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PLASMA EXTRACELLULAR VESICLES AS A SOURCE FOR BIOMARKERS OF RECURRENCE, PROGRESSION AND TREATMENT RESPONSE IN BLADDER CANCER

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Plasma Extracellular Vesicles as a source for biomarkers of recurrence, progression and treatment response in Bladder Cancer

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Abstract

Introduction

Bladder Cancer is a heterogeneous and common disease. It is categorized as NMIBC (70-80%) and MIBC (20-30%), with very different associated treatments and prognosis. In NMIBC the major problems are frequent recurrence and progression, while in MIBC, treatment failure is an important issue. In this context, liquid biopsies, and specifically EVs, have been gaining increasing attention, as they play key roles in cancer progression and their content mirrors the pathophysiological status of parent cells.

Goals

We sought to characterize a BLCa cohort, with emphasis on treatments performed and their outcomes (recurrence, progression), and to separate and concentrate EVs by a simple method, followed by full EV characterization.

Methods

Data was collected from medical records, clinical reports, and pathological reports. 6 out of 12 samples were chosen for separation and concentration of EVs, followed by characterization using NTA, Western-Blot, BCA and RNA extraction and quantification, with some of the remaining samples being used for EV detection using TEM.

Results

187 patients were initially diagnosed with NMIBC and 77 with MIBC. In the former group, there were 48 local recurrences and 22 progressions. 35 patients were treated with chemotherapy and 8 with ICI. Regarding EV Characterization, NTA disclosed particle size and distributions compatible with small EV. Western-blot allowed the clear demonstration of the presence of CD81, HSP70, ALIX, Albumin and UPKIIIa, and a weaker signal in some samples of Flotillin-1. TEM, resorting to sample dilution, also demonstrated intact EVs. Finally, RNA extraction and quantification disclosed the presence of RNA.

Discussion

The recurrence rate in the cohort was 25.7%, and the progression rate to any higher stage or grade was 11.7%. 61.9% of patients undergoing NAC had favorable response, while only 23,1% under palliative chemotherapy achieved favorable outcomes. In ICI, despite the small sample size, some patients seem to have very good responses, while others experience disease progression.

In this study we showed that UC continues to be an excellent option for EV isolation, providing a full characterization of samples. Furthermore, we demonstrated the presence of UPKIIIa in plasma

EVs of BLCa patients, which not only suggests an early “connection” and intercommunication between BLCa cells and the organism, but also that plasma can be a source of BLCa specific EVs.

This preliminary study disclosed that EVs’ are amenable to be separated and concentrated and may serve as biomarkers of recurrence, progression, and may allow for more accurate real-time monitoring.

Keywords: Bladder Cancer; Extracellular vesicles separation and concentration; Extracellular vesicles characterization; Recurrence; Progression; Biomarkers; RNA

Resumo

Introdução

O Cancro da Bexiga é uma doença heterogénea e comum. É categorizada em Cancro da Bexiga não musculo-invasivo (70-80%) e Cancro da Bexiga musculo-invasivo (20-30%), com tratamentos e prognostico associados muito diferentes. No Cancro da Bexiga não-musculo-invasivo, o principal problema são as recorrências e progressões frequentes, enquanto que no Cancro da Bexiga musculo-invasivo, a falência terapêutica é um importante problema. Neste contexto, as biópsias líquidas, e especificamente as vesículas extracelulares, tem vindo a ganhar atenção crescente, já que desempenham papeis fundamentais na progressão do cancro e os seus conteúdos refletem o estado fisiopatológico das células parentais.

Objetivos

Nós procuramos caraterizar uma coorte de Cancro da Bexiga, com enfase nos tratamentos realizados e os seus resultados (recorrência, progressão), e separar e concentrar vesículas extracelulares por um método simples, seguidas de uma caraterização completa das vesículas isoladas.

Métodos

Os dados foram obtidos de registos clínicos, relatórios clínicos e relatórios patológicos. 6 de 12 amostras foram escolhidas para separação e concentração de vesículas extracelulares, seguidas de caraterização usando NTA, Western-Blot, BCA e extração e quantificação de RNA, sendo algumas das amostras restantes usadas para deteção de vesículas extracelulares usando TEM.

Resultados

187 pacientes foram inicialmente diagnosticados com Cancro da Bexiga não musculo-invasivo e 77 com Cancro da Bexiga musculo-invasivo. No primeiro grupo, existiram 48 recorrências locais e 22 progressões. 35 pacientes foram tratados com quimioterapia e 8 com inibidores de *checkpoints* imunes. No que toca à caraterização, o NTA demonstrou tamanho e distribuição de partículas compatível com pequenas vesículas extracelulares. O Western-Blot permitiu a demonstração clara da presença de CD81, HSP70, ALIX, Albumina e UPKIIIa, com um sinal mais ténue de Flotilina-1 em algumas amostras. A TEM, recorrendo à diluição das amostras, também divulgou a presença de vesículas extracelulares. Finalmente, a extração e quantificação de RNA evidenciou a presença de RNA.

Discussão

A taxa de recorrência na coorte foi de 25,7%, e a taxa de progressão para qualquer estadio ou grau superior foi de 11,7%. 61,9% dos pacientes que realizaram quimioterapia neoadjuvante tiveram resposta favorável, enquanto que apenas 23,1% sob quimioterapia paliativa tiveram

resultados favoráveis. Nos inibidores de *checkpoints* imunes, apesar da pequena amostra, alguns pacientes parecem ter respostas muito boas, enquanto que outros têm progressão da doença.

Neste estudo, demonstramos que a ultracentrifugação continua a ser uma opção excelente para a separação e concentração de vesículas extracelulares, providenciando uma caraterização completa das amostras. Adicionalmente, demonstramos a presença de UPKIIIa nas vesículas extracelulares do plasma de pacientes com Cancro da Bexiga, o que não só sugere uma conexão e intercomunicação precoces entre as células do Cancro da Bexiga e o organismo, mas também que o plasma pode ser uma fonte de vesículas extracelulares específicas de Cancro da Bexiga.

Este estudo preliminar divulgou que as vesículas extracelulares são passíveis de serem separadas e concentradas e podem constituir biomarcadores de recorrência, progressão, poderão permitir uma monitorização em tempo real mais precisa.

Palavras-chave: Cancro Vesical; Separação e concentração de vesículas extracelulares; Caraterização de vesículas extracelulares; Recorrência; Progressão; Biomarcadores; RNA

List of Abbreviations

µg: Microgram

µL: Microliter

µm: Micrometer

ALIX: ALG-2-interacting protein X

APS: Ammonium Persulphate

BCA: Bicinchoninic acid assay

BCG: Bacille calmmete-guerin

BLCa: Bladder cancer

BMI: Body Mass Index

bp: Base pairs

BSA: Bovine serum albumin

BTA: Bladder tumor antigen

CD: Cluster of differentiation

cirRNA: Circular Ribonucleic acid

CIS: Carcinoma *in situ*

cm: Centimeter

DNA: Deoxyribonucleic acid

ECL: Enhanced luminol-based chemiluminescent substrate

ECOG: Eastern Cooperative Oncology Scale

EGFR: Epidermal growth factor receptor

EV: Extracellular vesicle

FU: Follow-Up

FU': Fluorescence units

g: Relative centrifugal force units

GC: Gemcitabine-Cisplatin scheme

HG: High-grade

HSP: Heat shock protein

ICI: Immune checkpoint inhibitors

IHQ: Immunohistochemistry

ILV: Intraluminal vesicle

IPOP: Portuguese Oncology Institute of Porto

ISEV: International Society for Extracellular Vesicles

kDa: Kilodalton

LG: Low-grade

LN: Lymph node

lncRNA: Long non-coding ribonucleic acid

MIBC: Muscle-invasive bladder cancer

min: Minute(s)

miRNA: Micro Ribonucleic acid

MISEV2018: Minimal information for studies of extracellular vesicles 2018

mL: Milliliter

mm: Milimeter

MMC: Mitomycin C

MVAC: Metotrexate-Vinblastin-Doxorubicin-Cisplatin scheme

NAC: Neoadjuvant chemotherapy

nm: Nanometer

NMIBC: Non-muscle-invasive bladder cancer

NMP22: Nuclear matrix protein 22

nt: Nucleotide

NTA: Nanoparticle tracking analysis

PBS: Phosphate-buffered saline

PBS-f: Filtered Phosphate-buffered saline

PCa: Prostate cancer

pg: Picogram

PUNLPM: Papillary Urothelial Neoplasm of Low Malignant Potential

PVDF: Polyvinylidene fluoride

RC: Radical cystectomy

RNA: Ribonucleic acid

RNAse - Ribonuclease

RT: Room Temperature

SDS: Sodium Dodecyl Sulfate

TEM: Transmission electron microscopy

TEMED: N,N,N',N' – Tetramethyl ethyllenediamine

TNM: Tumor, Node, Metastasis

TSG: Tumor Susceptibility

TURB: Transurethral resection of the bladder

UC: Ultracentrifugation

UPK: Uroplakin

UTI: Urinary Tract Infection

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Introduction

Epidemiology

Bladder cancer is the 10th most common cancer worldwide, accounting for approximately 549,000 new cases and about 200,00 deaths in 2018[1,2]. It is particularly incident in regions with high human development index, such as Europe and North America, where it ranks as the 4th most common cancer in men and 9th in women[2,3]. BLCa is 3-4 times more common in men than in women with incidence rates of 9,6 and 2,4 per 100.000[2,4,5], respectively. It affects mostly the elderly, with median ages at diagnosis of 69 years in males and 71 years in females[6] and, due to increased life expectancy, the incidence has risen over the last 20 years[4,7]. Thus, considering demographic trends, its global health burden is likely to increase in the future[3].

Tobacco smoking is the most important and well-established exogenous risk factor for BLCa, accounting for 50% of all cases (50-65% in males, 20-30% in females)[3,4,8-11]. The second most important risk factor, accounting for 20-25% of cases, is occupational exposure to carcinogens, namely aromatic amines, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons, by workers processing and/or handling aniline dyes, paints, rubber, metal and petroleum products[3,4,6,12-15]. In general, the latency period is long, ranging from 30 to 50 years, and is dose- and intensity-related[3,4,6]. Other risk factors reported are intake of high concentrations of arsenic in drinking water, exposure to ionizing radiation to the pelvis, and the use of cyclophosphamide and pioglitazone[3,16-19]. Chronic bladder inflammation, related or not to Schistosomiasis, also increases the risk of Bladder Cancer, albeit mostly associated with squamous cell carcinomas[3,12]. Genetics also influence the risk of BLCa, probably due to differences in carcinogenic detoxification, modulating the impact of other risk factors[3,4,20].

Pathology

BLCa is a heterogeneous disease originated in the urothelium and is the main cancer site in the urothelial tract (90-95% cases)[21]. Most cases (>90%) are of urothelial type, with others being mostly squamous cell carcinoma, adenocarcinoma, small-cell carcinoma, and sarcoma[1,5,12,22]. Tumors are classified according to their extension using the TNM staging system for Bladder Cancer (Table I)[23]. In broader terms, BLCa may be categorized, according to the extent of invasion, as NIMBC or MIBC, due to the clearly distinct prognosis[22]. Seventy to eighty percent of cases are NMIBC, which includes cancers confined to the mucosa (Ta and Tis) and those invading the lamina propria (T1). The remainder 20-30% are MIBC, which invade the muscle layer and beyond (Table I)[3,12,24-26]. Within NIMBC, approximately 70% of lesions are Ta, 20% T1 and 10% Tis[26]. The latter is a flat, aggressive

high-grade lesion with undefined borders and more likely to progress and spread[25]. Besides TNM staging, tumors are classified as either of low- or high-grade, on the basis of architectural and cytological atypia[27], which is the most important prognostic factor for NMIBC[22].

