Validation of a novel, sensitive and specific urine-based test for recurrence surveillance of patients with non-muscle invasive bladder cancer in a comprehensive multicenter study

Rui Batista[1,2,3,4]*, João Vinagre[1,2,3,5], Hugo Prazeres[1,2,3,5,6,7], Cristina Sampaio[1,2], Pedro Peralta[5], Paulo Conceição[5], Amílcar Sismeiro[5], Ricardo Leão[8,9], Andreia Gomes[8], Fredrico Furriel[9], Carlos Oliveira[8], João Nuno Torres[8], , Pedro Eufrásio[9], Paulo Azinhais[9], Fábio Almeida[10], Edwin Romero Gonzalez[11], Bohdan Bidovanets[12], Thorsten Ecke[13], P. Minnee[14], Álvaro Serrano Pascual[15], Rabehi Abdelmalek[16], Ainara Villafruela[17] Pastora Beardo-Villar[18] Nuno Fidalgo[19], Hakan Öztürk[20], Carmen Gonzalez-Enguita[21], Juan Monzo[21], Tomé Lopes[22], Luis Martínez-Piñeiro[23], Patricia Parra Servan[24], Santiago Moreno Perez De La Cruz[25], Mario Pual Sanchez Perez[25],Valdemar Máximo[1,2,4] Paula Soares[1,2,3,4,5]

[1] i3S -Institute for Research and Innovation in Health, Porto, Portugal

[2] Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

[3] U-Monitor Lda, Porto, Portugal

[4] Faculty of Medicine, Universidade do Porto, Porto, Portugal

[5] Department of Pathology, Faculty of Medicine, University of Porto, Porto, Portugal

[6] Urology Service, Portuguese Institute of Oncology, Coimbra, Portugal

[7] Pathology Service, Portuguese Institute of Oncology, Coimbra, Portugal

[8] Hospital de Braga, Braga, Portugal

[9] Hospital CUF Coimbra, Coimbra, Portugal

[10] Hospital Universitário Fernando Pessoa, Porto, Portugal

[11] Hospital Zafra-Llerena, Badajoz, Spain

[12] Ternopil Regional Oncology Center, Ternopil, Ukraine

[13] Department of Urology, Helios Hospital, Bad Saarow, Germany

[14] LangeLand ziekenhuis St. Anna Ziekenhuis, Zoetermeer, Netherlands

[15] Servicio de Urología. Hospital Clínico San Carlos, Madrid, Spain

[16] El Eulma, Algeria

[17] Hospital Universitario Donostia, San Sebastian, Spain

[18] Hospital Universitario de Araba, Vitória, Spain

[19] Hospital Garcia de Horta, Lisboa, Portugal

[20] Department of Urology, Medicalpark Izmir Hospital, Izmir, Turkey

[21] Hospital Univers. Fund. Jiménez Díaz, Madrid, Spain

[22] Hospital de Santa Maria, Lisboa, Portugal

[23] Hospital Universitário La Paz, Madrid, Spain

[24] Hospital de Mérida, Badajoz, Spain

[25] Hospital Universitário de Badajoz, Badajoz, Spain

*** Correspondence:**
Rui Batista
rbatista@ipatimup.pt

Keywords: Non-muscle invasive bladder cancer, TERT promoter mutation, FGFR3 mutation, Urinary test, Uromonitor®

This manuscript contains 7 figures and 3 tables. Supplementary Material contains 2 imagens and 7 tables

Abstract

Bladder cancer (BC), the most frequent malignancy of the urinary system, is ranked the sixth most prevalent cancer worldwide. 70-75% of all newly diagnosed patients with BC will present disease confined to the mucosa or submucosa, the non-muscle invasive BC (NMIBC) subtype. Of those, approximately 70% will recur after transurethral resection (TUR). Due to high rate of recurrence, patients are submitted to an intensive follow-up program maintained throughout many years, or even throughout life, resulting in an expensive follow-up, with cystoscopy being the most cost-effective procedure for NMIBC screening. Currently, the gold standard procedure for detection and follow-up of NMIBC is based on the association of cystoscopy and urine cytology. As cystoscopy is a very invasive approach, over the years, many different non-invasive assays (both based in serum and urine samples) have been developed in order to search genetic and protein alterations related to the development, progression and recurrence of BC. TERT promoter mutations and FGFR3 hotspot mutations are the most frequent somatic alterations in BC and constitute the most reliable biomarkers for (BC).

Based on these findings, we developed an ultra-sensitive, urine-based assay called Uromonitor®, capable of detecting trace amounts of TERT promoter and FGFR3 hotspot mutations, in tumor cells exfoliated to urine samples. Cells present in urine were concentrated by the filtration of urine through filters were tumor cells are trapped and stored until analysis, presenting long-term stability. Detection of the alterations was achieved through a custom made, robust and highly sensitive Multiplex Competitive Allele-Specific Discrimination PCR allowing clear interpretation of results.

In this study we validate a test for NMBIC recurrence detection, using for technical validation a total of 331 urine samples and 41 formalin-fixed paraffin-embedded tissues of the primary tumor and recurrence lesions from a large cluster of Urology Centers.

In the clinical validation we used 185 samples to access sensitivity/specificity in the detection of NMIBC recurrence vs cystoscopy/cytology and in a smaller cohort its potential as a primary diagnostic tool for NMIBC. Our results show this test to be highly sensitive and specific in detecting recurrence of BC in patients under surveillance of NMIBC.

1 Introduction

Bladder cancer is the most frequent malignancy involving the urinary system and affects approximately 4 times more males than females (Miyazaki and Nishiyama, 2017). Worldwide, bladder cancer is the seventh most diagnosed cancer in men; when considering both genders, it ranks the the tenth most diagnosed and the sixth position in prevalence (Ferlay J, 2012). Of all patients newly diagnosed with bladder cancer, around three quarters present disease confined to the mucosa or submucosa (Sanli et al., 2017), the so-called, non-muscle invasive bladder cancer (NMIBC) subtype (M. Babjuk, 2018). The remaining are classified as muscle invasive bladder cancer (MIBC) reflecting their capacity to infiltrate the muscle layer of the bladder (Alfred Witjes et al., 2017; Sanli et al., 2017). The current treatment for NIMBC is the transurethral resection (TUR); following TUR treatment, 70% of the NMIBC patients will recur after primary tumor removal and 10 to 20% will recur as muscle-invasive bladder cancer and with the capacity to progress and develop metastatic disease (Antoine G. van der Heijden, 2009; Kaufman et al., 2009; Chamie et al., 2013). This high rate of recurrence requires that patients are submitted to an intensive follow-up program. Major guidelines from European Association of Urology (EAU) and American Urological Association (AUA) recommend cystoscopy and urinary cytology that, depending from the grade, can be as often as every 3 months in the first 2 years, semi-annually during the subsequent 3 years and annually thereafter (Kassouf et al., 2016, Alfred Witjes et al., 2017; M. Babjuk, 2018).). This intensive follow-up is maintained throughout many years following the initial diagnosis and indicates bladder cancer as a type of cancer with the most expensive follow-up (Kamat et al., 2011; Yeung et al., 2014). Cystoscopy is invasive and uncomfortable for patients due to the technical requirements of the procedure, still, it renders the more accurate diagnosis method for bladder cancer (Geavlete et al., 2012). Contrarily to cystoscopy, the non-invasive urine cytology is an economical approach, easier to perform, and when high-grade tumors are considered, the sensitivity is high (84%). The major limitation of urine cytology is its overall sensitivity to detect low-grade tumors (as NMIBC) were the sensitivity decreases to 16%, precluding its use in detection of those lesions (Yafi et al., 2015). The combination of all these facts leads to the opportunity for developing new, alternative and minimally invasive methods to detect bladder cancer. As urine is in direct contact with the inner part of bladder, cells from the epithelium, including scammed cells from bladder tumors can exfoliate and be detected in urine and used to evaluate and monitor the presence of neoplasia in a non-invasive approach (Botezatu et al., 2000; Zwarthoff, 2008; Ralla et al., 2014; Critelli et al., 2016; Togneri et al., 2016). Over the years, many different non-invasive assays have been developed in order to search genetic and protein alterations well known to be involved in the development, progression and recurrence of bladder cancer, both in serum and urine samples, with the purpose to diagnose and monitor bladder cancer (Soloway et al., 1996; Fradet and Lockhard, 1997; Pode et al., 1999; Kruger et al., 2003; Tetu et al., 2005; Moonen et al., 2007; Halling and Kipp, 2008; Serizawa et al., 2011; Goodison et al., 2012; Kinde et al., 2012; Kinde et al., 2013; Allory et al., 2014; Bansal et al., 2014; Hurst et al., 2014; Ralla et al., 2014; Wang et al., 2014; Ellinger et al., 2015; Yafi et al., 2015; Springer et al., 2018). Some of these tests presented values of sensitivity and specificity higher than urinary cytology and achieved FDA-approval for bladder cancer diagnosis. Despite high sensitivities and specificities, all these molecular assays present inconvenient rates of false positive results (Hajdinjak, 2008; Dimashkieh et al., 2013; Gopalakrishna et al., 2017; Springer et al., 2018). False positive rates could result from several factors, including the presence of benign conditions as haematuria, cystitis, lithiasis, urinary tract infections, inflammation or even because of repeated instrumentation, such as cystoscopy (Parker and Spiess, 2011; Dal Moro et al., 2013). A meta-analysis about the performance of urinary biomarkers concluded that most of the available urinary biomarkers do not detect the presence of bladder cancer in a proportion of patients and allow false-positive results in others, more frequently in low-stage and low-grade tumors (Chou et al., 2015). So, more reliable biomarkers and assays are needed for earlier detection of bladder cancer recurrence, particularly in low-grade and low-

stage NMIBC. Recently, telomerase reverse transcriptase (TERT) promoter mutations emerged as a novel biomarker and detected in up to 80% of bladder cancer, independently of stage or grade (Rachakonda et al., 2013; Allory et al., 2014; Hurst et al., 2014; Hosen et al., 2015). TERT promoter (TERTp) mutations are the most common event across stages and grades in malignant bladder tumors, strongly suggesting its participation in the two major genetic pathways of urothelial tumorigenesis (Allory et al., 2014; Hurst et al., 2014). These features point TERTp mutations as a game changer in bladder cancer and pointed them to be considered as a useful urinary biomarker for disease monitoring and early detection of recurrence, even in low-grade NMIBC, where urinary cytology usually lacks sensitivity (Allory et al., 2014; Hurst et al., 2014; Vinagre et al., 2014; Descotes et al., 2017). TERTp mutations are not present in inflammatory or urinary infections, different from previously described urinary biomarkers (Raitanen et al., 2001; Chou et al., 2015; Descotes et al., 2017). TERTp mutations assumed a novel pivotal role, even surpassing the frequency of the oncogene-activating mutations in fibroblast growth factor receptor 3 (FGFR3) gene in NMIBC (Netto, 2011; Humphrey et al., 2016) one of the most relevant drivers of urothelial transformation. Cappellen et al., reported FGFR3 mutations in bladder cancer with a frequency of 35% and subsequent studies established this frequency in approximately half of the primary bladder tumors (Cappellen et al., 1999; Sibley et al., 2001); several studies report its presence in up to 80% regarding early stage and low-grade tumors, and as absent or a very rare event in high-grade and invasive tumors (Billerey et al., 2001; van Rhijn et al., 2003; Hernandez et al., 2006; Tomlinson et al., 2007; Pandith et al., 2013). FGFR3 assumes also an important role as a predictive biomarker due to the development of FGFR3 targeted therapies. KRAS mutations although found in a lower percentage (11.5%) of bladder cancers, are assuming a relevant position since the detection of KRAS mutations in conjunction with the previous alterations could improve the sensitivity of a biomarker panel (Alexander et al., 2012).

The uniqueness of TERTp mutations, mainly its location in a promoter region with a C:G base pair content >50% precluded that traditional methods using standardized conditions (conventional real-time assays or even next generation sequencing techniques) could be used with an efficient output.

