptide-signatures were built by Wilcoxon rank sum test joint to Support Vector Machine (described below), LASSO and Elastic-Net (ENET). The large proportion of zero/undetected-values either due to the absence of the protein marker or its abundance being below of the limit of detection (LOD) of the platforms was one of the main methodological issues to be fixed. The "Reverse" Kaplan-Meier (KM) method was applied to this end. The statistical power of those markers that fail to reach a high AUC was estimated as well as the added value of the biomarkers in comparison to cytology and cystoscopy.
The table below summarizes the results on the diagnostic performance of all markers in the ISBLaC study (CNIO). The studies on the CE-MS profiles are in more detail described below, as they formed the basis for the development of the UBC prototype kit.

Table 1. Diagnostic performance (AUC; sensitivity and specificity) of candidate markers in the ISBlaC cohort

Markers*	**AUC (95%CI)**	**Sensitivity**	**Specificity**
Proteinuria (mg/dL)	0.59 (0.55, 0.64)	0.6	0.58
Creatine (mg/dL)	0.53 (0.48, 0.57)	0.29	0.79
Haematuria (rbs_uL)$	0.60 (0.56, 0.63)	**0.88**	0.31
Markers assessed with uELISA			
TS1_ME (ng/mL)	0.59 (0.53, 0.65)	0.41	0.75
VDBP_ME (ng/mL)	0.61 (0.57, 0.66)	0.63	0.55
Markers assessed with ELISA			
SPARC_E (ng/mL)	0.61 (0.57, 0.65)	**0.85**	0.36
Markers assessed with MRM			
SPARC_MRM	0.54 (0.49, 0.58)	**0.98**	0.1
SLIT2_MRM	0.54 (0.5, 0.58)	**0.94**	0.15
PFN1_MRM	0.58 (0.53, 0.63)	**0.69**	0.48
VASP_MRM	0.56 (0.52, 0.6)	**0.94**	0.18
NMP22_MRM	0.55 (0.5, 0.6)	**0.77**	0.38
SPARC_MRM**	0.6 (0.56, 0.65)	**0.73**	0.48
SLIT2_MRM **	0.55 (0.51, 0.59)	**0.91**	0.22
PFN1_MRM **	0.6 (0.55, 0.65)	**0.78**	0.42
VASP_MRM**	0.5 (0.46, 0.55)	**0.79**	0.28
NMP22_MRM**	0.54 (0.5, 0.59)	**0.86**	0.29
CE-MS peptide score	0.82 (0.79, 0.86)	**0.69**	0.82

Diagnostic performance was also investigated after adjustment for age, gender, proteinuria, hematuria, indicating that creatinine, proteinuria and hematuria were associated with most of the markers (described also below). All the markers assessed with MRM and SPARC determined with ELISA had better sensitivity than cytology performance (Se=0.65 UBC high risk non-muscle invasive patients), while no marker had a specificity higher

than 0.95. A combined marker signature consisting of VitDBP-micorELISA, SPARC-ELISA, PFN1, SPARC, VASP and NMP22 determined with MRM was also tested. In this case, all marker values were log-10 transformed. A training and test set were randomly chosen by using 70% and 30% of samples respectively. The AUC in the test set was 0.73 (95%CI 0.65, 0.80), with sensitivity of 0.87 and specificity of 0.52. When creatinine, proteinuria, hematuria, age and gender were added to the model the AUC increased to 0.74 (95%CI: 0.67, 0.82). Nevertheless, still the performance was inferior to that of the CE-MS classifier hence the latter was selected for further development to a prototype kit.

Investigation of cofounders (WP3)

The potential confounder effects of urine levels of creatinine (mg/mL), total protein (mg/mL), and blood (haematuria, rbs/uL), as well as age and sex, were tested by assessing their correlations/associations with the markers of interest and by including/excluding them from logistic regression models. The variation in terms of diagnostic ability was estimated through the likelihood ratio test (LRT). The candidate markers considered were SPARC_E, ApoE_E, TS1_E, VDBP_E, ApoE_mE, TS1_mE, VDBP_mE, SPARC-mrm, SLIT2-mrm, PFN1-mrm, VASP-mrm, and NMP22-mrm, as well as CE-MS urine signature. In the Greek cohort, all markers assessed with micro-ELISA and ELISA were associated with proteinuria, while none of the markers evaluated with MRM showed any association. Adding proteinuria in the models increased significantly the diagnostic ability of all the markers assessed with MRM, while a borderline effect was observed for SPARC_E and TS1_E. No marker, with the exception of TS1_E and SPARC_mrm, was associated with creatinine levels. Adjustment for creatinine did not modify the diagnostic performance of most of the markers, except for SPARC_E, whose AUC increase from 0.53 to 0.7. Adjustment for age and gender increased significantly the diagnostic performance of PNF1_mrm by 8%. In the ISBlaC series, all markers showed a statistically significant association with proteinuria and all markers, with exception of TS1_E, were associated with creatinine. All markers, with exception of SLIT2_mrm and VASP_mrm, were associated with haematuria. Adding proteinuria to the models had a significant impact on the diagnostic ability of all the markers assessed with MRM: their AUC increased of more than 10%, with the exception of SPARC_mrm and PFN1_mrm. Adjustment for creatinine did not change the AUC of the markers. Adding haematuria increased significantly the diagnostic ability of all markers. Adjustment for age and gender did not affect significantly the diagnostic performance of the markers. The potential confounders varied the diagnostic ability mainly of MRM-determined markers, by increasing their AUC from 8% (PFN1_mrm) up to 31% (NMP22). In conclusion, creatinine, proteinuria, and haematuria were associated with most of the individual protein markers, suggesting that all of them need to be accounted for in assessing diagnostic ability of the markers. In particular, the strongest variation in terms of AUC was observed for MRM-determined markers. In contrast, none of the cofounders showed to have significant impact on the CE-MS model (described also below).