Recently, with the growing understanding of BLCa biology, different molecular subtypes have also been defined, based on gene expression patterns[22]. The most consensually recognized MIBC subtypes include luminal papillary, luminal non-specified, luminal unstable, stroma rich, basal/squamous and neuroendocrine-like[28]. Each has distinct differentiation patterns, oncogenic mechanisms, tumor microenvironments, response to treatment and prognosis[28-30]. For example, a luminal subtype, termed "p53-like", seems to be resistant to NAC[31], whereas basal tumors, which are enriched in EGFR and HIF-1, appear to be sensitive to EGFR inhibitors and bevacizumab[31-33].

Finally, urothelial carcinomas frequently undergo divergent differentiation, resulting in histological variants[22]. The frequency varies widely, but up to 60% of urothelial carcinomas may contain *foci* of squamous carcinoma and 10% may exhibit glandular differentiation[34]. Histological variants are generally diagnosed at an advanced stage, with worse prognosis – especially the micropapillary, plasmocytoid and sarcomatoid variants[35]. Radical cystectomy (RC) is the mainstay in the management of most variants[25].

Diagnosis and treatment

Hematuria, micro- or macroscopic, is the most common finding and is strongly associated with bladder cancer[4,22,36-38]. Additionally, carcinoma *in situ* (CIS) might be suspected in patients with irritative symptoms[25] whereas weight loss, skeletal pain and dependent edemas are common in locally advanced or metastatic disease[4]. Initial diagnostic evaluation of hematuria consists of cystoscopy and cross-sectional imaging[37]. The diagnosis of BLCa depends on TURB, the cystocopic examination of the bladder with complete removal of all visible lesions, ensuring *muscularis propria* representation, followed by histological evaluation of the sampled tissue[22,24,25].

After TURB, patients receive one instillation of intravesical chemotherapy within 24h[25] (MMC, Epirubicin), which reduces the risk of recurrence at 5-years by 14%[39]. Subsequently, patients are stratified into prognostic risk groups (Tables II), based on the likelihood of recurrence and/or progression, guiding the need of further adjuvant treatment and FU[25,40]. Several meta-analyses have clearly shown that adjuvant intravesical treatment decreases recurrence[41-45]. Intravesical chemotherapy, mainly using MMC, is an option in intermediate-risk patients, although it does not prevent progression to MIBC[22]. BCG, on the other hand, provides a significantly lower rate of relapse compared to chemotherapy, and is the only treatment that delays disease progression[41,45-49]. However, BCG induces more side effects than intravesical chemotherapy[45,48]. Additionally, performing a second TURB is strongly recommended in cases of incomplete tumor resection, no

muscularis propria representation or in T1 tumors[25], as there is a significant risk of disease persistence and understaging, especially in high-grade cases[37,50,51]. Furthermore, in patients with the highest risk (Table II) or with BCG-failure, RC should be offered, as these patients have significant risk of progression to MIBC, in which case the prognosis is dismal as 40% will die of BLCa[25,52].

In MIBC, RC with bilateral pelvic lymphadenectomy, preceded by NAC, is the gold standard for organ-confined (cT2,N0,M0) or locally advanced (cT3-4,N0-X,M0) disease[12,22]. MVAC and GC are common options, the later being widely used, given the lower toxicity but similar efficacy[12,53]. RC is a complex surgery, involving in men the removal of the prostate, seminal vesicles, and, eventually, in women the adjacent vagina and uterus[12]. Additionally, pelvic regional LN, below the bifurcation of the aorta, should be excised[12]. This surgery is associated with 90-day mortality of about 3-4%, but can be as high as 9%[54,55]

Despite treatment, about 50% of patients will relapse after RC, mostly within the first 2 years with distant metastasis[12,56]. Standard first-line treatment for patients with good kidney function is again chemotherapy (GC and MVC), with median OS of 12-14 months[57]. However, 50% are not eligible, and in these cases, Gemcitabine-Carboplatin is an option[12]. The increasing understanding of BLCa biology, particularly the observation of increased expression of CTLA4, PD-1 and PD-L1 in tumor surrounding immune cells has led to clinical trials and later approval of ICIs as second-line treatment (for those that progressed during or after chemotherapy), which are now the current standard[12]. Pembrolizumab, Atezolizumab and Nivolumab, not only are better tolerated by patients, but also disclose superior efficacy compared to cytotoxic chemotherapy[22].

Surveillance in NMIBC depends on risk and based on cystoscopy and cytology[25], whereas in MIBC follow-up should include CT scan every 6 months until 3 years and annually up to 5th-year[12].

Current problems and limitations

In NMIBC the major clinical problem currently is the high rate of recurrence (50-70%), and progression to MIBC (10-30% of cases), which often occurs even after 10 years of FU, demanding rigorous and, in intermediate- and high-risk patients, lifelong surveillance[40,52,58-60]. Currently, these patients cannot be prospectively identified[1]. This indicates that existing classification systems, based solely on clinical and pathological findings, are imperfect and do not depict the true heterogeneity and complexity of BLCa[1,22,40], creating two main issues. The first is that BLCa is the costliest cancer to treat on a per patient basis, representing an enormous financial burden to health care systems[40,61-63]. About 60-70% of costs are related to expensive and invasive cystoscopies and TURBs[37,64,65]. The second, is patient morbidity, because cystoscopies are frequently associated with

anxiety (58%), pain (59%), and side effects such as dysuria (50%), hematuria (19%) or urinary tract infection (3%)[66,67].

Therefore, the identification of cost-effective and noninvasive surveillance strategies has been a fundamental part of BLCa research.[64,68]. Urine cytology, while a reliable urine marker, because of its high specificity of 90-95%, discloses low sensitivity (30-50%) for BLCa detection (ranging from 16% for LG, to 84% for HG tumors) and, consequently, patients still need to undergo cystoscopy to check for recurrences during FU[21,69,70]. And, even though urine tests, such as BTA and NMP22, have been developed, depicting higher sensitivities (50-70%), specificity remains suboptimal (60-90%) and, hence, do not obviate the need for cystoscopy, and are not recommended for this purpose[21,25,70,71]. Therefore, the identification of accurate, easily obtainable, and cost-effective biomarkers for recurrence and progression would convey two main benefits in the care of these patients. First, it would help refine patient stratification, with a clearer understanding of which patients would benefit from further adjuvant therapy and which patients would effectively be at risk for overtreatment. Secondly, by serving as cost-effective monitor tools, they would reduce the need for invasive and expensive procedures. Consequently, biomarkers in NMIBC could effectively lead to improved clinical outcomes and quality of life[21,71,72].

On the other side of the spectrum is MIBC, an aggressive disease, where the rapid metastatic progression and high mortality (40-60% 5-year survival) are quite relevant[73,74]. Here, the major clinical problem is treatment failure due to inaccurate patient selection[1]. In non-responders, this delays effective therapy, leading to adverse prognosis, with unnecessary costs both economically and in the form of patient morbidity[1]. In NAC only about 30-40% patients disclose positive treatment response[1,75,76]. With ICI, despite some BLCa patients showing dramatic, complete, and durable responses, most relapse within the first few months of treatment or even fail to respond at all, with the overall response rates being only of about 15-25%[77-80]. One current limitation is the lack of tools to select patients that will most probably benefit from NAC or ICI[81]. Consequently, treatment is offered in a “one size fits all” manner and by trial and error, which is certainly a limiting factor in such a highly heterogeneous disease[1]. Accordingly, the identification of accurate and predictive biomarkers for non-responders *vs.* responders is one of the major challenges in MIBC research, which might help to improve patient outcome and avoid overtreatment in likely non-responders[1]. In this regard, molecular subtypes have been associated with different treatment response, and the use of biomarkers could not only allow us to identify them more easily, but also inform about the proportion of each subtype present in a single tumor – effectively, a complete tumor snapshot. Such information might prove valuable for appropriate and individualized therapy selection, with a higher likelihood of success and better oncological outcomes.

Liquid biopsies and extracellular vesicles (EVs)

As a repercussion to the above-mentioned issues, liquid biopsies have been gaining increased attention. Liquid biopsies correspond to sampling of biological fluids, such plasma and urine, and analysis of its contents in search of potential biomarkers, most commonly with the goal of improving cancer detection, follow-up, and prognostication[72,82]. Fundamentally, they are minimally invasive, rapid and have low-price, allowing serial and continuous monitoring, with a personalized snapshot of disease evolution – a fundamental pre-requisite of Precision Medicine[72,82,83]. Further, in Cancer, liquid biopsies are a very robust biomarker vehicle: not only are they an enriched source, but also more accurately reflect the phenotypic profile of all tumor subclones present in a patient, providing a wider perspective of the disease[82-84]. Therefore, liquid biopsies may provide real-time assessment of tumor burden, molecular profile, and genomics, including early evidence of recurrence[72,82]. In BLCa, while certain urine proteins[85,86] have been shown as differentially expressed in BLCa patients, the protagonist of liquid biopsies' biomarkers have been EVs.

EVs are a heterogenous population of lipid enclosed structures that may be classified into three main categories, according to the mechanisms by which they are formed: exosomes (30-150 nm diameter, by fusion of MVB with plasma membrane), microvesicles (100-1000 nm, by direct budding from plasma membrane) and apoptotic bodies (50-5000 nm, through shedding of dying cells)[87-89]. EVs can be produced by almost any kind of cell and are abundantly distributed in body fluids, including blood[24,82].

Exosomes, which have been gaining increasing interest, are constitutively generated by inward budding of late endosomes, forming ILVs and converting the endosome into a MVB, which then fuses with the plasma membrane and releases the ILVs into the extracellular space, now referred to "exosomes"[24,82,89]. During the budding process, certain proteins and cytosolic components are selectively enclosed within ILVs[83]. Accordingly, these contents reflect the pathophysiological status of the parental cell, including environmental conditions, developmental stages, specific phenotypes, and even epigenetic changes – a molecular signature of the parent cell[82,83,89-91]. Exosomes are particularly enriched in: tetraspanins (CD9, CD63, CD81, CD82, important in cell penetration and fusion events; HSP (HSP70, HSP90), involved in stress response; Proteins involved in exosome release (ALIX, TSG101)[87,89]. After release, exosomes display selectivity for tissues/cell homing, probably due to their high expression levels of adhesion molecules such as integrins and tetraspanins[89].

However, due to the considerable size overlap and because it is currently difficult to assign an EV to a particular biogenesis pathway, ISEV guidelines recommend the use of the generic term, EVs, and other operational terms, such as small EVs (< 100 nm) and large EVs (>100nm), since the term exosome may be misleading[92].

Nonetheless, EVs have, in recent years, emerged as novel and critical mediators of intercellular communication, both locally and distally[83,87,89]. They transport a multitude of molecules, including DNA, RNA, lipids, proteins, and metabolites[24,82,87,93], which are protected from degradation by the lipid bilayer-membrane[94,95]. These bioactive molecules have been shown to impact target cells by interacting with surface-bound ligands and signaling pathways, transferring activated receptors and cytokines and even through epigenetic reprograming, ultimately modulating recipient cells distal from their release[24,89]

EVs seem to play major roles in immune response[96], signal transduction[97] and antigen presentation[98], but it has been in Cancer where EVs play a role at multiple stages, that major interest has sparked[83]. EVs were suggested to be severely disturbed throughout multiple steps of tumorigenesis[24,82]. Namely, the transfer of pro-tumoral EV cargo has shown prominent roles in favoring cell proliferation[99], promoting angiogenesis[99], inducing drug resistance[82], modulating microenvironment in favor of cancer growth[87,100-106], and even in selecting and establishing premetastatic niches in specific tissue/organs[83,107].

