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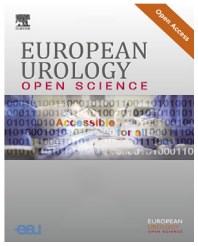

Trial Protocol

Prototol for the Prospective Sample Collection for Cancer of Bladder (ProCaB) Trial by the Cancer of the Bladder Leuven (CaBLe) Consortium

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Abstract

Non–muscle-invasive bladder cancer (NMIBC) is a heterogeneous disease categorized as low, intermediate, high, or very high risk, for which recurrence and progression rates and thus management strategies differ. Current molecular subclassification of bladder cancer (BC) is mainly based on data for muscle-invasive disease, with very few data for NMIBC. A more accurate classification system is needed for better stratification of NMIBC using multiomics and immunohistopathological molecular data alongside clinical data collected in a prospective cohort. ProCaB (Prospective Sample Collection for Cancer of Bladder) is a single-center non-interventional, prospective study recruiting all eligible patients diagnosed with BC in a tertiary center in the Flanders region of Belgium. Clinical data have been collected in a prospective registry since August 2013. Biosamples (blood, urine, and BC tissue) are collected from each patient at diagnosis and are stored at −80°C at BioBank UZ Leuven after appropriate processing according to the protocol. Multiomics (genomics, epigenetics, transcriptomics, proteomics, lipidomics, metabolomics) and immunohistopathology analyses will be performed on appropriate samples. The target is to enroll 300 patients over a 5-yr period, and all patients will be followed for 5 yr. The objective is to create a biobank of samples from patients diagnosed with BC for use in multiomics and immunohistopathological analyses. Results from these analyses, together with long-term clinical data, can be used for comprehensive multilayered molecular characterization of disease recurrence and progression in intermediate- and (very) high-risk NMIBC, identification of

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multibiomarker panels for better stratification, and identification of a patient subgroup that does not respond to bacillus Calmette-Guérin treatment.

This trial is registered on ClinicalTrials.gov as NCT04167332.

1. Introduction

Bladder cancer (BC) is the most common malignancy of the urinary tract, and its incidence varies between regions and countries. It is the ninth most common form of cancer diagnosed in both sexes worldwide and the sixth in the USA, while it ranks fifth in Europe, with an age-standardized rate of 17.0 cases per 100 000 individuals [1–3]. As life expectancy is expected to increase in Europe, the annual BC incidence is projected to reach 235 829 by 2030 [4]. In Belgium, 2364 new cases of BC were diagnosed in 2013. The 5-yr survival rate for the Belgian 2009–2013 cohort is ∼56% for males and ∼49% for females, and no clear trend in the survival rate over time has been observed. By 2025, the number of patients diagnosed with BC is expected to rise to 2900 [5].

In North America and Europe, the majority of BC cases (∼90–95%) are urothelial carcinoma. At initial presentation, 25% of patients are diagnosed with muscle-invasive BC (MIBC). These patients have aggressive disease, as approximately one-third have undetected metastases, while 25% have lymph node involvement [6]. The remaining 75% of BC cases present with disease confined to the mucosa or submucosa, termed non–muscle-invasive BC (NMIBC). Up to 70% of these cases will recur, and 15% will progress in stage and grade [7]. Therefore, patients with NMIBC are scheduled to undergo frequent monitoring, currently based on cystoscopy and cytology, which makes BC one of the costliest of all cancers to manage [8].

NMIBC includes heterogeneous subgroups that differ in recurrence and progression rates, so the European Association of Urology (EAU) guidelines recommend stratification into low, intermediate, high, and very high risk on the basis of clinically available prognostic factors. Patients with low-risk NMIBC have a low risk of disease progression and a low to moderate risk of recurrence, with excellent survival after defined treatment modalities [6]. Despite intensive treatment and follow-up schedules, consisting of transurethral resection of bladder tumor (TURBT) plus intravesical induction and maintenance bacillus Calmette-Guérin (BCG) therapy and follow-up cystoscopies, patients with intermediate- or high-risk NMIBC have a high risk of recurrence, a moderate to high risk of progression, and a BCG nonresponse rate of 40% [6].