With this goal in mind we developed an ultra-sensitive assay based on real-time PCR (with a proprietary reaction chemistry and probes), a urine-based test capable of detecting trace amounts of the most common alterations in NMIBC, TERTp and FGFR3 hotspot mutations, in urine samples.

2 Material and methods

All procedures described in this study were in accordance with national and institutional ethical standards and previously approved by Local Ethical Review Committees.

2.1 Sample collection

2.1.1 Urine samples

Urine samples were collected during routine urology appointments and previously to cystoscopic intervention. Urine samples were filtered through a 0.80µm nitrocellulose syringe filter containing a conservative storage buffer. Filters were stored at 2-8ºC for a maximum of 1 month until DNA extraction procedure.

2.1.2 Tissue samples

Formalin-fixed paraffin embedded (FFPE) tissues from primary tumor and/or from recurrent lesions from the cohort in study were retrieved from the files of the Instituto Português de Oncologia de

Coimbra Francisco Gentil, E.P.E (IPOC-FG). Clinicopathological and follow-up data were retrieved from the files of the Department of Pathology of IPOC-FG.

2.2 Cohort's characteristics

We studied a total of 372 samples (331 urine samples and 41 formalin-fixed paraffin-embedded (FFPE)) collected from 18 Urology Centers (supplementary Table 1). Technical validation of the assay was done in an independent setting where we studied a total of 334 samples from urine and FFPE (presented below). Clinicopathological and follow-up data were retrieved from the files of the centers involved in this study.

2.2.1 Urine and FFPE cohorts

The main aim of our study was to validate our new molecular panel in a non-invasive setting and for technical validation we analysed a total of 331 urine samples. The clinical validation, including sensitivity, sensibility, PPV and NPV, was done using data from 185 patients.
Thus, urine samples from 185 patients (77% males and 33% females), with a median age of 71 years (range 25-91) were used and were sub-divided in independent groups,(that may overlap samples) (Table 1). FFPE tissues of the primary tumor (n=9) and/or of the recurrence lesions (n=32) were also analysed.

2.2.2 Cystoscopy, Cytology and tumor resected evaluation

Cystoscopy was considered positive when the urologist considered the presence of a lesion deserving surgical treatment (despite the pathology result of the resected lesion).
Urine cytology and tissue pathology were performed by each Pathology Department from each centre. In 41 cases the diagnosis was confirmed in the histological examination of the lesion in the TUR.

2.3 DNA extraction

2.3.1 Urine samples

Filters used for urine filtration were stabilized at room temperature for 30 minutes. Upon filtration, quality DNA for further processing is obtained on filters that can be stored at 4ºC up to 3 months (Supplementary figure 1). In an inverted position, filters were attached to a 2 mL microcentrifuge tube and a cell lysis solution was injected through each filter and collected in microcentrifuge tube. Filtered lysates were incubated at 60ºC for 30 min with 30µL of Proteinase K and exposed to chaotropic lysis/binding buffer to release nucleic acids and protect the genomic DNA from DNases. The microcentrifuge tubes content were then processed according to the manufacturer's protocol of the Norgen® Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek Corp, Canada).

2.3.2 Tissue samples

DNA from FFPE tissues was retrieved from 10-µm cuts after careful manual dissection. Slides were deparaffinised in xylene (2x 10 minutes), followed by incubation in 100% alcohol (2x 5 minutes). Tumor tissue was removed from the slides to a 1.5 mL microcentrifuge tube. DNA extraction was performed using the Ultraprep Tissue DNA Kit (AHN Biotechnologie, Germany) according to the manufacturer's instructions. DNA extracted was quantified by spectrophotometry using Nanodrop ND-1000, and quality was assessed by analysis of 260/280nm and 260/230 nm ratios.

2.4 Urine testing workflow

The test is a custom-made full working procedure developed and optimized for the detection in a Real-Time PCR platform of oncogene hotspot mutations in bladder cancer tumor cells exfoliated to urine. (Figure 1).
Mutation detection is achieved by real-time PCR amplification curve analysis. Positive and negative mutation control samples are included in each run to ensure assay's validity. For TERTp -124C>T and -146C>T alterations screening, we developed an improved Real-Time Allelic Discrimination assay, with the use of Lock Nucleic Acid (LNA probes) (Figure 2A, 2B). LNA probes allowed to modulate the melting temperature on specific bases of the probe enhancing the possibility to achieve preferential melting temperatures in short probe sequences. This enhanced competitive probe strengthens allelic discrimination, allows higher stability on probe binding with the correct sequence, and higher binding melting temperature differences on the presence of a base pair mismatch (Supplementary Figure 2).
For FGFR3 mutations selected to screening, we designed for each mutation, a mutation allele primer, a wild-type allele blocker, a locus reverse primer and a fluorescent probe for real-time detection of the generated amplicon (Figure 2C, 2D). The use of a molecular blocker completely suppresses the amplification of the wild type allele in order to not interfere with the amplification of the mutant allele. By this technique we improved current detection thresholds enhancing the ability to detect a minimal quantity of altered cells in a large pool of cells without alterations. The screening of KRAS hotspot alteration was achieved through the use of a custom-made mutation detection procedure developed similarly to FGFR3 hotspot mutations detection procedure, rendering this method suitable for detection of mutations in bladder cancer tumor cells exfoliated to urine.
TERT FGFR3 and KRAS testing was performed in approximately 25 ng of DNA extracted from cells in each filtered urine, or from 25 ng of DNA extracted from FFPE tissues, either primary tumor and/or recurrent lesions. The extracted DNA was amplified and detected on a qPCR real-time machine, using the proprietary chemistry for amplification and detection as provided in the Uromonitor® test kit for the targeted nucleotide changes in TERTp and FGFR3 gene.

2.5 Uromonitor® technical validation

Uromonitor® precision was analysed by a reproducibility test. To achieve this, 10 samples were amplified and analysed using Uromonitor® test (8 samples harbouring mutations and 2 samples wild-type for the alterations of interest). These samples were amplified 5 times for each alteration, 1 week apart of each amplification, for 5 weeks. Uromonitor® accuracy was analysed by two independent tests. First, it was necessary to ensure that a test containing a sample without DNA or with DNA that doesn't harbour any of the alterations of interest, did not generate an analytical signal that may indicate a low concentration of mutation (analytical false positive). It was also necessary to access the accuracy of the results produced by Uromonitor® test comparing it to the standard method in the detection of the alterations in study (Sanger Sequencing). All the samples were validated by Sanger sequencing for the alterations in study.

2.5.1 Uromonitor® precision and accuracy in urine samples

To test accuracy in urine samples, 36 samples negative for all the mutations in study (status obtained by Sanger Sequencing) and 36 "Blank" samples (without DNA) were amplified for each alteration (false-positive testing).
Also, 252 blind tests from urine samples were analysed (73 tests for TERTp -124 assay, 72 tests for TERTp -146 assay, 55 tests for FGFR3 248 assay and 52 tests for FGFR3 249 assay).

2.5.2 Uromonitor® precision and accuracy in FFPE tissue samples

Uromonitor® test could also be used to screen FFPE samples in patients with a history of NMIBC. To test accuracy in FFPE tissue samples, 9 samples negative for all the mutations in study (status obtained by Sanger Sequencing), 36 "Blank" samples (without DNA) were amplified for each alteration (false-positive testing). Also, 483 tests from FFPE tissue samples were analysed (201 tests for TERTp -124 assay, 200 tests for TERTp -146 assay, 41 tests for FGFR3 248 assay and 41 tests for FGFR3 249 assay).

2.5.3 TERTp detection limit threshold

Uromonitor® includes TERTp alteration detection by Real-Time PCR by LNA Allelic discrimination probes. High GC content and thorough optimization of the amplified TERTp alterations characterize this innovative test. Since TERTp mutation detection by current methods have low sensitivity, a detection limit threshold was obtained for TERTp alterations included by the technology in the Uromonitor®. To achieve this, we performed 2-fold serial dilutions of genomic DNA containing the studied alteration (100% of mutated DNA) in genomic DNA Wildtype for the studied alterations. Serial dilutions were amplified for the corresponding detection assay. This procedure was repeated for both TERTp alterations that comprise the Uromonitor® test (figure 3).

2.5.4 Statistical analysis

Statistical analysis was performed using 21.0 SPSS Statistical Package (SPSS, Inc.,220 2003). Descriptive statistic was done and results are expressed as percentages and mean ± standard deviation.

3 Results

3.1 Genetic alterations technical validation

Uromonitor® precision was analysed, achieving a 100% concordance in a reproducibility test. In urine samples, accuracy tests (comparisons to Sanger sequencing), TERTp -124 assay achieved 100% and TERTp -146 assay 98.6%., FGFR3 248 assay 87.3% and FGFR3 249 assay 94.2%. Overall, Uromonitor® test presented a combined accuracy of 95.0%. Uromonitor® test accuracy In FFPE tissue samples, accuracy (comparison to Sanger Sequencing) achieved 98.5% for TERTp -124 assay, 99.5% for TERTp -146 assay, 90.2% for FGFR3 248 assay and 97.6% for FGFR3 249. For all assays, Uromonitor® achieved a combined 96.5% accuracy, (Supplementary Table 2). The test presented no false positives in samples without DNA (Blank samples). A combined accuracy lower than 100%, is justified by the detection of positivity by Real-Time PCR in samples for which Sanger sequencing fails to detect alteration due to lack of sensitivity.
In all the assays, the analytical limit detection threshold was 6.25% of mutant sequences in a background of wild-type DNA. The presence of altered DNA in less than 6.25% of the total DNA in the sample may not be detected.

3.1.1 Molecular characterization of urine samples

From the initial cohort of 331 urine samples, 304 were fully characterized for the alterations targeted by Uromonitor® test. From these, TERTp mutations were detected in 50.6% of cases (39.0% presented the -124C>T and 11.7% with the -146C>T) and FGFR3 mutations were detected in 49.4% of cases (31.2% at codon 248 and 18.2% at codon 249 of FGFR3 protein). Further correlations with clinical data were performed for 185 samples.

3.2 Clinical validation

3.2.1 Recurrence follow-up cohort

In the follow-up cohort (n=122), 28% (n=34) of the tumors recurred (confirmed by histology) of the TUR, whereas the remaining 72% (n=88) were negative for recurrence.

3.2.1.1 Comparison with cytology and cystoscopy methods

We analysed and compared follow-up recurrence detection of Uromonitor® in NMIBC in comparison to routinely used screening methods such as cystoscopy and/or cytology.
Uromonitor® sensitivity was 73.5% in the detection of TUR confirmed recurrence, with a specificity of 73.2% (Figure 4, Table 3, Supplementary Table 3). The values were comparable and similar to gold-standard cystoscopy performance that in the follow-up series presented values of 79.4% and 73.2% for sensitivity and specificity, respectively (Figure 4, Table 3, Supplementary Table 3).
Uromonitor® sensitivity performance was much higher than cytology (42.9% cytology sensitivity VS 73,5% Uromonitor® sensitivity (Figure 4, Table 3, Supplementary Table 3).
When cytology was combined with cystoscopy, they jointly achieve an increased sensitivity to 86.7% and a slightly decreased of specificity to 87.9% due to the increased rate of cytology false positives (Figure 4, Table 3, Supplementary Table 3). Although the combination with cytology presents an upgrade to cystoscopy per se, a greater benefit is obtained when combining Uromonitor® with cystoscopy, granting together a 100% sensitivity and 88.6% specificity, clearly demonstrating an upgrade in sensitivity and specificity relative to the "cystoscopy + cytology" screening method (Figure 4, Table 3, Supplementary Table 3).
To improve Uromonitor® test performance we analysed a subset of samples (Uromonitor®+KRAS follow-up cohort) for another oncogene activated in bladder cancer, KRAS hotspot alterations and compared follow-up recurrence detection to routinely used surveillance methods.
TERT/FGFR/KRAS hotspot increased sensitivity to 100% in the detection of TUR confirmed recurrence and with a specificity of 83.3 (Figure 4, Table 3, Supplementary Table 3). The values were higher when compared to cystoscopy performance that in the follow-up series achieved 79.4% and 73.2% for sensitivity and specificity, respectively (Figure 4, Table 3, Supplementary Table 3).
TERT/FGFR/KRAS sensitivity performed higher than cytology (42.9% sensitivity and 93.9% specificity) (Figure 4, Table 3, Supplementary Table 3).
When cytology was combined with cystoscopy, they jointly achieve an increased sensitivity of 86.7% and a slightly decreased specificity to 87.9% due to the increased rate of false positives (Figure 4, Table 3, Supplementary Table 3). Although this combination presents an interesting upgrade to cystoscopy per se, it does not achieve TERT/FGFR/KRAS molecular testing performance per se or molecular testing in combination with cystoscopy screening method (Figure 4, Table 3, Supplementary Table 3).