Regarding the different contents within EVs, miRNAs have received great attention, as they represent the largest component of EV-content[72,89]. miRNAs are a family of evolutionarily conserved small non-coding RNAs (18-22 bp) that epigenetically modulate a wide array of biological processes[68,108]. Specifically, by promoting translational repression or even miRNAs' degradation in a sequence specific manner, they can act as oncogenes (onco-miRNAs) or as or tumor suppressors (TS-miRNAs)[108,109]. In Cancer they were found to be heavily dysregulated, playing a role in several hallmarks of cancer, such as resisting cell death, inducing angiogenesis or activating invasion and metastasis[108,110]. Further, their transport inside EVs allows them to influence other cells, producing transient or even persistent phenotypic changes[89,98]. MiRNAs have clearly demonstrated a crucial contribution to BLCa development and progression[83,111-115]. miRNAs' biomarker potential has also been highlighted. Because their expression signatures are associated with tumor type, grade and clinical outcomes, EVs' miRNAs released by cancer cells may serve as a diagnostic and/or prognostic biomarker, which has been the focus of recent BLCa research[4,68,108]. Besides miRNAs, other EV constituents may be of value, namely lncRNAs and circRNAs, which are important epigenetic regulators[83,116], and dsDNA, which reflects the mutational status of parental cells[117].

Differential UC is the most reported strategy for EV separation and concentration[118]. The principle is that under certain centrifugal forces, EVs can be sequentially separated from other soluble components and precipitated at the bottom of a tube, based on their different size and density[72,118]. UC provides EVs with high purity, is easy to use and requires very little technical expertise[72]. Because EVs may be co-isolated with other components, MISEV2018 guidelines recommend the use of multiple and complementary techniques for EV detection and

characterization to establish the likelihood that biomarkers are associated with EVs and not with contaminants[92]. First, particle number and size distribution should be measured, using single particle analyzers, such as NTA[92,119]. Secondly, the presence of at least 3 positive EV protein markers should be demonstrated, including at least one transmembrane/lipid-bound extracellular protein and one cytosolic/periplasmic protein with lipid or membrane protein-binding ability. Additionally, at least one purity control, a co-isolated contaminant, should be tested (Table III)[92]. After lysis, these proteins are released from the EVs and can be demonstrated by western blot assay[92]. Thirdly, images of EVs, with their intact lipid bilayer, should be presented, to demonstrate that the analyzed material is more than just cell fragments. TEM is the most used method for this purpose[72,92]. Finally, quantification of the biomolecules present in EVs, such as total protein and RNA amount, and the different ratios (protein/particle, RNA/particle) can both provide useful measures of purity and demonstrate the presence of potentially useful biomarkers[92].

Goals

This project is included in a larger study exploring the potential of EVs present in liquid biopsy samples of BLCa patients as a source for noninvasive biomarkers, which may lead to the refinement of patient stratification, therapy selection and real-time monitoring.

Specifically, the goals of this project were:

- Characterize the BLCa cohort of patients that donated liquid biopsies for the purposes of cancer research, with an emphasis on treatments performed and their outcome (recurrence, progression)
- Separate and concentrate EV from plasma of patients using ultracentrifugation
- Fully characterize EV isolates, using NTA, BCA, Western-blot assay, TEM and RNA extraction and quantification

Methods

Clinical data

Clinical data was collected from a total of 292 BLCa suspects, that had kindly donated biofluids, blood and/or urine, to the Biobank of Portuguese Oncology Institute of Porto, for the purposes of cancer research, between 16/02/2015 and 27/10/2021. A total of 57 clinical and pathological variables were defined (Table IV). Variables were either dichotomic (Yes/No), polytomous (multiple possible results), or corresponded to date or time-lapse. All variables were classified as *Unknown* and treated as "missing" during data analysis if there was no information regarding it, except for *Previous pelvic radiotherapy, Other Non-Neoplastic Urologic Diseases* and *Chronic Kidney Disease* in which case it was assumed as "*No*". All variables not applicable to the patient in question were classified as "*Not applicable*". The information was gathered resorting to medical records, clinical reports, and pathological reports. Since there is no specific definition of Recurrence in the literature, it was considered if there was a new lesion during FU occurring at least 3 months after total removal of all evident tumors, as it is the timing recommended by EAU guidelines for a first cystoscopy for recurrence detection. Furthermore, the *date of 1st recurrence* was the first cystoscopy demonstrating a suspicious lesion, later histologically confirmed to be BLCa. All patients' records were revisited in April/May of 2022 to check for updates, namely further recurrence, progression, or more recent treatment outcomes. All data was stored in a Microsoft Office Excel® (Version 2107) Spreadsheet protected with a password. Graphics were generated using IBM SPSS Statistics® 26. This study was approved by IPO Porto's Ethics Committee (CES 221/020).

Blood processing and storage

Blood-containing tubes were centrifuged at 5000g and 4º for 30 min. The supernatant plasma was then transferred to Eppendorf's and stored in Eppendorf's at IPOP's Biobank until further use.

Case selection

As part of the larger study in which this project is included, a group of 3 healthy donor controls + 9 patients with BLCa were already being studied for cancer biomarkers in urine EV, group of which plasma had also been collected on the same instance and was available at IPO Porto Biobank. These patients presented "naïve tumors" - the collection of samples occurred before patients underwent any TURB and represent a wide range of disease stages. The characteristics of the 9 BLCa patients are displayed in Table V. Out of these 12 samples, 6 (of which 1 was a control) were initially chosen to be characterized, with the remaining 6 being used for additional experiments.

EV separation and concentration using UC

EVs were separated and concentrated using a modified version the original protocol described by Théry et al.[120], previously successfully used to separate and concentrate urine EVs at the

Epigenetics and Cancer Biology Group. Plasmas were slowly thawed within ice for 90 min. They were then centrifuged at 5.000g and 4º C for 15 min to pellet and exclude cells and cellular remnants. Then, for each sample, 2 mL of supernatant were transferred into new Eppendorf's, 1 mL into each, 8 µL of Thrombin were added in each, followed by gentle mixing and a 30 min waiting period to allow the Fibrin clot to form. Subsequently, 992 µL of filtered PBS1x were added to each Eppendorf, followed by a 20 min, 6.000 G 4ºC centrifugation, to deposit the clot. The 2 supernatants of each sample were now transferred into a 15 mL falcon and diluted in 10 mL of filtered PBS1x. Afterwards, samples were passed through a 0.22 µm pore membrane PVDF filter (Millipore, Burlington Massachusetts, United States). Subsequently, the filtered supernatant was ultracentrifuged in a Beckman Coulter 70Ti rotor (Beckman Coulter, United States, California, Brea) at 200.000 g for 52 min (4 min speeding-up time + 48 min at full gravitational force) at 10°C. Supernatant was then discharged and the wall-attached pellet was continually resuspended in 100µL of PBS1x for 3 minutes. The final volume, which we call in this study "EV isolate" was transferred into a 1,5 mL Eppendorf and either directly utilized for NTA and BCA protocols, or store at -80ºC for further use.

NTA

Single particle analyses were carried out through Nanoparticle Tracking Analysis using a Nanosight NS3000 system (Malvern Panalytical, Malvern, UK) equipped with a red laser. EV isolates were diluted in filtered PBS1x, to ratios variable for each sample, through trial and error, to obtain a minimum of 1000 *Total Valid Tracks* and 10-50 *Particles per frame* to ensure valid and optimal results, as recommended by the equipment manufacturer. The diluted sample was loaded in a 1 mL syringe. A continuous syringe pump flow of 3 was used and target temperature within the equipment was set to 25ºC for all experiments. Five videos of 30 seconds were recorded, with a screen gain of 9 and a camera level of 10. The captured particle motion was analyzed by the NTA Software 3.4 Build 3.4.4 to estimate particles' number and size distribution.

BCA

Total protein quantification from EV isolates was done using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, Massachusetts, United States) in Microplates, following manufacturer's instructions, which can be found here[121].

Western-Blot

The EV specific proteins' presence was demonstrated through a western-blot assay. To each 10 µL of EV isolate was first added 6 µL of dH_2O, adding up to 16 µL. As controls, PC3 cell line extract and whole blood were used, with volumes adjusted to 30 µg of protein (8.40 µL PC3 extract + 7.60 µL of dH20 and 0.56 µL of whole blood + 15.44 µL of dH20). To all these, 4 µL of loading/deposition

buffer (2-mercaptoethanol, glycerol, SDS, EDTA and bromophenol blue) was then added. The samples and controls were then incubated at 95 º C for 5 min to both rupture EVs and lead to protein denaturation. Afterwards, electrophoresis gels were prepared to run the samples: Stacking (795 µL dH_2O; 315 µl 0.5M Tris-HCl ph 6.8; 125 µL 40% Acrylamide; 12.5 µL SDS; 2.5 µL TEMED; 6.25 20% APS) and 10% Acrylamide Running/Resolving (1225 µL dH_2O; 625 µl 0.5M Tris-HCl ph 8.8; 625 µL 40% Acrylamide; 25 µL SDS; 7.5 µL TEMED; 12.5 µL 20% APS). Then samples were pipetted into the wells of the Stacking gel and then separated by gel electrophoresis. Then, the proteins were transferred from the gels to a PVDF membrane (Bio-Rad Laboratories, Hercules, California, United States), using a Trans-Blot ® Turbo TM transfer system (Bio-Rad Laboratories, Hercules, California, United States). Membranes were then blocked with a 2.5% BSA + 2.5% Milk powder (gram/mL), 0.1% Tween 20 (mL/mL) solution for 2h, RT. Subsequently, membranes cut according to the size ranges that included the proteins of interest, and were incubated for 1h, RT, with primary antibodies against CD9 (1:500; sc-13118, Santa Cruz Biotechnology, Dallas, Texas, United States), CD81 (1:500; sc-166029, Santa Cruz Biotechnology, Dallas, Texas, United States), Flotilin-1 (1:300 sc-74566, Santa Cruz Biotechnology, Dallas, Texas, United States), ALIX (1:300; sc-53540, Santa Cruz Biotechnology, Dallas, Texas, United States), UPKIIIa (1:500; sc-166808, Santa Cruz Biotechnology), TSG101 (1:500; sc-7964, Santa Cruz Biotechnology), Albumin (1:1000; sc-271605, Santa Cruz Biotechnology), HSP70 (1:500; sc-32239, Santa Cruz Biotechnology). Membranes were then washed for 5 min, 3 times. Afterwards, membranes were incubated with a secondary antibody (1:5000; 7076S, Cell Signalling Technology, Danvers, Massachusetts, United States) during 1h, at RT. Then, membranes were washed for 5 min, 6 times. Finally, membranes were exposed to Clarity Western-Blot ECL substrate (Bio-Rad Laboratories, Hercules, California, United States). Signal was then captured using a western blot film.

Negative-staining transmission electron microscopy

The demonstration of intact EV structure was done by transmission electron microscopy (TEM), using negative staining. 10 µL of EV isolate, non-diluted and diluted in a 2:1 and 5:1 ratio, were mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA). After a 2 min waiting period, 10uL of 1% uranyl acetate were added onto the grids and excess liquid was removed. Visualization was carried out on a JEOL JEM 1400 microscope (Tokyo, Japan) at 120 kV, with magnifications ranging from 25.000x to 100.000x. Images were recorded using the digital camera Orious 1100W (Tokyo, Japan).

RNA extraction and quantification

EV RNA was extracted from 100 µL of EV isolate. First, free RNA was eliminated using 0.2 µg/mL of RNAse A (Sigma; R5503, Kawasaki, Kanagawa, Japan), added and incubated at 37°C during 30min.