Assessment of biomarker prognostic value (WP3)

The prognostic value of candidate markers for risk of tumour relapse within the TransBioBC study was explored with the ISBlaC-1 cohort due to the prospective nature of this study and the availability of detailed follow-up data of its patients. Prognosis assessment was restricted to UBC patients whose cancer was diagnosed at baseline or within 6 months from baseline (e.g. time of urine collection). The total number of patients included in these analyses was 256: 188 with incident tumour and 68 with a prevalent tumour. Follow-up was updated in December 2015. The mean of follow-up for the whole series was 28 months with a median of 20.6 months (0.56 -78.7

months). The median figures for the "free-of-disease" patients were 41.8 months. Uncensored events (N=117) included tumour recurrences as first event, tumour progression as first event, and death due to cancer. Censored events (N=138) included free-of-disease patients and deaths due to other causes. There is a participant (incident tumour) with unknown follow-up. Kaplan-Meier (KM) curves, log-rank test, and Cox proportional-hazards models were applied in these analyses. Hazard ratios (HR) and 95% confidence interval (95%CI) were adjusted for patient and tumour characteristics, as well as potential confounders (urinary creatinine, protein, and hematuria). All markers were considered as continuous variables, after log10-tranformation and as categorical variables after grouping them according to their median. Age, risk group and tumour size were directly and significantly associated with tumour relapse. Higher values of both SPARC-ELISA and VDBP-microELISA were associated with increased risk of tumour relapse. Cox models were fitted to the data using the log-10 transformed biomarkers alone (unadjusted model) and adjusted first for creatinine, proteinuria and hematuria (model 1) and then further for the classical prognosticators age, gender, risk group and tumour size (model 2). Results are displayed in the table below. No marker showed an independent prognostic value for relapse free-survival (endpoint including recurrence, progression, or death). This result was confirmed also after adjustment for creatinine, proteinuria and hematuria and further adjustment for known clinical prognosticator.

Table 2. Prognostic estimates for each marker for event (defined as recurrence, progression or death) considering the marker as continuous variable.

	Model 1		**Model 2**	
Markers *	**HR (95%CI)[1]**	**p-value[1]**	**HR (95%CI) [2]**	**p-value[2]**
Proteinuria (ng/mL)	1.30 (0.87,1.95)	0.194		
Creatinine (ng/mL)	0.48 (0.27,0.85)	0.011		
Haematuria (rbs/uL)	1.25 (1.10,1.42)	< 0.001		
Markers assessed with uELISA				
TS1_ME (ng/mL)	1.04 (0.81,1.33)	0.753	0.98 (0.75,1.27)	0.853
VDBP_ME (ng/mL)	1.14 (0.94,1.38)	0.180	1.07 (0.85,1.34)	0.573
Markers assessed with ELISA				
SPARC_E (ng/mL)	1.17 (1.03,1.32)	0.018	1.06 (0.90,1.26)	0.475
Markers assessed with MRM				
SPARC_MRM	1.05 (0.79,1.39)	0.752	1.03 (0.76,1.40)	0.824
SLIT2_MRM	1.06 (0.78,1.42)	0.721	1.23 (0.90,1.69)	0.194
PFN1_MRM	1.03 (0.88,1.21)	0.704	1.04 (0.86,1.24)	0.708

VASP_MRM	1.02 (0.83,1.26)	0.843	1.07 (0.87,1.33)	0.512
NMP22_MRM	1.04 (0.88,1.23)	0.619	1.03 (0.85,1.24)	0.781
CE-MS Peptide profile	0.98 (0.70,1.38)	0.913	0.98 (0.70,1.38)	0.913

* log10-transformed markers levels; $ hematuria was considered as categorical variable

The prognostic value of the CE-MS derived biomarkers was also further investigated for tumour event (progression or recurrence), described below.

CE-MS peptide biomarkers (WP2)

CE-MS analysis was performed in a total of 1433 urine samples in Phase I and 712 in Phase II, as schematically depicted in the **Figure 3** below.

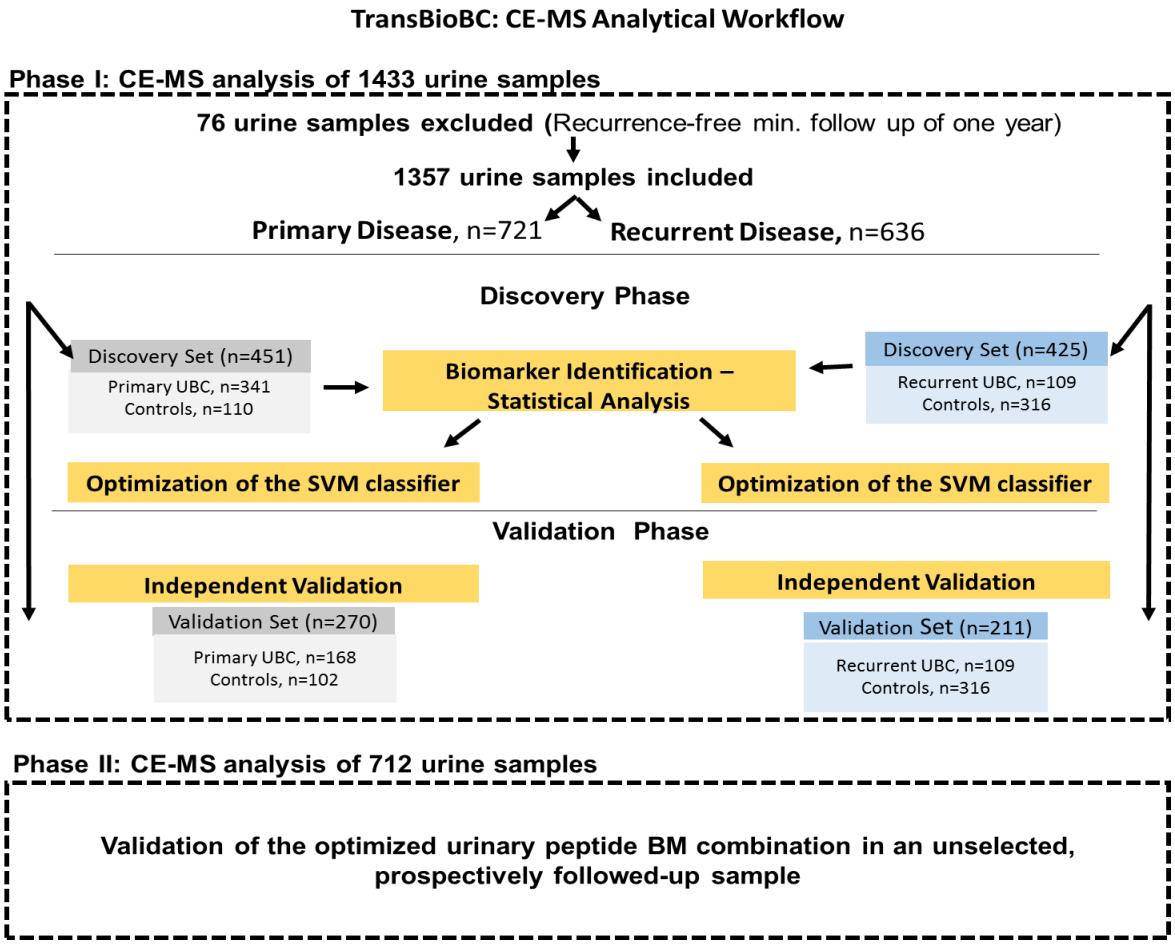

Figure 3. CE-MS study design and patient categorization for the development and the validation of the urinary peptide biomarkers.