3.2.2 Genetic alterations distribution in recurrences and initial diagnostic positive cases

Regarding the specific mutations detected in positive urine samples from the follow-up cohort, TERTp mutations were detected in 52.0% of cases (44.0% presented the -124C>T and 8.0% with the -146C>T) and FGFR3 mutations were detected in 40.0% of cases (28.0% at codon 248 and 12.0% at codon 249 of FGFR3 protein). A percentage of 8.0% of cases presented 2 concomitant alterations (2 cases with TERTp -124C>T and with alterations in codons 248 or 249 of FGFR3) (figure 5).
Regarding the specific mutations detected urine samples from initial diagnosis patients cohort positive for Uromonitor assay, TERTp mutations were detected in 35.7% of cases (21.4% presented the -124C>T and 14.3% the -146C>T mutation) and FGFR3 mutations were detected in 28.6% of cases (14.3% at codon 248 and 14.3% at codon 249 of FGFR3 protein). A percentage of 35.7% of cases presented TERTp and FGFR3 concomitant alterations (1 case with -124C>T & 248, 1 case with -124

& 249, 1 case with -146 & 249 and 2 cases with 248 & 249 alterations, (figure 5). Nine cases in the follow-up cohort and 14 cases in the initial diagnosis cohort did not present any of the TERTp or FGFR3 screened alterations (figure 5).

3.2.2.1 Correlation with stage/grade

Tumor stage information in cases positive for recurrence was available for 26 patients. The majority of recurrence positive cases were for stage Ta (50.0%), with T1 and Tis representing 27.0% and 19.2%, respectively. (Figure 6, Supplementary Table 4) Regarding the grade, the majority of recurrence positive tumours were high-grade (66.7%) being the remaining (33.3%) low-grade cases (Figure 7, Supplementary Table 5). In Cis/Tis recurrence positive patients, Uromonitor® achieved a 100% detection rate while in patients that recurred with a Ta tumor detection rate was of 53.8%. For T1 stage positive patients the detection rate was 71.4%. (Figure 6, Supplementary Table 6). In Low Grade recurrence positive patients, Uromonitor® achieved a 62.5% detection rate while in patients that recurred with a High Grade, Uromonitor® tumor detection rate was of 75%. (Figure 7, Supplementary Table 7). One case (3.8%) presented a hepatic metastasis, positively detected in the urine sample by Uromonitor® (Figure 6, Supplementary Table 4).

3.2.3 Initial diagnosis cohort analysis

3.2.3.1 Comparison with cystoscopy method

We analysed the diagnostic performance of the test in the initial diagnosis of bladder cancer in comparison to the usual screening methods such as cystoscopy and/or cytology.
Sensitivity was 50.0% in an initial diagnosis setting but with a specificity of 100% (Figure 4, Table 3, Supplementary Table 3). These values are low in comparison with cystoscopy's virtual sensitivity of 100%, although with a lower specificity of 88.6% (Figure 4, Table 3, Supplementary Table 3) are however much better than cytology which did not have sensitivity (0%) and achieved 86,7% specificity (Figure 4, Table 3, Supplementary Table 3).
We analysed the performance of Uromonitor®+KRAS in the initial diagnosis of bladder cancer in comparison to cystoscopy and/or cytology.
Uromonitor®+KRAS sensitivity was 93.3% in an initial diagnosis setting with a specificity of 80% (Figure 4, Table 3, Supplementary Table 3).

4 Discussion

TERT promoter mutations were firstly described in sporadic and familial melanoma (Horn et al., 2013; Huang et al., 2013) and since then they were reported in several cancers, such as central nervous system (43-51%), hepatocellular carcinoma (59%), thyroid (follicular cell-derived tumors) (10%) and notably in bladder cancer (59-80%) (Killela et al., 2013; Liu et al., 2013a; Liu et al., 2013b; Nault et al., 2013; Vinagre et al., 2013; Wu et al., 2014). For bladder cancer, the TERT promoter mutations are independent of stage or grade (Rachakonda et al., 2013; Allory et al., 2014; Hurst et al., 2014; Hosen et al., 2015) and were reported in both non-muscle and muscle invasive bladder cancer. As the current diagnosis and follow-up of patients with bladder cancer is highly invasive and expensive, new molecular markers are needed able to act in non-invasive approaches, in order to select an optimal treatment and follow-up for each patient (Kurth et al., 1995; Pandith et al., 2013; van Kessel et al., 2013). For this purpose, we developed a novel urine-based real-time assay, and in this study we present the technical and clinical performance of the detection of critical alterations in TERTp region and FGFR3 in DNA obtained from scammed cells of bladder present in urine. The main goal of the test is

to be able to predict recurrence in NMIBC per se or in combination with cystoscopy. In this study we analysed and compared its performance in the follow-up of recurrence and in an initial diagnosis setting in NMIBC and in comparison with routinely used screening methods such as cystoscopy and/or cytology. The first detected limitation of this study is the recurrence rate of only 28%. It would be expected a value ranging 60 to 70% as reported in the literature for NMINBC following TUR treatment [6-8]. The reason for this difference may reflect the restricted two year follow-up that patients were considered, or patient-related factors (age, gender, multiplicity, smoking status and adjuvant treatment) associated with recurrence frequency [68] that are not considered at this moment.

Sensitivity and specificity in the detection of TUR confirmed recurrence in the follow-up series is comparable to cystoscopy performance and in accordance with the literature that describes that about 20% of primary tumors lack TERTp and FGFR3 alterations, rendering an empirical detection rate around 80%. The remaining 20% can be attributed to tumors and recurrences that may undergo different tumorigenic pathways, other than the acquisition of TERTp and FGFR3 alterations that could preclude Uromonitor® testing capacity, like RAS mutations (see below).

Amongst the recurrence negative cases, the test was concordantly negative in 87.7% of cases. The mutational state of TERTp and FGFR3 genes in bladder cancer is considered a promising predictor for recurrence of NMIBC demonstrated by the association between FGFR3 mutation in primary tumor and later in recurrence events (Hernandez et al., 2006; Burger et al., 2008; Kompier et al., 2009; Miyake et al., 2010; Zuiverloon et al., 2010; van Rhijn et al., 2012). A decrease of the test specificity to 93.2% reflects the detection of three cases positive for FGFR3 mutations and three cases with TERTp mutations without evidence of recurrence by cystoscopy. These cases remained negative for recurrence during the 2 years follow-up, suggesting that they were false positives. TERTp mutations were extensively studied and are described in the literature as absent in normal tissue. Since a clear positive signal was obtained in the aforementioned cases, we hypothesize that Uromonitor® high sensitivity may be detecting microscopic lesions and that may predict the appearance of a macroscopic lesion in a longer-term period beyond the 2-years follow-up.

More than half of the cases presented at least one mutational event and it is reported that TERTp and FGFR3 mutations tend to occur more frequently together than per chance; the combination of both constitute a more reliable biomarker for NMIBC recurrence monitoring (Hosen et al., 2015; Critelli et al., 2016).

Uromonitor® sensitivity performance was higher than cytology, and if used as an adjunct to cystoscopy it allowed to achieve a 100% sensitivity and 88.6% specificity, an important upgrade in sensitivity and specificity in comparison to the cystoscopy and cytology combination. This data demonstrates that this test used together with cystoscopy at a routine level will lead to a cost-effectiveness increment. It can also be used per se without any decrease in performance relative to the current routinely used methods, such as cystoscopy or if cystoscopy is not routinely available.

Uromonitor® shown overall good performance in recurrence detection across all stages. In Cis/Tis tumors, that represent 20% of follow-up positive cases a peak performance was obtained with a 100% detection rate. For the majority of these Cis/Tis cases TERTp alterations were present in 4 out 5 cases (80.0%). Several authors reported that TERTp alterations are not associated to stage and FGFR3 hotspot alterations are rare in Cis/Tis tumors; this is concordant with our findings where only one patient presented a FGFR3 mutation.

This series was enriched for high-grade tumours which is in contrast to the majority of the reported series of NMIBC. This might have created a bias in the results and impacted the assay performance. Development of this test is actively based in TERTp/FGFR3 alterations detection that are more frequent in low-grade tumors; additionally, FGFR3 mutations are rare in high-grade tumours.

We also prospectively analysed and compared initial diagnosis performance in comparison to routinely used screening methods. In an initial diagnosis setting, sensitivity was lower in the detection of disease, compared to standard cystoscopy that is considered to virtual hold a 100% sensitivity (a value due to

the absence of confirmation of tumor existence on cystoscopy negative cases) and 88,6% specificity. Still, it should be taken into consideration that this test was not developed for initial diagnosis. Amongst the negative cases, the Uromonitor® test was concordant in 100% of cases being highly specific. In comparison with another routine assay, Uromonitor® largely surpasses cytology's sensitivity that could not detect any new-BC positive case (0% sensitivity).

Nevertheless, taking into consideration that Uromonitor® main performance is aimed for low-grade tumors, these series gave us the opportunity to improve Uromonitor® performance in a high-grade tumors enriched setting. To this purpose, we included a new biomarkers in the test in order to improve Uromonitor® detection rate and testing capabilities over-all; we want to achieve and maintain the high sensitivity and specificity that our test offers. This led to the KRAS hotspot alterations inclusion, and this preliminary data demonstrated that, either for follow-up or initial diagnosis cases, Uromonitor®+KRAS improves significantly, reaching a 100% sensitivity in follow-up detection and 93.3% in initial-diagnosis detection and with an overall performance of 95.2% regardless of grade. With the inclusion of KRAS hotspot mutation screening together with Uromonitor®, this preliminary data presents this non-invasive approach as a true alternative to cystoscopy for NMBIC follow-up or even as a population screening and/or initial diagnosis for bladder cancer. It is also noteworthy to mention that the inclusion of FGFR3 in Uromonitor® test, rises is usefulness as biomarker test for targeted therapy.

During the course of this study, an interesting case demonstrated the capacities of these novel tests in providing new information regarding disease progression. In a patient free of local disease a TERTp mutation was detected in urine. Although the patient did not present BC at the time, it had a hepatic metastasis. Further analysis confirmed that the hepatic metastasis presented a TERTp mutation. It will be worthwhile to investigate the usefulness of this test technology in the screening of other tumors and metastasis, namely those harbouring TERTp mutations, in urine samples.

In terms of the Uromonitor® performance in comparison with other available options it presented improved features. Reviewed by Sapre et al. (Sapre et al., 2014) the sensitivity of other available options ranges from 50.0% to 96.6%, and the tests are based in different methodologies approaches, some more technically challenging and maintaining invasive requirements for the procedure. Avoidance of invasive procedures for the patients was a concern in the development of this test since morbidity of cystoscopy is often underestimated and can impact on patient adherence, with surveillance rates as low as 40% (Schrag et al., 2003). The fact that the test is conducted in urine renders it safer for patient use and with better acceptance in comparison with conventional cystoscopy. Another important aspect in the development of Uromonitor® was the implementability across different centers or laboratories. For this implementability we concentrated on three pillars: ease of use, costs and response time. Being a real-time PCR-based method, it is already well implemented in most laboratories not requiring a specialized technician to execute the test, with affordable equipment and reduced costs, and most determinately, with the capacity to output a result in 6 hours. If we compare this approach with next generation sequencing (NGS)-based method it is promptly detected that such a fast response is not possible as it is required to have a sample and library preparation, failing the short-time frame response, the costs would increase with run and equipment requirements and NGS equipment is not widely available.