Afterwards, a 1U/µL of RNAse A inhibitor (Thermofisher Scientific, United States, Massachusetts, Waltham) was added to the solution, and incubated at 37°C for 30 min, to inhibit further RNAse action. Then RNA was extracted from EVs using miRNeasy Serum/Plasma (Quiagen, Germany, Hilden), following manufacturer's instructions, which can be found here[122]. RNA quantification and profiling was carried out in a Bioanalyzer 2100 Expert (Agilent Technologies, Santa Clara, California, United States) using Eukaryote Total RNA Pico assay (Agilent Technologies, Santa Clara, California, United States). Notably, the assay only quantifies Small RNA between 6 and 150 nt.

Results

Of 292 patients, 28 were excluded (Figure 1): 9 were initially mistaken for bladder cancer (endometriosis implant, bladder wall thickening, radiation cystitis, among others), but latter ended up having benign conditions; 9 patients continued their follow-up at other institutions, and never did TURB at IPOP; 4 patients presented other non-urothelial neoplasia's involving the bladder (urachal adenocarcinoma, rectum adenocarcinoma that invaded the bladder wall, sigmoid colon adenocarcinoma that invaded the bladder wall and Non-Hodgkin lymphoma involving the bladder wall); 2 had upper tract urothelial carcinoma; 1 had a prostatic urethra only urothelial carcinoma; 1 had an inverted papilloma; 1 patient was too frail to undergo TURB for diagnosis and was treated symptomatically; 1 patient deceased before TURB was performed to confirm the diagnosis. Characteristics of the cohort can be seen in Figures 2 – 5, including some risk factors.

Thus, 187 patients disclosed NMIBC at diagnosis, and the distribution of histological types is depicted in Figure 6. It comprises 103 Ta, 80 T1, and 4 Tis. Regarding first treatment, results are shown in Figures 7 & 8. In this same cohort, there were a total of 48 patients with locally recurrent disease during FU, ranging from 1 to 5 recurrences for the same patient, as depicted in Figure 9. Of note, in this Figure are shown recurrences up to 1100 days (about 3 years) of FU, even though there were 5 recurrences beyond that (1208, 1767, 2560, 3821 and 4311 days). Furthermore, 22 patients experienced any form of disease progression (Figure 10).

In this group, 77 patients were initially diagnosed with MIBC. The histological types of MIBC are displayed in Figure 11. In 4 patients histological type remained unknown, due to patient frailty and advanced disease at first presentation (diagnosis was based on the evidence of a large bladder lesion, and in 2 cases complemented with biopsy of a metastasis). Concerning definitive treatment and progressions, results are shown in Figure 12. Of note, all NAC consisted of GC, except for one patient who preferred to be treated with Chemotherapy at another hospital, and the scheme used is unknown; out of the 8 patients that underwent palliative chemotherapy, 4 did GC, 3 Carboplatin-Gemcitabine and 1 MVAC; 1 patient preferred, after TURB, to be managed at another institution, with no information regarding treatment performed.

Thirty-five patients at IPO Porto were treated with Chemotherapy (29 already presenting with MIBC and 6 progressing from NMIBC). Among the 21 NAC patients (with GC): 9 showed a size reduction with residual or no tumor at cystectomy; 4 had a size reduction only; in 4 patients the size of the neoplasia was kept stable; 2 had a size increase during treatment; and 2 patients did only 1 and 2 cycles due to marked toxicity. In one patient that was treated with adjuvant chemotherapy, no recurrence was observed during treatment, but rather after its end. Finally, in the 13 patients that were under palliative chemotherapy (4 from progressing from NMIBC, 1 progressing after RC

only, and 7 as the first treatment): 2 showed size reduction only; 1 had complete nodal response and a primary lesion size reduction; in 2 the size of lesions was kept stable; 3 patients' lesions increased in size while under chemotherapy. In 5 patients, follow-up data was not sufficient to assess the response, either because only 1-2 cycles were performed, or, in one patient, chemotherapy was carried out at another institution.

Concerning immunotherapy, 9 patients underwent treatment with ICI: 8 were treated with Pembrolizumab, all as 2nd line treatment, except for one patient, in which it was 1st line treatment. Furthermore, one patient was treated with Avelumab as 2nd line treatment. Concerning the outcomes of ICI therapy, in 1 case the patient had just started treatment with ICI (1 cycle only) as of the writing of this manuscript. Concerning the remaining 8 cases: 2 had initially a favorable response to pembrolizumab, with disease stability, but later disease progression occurred, albeit they are still alive after 12 and 20 months under ICI; 1 treated with avelumab had slow disease progression, and died after 8 months; 1 case treated with pembrolizumab as 1st line also showed very favorable response, which is maintained 9 months after treatment started; in 2 cases, patients did cycle 1 and 2, but disease progressed and died; 1 case had disease progression with death after 4 months; 1 patient had disease progression after 3 months and started 3rd line chemotherapy.

Finally, 25 out of all the male patients that underwent cystoprostatectomy for bladder cancer (47) disclosed incidental PCa (Table VI), whereas PIN was present in 16 specimens. Histologically, all PCa cases were acinar adenocarcinomas, except one, which was an acinar adenocarcinoma with mucin producing areas. Eleven had a tumor with maximum Gleason score of 3+3, 11 of 3+4 and 1 of 4+3. Number of *foci* ranged from 1 to 7, and largest dimension from 0.1 cm to 2.3 cm.

As of April/May of 2022, of the 265 patients initially treated at IPO Porto, 56 had deceased, regardless of being or not still FU at our institution, 34 had been discharged (of which 3 later died), either due to lack of evidence of recurrence of NMIBC or to continue treatment elsewhere. One hundred and seventy-seven are still being actively FU at IPOP, 141 due to NMIBC and 36 in the context of MIBC. The distribution of patient status at last FU can be seen in Figure 13.

Regarding full EV characterization, results are shown in Tables VII & VIII and Figures 14 – 24. Western blot of CD81, HSP70 and Alix were depicted in all samples. CD9, while not showing a band with the correct MW (besides the positive control), presented a band with 30 kDa, both in the control and cases samples. TSG101, showed a strong signal at around 60 kDa, and at its MW of 45 kDa, Case 3 sample presented a subtle but unspecific band. Flotilin-1 was expressed with correct MW (47 kDa) in the sample of Case 3 as well, in the Control and Case 1, although with lower expression. Albumin, a category 3 protein, was present at the correct MW. Finally, UPKIIIa, while not part of the MISEV2018 guidelines, was tested as well.

Discussion and conclusions

Considering the clinical challenges previously enunciated concerning BLCa patients management, this study sought to determine the feasibility of using EVs isolates as a source of biomarkers to improve disease detection, monitoring, and risk stratification. It should be emphasized that this is part of a larger project ongoing at the Cancer Biology and Epigenetics Group of IPO Porto's Research Center that is exploring the potential of EVs present in liquid biopsy samples of BLCa patients.

The cohort of patients characterized for the purposes of this study included 48 out of 187 NMIBC patients which experienced recurrence (25.7%), a lower rate than expected based on published data. This may be explained by a shorter FU of some patients, particularly those diagnosed more recently, and/or a superior success rate in patient management, due to strict adherence to treatment guidelines of NMIBC patients. In the case of cancer progression, 22 patients progressed to any stage or grade above the one initially evident (11.7%). Notably, the term "progression" generally refers to upstaging from NMIBC to MIBC, that in this cohort was disclosed in 12 cases (6.4%), which is even lower, and once again, can be explained by the same reasons.

In the MIBC patients, 13 out of the 21 patients that underwent NAC, showed at least a size reduction when undergoing treatment (61.9%). Nonetheless, of the 13 patients treated with palliative intention, only 3 (23.1%) showed a favorable response. In the ICI group, conclusions are difficult to draw due to the reduced sample size, but it seems that some patients have very good responses, while others (about 50%) experience disease progression regardless of treatment. A larger sample is needed to take into consideration confounding factors such as different frailty status, disease stages and previous treatments, and provide more definitive conclusions.

An interesting finding was that 54.3% of male patients undergoing cystectomy and that did not have previously diagnosed PCa cancer were incidentally diagnosed with the disease. This is in line with the 40% reported in previous studies[22,123]. However, given the size, Gleason score and age of patients, it is unlikely that this cancer will affect their survival, or would have even affected were it not detected in this context.

Finally, it is worth noting that on Figure 13 data concerning *Death with evidence of disease* and *Death from the disease* is shown. Even though it was attempted by the author based on available clinical data, particularly the likely contribution of BLCa to the patient's deterioration and eventual demise, it is a highly subjective assumption, and these two parameters may be jointed as one for a more objective view. The major limitation of the database was the quality and quantity of information present in medical records, with lack of data for some variables.

Regarding EV separation and concentration, we showed in this study that UC continues to be an excellent option for isolating EVs in an efficient manner, even with all the innovative methods now available. Despite advances in the EV field, the challenge of efficient EV isolation is far from solved, particularly in biofluids, due to their complexity and variability[118]. Plasma, which despite being very attractive due to inherent richness in EVs, is a very complex medium, making high-yield EV isolation challenging[118]. UC remains the most widely used method, with high recovery but low specificity[92]. It offers some clear advantages, such as high processing capacity, low cost and requiring little technical expertise, but has clear limitations, including vesicle rupture, high protein contamination in the pellet, and is time consuming[89,118]. Due to the lack of a gold-standard, the choice will depend on the goals of the study[72,92]. In this case, the goal was to use a more accessible method, cost- and availability-wise, enabling an easier translation into the clinics. Additionally, the protocol was kept simple, with only one ultracentrifugation, as each additional step, while potentially increasing purity of the sample, further increases costs, time and reduces yield due to vesicle rupture[118]. One additional limitation might be that the available Ultracentrifuge only reaches 10◦C, while some protocols require 4◦C). However, this would unlikely affect the outcome.

Regarding NTA, overall, the graphics show a distribution, mean and mode of particle sizes compatible with the presence of EVs, particularly smaller EV, which may correspond to exosomes. Further, while only one control case was studied, its size distribution is slightly different from the cancer cases, showing a more uniform size distribution. Although this suggests that cancer cases might tend to have smaller EV sizes circulating in plasma, higher number of cases and controls are needed to test such a hypothesis. NTA does, however, have some drawbacks, as nanoparticles > 400 nm and <50 nm in size are not accurately quantified[89,92], which might underestimate the vesicle count.

We were also able to perform a full EV characterization. Currently, in the literature, even with all the attention that EVs have attracted in recent years, there are few publications providing a complete EV characterization, in accordance with the MISEV 2018 guidelines. By Western-Blot, the positivity for CD81, ALIX and HSP70 fulfill the MISEV minimal requirements, although some bands were rather weak. Additionally, the presence of an intense band, particularly in CD9, at a slightly higher MW suggests the presence of a glycosylated form of the protein, albeit this remains hypothetical. Surprisingly, UPKIIIa showed a very strong signal in all samples with correct MW. Although, we did not have any positive control for this protein marker, this suggests that EVs, potentially released by BLCa, reach the circulation, even in cases of NMIBC (Case 1 and Case 4). Therefore, this, not only suggests an early “connection” and intercommunication between cancer cells and the rest of the organism, but also that plasma can be a source of BLCa specific EVs, even at early stages of the disease.

Another potential limitation of the Western blot relates to the volume of sample used, as it was not adjusted to the amount of protein quantified by BCA, but rather to a volume of 10 µL for all samples. This was done because there is considerable non-EV protein contamination with this technique, which is overestimated by BCA[92], and may vary considerably. As, currently, there is no standard Western blot control for EVs, the use of the same volume for all patients might endow more consistent results.