In all cases, sample preparation was conducted based on the ISO 13485 protocol and the CE-MS data acquisition was performed according to SOPs applied to routine diagnostics. The analytical workflow is schematically depicted in the Figure 4, below.

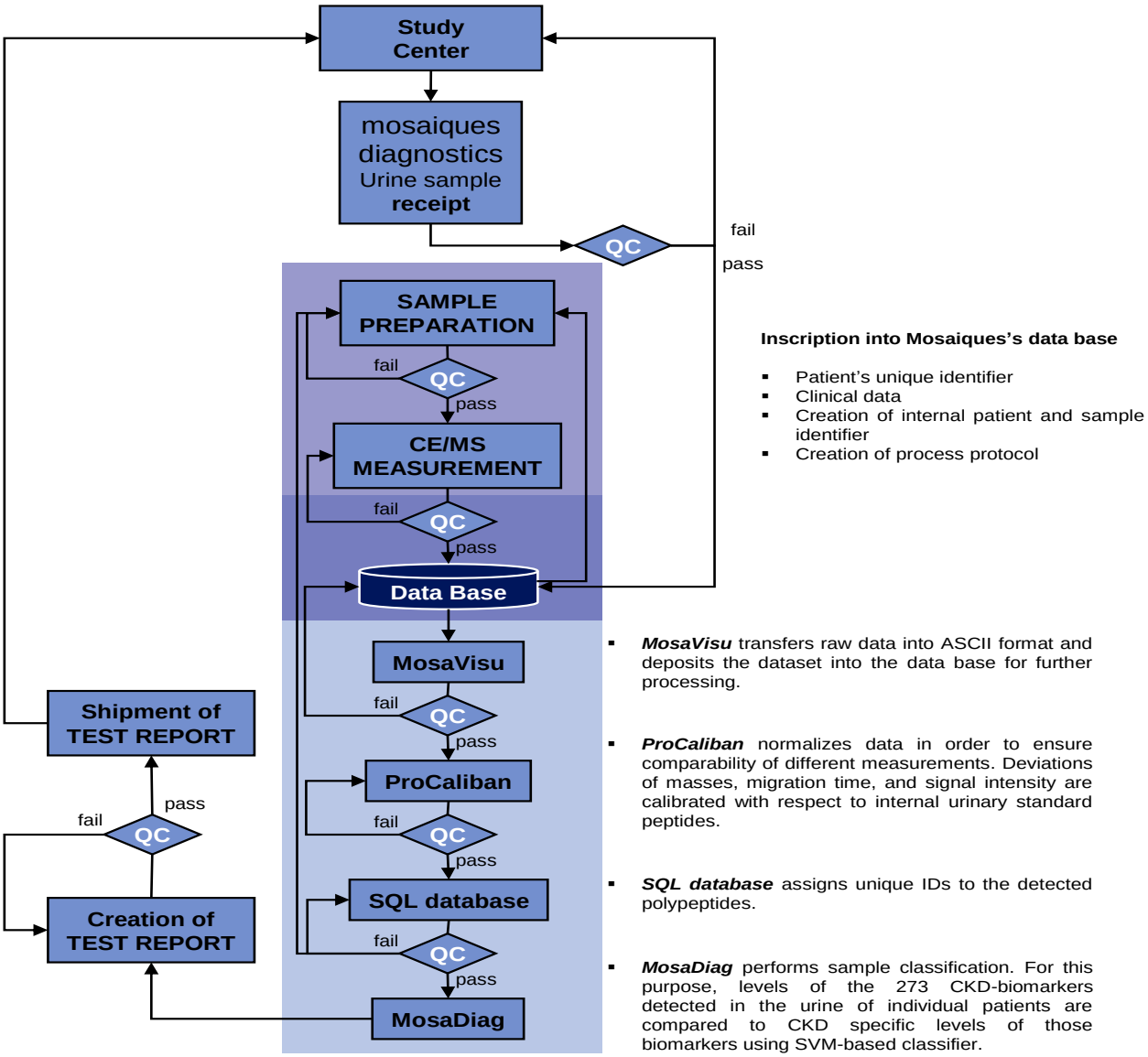

Figure 4. CE-MS analytical workflow for the development and the validation of the urinary peptide biomarkers.

For the initial large-scale case/control design the 1433 urine samples originating from 5 clinical centers (CNIO-ISBLaC, Hannover Medical School, Erasmus Medical Center, Laikon Hospital-Greece, Virginia University) included patients with primary and relapsed/recurrent urothelial cancer. The urine samples were collected from patients presenting with suspicion of primary disease (N=732) or those suspicious for a relapsed/ recurrent bladder tumour during the follow-up monitoring (N=701).

Accordingly, the diagnostic accuracy for the CE-MS derived biomarkers was estimated in two disease groups, primary/ incident BC and relapsed/ prevalent BC (Figure 3). Out of 1433 urine samples, a total of 1357 were included for the establishment and the initial independent validation of the CE-MS derived biomarkers while 76 were initially excluded, as not applicable for the diagnostic setting (minimum follow-up of recurrence free patients was set at one year to rule out false negatives). An initial application of the previously defined classifier (Theodorescu D. *et al. Lancet Oncol* 7(3), 230-40;2006) including 192 discriminatory peptides, was evaluated using the ISBlaC cohort (n=531; 251 cases, 280 controls). The results supported highly significant association of the previously developed classifier with UBC ($p<0.0001$). However, the accuracy rates appeared sub-optimal, with 73% sensitivity and 51% specificity in UBC detection. This moderate performance is most likely indicative of the fact that the classifier was established using mostly high grade UBC cases. To enhance the performance, especially given the high number of analyzed samples, statistical analysis at the peptide level was conducted using non-parametric Wilcoxon rank sum test. The 1357 samples were randomly divided into a training and a test set, according to the "2/3 and 1/3 rule" to allow for validation of the biomarker panel in an independent test set. Two cross-sectional studies were conducted to establish and validate urinary-based biomarker panels for detecting primary (n=721) and relapsed (n=636) UBC. For detecting primary UBC, 721 urine samples (509 cases and 212 controls) were randomly assigned to the study discovery (n=451) and validation arms (n=270). Comparisons between groups – 341 UBC cases and 110 controls, were performed in the discovery set using the Wilcoxon test. Statistical comparisons enabled the identification of 321 peptides significantly associated with the primary outcomes of interest. To eliminate potential center bias, the biomarkers were further analyzed for their correlation with primary UBC across the various participating clinical centers. Of 321 peptides, 116 significantly associated peptides displayed similar regulation across all centers and were thus selected for further assessment. A Support vector adaptive machine (SVM) learning algorithm was adopted to develop a biomarker panel based on these peptides. Subsequent validation of the 116 biomarker panel was conducted in the test set of 270 samples (including 168 primary UBC cases and 102 urological controls) which resulted in an AUC of 0.87. At the optimal cut-off -0.27, the biomarkers' sensitivity and specificity was 91% and 68%, respectively. The 116 biomarker panel enabled significant discrimination according to disease stage for the 168 UBC cases, particularly between NMIBC (n=115, $p<0.0001$) and MIBC (n=53, $p<0.0001$).