Overall, this study demonstrates that Uromonitor® represents a highly sensitive and specific urine test in detecting recurrence of NMIBC. The test could potentially alternate with cystoscopy or be used as a complement to cystoscopy in surveillance programs, diminishing the risk of missing recurrences, and with the benefit of alleviating the number of cystoscopy procedures that patients require to undergo. The rate of Uromonitor® false positives was similar to the rate of cystoscopy false positives. Our results prompt us to validate these findings in an enlarged robust independent series, in an ongoing study with a design that includes a group of benign conditions (renal lithiasis, urinary infections,

hyperplasia of the prostate and other). We intent to further test it to assess its cost-effectiveness and to determine its value in patient's follow-up.

5 Author Contributions

RB performed all studies and wrote the draft of the manuscript. HP participated in the genetic analysis. JV, HP, VM and PS supervised the entire project and gave critical comments on the manuscript. CS participated in the genetic analysis in the experimental design and managed the literature searches. PP, PC, AS, RL, AG, FA, EG, BB, TE, PM, ASP, RA, AV, PB, NF, HO, MG, JM, TL, LM, PPS, SC, AP and MP contributed to the sample and data collection for the study. All authors read and approved the final manuscript.

6 Funding

This study was supported by FCT ("Portuguese Foundation for Science and Technology") through a PhD grant to Rui Batista (SFRH/BD/111321/2015). Further funding was obtained from the project "Advancing cancer research: from basic knowledge to application" NORTE-01- 0145-FEDER-000029: "Projetos Estruturados de I & D & I", funded by Norte 2020—Programa Operacional Regional do Norte. This article is a result of the project 31438 (The other faces of Telomerase: Looking beyond tumor immortalization), supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI) and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior.

7 Conflict of Interest

RB, JV, HP and PS are the founders of U-Monitor Lda, owner of the Uromonitor® product. This company has licensed technologies from Ipatimup that are related to the work described in this paper (International Patent PCT/PT2016/050007 – Method, sequences, compositions and kit for detection of mutations in the htert gene promoter). The remaining authors declare that they have no conflict of interest.

8 Acknowledgments

We are grateful for the Cancer Signalling and Metabolism group for the indirect participation in this study, especially to Marcelo Correia for the manutention of Imortalized Cell lines important to this study.

9 References

Alexander, R.E., Lopez-Beltran, A., Montironi, R., MacLennan, G.T., Chen, G.R., Post, K.M., et al. (2012). KRAS Mutation Is Present in a Small Subset of Primary Urinary Bladder Adenocarcinomas. Laboratory Investigation 92, 188a-188a.

Alfred Witjes, J., Lebret, T., Comperat, E.M., Cowan, N.C., De Santis, M., Bruins, H.M., et al. (2017). Updated 2016 EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer. Eur Urol 71(3), 462-475. doi: 10.1016/j.eururo.2016.06.020.

Allory, Y., Beukers, W., Sagrera, A., Flandez, M., Marques, M., Marquez, M., et al. (2014). Telomerase reverse transcriptase promoter mutations in bladder cancer: high frequency across stages, detection in urine, and lack of association with outcome. Eur Urol 65(2), 360-366. doi: 10.1016/j.eururo.2013.08.052.

Antoine G. van der Heijden, J.A.W. (2009). Recurrence, Progression, and Follow-up in Non-Muscle-Invasive Bladder Cancer. European Urology Supplements, 556-562.

Bansal, N., Gupta, A., Sankhwar, S.N., and Mahdi, A.A. (2014). Low- and high-grade bladder cancer appraisal via serum-based proteomics approach. Clin Chim Acta 436, 97-103. doi: 10.1016/j.cca.2014.05.012.

Billerey, C., Chopin, D., Aubriot-Lorton, M.H., Ricol, D., Gil Diez de Medina, S., Van Rhijn, B., et al. (2001). Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. Am J Pathol 158(6), 1955-1959. doi: 10.1016/S0002-9440(10)64665-2.

Botezatu, I., Serdyuk, O., Potapova, G., Shelepov, V., Alechina, R., Molyaka, Y., et al. (2000). Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 46(8 Pt 1), 1078-1084.

Burger, M., van der Aa, M.N., van Oers, J.M., Brinkmann, A., van der Kwast, T.H., Steyerberg, E.C., et al. (2008). Prediction of progression of non-muscle-invasive bladder cancer by WHO 1973 and 2004 grading and by FGFR3 mutation status: a prospective study. Eur Urol 54(4), 835-843. doi: 10.1016/j.eururo.2007.12.026.

Cappellen, D., De Oliveira, C., Ricol, D., de Medina, S., Bourdin, J., Sastre-Garau, X., et al. (1999). Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 23(1), 18-20. doi: 10.1038/12615.

Chamie, K., Litwin, M.S., Bassett, J.C., Daskivich, T.J., Lai, J., Hanley, J.M., et al. (2013). Recurrence of high-risk bladder cancer: a population-based analysis. Cancer 119(17), 3219-3227. doi: 10.1002/cncr.28147.

Chou, R., Gore, J.L., Buckley, D., Fu, R., Gustafson, K., Griffin, J.C., et al. (2015). Urinary Biomarkers for Diagnosis of Bladder Cancer: A Systematic Review and Meta-analysis. Ann Intern Med 163(12), 922-931. doi: 10.7326/M15-0997.

Critelli, R., Fasanelli, F., Oderda, M., Polidoro, S., Assumma, M.B., Viberti, C., et al. (2016). Detection of multiple mutations in urinary exfoliated cells from male bladder cancer patients at diagnosis and during follow-up. Oncotarget 7(41), 67435-67448. doi: 10.18632/oncotarget.11883.

Dal Moro, F., Valotto, C., Guttilla, A., and Zattoni, F. (2013). Urinary markers in the everyday diagnosis of bladder cancer. Urologia 80(4), 265-275. doi: 10.5301/urologia.5000041.

Descotes, F., Kara, N., Decaussin-Petrucci, M., Piaton, E., Geiguer, F., Rodriguez-Lafrasse, C., et al. (2017). Non-invasive prediction of recurrence in bladder cancer by detecting somatic TERT promoter mutations in urine. Br J Cancer 117(4), 583-587. doi: 10.1038/bjc.2017.210.

Dimashkieh, H., Wolff, D.J., Smith, T.M., Houser, P.M., Nietert, P.J., and Yang, J. (2013). Evaluation of urovysion and cytology for bladder cancer detection: a study of 1835 paired urine samples with clinical and histologic correlation. Cancer Cytopathol 121(10), 591-597. doi: 10.1002/cncy.21327.

Ellinger, J., Muller, S.C., and Dietrich, D. (2015). Epigenetic biomarkers in the blood of patients with urological malignancies. Expert Rev Mol Diagn 15(4), 505-516. doi: 10.1586/14737159.2015.1019477.

Ferlay J, S.I., Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. (2012). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; .

Fradet, Y., and Lockhard, C. (1997). Performance characteristics of a new monoclonal antibody test for bladder cancer: ImmunoCyt trade mark. Can J Urol 4(3), 400-405.

554 Geavlete, B., Jecu, M., Multescu, R., and Geavlete, P. (2012). Narrow-band imaging cystoscopy in
555 non-muscle-invasive bladder cancer: a prospective comparison to the standard approach. Ther Adv
556 Urol 4(5), 211-217. doi: 10.1177/1756287212454181.
557 Goodison, S., Chang, M., Dai, Y., Urquidi, V., and Rosser, C.J. (2012). A multi-analyte assay for the
558 non-invasive detection of bladder cancer. PLoS One 7(10), e47469. doi:
559 10.1371/journal.pone.0047469.
560 Gopalakrishna, A., Fantony, J.J., Longo, T.A., Owusu, R., Foo, W.C., Dash, R., et al. (2017).
561 Anticipatory Positive Urine Tests for Bladder Cancer. Ann Surg Oncol 24(6), 1747-1753. doi:
562 10.1245/s10434-016-5763-5.
563 Hajdinjak, T. (2008). UroVysion FISH test for detecting urothelial cancers: meta-analysis of diagnostic
564 accuracy and comparison with urinary cytology testing. Urol Oncol 26(6), 646-651. doi:
565 10.1016/j.urolonc.2007.06.002.
566 Halling, K.C., and Kipp, B.R. (2008). Bladder cancer detection using FISH (UroVysion assay). Adv
567 Anat Pathol 15(5), 279-286. doi: 10.1097/PAP.0b013e3181832320.
568 Hernandez, S., Lopez-Knowles, E., Lloreta, J., Kogevinas, M., Amoros, A., Tardon, A., et al. (2006).
569 Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder
570 carcinomas. J Clin Oncol 24(22), 3664-3671. doi: 10.1200/JCO.2005.05.1771.
571 Horn, S., Figl, A., Rachakonda, P.S., Fischer, C., Sucker, A., Gast, A., et al. (2013). TERT promoter
572 mutations in familial and sporadic melanoma. Science 339(6122), 959-961. doi:
573 10.1126/science.1230062.
574 Hosen, I., Rachakonda, P.S., Heidenreich, B., de Verdier, P.J., Ryk, C., Steineck, G., et al. (2015).
575 Mutations in TERT promoter and FGFR3 and telomere length in bladder cancer. Int J Cancer 137(7),
576 1621-1629. doi: 10.1002/ijc.29526.
577 Huang, F.W., Hodis, E., Xu, M.J., Kryukov, G.V., Chin, L., and Garraway, L.A. (2013). Highly
578 recurrent TERT promoter mutations in human melanoma. Science 339(6122), 957-959. doi:
579 10.1126/science.1229259.
580 Humphrey, P.A., Moch, H., Cubilla, A.L., Ulbright, T.M., and Reuter, V.E. (2016). The 2016 WHO
581 Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder
582 Tumours. Eur Urol 70(1), 106-119. doi: 10.1016/j.eururo.2016.02.028.
583 Hurst, C.D., Platt, F.M., and Knowles, M.A. (2014). Comprehensive mutation analysis of the TERT
584 promoter in bladder cancer and detection of mutations in voided urine. Eur Urol 65(2), 367-369. doi:
585 10.1016/j.eururo.2013.08.057.
586 Kamat, A.M., Karam, J.A., Grossman, H.B., Kader, A.K., Munsell, M., and Dinney, C.P. (2011).
587 Prospective trial to identify optimal bladder cancer surveillance protocol: reducing costs while
588 maximizing sensitivity. BJU Int 108(7), 1119-1123. doi: 10.1111/j.1464-410X.2010.10026.x.
589 Kassouf, W., Traboulsi, S.L., Schmitz-Drager, B., Palou, J., Witjes, J.A., van Rhijn, B.W., et al. (2016).
590 Follow-up in non-muscle-invasive bladder cancer-International Bladder Cancer Network
591 recommendations. Urol Oncol 34(10), 460-468. doi: 10.1016/j.urolonc.2016.05.028.
592 Kaufman, D.S., Shipley, W.U., and Feldman, A.S. (2009). Bladder cancer. Lancet 374(9685), 239-
593 249. doi: 10.1016/S0140-6736(09)60491-8.
594 Killela, P.J., Reitman, Z.J., Jiao, Y., Bettegowda, C., Agrawal, N., Diaz, L.A., Jr., et al. (2013). TERT
595 promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low
596 rates of self-renewal. Proc Natl Acad Sci U S A 110(15), 6021-6026. doi: 10.1073/pnas.1303607110.
597 Kinde, I., Munari, E., Faraj, S.F., Hruban, R.H., Schoenberg, M., Bivalacqua, T., et al. (2013). TERT
598 promoter mutations occur early in urothelial neoplasia and are biomarkers of early disease and disease
599 recurrence in urine. Cancer Res 73(24), 7162-7167. doi: 10.1158/0008-5472.CAN-13-2498.
600 Kinde, I., Papadopoulos, N., Kinzler, K.W., and Vogelstein, B. (2012). FAST-SeqS: a simple and
601 efficient method for the detection of aneuploidy by massively parallel sequencing. PLoS One 7(7),
602 e41162. doi: 10.1371/journal.pone.0041162.