TEM was first carried out with the standard preparation of a 1:1 dilution. However, as apparent in the first 4 captures (figures 32 – 35), although EVs can be seen, they appear to be overshadowed and merged in a dense material, most likely protein contamination, limiting their visualization. Upon this result, a dilution of 1:2 and then 1:5 was tested. As observed in figures 36 – 39, a higher number of EVs are now visible and in a much clear fashion. Further, there seems to be a marked predominance of small EVs, < 50 nm, and even < 30 nm. Considering the limitations of this technique, it causes dehydration of EV, potentially causing shrinking and altering their morphology[92,124] , which in this case may explain the large number of EVs reduced size.

Finally, regarding RNA quantification (Figures 26 – 31), all cases showed, small, but appreciable quantities of RNA, with variable proportions of miRNA (here, quantified between 6 and 40 nucleotides). This RNA, contained within EVs, has the potential to be used for discovery and validation of BLCa biomarkers.

BLCa is a very heterogeneous disease, exhibiting vast histological and molecular intra- and inter-tumor heterogeneity, with the multiple subtypes eventually coexisting within the same tumor[1]. As pointed both in the introduction and shown in the data collected, there are two critical issues regarding BLCa, at present: in NMIBC, unpredictable recurrences and progressions; in MIBC, the lack of treatment response prediction tools. EVs, and particularly, plasma EVs, separated and concentrated through a simple and accessible method, might be a venue to follow. Not only because they are extremely resistant to adverse physical conditions[72], but also are secreted in much higher numbers by tumor cells compared to non-tumor cells[82]. Because they mirror their parent cells, and since all cells in the tumor release EVs, their protein and/or RNA and/or DNA may better portray tumor genetics, epigenetics, and molecular and phenotypic profile.

In conclusion, this preliminary study disclosed that not only EVs may serve as biomarkers predictive of recurrence, progression, and therefore improve patients' management, but also, their contents (e.g., microRNAs) are amenable to be separated and concentrated, allowing for more accurate, real-time monitoring, providing critical clinical data on how the tumor is progressing.

Appendix

Tables

Table I - BLCa TNM staging system. Adapted from Amin MB, E. S. *et al.*(2017)[23].

T – primary tumor			
Tx	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
Ta	Noninvasive papillary carcinoma		
Tis	Carcinoma in situ		
T1	Tumor invades subepithelial connective tissue		
T2	Tumor invades muscle layer		
T2a	Tumor invades superficial/inner muscle layer		
T2b	Tumor invades deep/outer muscle layer		
T3	Tumor invades perivesical tissue		
T3a	Tumor invades perivesical tissue microscopically		
T3b	Tumor invades perivesical tissue macroscopically		
T4	Tumor invades prostate stroma, seminal vesicles, uterus, vagina		
T4a	Tumor invades pelvic wall or abdominal wall		
T4b	Tumor invades prostate stroma, seminal vesicles, uterus, vagina, pelvic wall or abdominal wall		
N – regional lymph nodes			
Nx	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastases in a single LN in the true pelvis (hypogastric, obturator, external iliac or presacral)		
N2	Metastases in multiple LN in the true pelvis (hypogastric, obturator, external iliac or presacral)		
N3	Metastases in common iliac lymph node (s)		
M – distant metastasis			
M0	No distant metastasis		
M1a	Nonregional lymph nodes		
M1b	Other distant metastases		
Stage grouping			
Stage 0a	Ta, N0, M0	Stage IIIA	T3a – T4a, N0, M0 *OR* T1-T4a, N1, M0
Stage 0is	Tis, N0, M0	Stage IIIB	T1 – T4a, N2 – N3, M0
Stage I	T1, N0, M0	Stage IVA	T4b, any N, M0 OR any T, any N, M1a
Stage II	T2a or T2b, N0, M0	Stage IVB	any T, any N, M1b

Table II - NMIBC patient risk groups, as established by the EAU and AUA for management of NMIBC, and recommended Treatment and FU according to the EAU. Adapted from Babjuk *et al.* (2019)[25] and Chang SS *et al.* (2016)[125].

Low - risk	Intermediate - risk	High - risk
According to the EAU		
All the following characteristics: Primary, Solitary, Ta LG (including PUNLMP), < 3 cm and no CIS	All not defined in the two adjacent categories. For example: - Ta LG single large (> 3cm) - Ta LG multiple small (< 3cm) - Ta LG multiple large (> 3cm) - Ta LG recurrent	T1 HG Presence of CIS Ta LG multiple, recurrent, and large (> 3 cm) (all features)
		Subgroup of highest risk: - T1 HG with bladder CIS - T1 HG with prostatic urethra CIS - T1 HG multiple or large - T1 HG recurrent - Lymphovascular invasion - Variant histology of UCa (some forms)
According to the AUA		
Any PUNLMP LG: - Ta and - ≤ 3cm and - Solitary	LG: - T1 or - > 3 cm or - Multifocal or - Recurrence within 1 year HG: - Ta and - ≤ 3cm and - Solitary	HG: - T1 or - > 3 cm or - Multifocal or - CIS
		Very high - risk features (any) - BCG unresponsive - Variant histology - Lymphovascular invasion - Prostatic urethral invasion
Recommended additional treatment		
One immediate instillation of intravesical chemotherapy after TURB	1-year intravesical BCG: induction + maintenance at 3,6 and 12 months OR Intravesical chemotherapy for a maximum of 1 year	1 to 3-year intravesical BCG: induction + maintenance at 3,6, 12, 18, 24, 30, 36 months (Consider radical cystectomy in highest risk)
Recommended FU		
Cystoscopy at 3 months, 12 months, then yearly for 5 years	Cystoscopy at 3 months, and then an in-between follow-up scheme using cystoscopy	Cystoscopy every 3 months for 2 years, every 6 months until 5 years, and then yearly Yearly upper tract imaging (CT-IVU or IVU)

Table III - Protein content-based EV characterization, according to MISEV 2018 guidelines. Adapted from Thery, C. *et al.* (2018)[92]

Category 1	Category 2	Category 3
Transmembrane or GPI- anchored proteins associated to plasma membrane and/or endosomes. **Demonstrates the lipid bilayer structure specific of EVs**	**Cytosolic proteins presumably actively incorporated into EVs due to the ability of bind membranes or cytosolic sequences of transmembrane proteins.** **Demonstrates that presence of lipid bilayers enclosing intracellular material**	**Major components of non-EV co-isolated structures** **Helps access the degree of purity of the EV preparation**
1a - non-tissue specific: -Tetraspanins (CD63, CD81, CD82) -MHC class 1 (*HLA-A/B/C, H2-K/D/Q*) -Integrins (*ITGA/ITGB*), Transferrin receptor, LAMP1/2, Complement binding proteins CD55 and CD59 1b - cell/tissue specific: - CD37 and CD53 (leucocytes), CD9 (absent from NK and B cells) - CD45 (immune cells), CD42a (platelets), MHC class II (HLA – DR/DP/DQ, H2-A)	2a – with lipid or membrane protein-binding ability: - ESCRT-I/II/III (*TSG101, CHMP*) and accessory proteins ALIX, VPS4A/B - Flotillins-1 and 2, Caveolins, HSC70 (*HSPA8*) 2b – promiscuous incorporation in EVs - HSP70 (*HSPA1A*) - Cytoskeleton proteins actin (*ACT*) and tubulin (*TUB*) - Enzymes (*GAPDH*)	3a – proteins (produced by liver, abundant in plasma) - Apolipoproteins A1/2 and B (*APOA1/2 and APOB*) - Albumin (*ALB*)

Table IV - List of all the clinical and pathological variables collected during the study.

Variable		Values
Birth date		Date
Gender		Male/Female
Relevant profession		See Figure 2
Weight	BMI	Value
	BMI Class	Underweight – Obesity class 3
Smoker		See Figure 3
Initial ECOG		0-4
First IPOP Urology's appointment		Date
Previous Pelvic Radiotherapy		Yes/No
Malignant Neoplasia		Type
		Date of diagnosis
Other Non-Neoplastic Urological Diseases		See Figure 4
Chronic Kidney Disease		Stage
First TURB		Date
Diagnosis	Date	Date
	Age	Age
Tumor	Size of initial neoplasia	Value in cm
	Histological classification	See Figures 6 & 11
	Multifocality	Yes/No
	Concomitant CIS	Yes/No
	LV invasion	Yes/No
Initial TNM	T	Tx – T4b
	N	Nx – N3
	M	M0 – M1b
Metastasis location (diagnosis)		Location
Stage grouping		I – IV
Initial EAU NMIBC Group Risk		Low risk Intermediate risk High risk
Initial AUA NMIBC Group risk		Low risk Intermediate risk High risk

(Cont.)

Variable		Values
First Treatment	Date	Date
	Type	See Figures 7, 8 & 12
Systemic chemotherapy for BLCa	Type	GC MVAC Adjuvant Gemcitabine-Cisplatin Palliative Gemcitabine-Cisplatin Palliative Gemcitabine-Carboplatin Palliative Chemotherapy, unknown scheme
	Starting date	Date
	Nr of cycles	Value
	Finishing date	Date
	Treatment outcome	Size reduction with residual/ no tumor Size reduction only Stable size Size increase/no response Complete ganglionar response + parcial local response No evidence of recurrence during adjuvant treatment
Cystectomy	Date	Date
	Margin status	R0 – R2
PCa on RC	Diagnosis	No Yes PIN+ Previous diagnosis
	Histology	Acinar adenocarcinoma Acinar adenocarcinoma with mucous producing areas Previous diagnosis but no current evidence of cancer
	Number of *foci*	Value
	Biggest *foci*	Value in cm

(Cont.)

Table IV (cont.) - List of all the clinical and pathological variables collected during the study

Variable		Values
ICI	IHQ	Not done, Positive result, Negative Result
	ICI	Pembrolizumab Avelumab
	Starting date	Date
	Nr of cycles	Number of cycles
	Treatment outcome	Size reduction with residual tumor Size reduction only Stable size Size increase/no response
Recurrence	Date of 1st recurrence	Date
	Time to 1st recurrence	Time value
	Number of recurrences	Value
Progression	Progression	Yes/No
	Date of 1st progression	Date
	Type	See Figure 11
	New treatment	ICI Palliative/Continued Care TURB + MMC Scheme TURB + BCG Scheme Palliative Chemotherapy Surveillance Cystectomy Palliative RT
Last FU (Urology, Oncology, Cystoscopy appointments)		Date
Discharge Date		Date
Death date		Date
Status at last FU		See Figure 13

Table V - Overview of cases selected for the study.

Case	Age at diagnosis	Gender	Stage (Grade)	Smoking status	Collecting date	Plasma collected while fasting
1	75	Male	T1N0M0 (HG)	Non-smoker	07/09/2020	No
2	60	Male	T2N0M0	Former smoker	06/11/2020	Yes
3	68	Female	T4aN0M0	Non-smoker	17/12/2020	Yes
4	62	Male	TaN0M0 (LG)	Active smoker	26/03/2021	No
5	76	Male	T3bN0M0	Former smoker	23/06/2021	Yes
6	61	Male	TaN0M0 (HG)	Former smoker	01/08/2019	Yes
7	75	Female	TaN0M0 (HG)	Active smoker	20/08/2019	Yes
8	61	Male	TaN0M0 (LG)	Former smoker	30/10/2020	Yes
9	75	Female	TaN0M0 (LG)	Non-smoker	20/05/2021	No

Table VI - Overview of patients incidentally diagnosed with PCa in the context of cystoprostatectomy.