Along the same lines, for the investigation of recurrent UBC, in the respective discovery set (n=427), 328 peptides were significantly associated with recurrent UBC. Investigation of the consistency of the peptides across the different centers rendered 106 peptides that were subsequently retained in the final biomarker panel. The 106 peptide biomarker panel was validated in the 211 samples forming the test set (including 55 UBC recurrent cases and 156 recurrent controls), with an AUC of 0.75. At the ideal cut-off of -0.63, sensitivity and specificity values were 88% and 53%, respectively. As in the case of primary UBC (116 peptide biomarker panel), the biomarker panel for detecting UBC recurrence presented significant discriminatory ability between patients presenting with NMIBC (n=41, $p<0.0001$) and MIBC (n=14, $p<0.0001$), respectively.

Based on the specific disease context, special emphasis was given on the assessment of the diagnostic capability of the panel for low risk UBC. A urinary biomarker panel with adequate performance is expected to reduce the monitoring cystoscopies in the surveillance group (recurrence cohort). The 106 peptide biomarker panel was thus further evaluated for its diagnostic capability in detecting low risk recurrent UBC in 182 samples from low risk patients (26 UBC recurrent cases and 156 recurrent controls) out of the 211 samples of the test set. In this sub-population an AUC of 0.72 was achieved. Importantly, cytological examination was available for 88 out of these 182 low risk recurrent samples. When examining the performance of the classifier in these samples only, the 106 peptide biomarker panel outperformed cytology with an AUC of 0.79, compared to 0.64 for cytology. Combination of both tests increased the diagnostic accuracy, resulting in an AUC of 0.90.

To investigate impact of cofounders, measurements of total urinary protein, levels of hematuria and urinary creatinine were obtained in MOS for the ISBLaC samples. Out of the 211 samples of the validation test set

(prevalent disease context of use), 86 were derived from the ISBlaC Series. In this later set and following respective analysis, the 106 peptide panel was not significantly affected by hematuria or urinary creatinine levels. The results of the first phase study describing the 116-(primary disease) and 106-(surveillance) models were published in Clinical Cancer Research (Frantzi M. et al. *Clin Cancer Res*. 22(16), 4077-86, 2016).

Prognostic value of the CE-MS peptides

A preliminary analysis of the prognostic value of the peptide classifiers for tumor relapse (recurrence-progression) was performed using the ISBLaC cohort. For this analysis, 215 patients were considered, with 94 out of 215 patients derived from the primary/ incident group and 121 out of 215 patients from prevalent/ recurrent group. Positive and negative scores were separately assessed based on the published cut-off criteria of -0.27 and -0.63 for primary/ incident and recurrence/ prevalent disease (Frantzi M. et al. *Clin Cancer Res*. 22(16), 4077-86, 2016). Kaplan-Meier (KM) curve analysis and Cox proportional-hazards ration analysis were performed. Hazard ratios (HR) and 95% confidence interval (95% CI) were estimated.
Of the 94 patients of the primary/incident group, 67 were considered as uncensored events (developed tumors) and 27 censored (disease free). When considering as predictive factor the classification score -positive (>-0.27) and negative (<-0.27), the Hazard ratio values for the former (positive score) was found significantly different to the latter (negative score) in the prediction of the tumour event (p=0.0014).
Along the same lines, the CE-MS Peptide marker panel for detecting recurrence, consisting of 106 biomarkers was analysed in the 121 patients of test set including 55 uncensored events and 66 censored. The classification scores were assessed as two groups: positive (>-0.63) and negative (<-0.63) based on the predefined cut off differing significantly (p=0.01) in the respective Hazard ratio values. Due to the small sample sizes these results have to be confirmed in larger datasets.

Establishment of the Optimal Diagnostic Nomogram (WP3)

The added value of the combination of the urinary biomarkers with clinical variables was further investigated. Combination of the 106 peptide biomarker panel with clinical variables, such as age and gender, resulted in no improved overall AUC of 0.75 in the test set of samples (n=211), although a slight, yet insignificant improvement in the specificity. Like the peptide biomarker panel, the nomogram was not significantly affected by hematuria, urinary total protein and urinary creatinine. Data on supplementary treatment (e.g. BCG or chemotherapy) administered prior to the urine collection were available for a total of 123 out of 211 patients included in the validation set. The results from the logistic regression analysis indicate that the classification score is the only significant predictor of disease status ($p<0.0001$).
Direct comparative assessment between the urinary CE-MS-based Nomogram and cytology was feasible, based on the available data for 111 out of 211 samples, included in the validation phase. In this patient groups the Nomogram performed better than cytology with an AUC of 0.78 compared to an AUC of 0.63 for cytology. Combination of both tests increased the diagnostic accuracy, as assessed by the given AUC of 0.84, compared to the performances of any single test alone. The difference between the AUC values was shown to be statistically significant (p=0.0268). When investigating only the low risk patients under surveillance (86 out of the 111 from above), the nomogram resulted in an estimated AUC of 0.76 showing superior performance compared to cytology, with the latter detecting UBC with an AUC of 0.67. Combination of both cytology and the Nomogram's score resulted in an estimated AUC of 0.90.

Sample size power calculations for the phase II study (WP3)

For **primary cancer diagnosis** and **surveillance markers**, we considered the following real sensitivity (Se) and specificity (Sp) of the cytology test as a reference.

UBC Cytology: Se=26% and Sp=95%

Low Risk-Non Muscle Invasive Bladder Cancer Cytology: Se=26%, Sp=95%

High Risk- Non Muscle Invasive Bladder Cancer Cytology: Se=65%, Sp=95%

Calculations were based on a binomial test for a single proportion, assuming a single-sided type I error of 5% (alpha=0.05) and a power of 90% (beta=0.10) in a one-sided test. **Table 3** displays the sample size figures needed to reach a power of 90% according to different scenarios for sensitivity (Se) and Specificity (Sp) and 10% of dropout.