Kompier, L.C., van der Aa, M.N., Lurkin, I., Vermeij, M., Kirkels, W.J., Bangma, C.H., et al. (2009). The development of multiple bladder tumour recurrences in relation to the FGFR3 mutation status of the primary tumour. J Pathol 218(1), 104-112. doi: 10.1002/path.2507.

Kruger, S., Mess, F., Bohle, A., and Feller, A.C. (2003). Numerical aberrations of chromosome 17 and the 9p21 locus are independent predictors of tumor recurrence in non-invasive transitional cell carcinoma of the urinary bladder. Int J Oncol 23(1), 41-48.

Kurth, K.H., Denis, L., Bouffioux, C., Sylvester, R., Debruyne, F.M., Pavone-Macaluso, M., et al. (1995). Factors affecting recurrence and progression in superficial bladder tumours. Eur J Cancer 31A(11), 1840-1846.

Liu, X., Bishop, J., Shan, Y., Pai, S., Liu, D., Murugan, A.K., et al. (2013a). Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocr Relat Cancer 20(4), 603-610. doi: 10.1530/ERC-13-0210.

Liu, X., Wu, G., Shan, Y., Hartmann, C., von Deimling, A., and Xing, M. (2013b). Highly prevalent TERT promoter mutations in bladder cancer and glioblastoma. Cell Cycle 12(10), 1637-1638. doi: 10.4161/cc.24662.

M. Babjuk, M.B., E. Compérat, P. Gontero, A.H. Mostafid, J. Palou, B.W.G. van Rhijn, M. Rouprêt, S.F. Shariat, R. Sylvester, R. Zigeuner, O. Capoun, D. Cohen, V. Hernández, V. Soukup (2018). EUA Guidelines on Non-muscle-invasive Bladder Cancer. Edn. presented at the EAU Annual Congress Copenhagen 2018. ISBN 978-94-92671-01-1.

Miyake, M., Sugano, K., Sugino, H., Imai, K., Matsumoto, E., Maeda, K., et al. (2010). Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicator of tumor recurrence in non-muscle invasive bladder cancer. Cancer Sci 101(1), 250-258. doi: 10.1111/j.1349-7006.2009.01334.x.

Miyazaki, J., and Nishiyama, H. (2017). Epidemiology of urothelial carcinoma. Int J Urol 24(10), 730-734. doi: 10.1111/iju.13376.

Moonen, P.M., Merkx, G.F., Peelen, P., Karthaus, H.F., Smeets, D.F., and Witjes, J.A. (2007). UroVysion compared with cytology and quantitative cytology in the surveillance of non-muscle-invasive bladder cancer. Eur Urol 51(5), 1275-1280; discussion 1280. doi: 10.1016/j.eururo.2006.10.044.

Nault, J.C., Mallet, M., Pilati, C., Calderaro, J., Bioulac-Sage, P., Laurent, C., et al. (2013). High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. Nat Commun 4, 2218. doi: 10.1038/ncomms3218.

Netto, G.J. (2011). Molecular biomarkers in urothelial carcinoma of the bladder: are we there yet? Nat Rev Urol 9(1), 41-51. doi: 10.1038/nrurol.2011.193.

Pandith, A.A., Shah, Z.A., and Siddiqi, M.A. (2013). Oncogenic role of fibroblast growth factor receptor 3 in tumorigenesis of urinary bladder cancer. Urol Oncol 31(4), 398-406. doi: 10.1016/j.urolonc.2010.07.014.

Parker, J., and Spiess, P.E. (2011). Current and emerging bladder cancer urinary biomarkers. ScientificWorldJournal 11, 1103-1112. doi: 10.1100/tsw.2011.104.

Pode, D., Shapiro, A., Wald, M., Nativ, O., Laufer, M., and Kaver, I. (1999). Noninvasive detection of bladder cancer with the BTA stat test. J Urol 161(2), 443-446.

Rachakonda, P.S., Hosen, I., de Verdier, P.J., Fallah, M., Heidenreich, B., Ryk, C., et al. (2013). TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. Proc Natl Acad Sci U S A 110(43), 17426-17431. doi: 10.1073/pnas.1310522110.

Raitanen, M.P., Kaasinen, E., Lukkarinen, O., Kauppinen, R., Viitanen, J., Liukkonen, T., et al. (2001). Analysis of false-positive BTA STAT test results in patients followed up for bladder cancer. Urology 57(4), 680-684.

Ralla, B., Stephan, C., Meller, S., Dietrich, D., Kristiansen, G., and Jung, K. (2014). Nucleic acid-based biomarkers in body fluids of patients with urologic malignancies. Crit Rev Clin Lab Sci 51(4), 200-231. doi: 10.3109/10408363.2014.914888.

Sanli, O., Dobruch, J., Knowles, M.A., Burger, M., Alemozaffar, M., Nielsen, M.E., et al. (2017). Bladder cancer. Nat Rev Dis Primers 3, 17022. doi: 10.1038/nrdp.2017.22.

Sapre, N., Anderson, P.D., Costello, A.J., Hovens, C.M., and Corcoran, N.M. (2014). Gene-based urinary biomarkers for bladder cancer: an unfulfilled promise? Urol Oncol 32(1), 48 e49-17. doi: 10.1016/j.urolonc.2013.07.002.

Schrag, D., Hsieh, L.J., Rabbani, F., Bach, P.B., Herr, H., and Begg, C.B. (2003). Adherence to surveillance among patients with superficial bladder cancer. J Natl Cancer Inst 95(8), 588-597.

Serizawa, R.R., Ralfkiaer, U., Steven, K., Lam, G.W., Schmiedel, S., Schuz, J., et al. (2011). Integrated genetic and epigenetic analysis of bladder cancer reveals an additive diagnostic value of FGFR3 mutations and hypermethylation events. Int J Cancer 129(1), 78-87. doi: 10.1002/ijc.25651.

Sibley, K., Cuthbert-Heavens, D., and Knowles, M.A. (2001). Loss of heterozygosity at 4p16.3 and mutation of FGFR3 in transitional cell carcinoma. Oncogene 20(6), 686-691. doi: 10.1038/sj.onc.1204110.

Soloway, M.S., Briggman, V., Carpinito, G.A., Chodak, G.W., Church, P.A., Lamm, D.L., et al. (1996). Use of a new tumor marker, urinary NMP22, in the detection of occult or rapidly recurring transitional cell carcinoma of the urinary tract following surgical treatment. J Urol 156(2 Pt 1), 363-367.

Springer, S.U., Chen, C.H., Rodriguez Pena, M.D.C., Li, L., Douville, C., Wang, Y., et al. (2018). Non-invasive detection of urothelial cancer through the analysis of driver gene mutations and aneuploidy. Elife 7. doi: 10.7554/eLife.32143.

Tetu, B., Tiguert, R., Harel, F., and Fradet, Y. (2005). ImmunoCyt/uCyt+ improves the sensitivity of urine cytology in patients followed for urothelial carcinoma. Mod Pathol 18(1), 83-89. doi: 10.1038/modpathol.3800262.

Togneri, F.S., Ward, D.G., Foster, J.M., Devall, A.J., Wojtowicz, P., Alyas, S., et al. (2016). Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA. Eur J Hum Genet 24(8), 1167-1174. doi: 10.1038/ejhg.2015.281.

Tomlinson, D.C., Baldo, O., Harnden, P., and Knowles, M.A. (2007). FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer. J Pathol 213(1), 91-98. doi: 10.1002/path.2207.

van Kessel, K.E., Kompier, L.C., de Bekker-Grob, E.W., Zuiverloon, T.C., Vergouwe, Y., Zwarthoff, E.C., et al. (2013). FGFR3 mutation analysis in voided urine samples to decrease cystoscopies and cost in nonmuscle invasive bladder cancer surveillance: a comparison of 3 strategies. J Urol 189(5), 1676-1681. doi: 10.1016/j.juro.2012.11.005.

van Rhijn, B.W., van der Kwast, T.H., Liu, L., Fleshner, N.E., Bostrom, P.J., Vis, A.N., et al. (2012). The FGFR3 mutation is related to favorable pT1 bladder cancer. J Urol 187(1), 310-314. doi: 10.1016/j.juro.2011.09.008.

van Rhijn, B.W., Vis, A.N., van der Kwast, T.H., Kirkels, W.J., Radvanyi, F., Ooms, E.C., et al. (2003). Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 21(10), 1912-1921. doi: 10.1200/JCO.2003.05.073.

Vinagre, J., Almeida, A., Populo, H., Batista, R., Lyra, J., Pinto, V., et al. (2013). Frequency of TERT promoter mutations in human cancers. Nat Commun 4, 2185. doi: 10.1038/ncomms3185.

Vinagre, J., Pinto, V., Celestino, R., Reis, M., Populo, H., Boaventura, P., et al. (2014). Telomerase promoter mutations in cancer: an emerging molecular biomarker? Virchows Arch 465(2), 119-133. doi: 10.1007/s00428-014-1608-4.

Wang, K., Liu, T., Ge, N., Liu, L., Yuan, X., Liu, J., et al. (2014). TERT promoter mutations are associated with distant metastases in upper tract urothelial carcinomas and serve as urinary biomarkers detected by a sensitive castPCR. Oncotarget 5(23), 12428-12439. doi: 10.18632/oncotarget.2660.
Wu, S., Huang, P., Li, C., Huang, Y., Li, X., Wang, Y., et al. (2014). Telomerase reverse transcriptase gene promoter mutations help discern the origin of urogenital tumors: a genomic and molecular study. Eur Urol 65(2), 274-277. doi: 10.1016/j.eururo.2013.10.038.
Yafi, F.A., Brimo, F., Steinberg, J., Aprikian, A.G., Tanguay, S., and Kassouf, W. (2015). Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. Urol Oncol 33(2), 66 e25-31. doi: 10.1016/j.urolonc.2014.06.008.
Yeung, C., Dinh, T., and Lee, J. (2014). The health economics of bladder cancer: an updated review of the published literature. Pharmacoeconomics 32(11), 1093-1104. doi: 10.1007/s40273-014-0194-2.
Zuiverloon, T.C., van der Aa, M.N., van der Kwast, T.H., Steyerberg, E.W., Lingsma, H.F., Bangma, C.H., et al. (2010). Fibroblast growth factor receptor 3 mutation analysis on voided urine for surveillance of patients with low-grade non-muscle-invasive bladder cancer. Clin Cancer Res 16(11), 3011-3018. doi: 10.1158/1078-0432.CCR-09-3013.
Zwarthoff, E.C. (2008). Detection of tumours of the urinary tract in voided urine. Scand J Urol Nephrol Suppl (218), 147-153. doi: 10.1080/03008880802283953.