Age at Cystectomy	Cystectomy date	Histology	Number of *foci*	Biggest *foci*	Highest Gleason
75	17/03/2016	Acinar Adenocarcinoma with mucous producing areas	2	2,3 cm	3 + 4
68	22/11/2016	Acinar Adenocarcinoma	2	1 cm	3 + 4
61	17/01/2017	Acinar Adenocarcinoma	1	0,4 cm	3 + 3
70	08/09/2017	Acinar Adenocarcinoma	5	0,5 cm	3 + 3
71	20/02/2018	Acinar Adenocarcinoma	2	0,5 cm	3 + 3
70	15/05/2019	Acinar Adenocarcinoma	5	1,2 cm	3 + 4
76	15/10/2019	Acinar Adenocarcinoma	4	1,2 cm	3 + 4
65	07/02/2020	Acinar Adenocarcinoma	1	1,2 cm	3 + 4
70	21/02/2020	Acinar Adenocarcinoma	1	0,1 cm	3 + 3
66	08/04/2020	Acinar Adenocarcinoma	1	0,2 cm	3 + 3
59	24/04/2020	Acinar Adenocarcinoma	2	0,1 cm	3 + 3
79	11/05/2020	Acinar Adenocarcinoma	3	1,6 cm	4 + 3
72	12/06/2020	Acinar Adenocarcinoma	1	0,3 cm	3 + 3
58	17/06/2020	Acinar Adenocarcinoma	2	0,3 cm	3 + 3
81	03/07/2020	Acinar Adenocarcinoma	7	0,4 cm	3 + 4
64	25/09/2020	Acinar Adenocarcinoma	1	0,1 cm	3 + 3
70	14/10/2020	Acinar Adenocarcinoma	2	1,1 cm	3 + 4
79	20/05/2021	Acinar Adenocarcinoma	1	0,4 cm	3 + 4
82	08/07/2021	Acinar Adenocarcinoma	1	0,3 cm	3 + 3
75	09/09/2021	Acinar Adenocarcinoma	3	0,3 cm	3 + 3
79	11/11/2021	Acinar Adenocarcinoma	5	1,2 cm	3 + 4
80	25/11/2021	Acinar Adenocarcinoma	4	1,2 cm	3 + 4
56	06/01/2022	Acinar Adenocarcinoma	6	2,1 cm	3 + 4

Table VII – Initial plasma and final EV isolate Volumes, Particle Concentration obtained via NTA and calculated Particle Concentrations on EV isolate and Plasma samples. EV – Extracellular vesicle; NTA – Nanoparticle tracking analysis; µL – Microliter; mL – Milliliter; E – Exponential number.

Sample	Particle Concentration (Particle / mL NTA fluid)	NTA Dilution Factor	Particle Concentration (Particle / mL EV isolate)	Particle Concentration (Particle / µL EV isolate)
Control 1	4,40E+08	3 : 1000	1,47E+11	1,47E+08
Case 1	3,13E+08	4 : 1000	7,83E+10	7,83E+07
Case 2	7,35E+08	1,5 : 1000	4,90E+11	4,90E+08
Case 3	2,43E+08	1,5 : 1000	1,62E+11	1,62E+08
Case 4	4,09E+08	3 : 1000	1,36E+11	1,36E+08
Case 5	6,56E+08	3 : 1000	2,19E+11	2,19E+08

Table VIII – Protein concentrations, protein to particle ratios, EV RNA concentrations and EV RNA to particle ratios. On case 5 RNA quantification was not done due to the plasma sample being markedly hematic, which would have most likely led to inaccurate quantification results. EV – Extracellular vesicle; RNA – Ribonucleic acid; µg – Picogram; mL – Milliliter; E- Exponential number.

Sample	Protein Concentration (µg/µL EV isolate)	Protein : Particle Ratio (ug / Particle)	EV RNA concentration (ng / mL Plasma)	EV RNA : Particle Ratio (ng / Particle)
Control 1	11,86	8,08E-08	152,4	2,08E-08
Case 1	9,89	1,26E-07	102,6	2,62E-08
Case 2	7,55	1,54E-08	166,7	6,80E-09
Case 3	12,24	7,56E-08	250,9	3,10E-08
Case 4	10,37	7,61E-08	287,0	4,21E-08
Case 5	25,83	1,18E-07	Not Done	Not Done

Figures

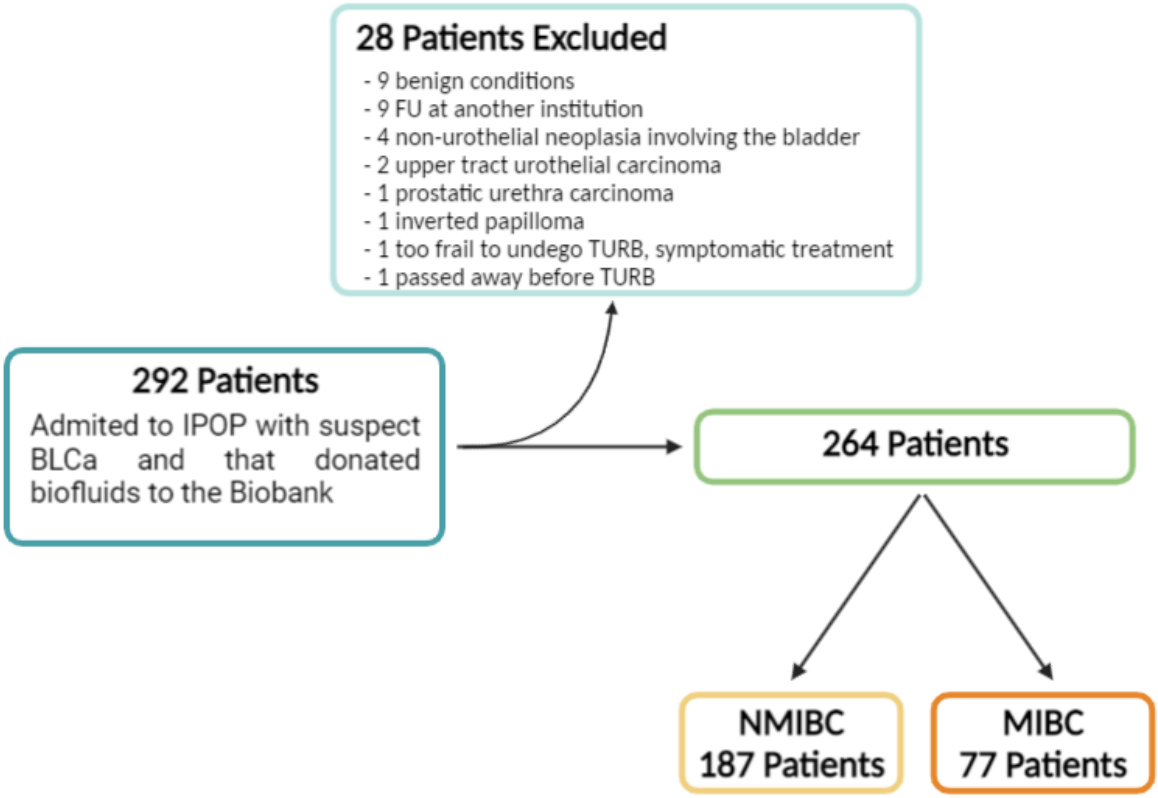
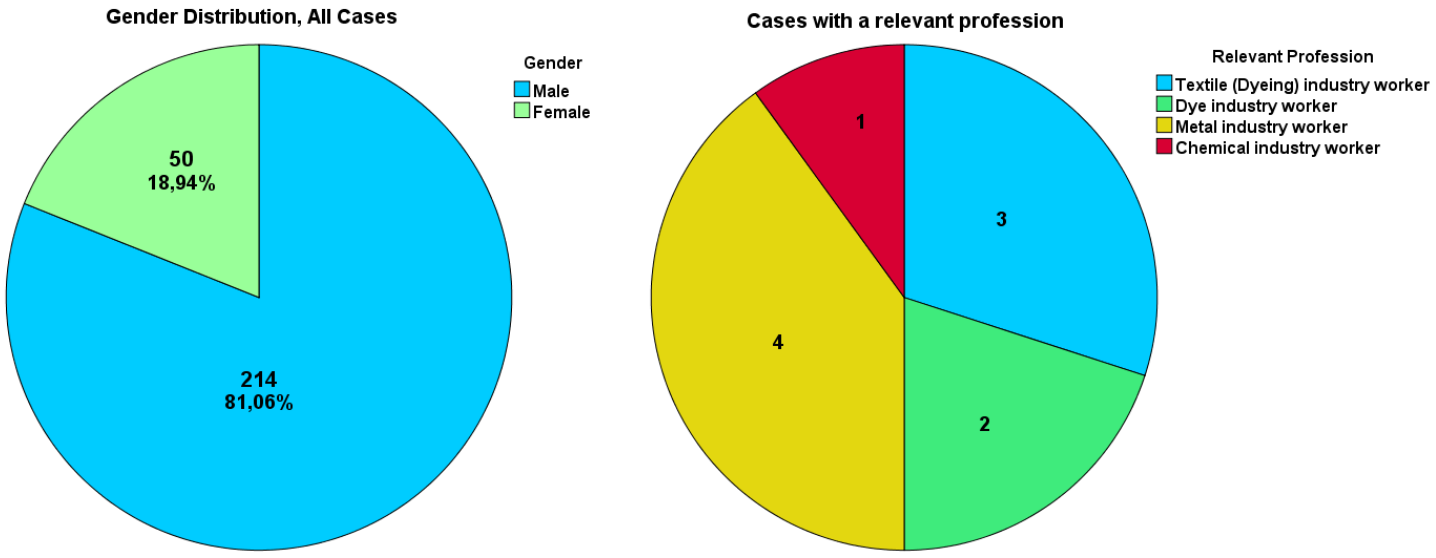


Figure 1 - Overview of Database. IPOP - Portuguese Oncology Institute of Porto; BLCa – Bladder cancer; FU – Follow-up; TURB – Transurethral resection of the Bladder; NMIBC – Non-muscle-invasive Bladder Cancer; MIBC – Muscle-invasive Bladder cancer.



Figures 2 – Overview of BLCa Cohort: Gender distribution and Cases with a relevant profession.

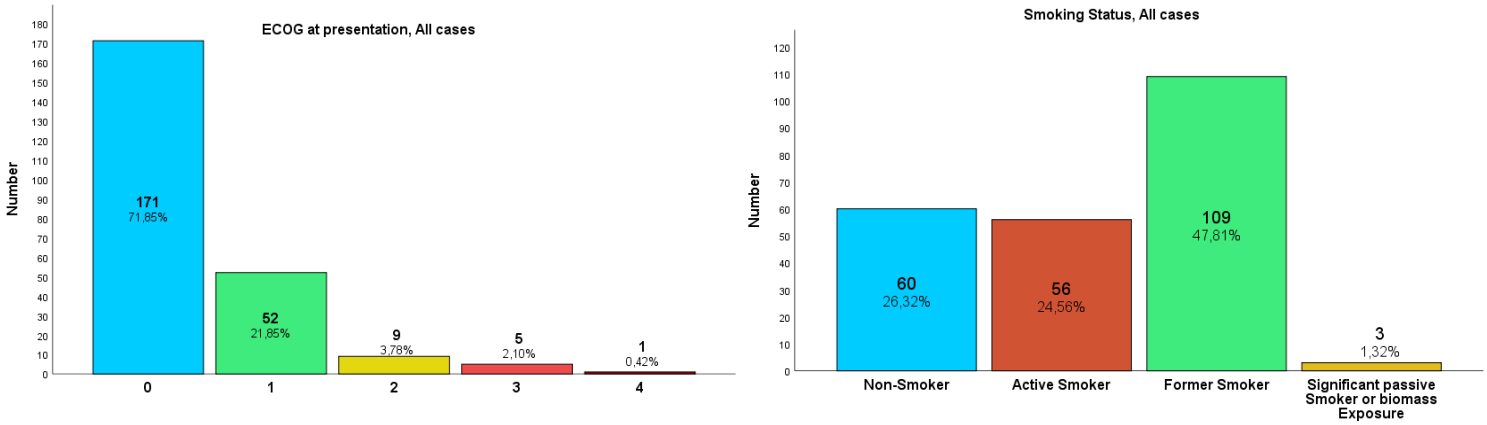


Figure 3 – Overview of BLCa Cohort: ECOG at presentation and Smoking Status. ECOG – Eastern Cooperative Oncology Group.