Existing scoring systems developed by the European Organization for Research and Treatment of Cancer and Club Urologico Español de Tratamiento Oncologico are based on clinicopathological features alone and have failed to accurately predict the risk of disease recurrence and progression in the short and long term in external validation studies [9–12]. The dichotomized model of papillary versus nonpapillary BC does not fully address the heterogeneity of the disease [13,14]. Recent intense molecular research has revealed different molecular subtypes of BC that are similar to those in breast cancer and facilitated a better understanding of the heterogeneity of BC [15–22]. However, these subclassifications are based on RNA analyses performed mainly for retrospective MIBC cohorts, with very few data for NMIBC. Comprehensive transcriptome profiling has revealed the presence of three biological subclasses for NMIBC (UROMOL class 1, 2, and 3), which differs from MIBC profiles [23]. A recent update for this cohort revealed four subclasses (UROMOL2021 class 1, 2a, 2b, and 3) [24], but correct discrimination of high-risk tumors from less aggressive tumors remains challenging. There is a lack of predictive and prognostic tools that can specifically stratify NMIBC according to pathological and molecular properties.

There is a need to develop a more accurate scoring system considering the NMIBC treatment options available after TURBT. This would allow better stratification for treatment selection and further follow-up scheduling on the basis of better prediction of response to a particular treatment.

2. Hypothesis and objective

2.1. *Hypothesis*

In light of the facts discussed above, we hypothesized that multiomics data (genomics, epigenetics, transcriptomics, proteomics, lipidomics, and metabolomics) and immunohistopathology data would allow us to determine the characteristics of NMIBC in a more detailed and multidimensional fashion.

With the goal of improving the prediction model for recurrence and progression, stratifying patients properly, and guiding therapeutic interventions in a personalized manner, we propose a comprehensive assessment of the genomic, epigenomic, transcriptomic, proteomic, lipidomic, metabolomic, and immunohistopathological characteristics of NMIBC to represent the broad spectrum of NMIBC phenotypes.

2.2. *Objective*

To test the hypothesis outlined above, biosamples need to be collected from patients diagnosed with BC, which is the objective of this study: to create a biobank of blood, urine, and BC tissue samples.

3. Design

3.1. Consortium

In November 2015, a joint venture was started between several clinical research teams in University Hospitals Leuven and fundamental research groups from KU Leuven, all focused on diagnosing and treating BC. Within this consortium, the unique expertise and knowledge of each participating team make it possible to bridge the gap between the bench and the clinic, thereby enabling genuine translational research. In September 2016, this multidisciplinary collaboration was formalized and named the CaBLe (Cancer of the Bladder Leuven) Consortium. The CaBLe Consortium then established collaborations with several other academic centers and small-medium enterprises (SMEs) abroad. A steering committee has been set up to assess the scientific content and feasibility of projects on biomarker research.

In August 2013, the Department of Urology in UZ Leuven and two other nonacademic hospitals established a specific prospective registry for NMIBC (NCT03973671) that included electronic case report forms for surgery, bladder instillations, multidisciplinary team meetings, and follow-up, all implemented within the electronic patient file systems of the participating hospitals [25]. Seven more hospitals later joined this registry, and more than 600 unique patients are prospectively registered yearly under the umbrella of a local hospital network (Vlaams Ziekenhuisnetwerk – KU Leuven [VZNKUL]).

3.2. Study design

In light of the unmet needs and our hypothesis, we decided to prospectively collect biosamples from patients with NMIBC. ProCaB (Prospective Sample Collection for Cancer of Bladder) is a single-center, non-interventional, prospective study that will recruit all patients diagnosed with NMIBC. The trial has been registered on ClinicalTrials.gov as NCT04167332. Each patient enrolled in the study will be automatically enrolled in the above-mentioned prospective registry for collection of demographic and clinical data. Therefore, in accordance with EU general data protection regulations, each enrolled patient provides two separate signed informed consent forms.

3.3. Patient population

Patients (male and female) aged $\geq$18 yr with a clinical diagnosis of BC (symptoms and/or imaging) are eligible to participate in the study. Patients who give informed consent for collection of biological samples before surgery are included in the study. The target is to enroll a total of 300 patients over a 5-yr period.