Table 3: Sample size figures considering a power of 90% and different Sensitivities/Specificities

Scenario Se/Sp	Reference Se/Sp	BMx Se/Sp	Dropout	Sample size
Se-1	26%	80%	10%	6
Se-2	26%	90%	10%	4
Se-3	65%	80%	10%	68
Se-4	65%	90%	10%	20
Se-5	80%	90%	10%	95
Sp-1	95%	96%	10%	3302
Sp-2	95%	97%	10%	728
Sp-3	95%	98%	10%	276
Sp-4	95%	99%	10%	123
Sp-1	95%	100%	10%	38

Se: sensitivity, Sp: specificity, BMx: biomarker

According to these calculations, the TransBioBC study is powered (Power>90%) in the comparisons to the cytology test performance according to the different scenarios for bladder cancer primary diagnosis and surveillance when the biomarker Se and Sp are >80% and 95%, respectively and the AUC>0.6.

The table below (**Table 4**) displays the required sample sizes for observing a higher AUC level with respect to the reference values, with alpha=0.05, 1 side test (H1: AUC>refAUC) and 90% of power (beta=0.10) in a two-sided test (alpha=0.05) and 10% of dropout.

Table 4: Required sample size to obtain AUC> than that of reference (cytology)

Scenario AUC	Reference Se/Sp	BMx Se/Sp	Dropout	Sample size
AUC-1	50%	60%	10%	257
AUC-2	50%	70%	10%	63
AUC-3	50%	80%	10%	27
AUC-4	50%	90%	10%	14

Considering the results from the CE-MS peptide models (Frantzi et al., *Clin Cancer Res*. 22, 4077, 2016) for the urinary-based peptide biomarker panel with 116 peptides markers that presented a Se=91% and Sp=68% for bladder cancer **primary diagnosis,** measured in a set of 270 samples (168 UBC cases and 102 controls), the phase I study has a power >95% for the Se estimate when compared to the cytology performance. Regarding specificity, the study would have had 90% of power to observe a specificity of 98% compared to the cytology performance. As for AUC, the observed AUC was 0.87 (95%CI 0.83-0.91) and the study has a power >95% for the AUC estimate when compared to AUC=0.5.

Regarding the results obtained by Frantzi et al. [Clinical Cancer Research 2016] for the urinary-based peptide biomarker panel with 106 peptides markers that presented a Se=87% and Sp=51% for surveillance cut-off of -0.63 and a Se=76% and Sp=61% **for surveillance cut-off** of -0.43 when measured in a sample size of **211** samples (55 UBC cases and 156 controls), the study has a power >99% when Se=88% as well as when Se=76%. Regarding specificity, the study would have had 78% (98%) of power to observe a specificity of 98% (99%) compared to the cytology performance. As for AUC, the observed AUC was 0.75 (95%CI 0.68-0.80) and the study has a power >99% for the AUC estimate when compared to AUC=0.5.

Second phase validation study (WP2-WP3)

As per the TransBioBC plan, further validation of the optimal models defined in the phase I study was seeked through a second phase prospective validation. A total of 712 samples were received from two clinical centers-Radboud University Nijmegen (Netherlands) and University of Barcelona (Spain) All samples have been processed by CE-MS (MOS), analyzed by the established cut-off in phase I study, nevertheless unblinding and reporting of the data in a manuscript is expected after the end of the project due to small delays in the clinical centers when compiling the needed follow-up information (as a result of a lack of funding for this purpose).

Clinical implementation (WP4-WP5)
Health economic calculations (WP4)

Health economic calculations, including cost efficiency analysis and development of Markov Models were carried out with the TreeAge Software Package for the primary and recurrence groups. Models were generated based on the diagnostic and follow-up procedures recommended by the EAU guidelines. Furthermore, sensitivity analyses were carried out, including the sensitivity, specificity and pricing of the biomarker based assay as well as the current diagnostic work-up of cystoscopic procedure.
For detection of primary UBC, and for the specificity / sensitivity scenario close to the one reported in the Primary biomarker setting (116 peptide biomarker panel; 91% sensitivity; 68% specificity), the average number of cystoscopies per patient decreases to 0.5, from 1.8 (single cystoscopy scenario) and 2.3 (double cystoscopy scenario) enhancing thus the patients' compliance during UBC diagnosis. Collectively, the biomarker / cystoscopy-based diagnosis represents the optimal approach, preferred over the cystoscopy-based diagnostic procedures.

For the surveillance group, the costs per patient and per UBC detected case, the number of biomarkers used, the number of cystoscopies used and number of true-positive diagnosis for each diagnostic procedure, were considered in the analysis. The model that was used for the cost-effectiveness calculations was designed to yield characteristics (e.g. cost per UBC case, cost per patient, average number of cystoscopies required) until the first true-positive UBC recurrence is detected. Subsequent recurrences are not covered in this model, since re-recurrence rates for UBC cannot be estimated. The cost-effectiveness calculations indicate that the biomarker / cystoscopy-based follow-up is favoured over the cystoscopy only based procedure, since the cost per patient decreases by approximately 14%, the cost per UBC case by approximately 13% and the average number of cystoscopies by approximately 52%. Moreover, the average number of cystoscopies decreases by about 4 cystoscopies per patient during the follow-up, thereby increasing the patients' compliance during bladder cancer surveillance. In summary, a non-invasive biomarker classifier is highly suited for its application during the follow-up of UBC patients, as it demonstrates cost-effectiveness compared to the state of the art diagnosis.

Pre-analytical Biomarker Validation (WP4)

Pre-analytical biomarker validation of the applied CE-MS method was performed by MOS according to the FDA requirements for Industrial Biomedical Method Validation. In brief, intermediate precision, repeatability and stability were monitored. The pre-analytical stability of the biomarkers at various temperatures, storage conditions before and after sample preparation, frost/ defrost cycles is reported, using the validated analytical platform of CE-MS for both the 106 and 116 biomarker peptide panel. For the CE-MS analysis, three types of reference standards are used: a) a mixture of seven peptides of known concentration that are normally employed for the calibration of the MS instrument (every two days), b) an array of 29 internal standards (naturally occurring urinary peptides; this array is preferred over single compounds as internal reference standards, as it ensures higher stability), c) a standard urine sample that is used as quality control (daily). The typical method development and establishment for a bioanalytical method, as proposed by the FDA guidelines for Analytical Validation, include determination of (1) selectivity, (2) accuracy, precision, recovery, (3) calibration curve, and (4) stability of the biomarkers. For selectivity, analyses of blank samples of the appropriate biological matrix (in this case urine) is being performed to investigate potential interference. Selectivity is ensured at the lower limit of detection (LOD). For CE-MS analysis, the LOD was determined for five different peptides, by employing reference signals of synthetic peptides that were labelled with stable isotopes, as previously described by Jantos-Siwy et al. *J. Proteome. Res.* 8, 268, 2009). To account for analytical variability in the assessment of the analytical LOD (aLOD), the standard deviation (SD) was incorporated to the aLOD value, resulting in a final LOD (fLOD). LOD was determined in triplicate measurements for five different peptides. The calculation of LOD for these five peptides resulted in a mean value of 1.5 fmol.