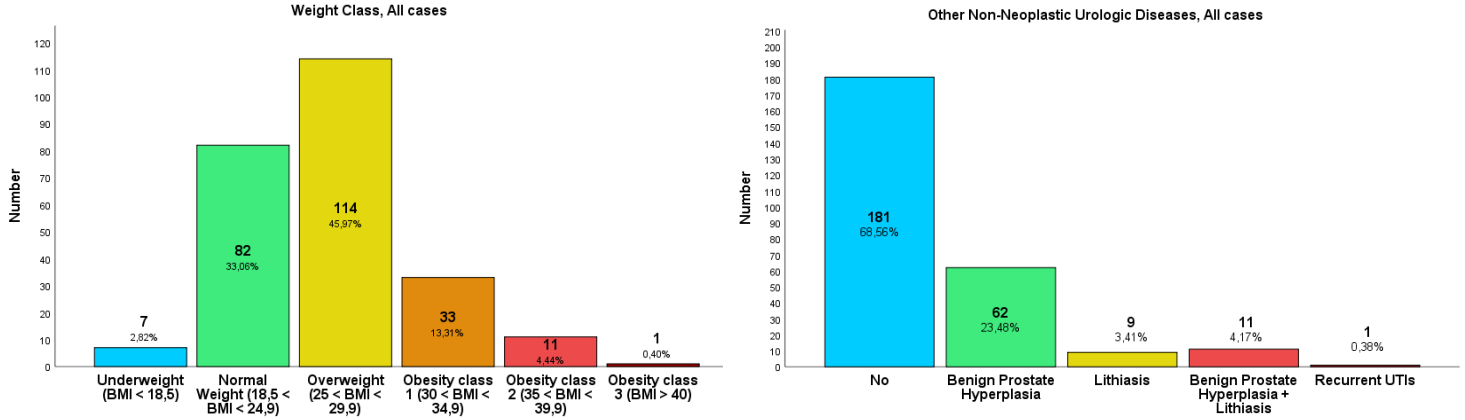


Figure 4 – Overview of BLCa Cohort: Weight Class and Other Non-Neoplastic Urologic Diseases. BMI – Body Mass Index; UTIs – Urinary Tract Infections.

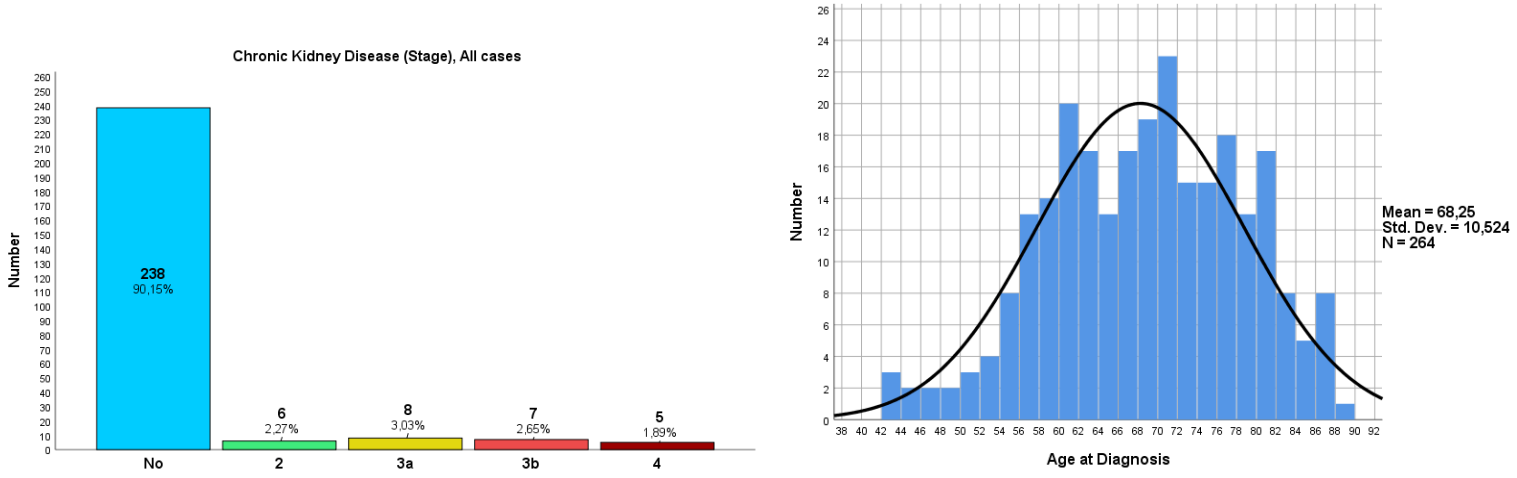


Figure 5 – Overview of BLCa Cohort: Chronic Kidney Disease (Stage) and Age at Diagnosis.

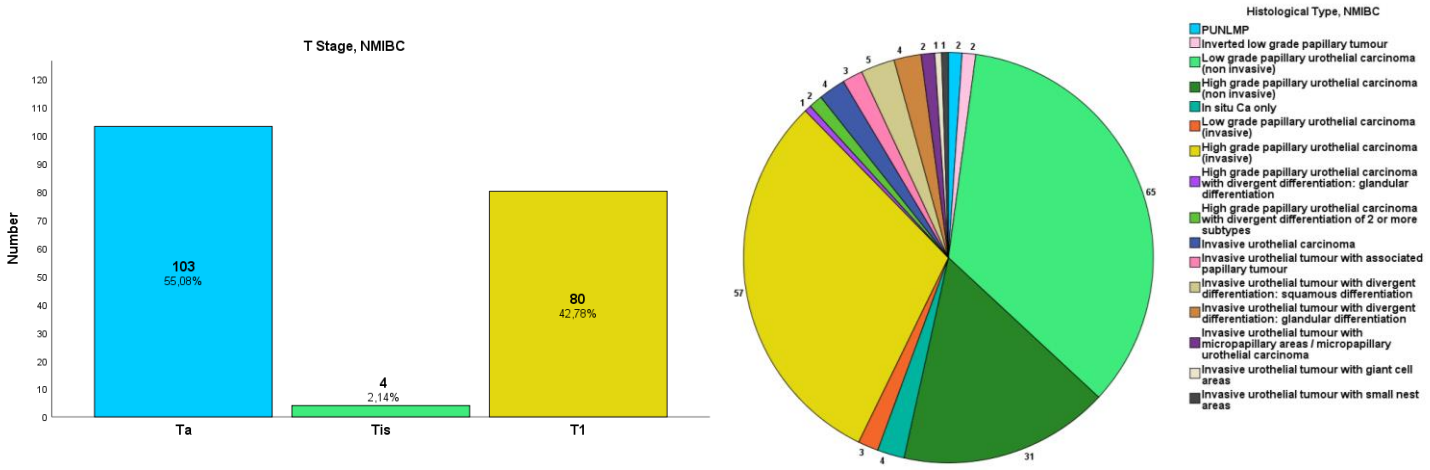


Figure 6 – NMIBC, T stage and Histological Type, at diagnosis. NMIBC – Non-muscle-invasive bladder cancer; PUNLMP – Papillary urothelial neoplasm with low malignant potential.

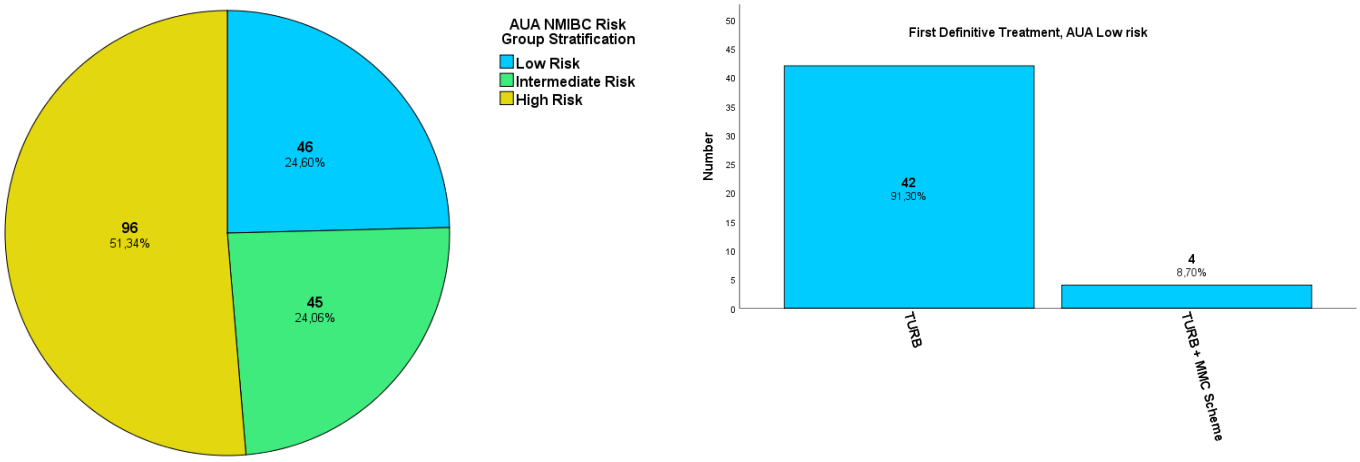


Figure 7 – NMIBC, AUA Group Risk at diagnosis and First Definitive Treatment done on patients of Low risk. NMIBC – Non-muscle-invasive bladder cancer; AUA – American Urology Association; TURB – Transurethral resection of the bladder; MMC – Mitomycin C.

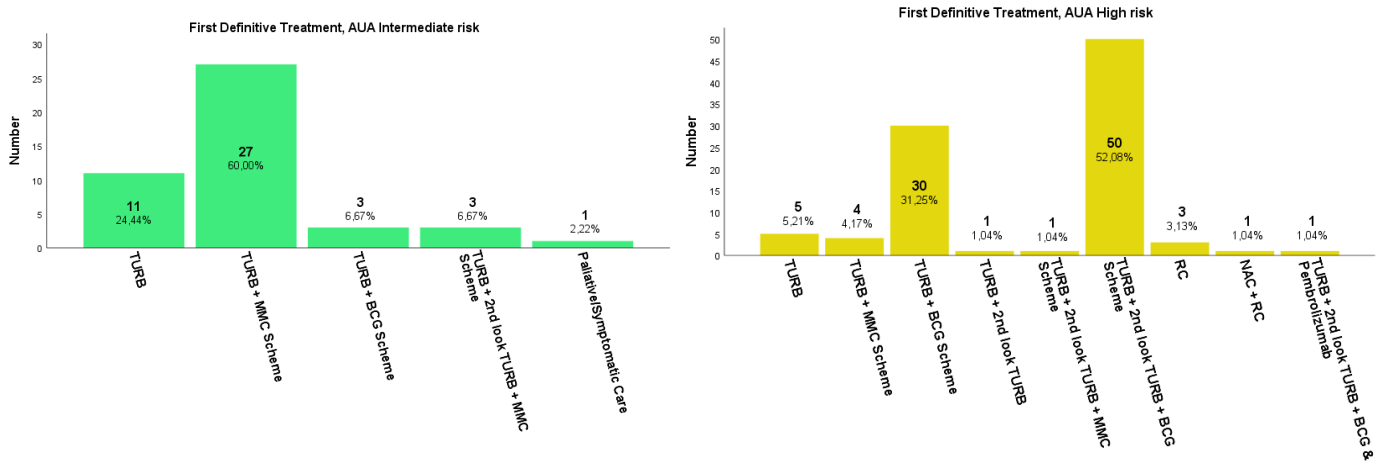


Figure 8 – NMIBC, First Definitive Treatment done on patients of Intermediate and High risk. NMIBC – Non-muscle-invasive bladder cancer; AUA – American Urology Association; TURB – Transurethral resection of the bladder; MMC – Mitomycin C; BCG – Bacillus Calmette – Guerin; RC – Radical cystectomy; NAC – Neoadjuvant chemotherapy.