3.4. Treatment and follow-up

All patients receive treatment and follow-up according to the standard of care recommended in the EAU guidelines for their risk group [6]. The follow-up period is scheduled for 5 yr, as most recurrence or progression events occur during this time.

3.5. Data collection and management

To comply with local privacy laws, all demographic and clinical data (for initial diagnosis and following recurrence and/or progression events) are prospectively stored in the registry mentioned above, which is implemented in the Klinisch Werkstation (KWS) electronic patient file system and protected by firewalls [25]. All patients receive a study identification number, and names (and any other identifying data) are removed from the biosamples collected; therefore, the omics and pathology laboratories will be blinded to the identity and clinical status of the patients. Only the Department of Urology in UZ Leuven keeps the correspondence between the study identification numbers and patient names in a secured database.

3.6. Sample collection

Blood, urine, and BC tissue samples are collected from every trial participant at initial and recurrent diagnosis. Blood and urine are collected by nurses in the inpatient department in the morning before TURBT. After processing by dedicated personnel, the samples are sent to BioBank UZ Leuven (ISO 15189 certified), which has continuously monitored and alarmed freezers and a robotic handling and storage system, and has implemented the Standard Preanalytical Code (SPREC) system.

3.6.1. Blood collection

On the morning before TURBT (between 07:00 and 11:00 h), blood is taken from patients into five separate tubes (4 $\times$ 10-ml EDTA tubes for genomics, epigenetics, transcriptomics, and proteomics; 1 $\times$ 6-ml EDTA tube for lipidomics and metabolomics). The collection date and time are recorded on a urine/blood sampling form by the nurse. All EDTA tubes are inverted gently ten times after blood collection and then immediately placed in a refrigerator at +4°C in an upright position until centrifugation. All blood samples are centrifuged within 4 h of collection, and the centrifuge date and time are recorded on a blood processing and storage form. After processing all blood samples according to the specific protocol for each analysis, all plasma/pellet/whole blood samples are sent in cryovials to BioBank UZ Leuven to be stored at −80°C. Related data, including the date and time of sample acceptance, are recorded in the BioBank UZ Leuven registry system to calculate the SPREC for each sample.

3.6.2. Urine collection

A total of 230–250 ml of urine is collected into two tubes (1 $\times$ 150 ml and 1 $\times$ 100 ml) from the first morning void (between 07:00 and 11:00 h) before TURBT. The patient should be sufficiently hydrated. The collection date and time are recorded on a urine/blood sampling form by the nurse. A dipstick test is performed immediately after collection to exclude any urinary tract infection. The urine samples are immediately stored at +4°C in a refrigerator and processed within 2 h after collection, with the centrifuge date and time recorded on a urine processing and storage form. After processing all urine samples according to the specific protocol for each analysis, all samples are sent in

cryovials to BioBank UZ Leuven to be stored at −80°C. Related data, including the date and time of sample acceptance, are recorded in the BioBank UZ Leuven registry system to calculate the SPREC for each sample.

3.6.3. Tissue collection

During TURBT, if the tumor is large enough (>10 mm in diameter), a large chip (that aims to include the tumor stalk) is cut with a resectoscope. A small piece of this tumor sample is cut with a scalpel by a urologist. The larger part of the sample is placed in a cryovial, which is immediately put into a small liquid nitrogen container. The cryovial is then sent to BioBank UZ Leuven to be stored at −80°C. The date and time of collection and acceptance are recorded on a tissue sampling and storage form'. The small piece is used as a "mirror image" of the stored tumor by a uropathologist for histopathological diagnosis according to hematoxylin-eosin staining of slides prepared from a formalin-fixed paraffin-embedded (FFPE) block of the sample. Cryosectioning of frozen samples will be performed by a uropathologist before omics analyses to obtain parts enriched in tumor cells.