10 Figure legends

Figure 1 – Urine testing Workflow. In patients under surveillance for NMBIC, a minimum of 10ml of urine is collected before cystoscopy. 10ml of urine is then filtered through a 0.8um filter and stored at 4ºC. DNA extraction and Uromonitor® test is then performed. If a positive result is obtained, confirmatory cystoscopy and transurethral resection of eventual recurrences is recommended. If a negative result is obtained, it is recommended that test should be repeated on next Follow-up appointment

Figure 2 – Technical principles of the test. A – Real-Time qualitative method optimized for TERTp c.1-124C>T detection. Two competitive fluorescent probes targeting normal (WT- c.1-124C) and mutated (Mut- c.1-124C>T) alleles incorporating Locked Nucleic Acid bases are used to detect the mutations. B – Real-Time qualitative method optimized for TERTp c.1-146C>T detection. Two competitive fluorescent probes targeting normal (WT- c.1-146C) and mutated (Mut c.1-146C>T) alleles incorporating Locked Nucleic Acid bases are used to detect the mutations. C – Real-Time qualitative method optimized for FGFR3 c.742C>G detection. A mutation allele specific primer, a phosphorylated wild type allele blocker that completely suppresses the amplification of the wild type allele, a locus reverse primer and a fluorescent probe for Real-Time detection of the generated amplicon are used. D – Real-Time qualitative method optimized for FGFR3 c.746C>T detection. A mutation allele specific primer, a phosphorylated wild type allele blocker that completely suppresses the amplification of the wild

type allele, a locus reverse primer and a fluorescent probe for Real-Time detection of the generated amplicon are used.

Figure 3 – A. Serial dilution detection of TERTp c.1-124C>T. TERTp c1-124C>T mutated DNA with 50% WT/Mutation ratio was diluted in a 2-fold dilution (8 dilutions) in WT DNA. Detection limited was fixed at the presence of 6.25% of TERTp c.1-124C>T alteration in the total DNA in a reaction with 25ng of total DNA. Below this threshold, mutation detection is not guaranteed; B. Serial dilution detection of TERTp c.-146C>T. TERTp c1-146C>T mutated DNA with 50% WT/Mutation ratio was diluted in a 2-fold dilution (8 dilutions) in WT DNA. Detection limited was fixed at the presence of 6.25% of TERTp c.1-146C>T alteration in the total DNA in a reaction with 25ng of total DNA. Below this threshold, mutation detection is not guaranteed.

Figure 4 – Different screening methods performance in NMIBC Follow-up Recurrence detection, in NMIBC Diagnosis

Figure 5 – Mutation distribution across Follow-up cohort (A) and Initial-Diagnosis cohort (B).

Figure 6 - Cohort's tumor stage distribution and Uromonitor Performance in Recurrence detection across tumor stages

Figure 7 - Cohort’s tumor grade and Uromonitor performance in Recurrence detection across tumor grade

Table 1 – Clinical Validation cases information and clinicopathological data

	CHARACTERISTIC	TOTAL CASES (N=185)
AGE - YEARS		
	Median age (range)	71 (25-91)
AGE CLUSTER – Nº (%)		
	20-39	9 (4,9)
	40-59	39 (21,3)
	60-79	102 (55,7)
	80+	33 (18,0)
GENDER - Nº (%)		
	Female	41 (23,2)
	Male	136 (76,8)
SMOKING STATUS- Nº (%)		
	Yes/Former	45 (39,5)
	No	69 (60,5)
DISEASE STATUS- Nº (%)		
	Primary	122 (65,9)
	Recurrence	63 (34,1)
STAGE- Nº (%)		
	Cis/Tis	5 (9,8)
	Ta	32 (62,7)
	T1	12 (23,5)
	T2	1 (2)
	Hep.Met	1 (2)
GRADE- Nº (%)		
	Low Grade	25 (51)
	High Grade	24 (49)
URINE CITOLOGY - Nº (%)		
	Positive/Atypical Citology	12 (14,3)
	Negative Citology	72 (85,7)
CYSTOSCOPY- Nº (%)		
	Positive	65 (35,2)
	Negative	120 (64,8)

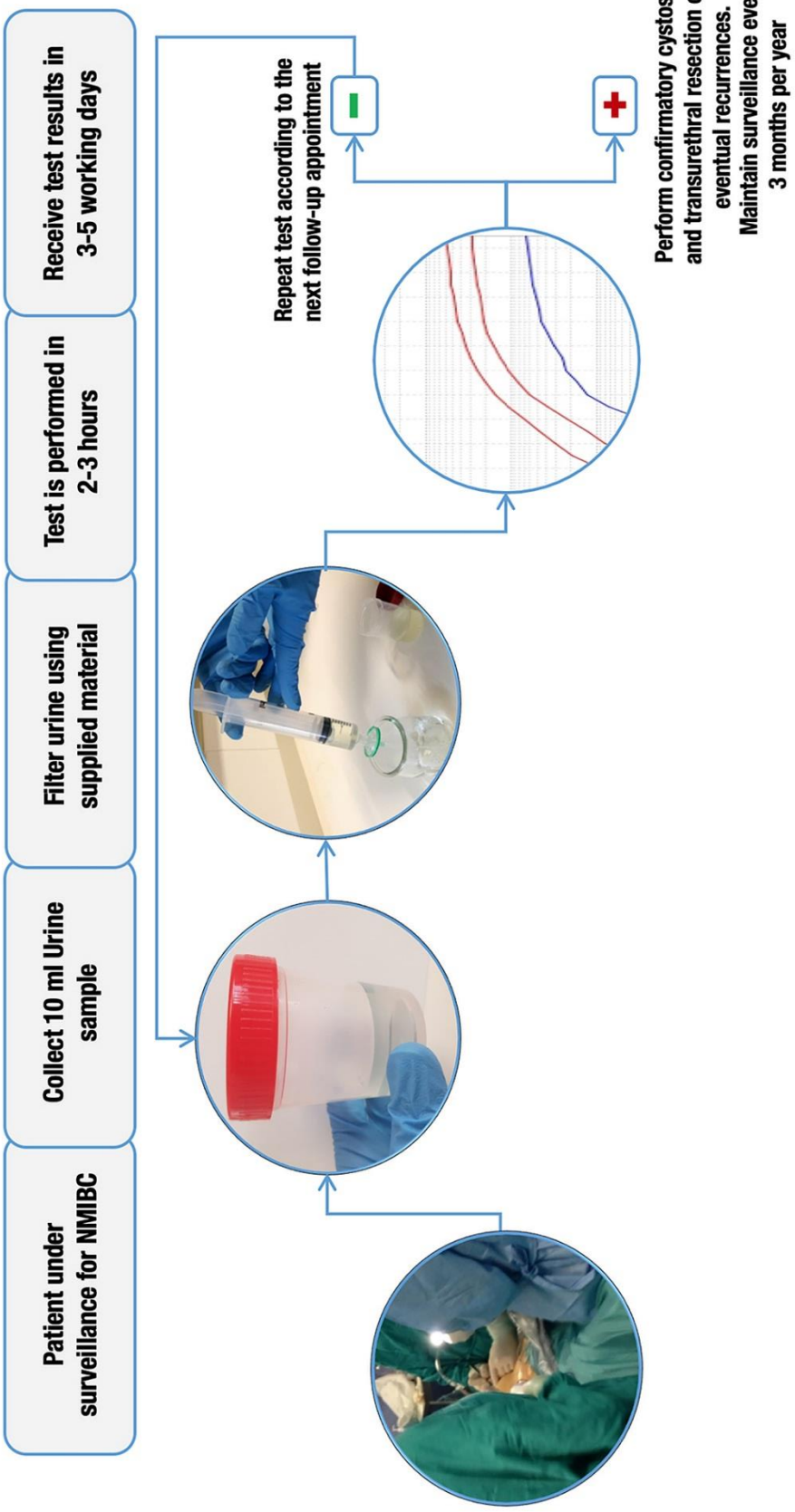
Table 2 - Cohort's used in this study

COHORT NAME	COHORT DESIGNATION	NUMBER OF SAMPLES
FOLLOW-UP COHORT	Urine samples from patients under follow-up for NMIBC	122
INITIAL DIAGNOSIS COHORT	Urine samples from patients screened for bladder cancer	63
TUMOR SAMPLES COHORT	FFPE samples from primary tumors and recurrence from patients under follow-up for NMBIC.	41
UROMONITOR + *KRAS* FOLLOW-UP COHORT	Urine samples from patients under follow-up for NMIBC screened for both Uromonitor® and KRAS hotspot alterations.	24
UROMONITOR + *KRAS* INITIAL DIAGNOSIS COHORT	Urine samples from patients screened for bladder cancer for both Uromonitor® and KRAS hotspot alterations.	25

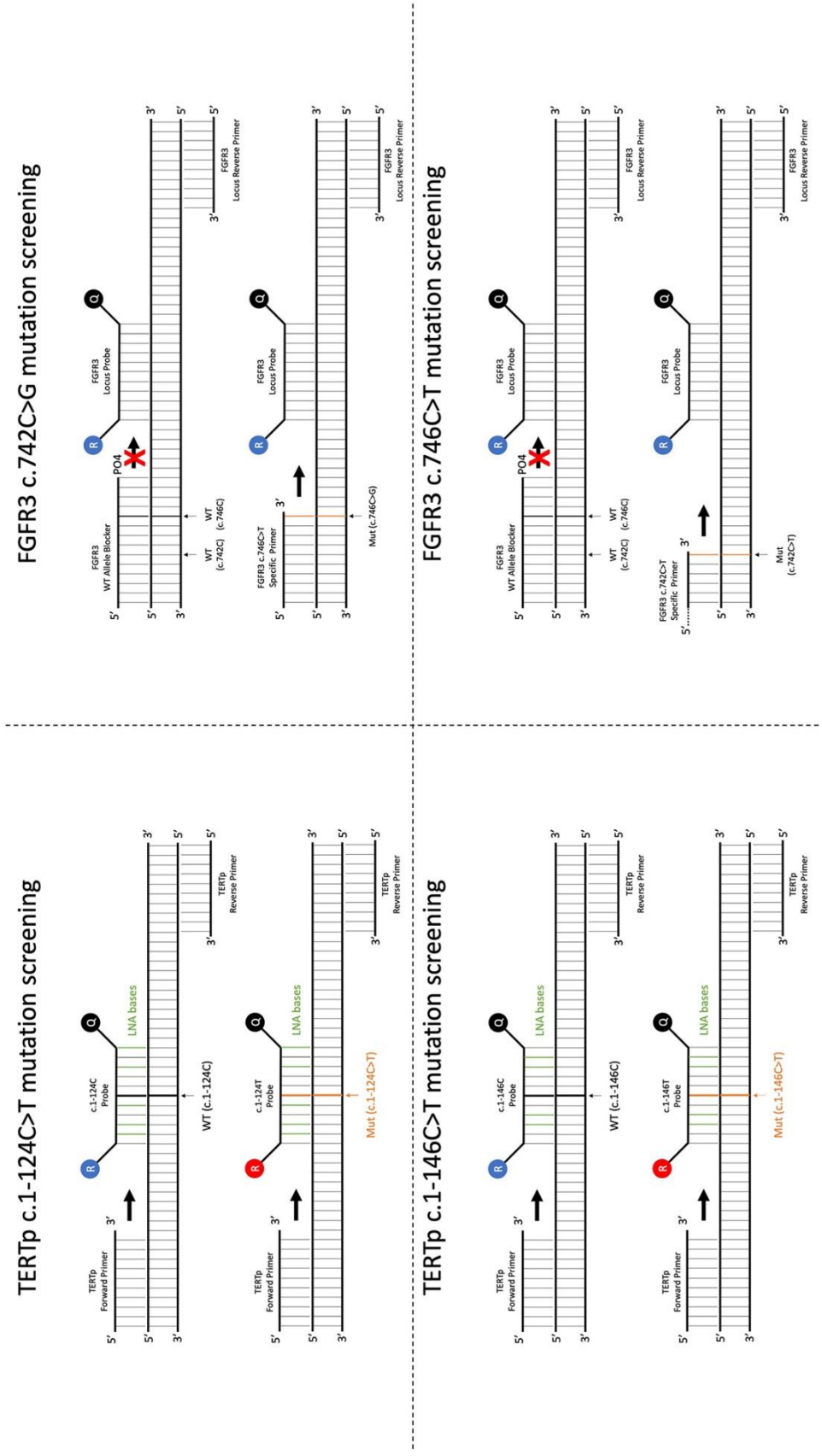
Table 3 – Different screening methods performance in NMIBC Follow-up Recurrence detection and in NMIBC Initial diagnosis