Performance and stability was assessed on the level of final SVM classification results of the CE-MS BC classifiers (106 peptide and 116 biomarker panel) by using established methodologies as published in the past (Mischak et al, *Clin. Biochem*. 46, 432, 2013). This procedure allowed the assessment of not only variances of the molecular biomarker pattern but also data processing variances and variation based on the classification algorithm. A set of experiments for platform validation was performed, as following:

- Investigation of repeatability for assessing intra-assay precision,
- Investigation of intermediate precision for assessing inter-assay variability,
- Investigation of temperature stability,
- Investigation of post- preparation stability,

Investigation of frost/defrost stability

The intra-assay precision was assessed by the intra-assay coefficient variance, indicated by the percentage of relative standard deviation, as estimated above (% RSD). Based on the above calculations, intra-assay coefficient variance was estimated at 10.5%, 10.9% and 9.8% for the three samples investigated. This results in an average CV value of 10.4%, less than 15%, which is generally acceptable, according to the guidelines. The average intermediate precision or within-laboratory precision was assessed and found to be 11.4%. Based on the above calculations, inter-assay coefficient variance was higher than the intra-assay coefficient variance, as expected, but less than 15%. The value is generally acceptable, according to the guidelines. The assessment of the biomarkers' stability was performed after: a) Urine storage at RT, b) Urine storage at 4 ^{o}C, c) Repeated frost/defrost cycles of urine, and d) Prolonged post-preparation storage. For storage at 4 ^{o}C the analysis resulted in an average coefficient variance value of 14.6%, for Urine Storage at room temperature the coefficient variance was estimated at 9.7% and for Repeated frost/defrost cycles of urine, the coefficient variance was estimated at an average coefficient variance value of 10%. For prolonged post-preparation storage, the coefficient variance was estimated at 12.6%. All values are below the required limits, hence acceptable.

The standard procedures for clinical routine use of the product were developed jointly with collaborating clinical centers. Specifically, the use of Monovette tubes with boric acid as stabilizer was examined to enable shipment without dry-ice. For this purpose, urine samples were collected from 7 patients presenting with primary bladder cancer at the outpatient clinical centre in Laikon Hospital, Greece, after the appropriate ethical approval being in place. The urine was initially collected in a sterile urine container and further aliquoted three times at equal volumes in three Urine Monovette tubes. The mean classification scores were evaluated regarding the stability of the biomarkers of the BC classifier (peptide biomarker panel). Based on the experimental investigation of three different sampling, storage and shipping procedures, the following standardised sampling, shipping and storage procedures were established: Urine Monovette tubes, including boric acid will be applied for the sampling of the urine samples, normal Express Shipment without dry ice was selected as preferable as shipping procedure and storage at room temperature or at the 4^{o}C until the time of shipment is recommended.

At the same time a manual was developed. Based on: a) the assay performance of the optimal nomogram, including the CE-MS derived biomarkers in combination with demographical data such as age and gender, and b) the pre-analytical stability of the CE-MS biomarkers at various temperatures, storage conditions and frost/ defrost cycles, the standardization of the operational procedure, sampling and shipping was established. The purpose was to provide with manuals ready to be incorporated in the Prototype Kit. For the optimal combination of the CE-MS derived biomarkers, sampling materials and manuals were developed on the basis of the analytical performance and the stability of the CE-MS derived biomarkers.

<u>Development of a UBC prototype kit (WP5)</u>

Based on the above standardised procedures a detailed manual is included in the Prototype kit.

A prototype in-vitro diagnostic device (IVD) for application in clinical trials was launched. The Kit was developed based on the CE-MS derived biomarkers including:

1) standardised sampling materials, such as cups and monovettes,
2) a detailed user manual for sampling, storage and shipment,
3) technical specifications of the sampling kit based on reproducibility and stability data

Within the TransBioBC Project, a prototype in-vitro diagnostic device (IVD) was launched based on the

diagnostic nomogram that was established. The IVD prototype kit refers to an optimal classifier consisting of combination of different peptide biomarkers derived from CE-MS analysis in combination with clinical variables, like age and gender. The intended use of the IVD prototype kit is the detection of primary UBC or recurrence and monitoring bladder cancer patients under surveillance. The prototype in-vitro diagnostic device (IVD) was based on the CE-MS derived urinary biomarkers.
The sampling kit is shipped to customers on demand and the analysis is performed centrally (in MOS) upon return. Sample analysis as well as data processing is performed in a quality controlled laboratory environment based on a CE-MS based assay for urinary biomarkers (MOS). Sample preparation is conducted according to the ISO 13485 protocol and the CE-MS data acquisition was performed according to the SOP which is applied to routine diagnostics. Detailed instructions have been formed to ensure the correct application of the sampling kit. Improper handling of the IVD TransBioBC Kit may affect the test result. The instructions for urine sampling and shipping have been formed based on the standard procedures for clinical routine use.
Midstream urine collected during the second morning urination is required. The patient is requested to discard the initial flow of urine before collecting the specimen in the sterile container. The manual, apart from the English language, has been translated into German and Greek.

Collectively, the vast majority of the main objectives of TransBioBC were reached. Some markers, especially those measured by immunoassay- based techniques, did not provide sufficient accuracy rates for a diagnostic test, likely associated to suboptimal antibody specificities in combination to confounding effects by hematuria. Mass spectrometry-based (MRM) assays appear to provide a good alternative of higher specificity in marker measurement. Multi-parametric classifiers measured by mass spectrometry (CE-MS) proved to be more robust and accurate diagnostic means likely due to better reflecting inter-individual variability and tumor heterogeneity. Importantly, TransBioBC lead to the establishment of a prototype kit to be used in bladder cancer surveillance, specifically detection of tumor relapse. Some indications supporting a prognostic value of the classifier exist, however, this objective could not be fully met due to insufficient sample sizes. Nevertheless, a diagnostic prototype kit for use in UBC surveillance has been developed following high impact scientific publications of the TransBioBC results, extensive dissemination activities to multiple stakeholders have been performed, a network of clinical centers supporting the kit use has been established and discussions with pharma companies for establishing use in drug design are ongoing, collectively attesting the success of this project.