3.7. Considerations for power analysis

According to the number of patients recorded in the registry [25], an average of 270 TURBTs are performed annually at our center, of which two-thirds are for new patients. Assuming that 25% have MIBC, 135 NMIBC cases are expected to be registered each year. We also foresee that of these patients, 30% will have a tumor smaller than 1 cm, 20% will be unwilling to participate, and 10% will be lost to follow-up, which will leave an average of 68 patients per year to include in the study. An extra 10% of patients could be lost for unexpected reasons, so we expect a final total enrollment of 306 patients over a period of 5 yr. According to the registry records, 65% of these patients are expected to have high-risk disease. Assuming that only 60% of these BCG-indicated cases actually receive BCG (given the shortage) and that 40% will be unresponsive, we foresee including 45–47 BCG-unresponsive patients during the study period.

3.8. Schedule for analyses

With the assumption that sample collection might extend to the end of 2026 because of the delays that have already occurred, we expect to start DNA and RNA extraction from the samples in mid-2026, and library preparation would be completed by the end of 2027 (with an assumed plan of performing 16–24 samples per batch, one batch per month, 12–18 batches in total). Sequencing of the libraries and primary/secondary analyses of the outputs could be finalized in the following year. Depending on the future research questions and/or opportunities in participating (international) research collaborations, tertiary analyses can be performed afterwards. Any analysis of the association of potential signatures with oncological outcomes can be performed when the follow-up data for participants is collected by the end of 2030 (or 2031 in case of a delay).

3.9. Patient and public involvement

This study protocol was written without patient involvement. Patients were not invited to comment on the study design, to consult on the development of patient-relevant outcomes, or to interpret the results. Patients were also not invited to contribute to the writing or editing of this document for readability or accuracy.

3.10. Ethics and dissemination

The study complies with the Declaration of Helsinki and the principles of good clinical practice. The study protocol, informed consent forms, and biosample collection have been approved by the ethics committee of UZ/KU Leuven (reference S59371; April 19, 2018) and UZ Leuven BioBank. Patients who agree to participate in this study provide written informed consent.

The study results will be disseminated to clinicians, patients, and funders via publication in peer-reviewed journals, presentations at national and international scientific meetings, and social media.

Owing to ethical restrictions, only anonymized data will be available to the steering committee on request. Anonymized patient data can be requested for scientific studies. The steering committee of the CaBLe Consortium will evaluate all such requests.

4. Discussion

The aim of this project is to create a biobank of blood, urine, and BC tissue samples from patients diagnosed with BC alongside prospectively collected clinical data. The samples collected will be used to perform multiomics and immunohistopathological analyses. Results from these analyses will allow us to determine the molecular characteristics of NMIBC pathophysiology, with a focus on NMIBC stratification and the mechanisms underlying nonresponsiveness to BCG treatment, which will facilitate a better understanding of the characteristics and the broad spectrum of NMIBC phenotypes. This could potentially allow for better and more reliable risk stratification of patients in comparison to the current risk calculators. A comprehensive depiction of the mechanism(s) of nonresponsiveness to intravesical BCG treatment and the molecular alterations underlying NMIBC heterogeneity could reveal unique molecular pathways that might guide future drug development by identifying novel therapeutic targets. Better stratification of NMIBC will help in tailoring treatment in a more personalized way, which in turn will have a significant direct impact on survival and patients' quality of life. However, these aims should be addressed in future studies for which specific research questions should be formulated and specific study protocols will need to be written.

Although prospective clinical data collection has been running in seven other nonacademic Flemish hospitals, collection of biosamples has only been planned (and started) in the Department of Urology in UZ Leuven. We are planning the infrastructure for sample collection and delivery to UZ Leuven BioBank for centers that want to participate in this project. This will undoubtedly increase patient recruitment,

which in turn will increase the study power and shorten the time to reach the target patient number.

A similar, prospective, multicenter, clinical cohort study (COBLAnCE) has been initiated in France in 13 hospitals, with planned enrollment of 2000 patients that started in December 2012 [26]. Over a recruitment period of 6 yr (2012–2018), 1800 patients with BC (1195 [66.4%] with NMIBC) were included and will be followed up for 6 yr [27]. Different from our study, toenail clippings and data on quality of life and resource use are being collected in COBLAnCE. Even though we will have several samples from MIBC patients, as the samples are collected before histopathological diagnosis, our study is mainly focused on NMIBC. However, we plan to use the samples and data from NMIBC and MIBC patients to establish collaborations with other groups.