FOLLOW-UP COHORT						
	UROMONITOR	**CYSTOSCOPY**	**CYTOLOGY**	**CYSTOSCOPY + CYTOLOGY**	**UROMONITOR + CYSTOSCOPY**	**UROMONITOR + KRAS**
SENSITIVITY	73.5	79.4	42.9	86.7	100.0	100
SPECIFICITY	93.2	93.2	93.9	87.9	86.4	83.3
ACCURACY	87.7	89.3	78.7	87.5	90.2	87.5
PPV	80.6	81.8	75.0	76.5	73.9	66.7
NPV	90.1	92.1	79.5	93.5	100.0	100
INITIAL DIAGNOSIS COHORT						
	UROMONITOR	**CYSTOSCOPY**	**CYTOLOGY**	**CYSTOSCOPY + CYTOLOGY**	**UROMONITOR + CYSTOSCOPY**	**UROMONITOR + KRAS**
SENSITIVITY	50.0	100.0	0.0	100.0	100.0	93.3
SPECIFICITY	100.0	88.6	86.7	86.7	88.6	80.0
ACCURACY	77.8	93.7	70.3	89.2	93.7	88.0
PPV	100.0	87.5	0.0	63.6	87.5	87.5
NPV	71.4	100.0	78.8	100.0	100.0	88.9

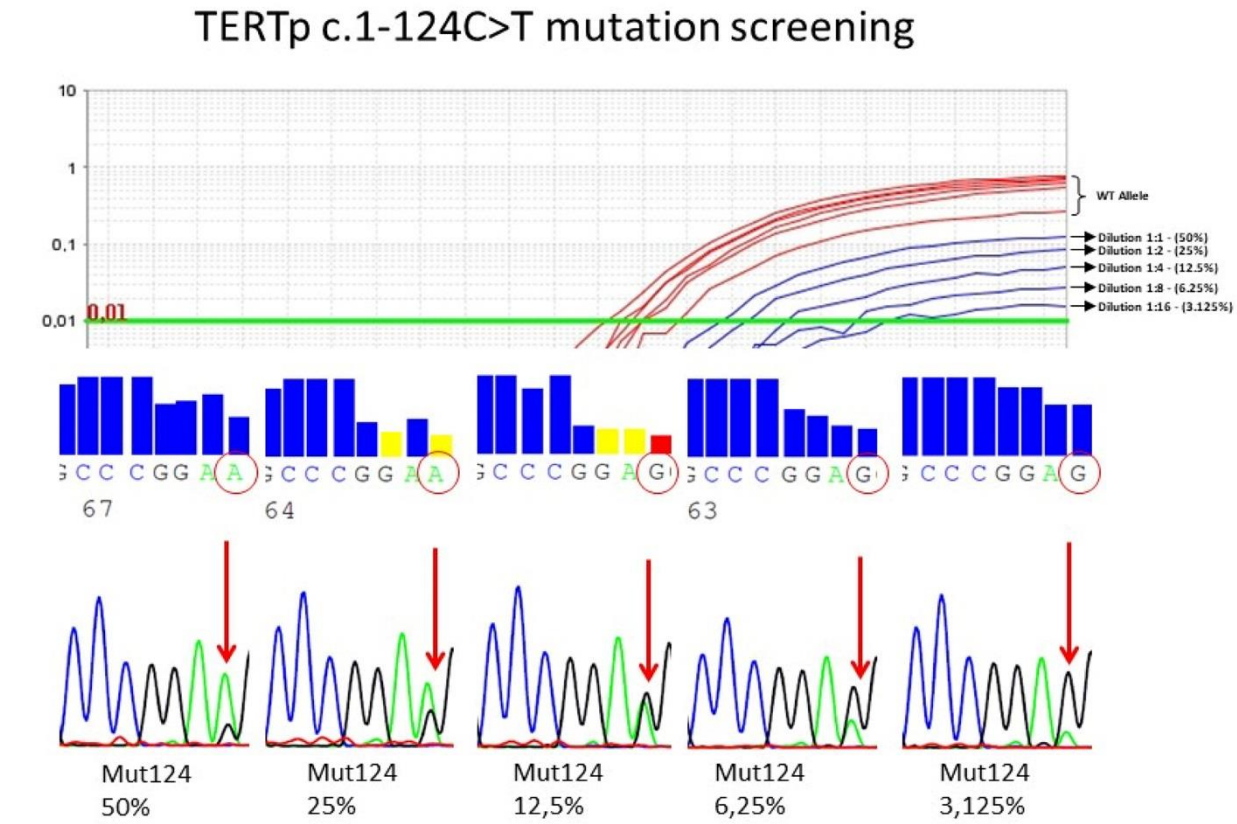
770

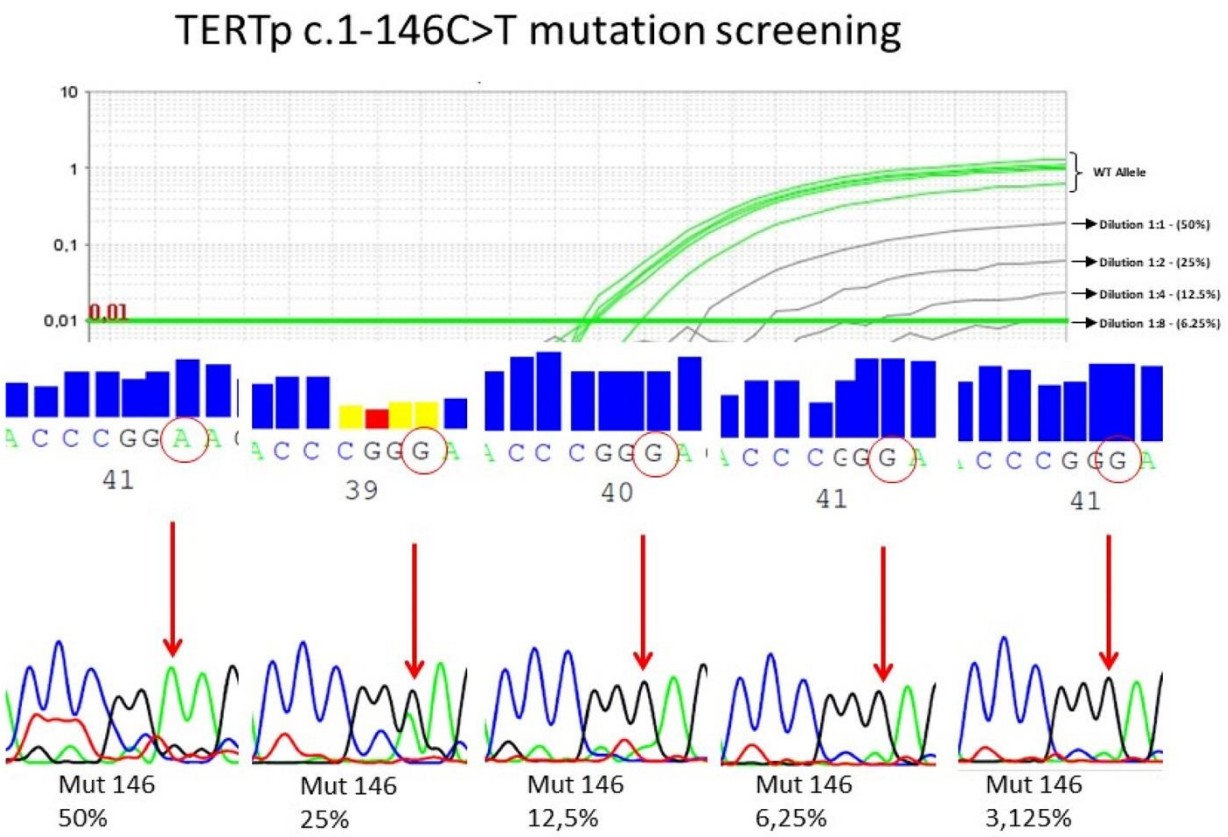

Patient under surveillance for NMIBC
Collect 10 ml Urine sample
Filter urine using supplied material
Test is performed in 2-3 hours
Receive test results in 3-5 working days
Repeat test according to the next follow-up appointment
Perform confirmatory cystoscopy and transurethral resection of the eventual recurrences. Maintain surveillance every 3 months per year