Description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of the results

Long term Societal-Economic Impact

TransBioBC focused on the development of biomarker (BM) classifiers for the non-invasive detection of UBC. Surveillance of recurrence is currently conducted by cystoscopy, an invasive procedure associated with high economic cost, high patient discomfort and, as a result, low patient compliance. As a result, an urgent need for improvement in guiding UBC management exists. Development of non-invasive, urine based BM assays to assist in the BC surveillance program is considered a very promising approach towards a significant improvement. The main aim and results of **TransBioBC,** the availability of a non-invasive urine test guiding management and treatment of patients at risk of UBC, delivers exactly such a product.

The developed test particularly targets patients with low grade UBC, as these represent the largest BC subtype and also the group that would benefit the most from improvement in surveillance and prognosis. The currently available tests to assess recurrence are of poor sensitivity. Due to the low associated risk for progression in this group of patients lower specificities in disease detection can be acceptable (Schmitz-Dräger BJ et al *Urol Oncol.* 2014;32(7):1061-8). The specific tangible impact of the TransBioBC non-invasive BM classifier would be: (1) to reduce the number of surveillance cystoscopies performed during the monitoring of patients treated for BC, and/or 2) increase cystoscopy accuracy rates by guiding physicians towards a more thorough investigation of the bladder, in view of a positive BM test. The evident benefit for the patient is reduction of the need for invasive cystoscopy, and improved accuracy of cancer detection, which is a pre-requisite for improved, targeted, personalized intervention.

Socio-economic impact:

Based on the presented UBC epidemiological data it is safe to state that UBC generates the highest cost per patient of all tumors in the western world, mostly due to the need to constantly monitor patients with UBC generally for recurrence, for many years. The decrease in the number of unnecessary cystoscopies (resulting from the development of the urine-based classifier) is expected to result in a net decrease in UBC associated costs, also supported by the cost effectiveness calculations performed in the context of TransBioBC, while at the same time increasing quality of life.

Increase SME competitiveness: The validated BMs will have a major impact on MOS business plans and increase significantly its competitiveness, as they are already available as prototype kit and can now be exploited as product. Based on the number of needed tests per year, UBC surveillance represents a significant market. In parallel, usage of the validated BMs in clinical trials involving pharmaceutical industry as customers is also currently pursued as part of the MOS business plans which collectively further enhances the competitiveness of this SME.

Effective use of European resources. The successful clinical implementation of findings and technologies developed to a large extent within EU projects, brings European research at the forefront of the highly competitive biomarker research field: TransBioBC will serve as a show-case for the successful translation of knowledge into innovative products, inspiring further development and similar integrative efforts in the field. The platform and

approach established in TransBioBC will also serve as a basis and demonstrator project to initiate similar enterprises in associated areas and market segments, further **strengthening the European position in biomarker research and personalized medicine.**

In parallel, direct scientific and societal impact from TransBioBC include:

Scientific impact: The scientific impact of TransBioBC has been prominent as evaluated based on positive feedback received from the publications, (of note : the manuscript by Frantzi et al. reporting on the CE-MS-based biomarker assays developed in TransBioBC was highlighted in *Nature Reviews Urology* 13, 240–241, 2016) and during the numerous presentations of the TransBioBC partners to international scientific conferences of relevant professional societies, disease specific networks of investigators (such as the International Bladder Cancer Network), as well as meetings of other EU-funded projects in which TransBioBC partners (MOS and BRFAA) participate. On the latter, special emphasis was placed on readily disseminating important know-how and foreground acquired in Transbiobc, so as to maximize efficient use of European resources. Important foreground on ELISA assay limitations in urine, points of caution during assay performance characterization, as well as developed know-how on MRM assays had immediate impact on other projects (such as iMODE-CKD, SysVasc) where biomarkers are developed and evaluated. Of note, in the course of TransBioBC 5 review articles and 5 original research articles were published and co-authored by multiple TransBioBC partners, with one more research article being submitted and at least two more foreseen (on the CE-MS phase II study and biomarker measurements by MRM).

For the SME partner DS, even though the quantified markers did not exhibit the needed diagnostic accuracy to make it into the prototype kit, important foreground was still developed, providing a real "clinical" test case for the platform, never before investigated. For the SME partner MOS, the foreground of the UBC prototype kit is a major project output of significant expected impact as aforementioned, boosting the competitiveness and expanding significantly the business plans of the company.

In terms of direct social and educational impact, TransBioBC has resulted in the hiring of five personnel by BRFAA (3 female, 2 male). On 3 different dates (04/03/15, 24/11/2015, 02/03/2016) 60 students from Greek High-schools along with 3 biology teachers took a guided tour of the BRFAA proteomics facility and then attended a lecture on epidemiology and diagnosis of bladder cancer by Dr. Ieronymos Zoidakis. Particular emphasis was put on the correlation of smoking and bladder cancer incidence. The total number of students who attended this dissemination activity is 180 and positive feedback was received from both students and teachers. In parallel TransBioBC formed the basis of two masters' thesis involving MRM assays in urine (BRFAA). The one (by Mr P Savvopoulos) was completed in the course of the project and a second one (Mr Paolo Diasbastos student from U Aveiro funded by the Erasmus program) is ongoing and is expected to be completed in January 2017.

The project has resulted in the hiring of 3 scientists (2 female, 1 male) at MOS, and 4 at BRFAA (3 female, 1 male) and the establishment of intense collaborations with clinical centers, which is an extremely highly valuable asset for the implementation of the tests developed in TransBioBC. Jointly with the clinical centers also companies involved in drug development were approached, expressed substantial interest, and it is expected that the CE-MS-based test will be implemented in at least two clinical trials in the context of UBC treatment.

In conclusion, TransBioBC had significant outputs providing important know-how on biomarker assay establishment and clinical implementation, educating young people on (prevention of) bladder cancer, and, most

importantly, with scientific results that enabled the development of a UBC prototype kit to be used in UBC surveillance, expected to contribute to a more effective management of this disease.

Dissemination Activities

Successfully completed scientific dissemination activities include:

1) A public TransBioBC website was developed, providing information on BC in three major sections addressing different target groups, including the general public and patients, physicians and researchers, and industry members (www.transbiobc.org).

2) Information dissemination material (logo and leaflets) have been developed and have been disseminated through the TransBioBC website.

3) Participation in Urological Conferences (ESUR, IBCN) in several EU countries and USA (summarized also below).

4) Networking with other scientists and R&D initiatives in the field.

5) Networking with patient organizations and support groups.

6) Publication of scientific articles in international peer-reviewed journals (summarized below).