Another multicenter, large-scale, population-based biobanking infrastructure (UROSCAN biobank) was established in 2013 among nine hospitals in the Southern Healthcare Region in Sweden, and 1825 patients with BC (90% primary disease) had been included up to mid-2021 [28]. Since 2018, the biobanking has been coupled to sequencing (UROSCANSEQ), with real-time transcriptomic profiling performed for 605 patients, so molecular subtype classification according to the Lund taxonomy was available for 553 samples [15]. Using results from this biobanking and sequencing, the group plans to investigate several questions in a population-based and adequately powered setting. These research questions include prediction of patients with T1 disease who have a higher risk of progression for whom an early radical cystectomy (RC) could be recommended, and identification of the subgroup of patients with MIBC for whom neoadjuvant chemotherapy would be ineffective or not necessary so that upfront RC could be performed.

Our aim to begin enrollment in January 2020 was disrupted by the COVID-19 pandemic, so the first patient was enrolled in September 2020, followed by another pause because of the second wave of the pandemic. To date, 82 patients have been included. FFPE BC tissue samples (95 NMIBC, seven MIBC) archived before initiation of this study have been used to generate genomic data (TruSight Oncology 500 targeted gene panel; Illumina, San Diego, CA, USA) and transcriptomic data (bulk RNA sequencing; CORALL FFPE Whole Transcriptome kit, Lexogen, Vienna, Austria) that have been used in the retrospective part of the ATHENA study [29]. In the prospective part of ATHENA, FFPE samples collected during the ProCaB study will be used. Moreover, single-nucleus sequencing will be performed on the 16 fresh-frozen tissue samples collected within this study.

Bessa et al [30] identified the priorities for BC research in a collaboration involving health care professionals and patients. Out of ten unanswered research questions, the items ranked second, third, and fourth were on developing biomarkers for better stratification of NMIBC, predicting response to therapies, and selecting high-risk patients for effective radical treatments. We hope that this study will collect important clinicopathological data and biological samples, and the multiomics and immunohistopathological analyses performed on these samples will have the potential to answer these questions.

We foresee that the target analyses performed after data collection will include the following:

1. Participating either in the discovery phase or as an external validation cohort for the classification system being developed by the NMIBC Consensus Classification Consortium with bulk RNA sequencing data;
2. External validation of the Bladder Cancer Prognosis Programme RNA classification developed by Goel et al [31] with combined exome and transcriptome sequencing data;
3. Differentiation of BCG nonresponders from BCG responders using single-nucleus sequencing data; and
4. Better stratification (with or without the creation of new molecular subgroups) of NMIBC tumors according to multiomics data from transcriptome, exome, and methylation sequencing.

We hope that results from these analyses will allow better stratification of patients for more appropriate decisions on adjuvant intravesical therapies, such as which intermediate-risk subgroups should receive BCG and chemotherapy, which subgroups can avoid intravesical treatment, and which T1 or high-risk subgroups might not respond to BCG treatment and/or have a higher risk of progression to MIBC and should be counseled regarding early RC. The results may also help in selecting biomarkers that could be safely and effectively used in patient follow-up to reduce the frequency and number of control cystoscopies, which would ultimately minimize the economic and psychological burden of NMIBC treatment.

5. Summary

ProCaB is a single-center non-interventional prospective study that will collect urine, blood, and tumor samples from eligible patients diagnosed with BC, along with routine clinicopathological and follow-up data. The samples and data will be used in the future for elucidation of disease heterogeneity, better patient stratification, and the development of novel markers.

Author contributions: Frank Van der Aa had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Akand, Joniau, Van der Aa.
Acquisition of the data: Akand.
Analysis and interpretation of data: Akand, Joniau, Van der Aa.
Drafting of the manuscript: Akand
Critical revision of the manuscript for important intellectual content: Van der Aa, Joniau, Van Cleynenbreugel, Gevaert, Muilwijk.
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Supervision: Van der Aa, Joniau.
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***Ethics considerations*:** The study protocol has been approved by the ethics committee of UZ/KU Leuven (reference S59371; April 19, 2018) and UZ Leuven BioBank. Patients who agree to participate in this study provide written informed consent.

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