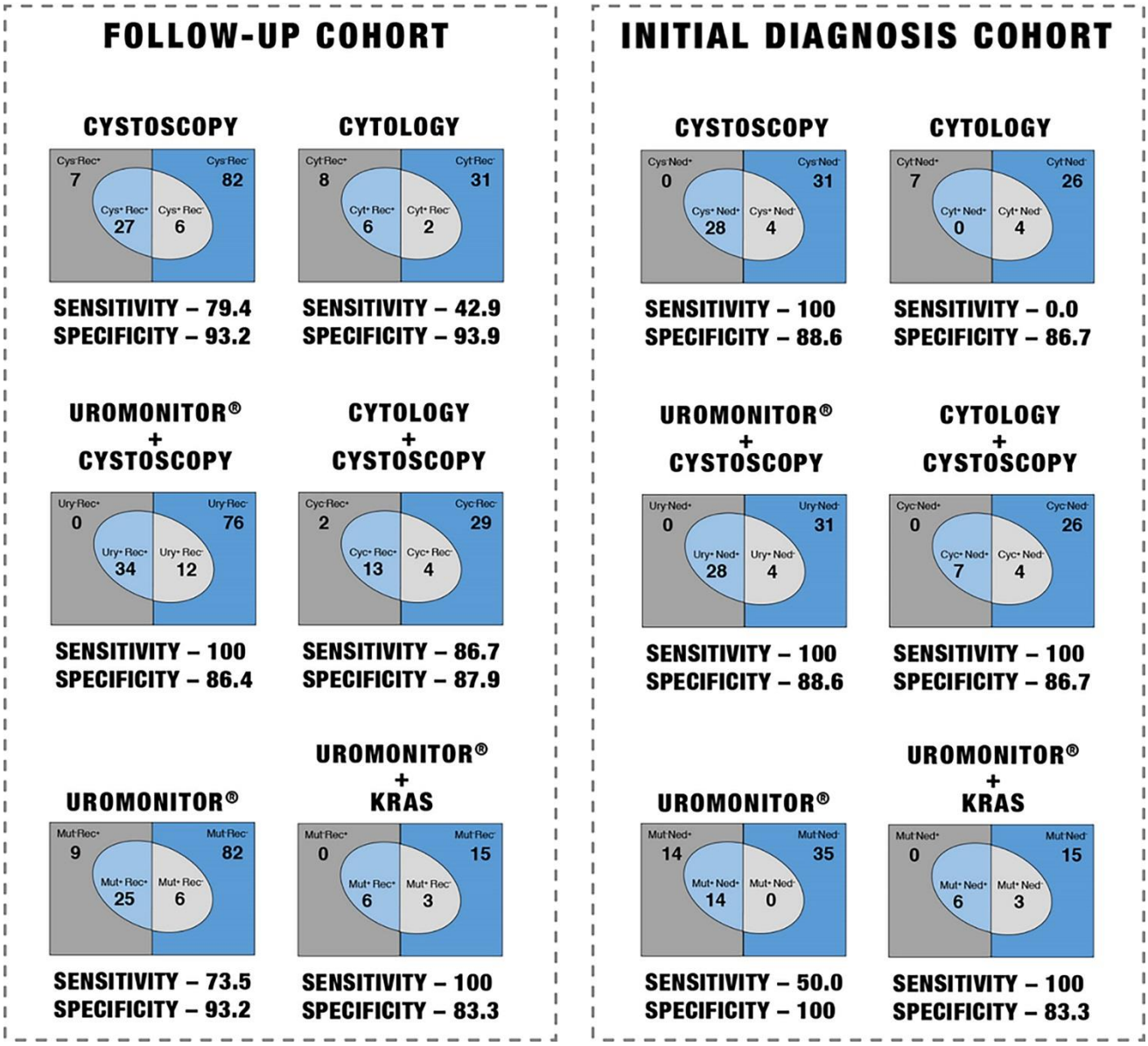
771

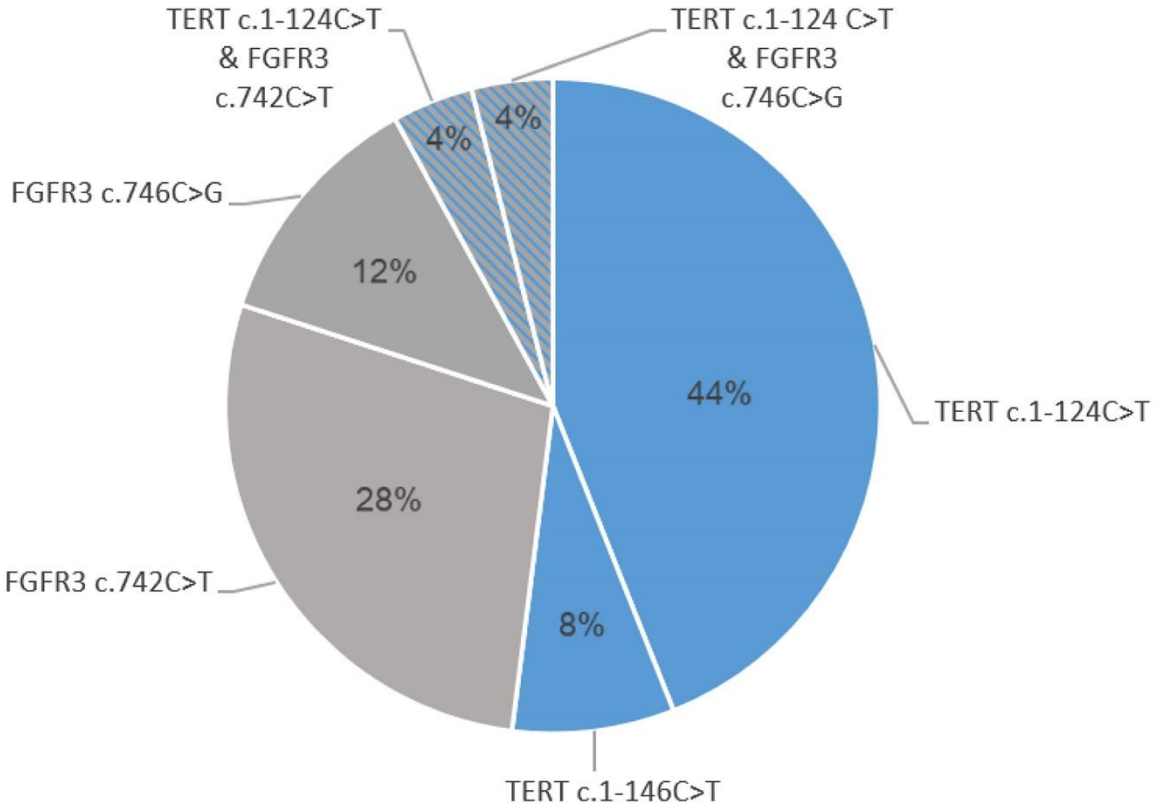


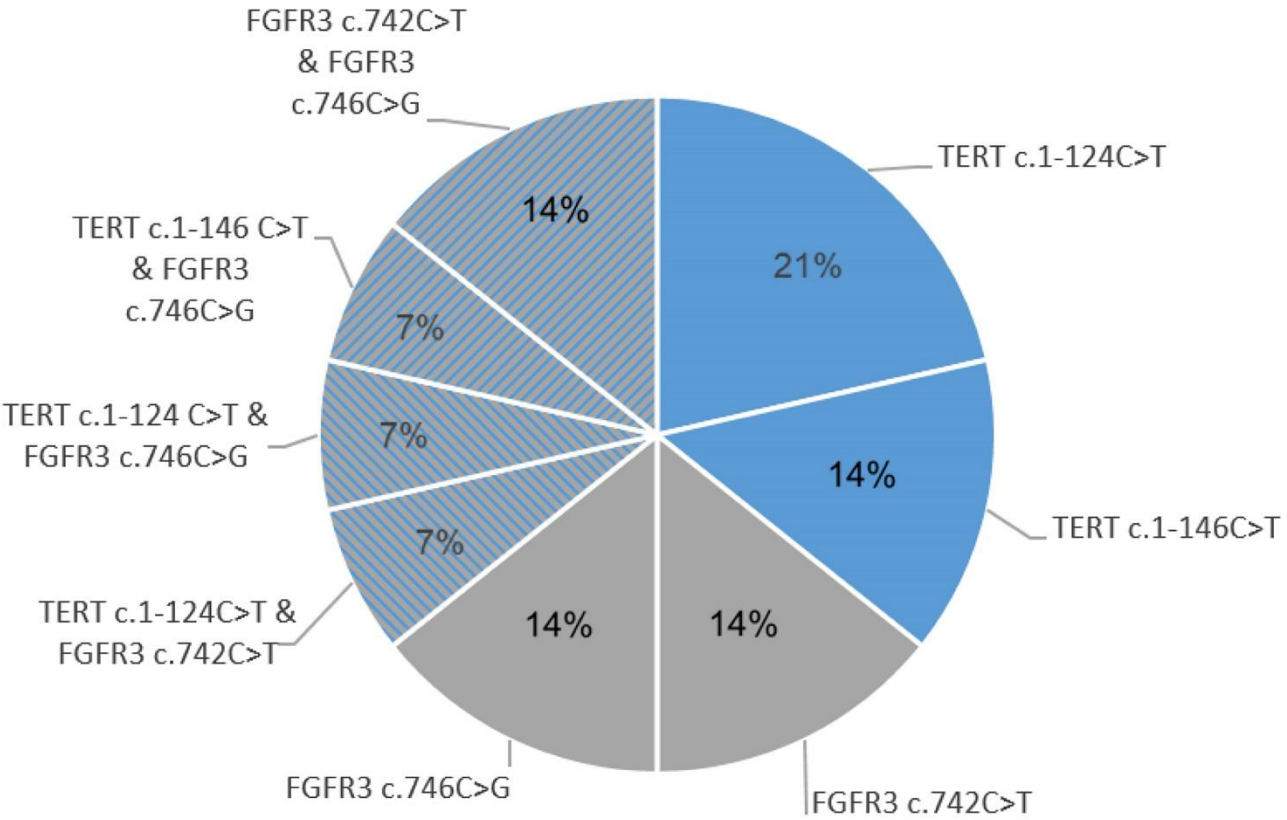
772


TERTp c.1-124C>T mutation screening
10
1
0,1
0,01
0.01
WT Allele
Dilution 1:1 - (50%)
Dilution 1:2 - (25%)
Dilution 1:4 - (12.5%)
Dilution 1:8 - (6.25%)
Dilution 1:16 - (3.125%)
67
64
63
Mut124
50%
Mut124
25%
Mut124
12,5%
Mut124
6,25%
Mut124
3,125%

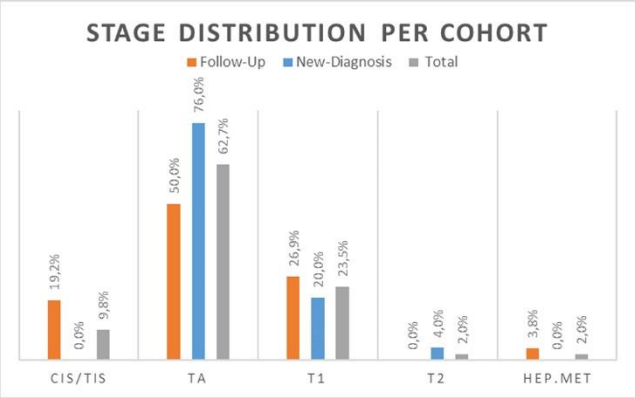

TERTp c.1-146C>T mutation screening
10
1
0,1
0,01
0.01
WT Allele
Dilution 1:1 - (50%)
Dilution 1:2 - (25%)
Dilution 1:4 - (12.5%)
Dilution 1:8 - (6.25%)
41
39
40
41
41
Mut 146
50%
Mut 146
25%
Mut 146
12,5%
Mut 146
6,25%
Mut 146
3,125%

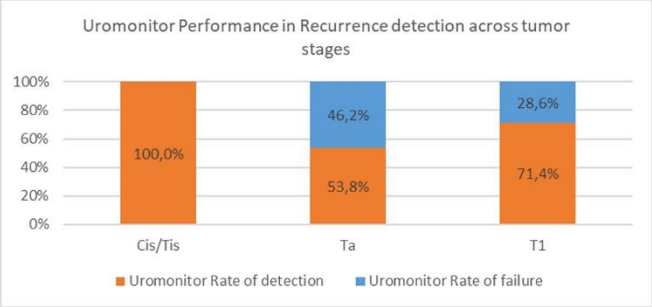



TERT c.1-124C>T & FGFR3 c.742C>T
TERT c.1-124 C>T & FGFR3 c.746C>G
4%
4%
FGFR3 c.746C>G
12%
44%
TERT c.1-124C>T
28%
FGFR3 c.742C>T
8%
TERT c.1-146C>T


FGFR3 c.742C>T & FGFR3 c.746C>G
TERT c.1-124C>T
14%
21%
TERT c.1-146 C>T & FGFR3 c.746C>G
7%
TERT c.1-124 C>T & FGFR3 c.746C>G
7%
14%
TERT c.1-146C>T
7%
TERT c.1-124C>T & FGFR3 c.742C>T
14%
14%
FGFR3 c.746C>G
FGFR3 c.742C>T

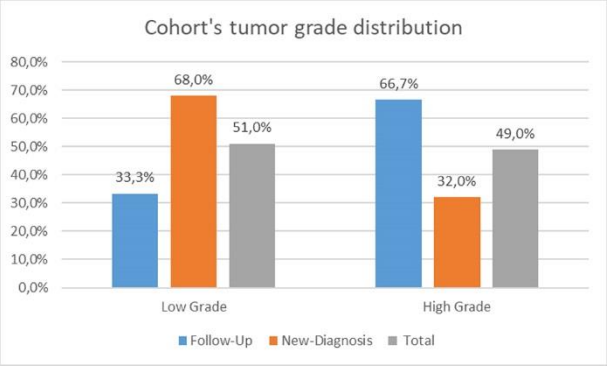
796

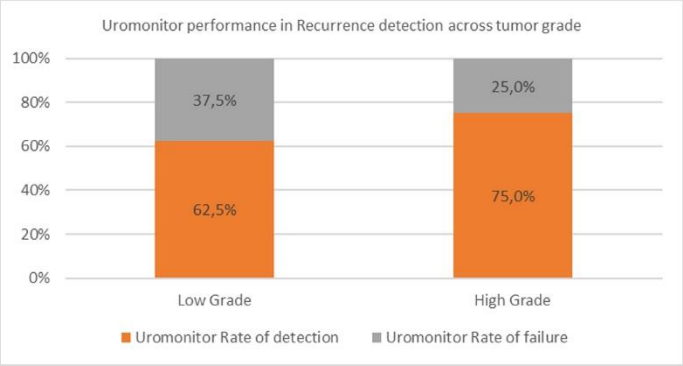




813

814


Cohort's tumor grade distribution
80,0%
70,0%
60,0%
50,0%
40,0%
30,0%
20,0%
10,0%
0,0%
33,3%
68,0%
51,0%
66,7%
32,0%
49,0%
Low Grade
High Grade
Follow-Up
New-Diagnosis
Total


Uromonitor performance in Recurrence detection across tumor grade
100%
80%
60%
40%
20%
0%
37,5%
62,5%
25,0%
75,0%
Low Grade
High Grade
Uromonitor Rate of detection
Uromonitor Rate of failure