Dissemination of the proposed Biomarkers

In TransBioBC and as a main output, a prototype kit was developed. A significant expansion of the dissemination activities was organized to incorporate scientific but also marketing exploitation of the TransBioBC results. Scientific audience was targeted through highly ranked publications as well as the medical society, pharma companies and patient groups through presentation to respective meetings, a TransBioBC flyer summarizing the case-control study results and personal contacts. In brief:

1st Target Group: Patients/ General Public

- A brochure for the Public in lay terms was generated to inform the patients for the proposed biomarker combination, the potential benefit for them and the diagnostic accuracy. The technology used is also briefly described. Brochures in english and german were generated.
- The brochure was disseminated to Patients' Organizations via emails and during meeting presentations.
- A newsletter is also under development and planned to be released in a Journal, which is published by a German Bladder Cancer Organization (Selbsthilfe-Bund Blasenkrebs e.V, named "Die Harnblase".

Figure 5. Brochure targeting to inform the Public about the Biomarkers and dissemination activities

2nd Target Group: Health Professionals (Urologists/ Physicians)

- ❑ A network between ten clinical centers has been created in Germany, including the clinical centers listed below :

<u>Network of German Urological Clinics:</u>

1. KRH Hospital Siloah-Oststadt-Heidehaus, Hannover, Germany: Prof. Dr. med. Christoph Wiesner.
2. KRH Klinikum Großburgwedel: Dr Joachim Stein
3. KRH Klinikum Robert Koch Gehrden: Chefarzt PD Dr. med. Marcus Schenck
4. Hannover Medical School: Prof. Dr. med. Markus A. Kuczyk
5. Lubeck Klinik für Urologie: Prof. Dr. med. Axel S. Merseburger
6. Vinzenzkrankenhaus Urologie: Dr. Lutz Neuhaus
7. Klinik für Urologie und Uroonkologie am Klinikum Braunschweig: Prof. Dr. med. Peter Hammerer
8. Klinik und Poliklinik für Urologie, Greifswald: Univ.-Prof. Dr. med. Martin Burchardt
9. Urologische Klinik und Poliklinik - Universität Rostock: Prof. Dr. Oliver Hakenberg
10. Magdeburg: Prof. Dr. Martin Schostak, Universitätsklinik für Urologie und Kinderurologie

- ❑ Clinical Guidance regarding the outpatient clinics is provided by Professor Merseburger, TransBioBC SAB member and Director of Department of Urology in Lübeck University (UKSH).

- ❑ The biomarkers (based on the CE-MS analysis) was offered to the Patients visiting the above outpatient clinics. This approach aims to familiarize the Patients and the Clinicians with the Proteomics Analysis.

3rd Target Group: Pharmaceutical Industry

- ❑ A brochure was produced, aiming at informing the Pharmaceutical Companies about the Consortium and the proposed biomarkers. The technology used is briefly described..

- ❑ A database including the top 100 pharmaceutical companies that perform clinical trials in the oncological field was created.

- ❑ Based on the Database entries, contacts have been established with: Roche, Merck Serono, MSD, Novartis, Pfizer, GlaxoSmithKline, Astellas, Astrazeneca, Lilly, BMS, J&J, Bayer. These contacts have resulted in several companies indicating interest, and currently two clinical trials proposed where the CE-MS-derived biomarkers are to be implemented.

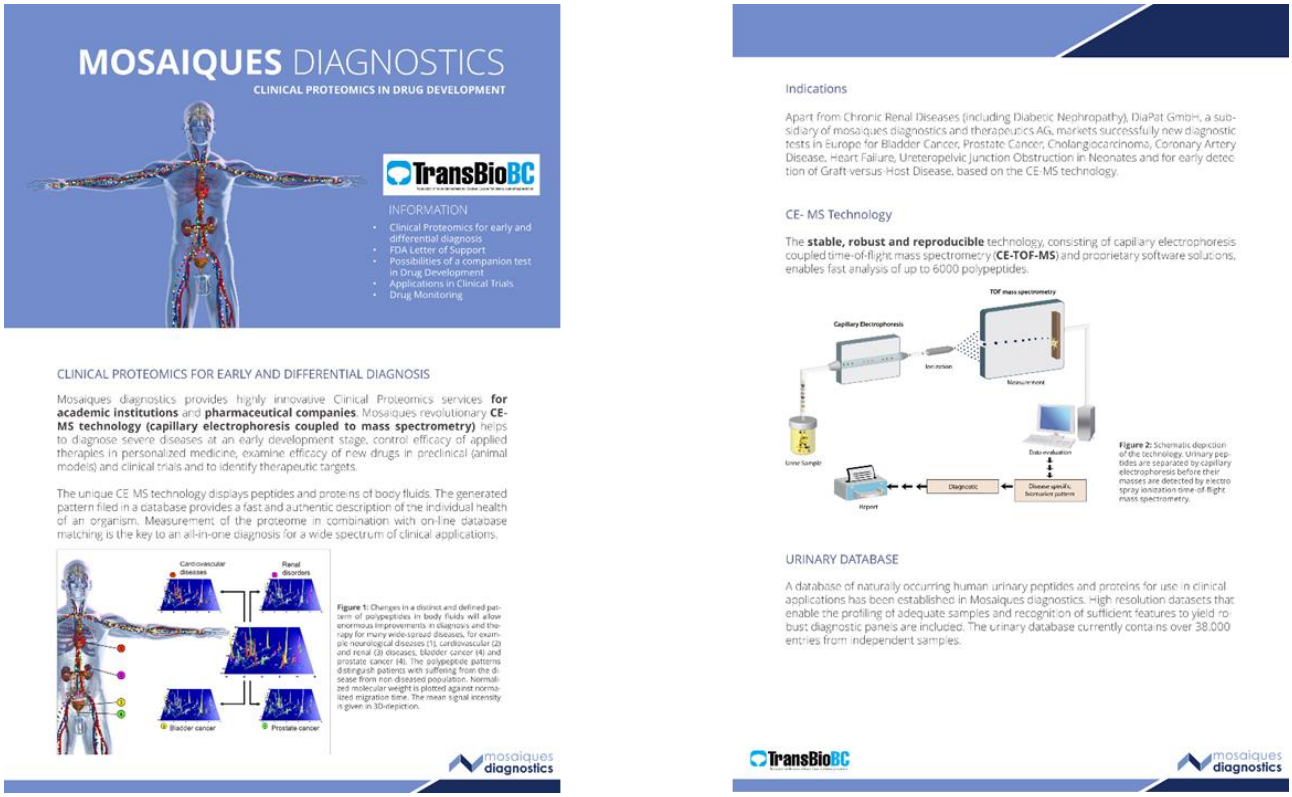

Figure 6. Brochure targeting to inform the pharmaceutical industry about the Biomarkers and dissemination activities

Figure 7. IVD TransBioBC Prototype Kit shipping components: a Urine Monovette tube with plastic syringe (Sarstedt), b) a sponge, c) plastic container (box), d) a plastic bag for shipment of biological material