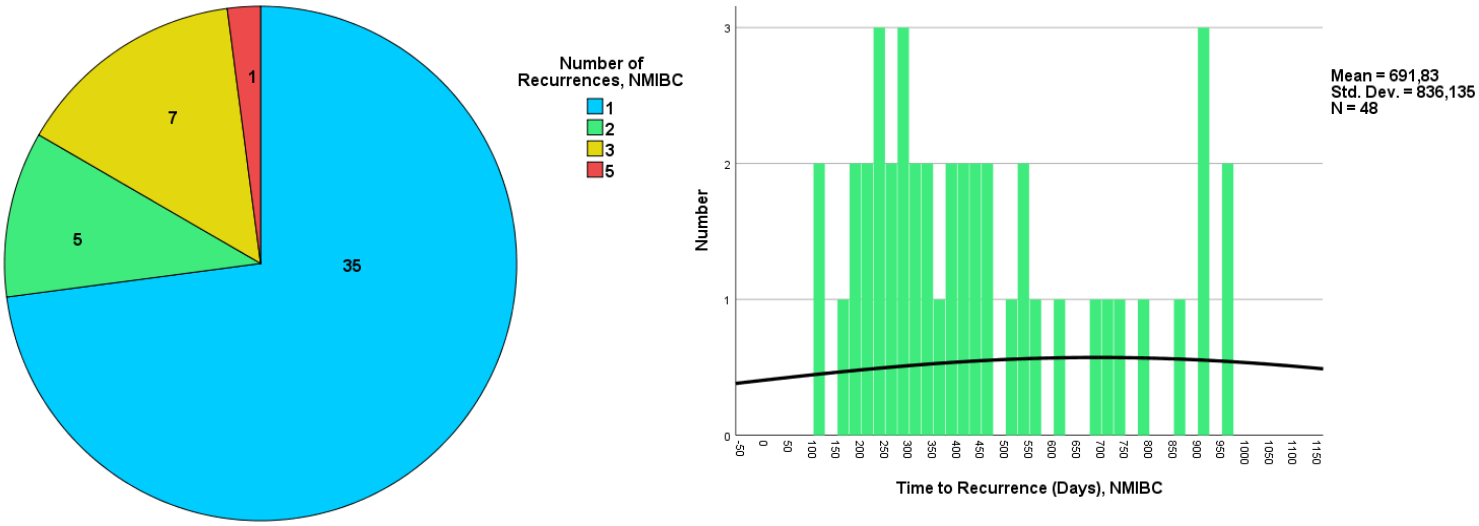


Figure 9 – NMIBC, Number of recurrences and time gap from diagnosis to recurrence. NMIBC – Non-muscle-invasive bladder cancer.

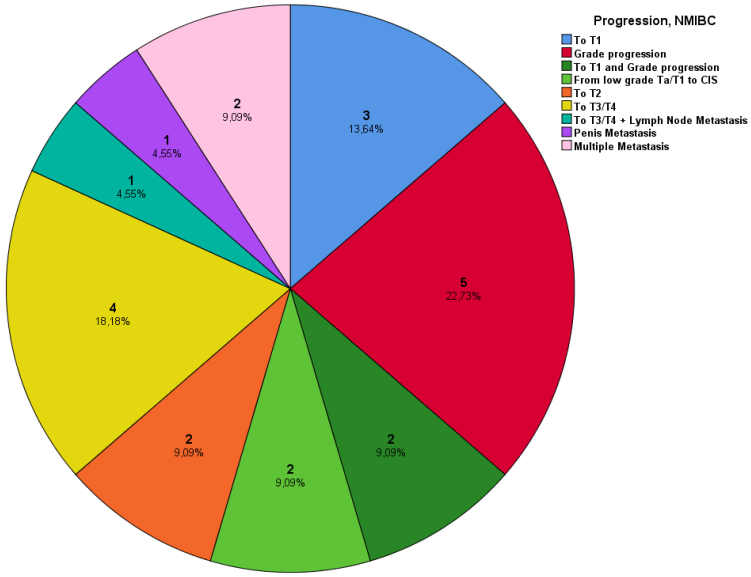


Figure 10 – NMIBC, Progression and type of progression. NMIBC – Non-muscle-invasive bladder cancer; CIS – Carcinoma *in situ.*

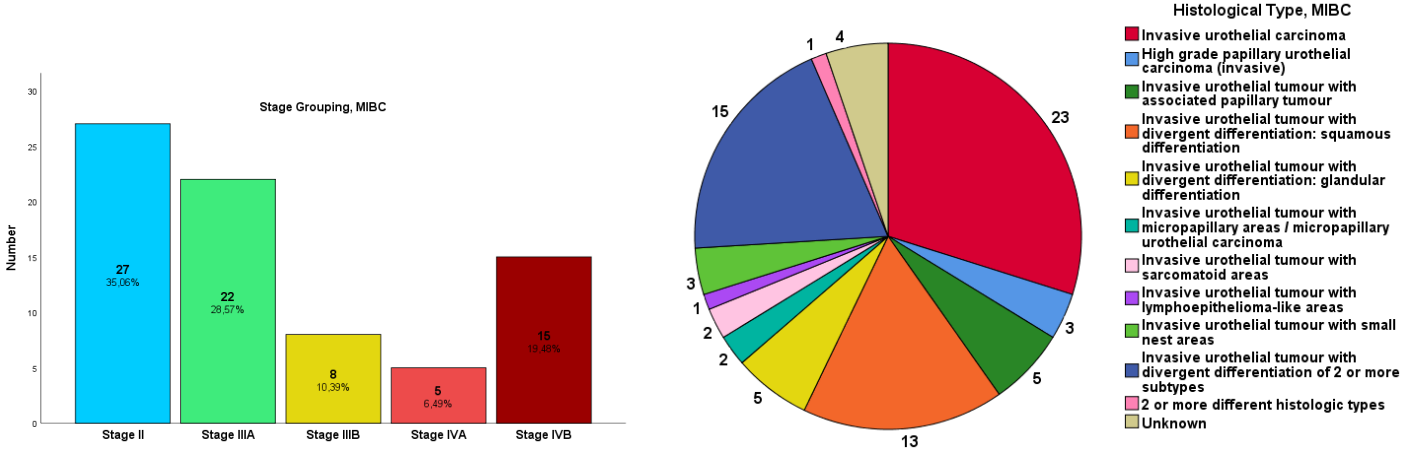


Figure 11 – MIBC, Stage Grouping and Histological classification, at diagnosis. MIBC – Muscle-invasive bladder cancer.

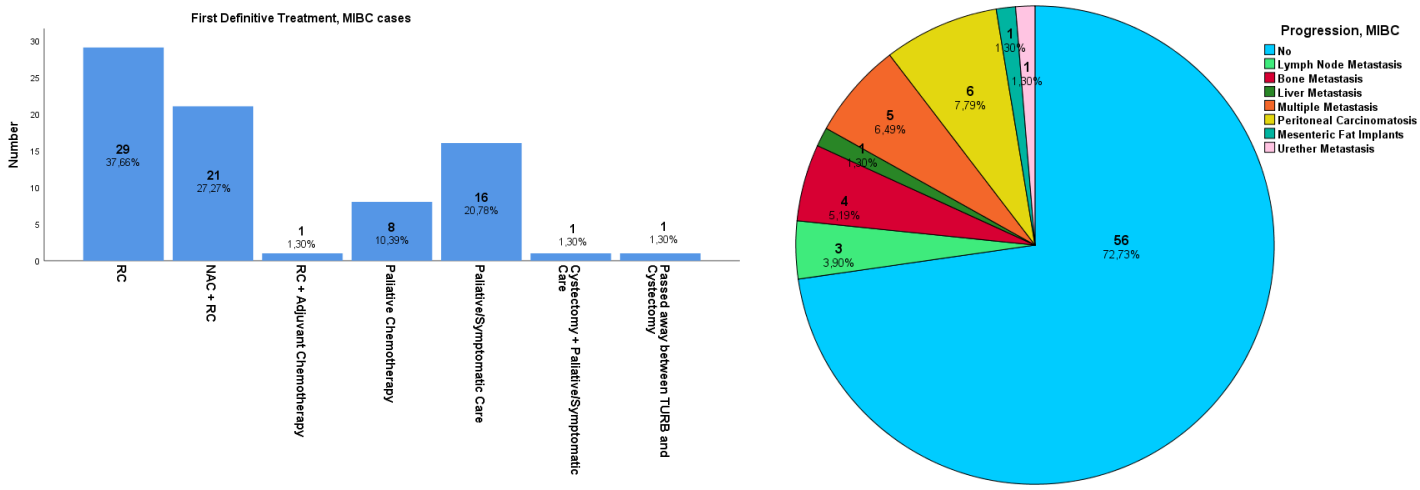


Figure 12 – MIBC, First Definitive Treatment and Progressions. MIBC – Muscle-invasive bladder cancer; RC – Radical Cystectomy; NAC – Neoadjuvant Chemotherapy.

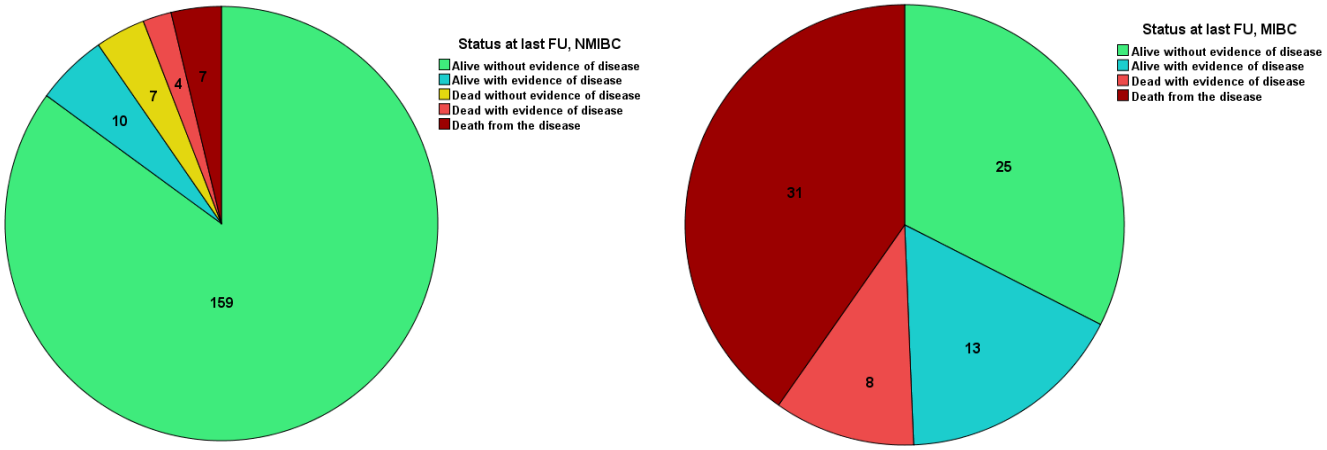


Figure 13 – Status at last FU in NMIBC and MIBC group. NMIBC – Non-muscle-invasive Bladder Cancer; MIBC – Muscle-invasive Bladder Cancer.

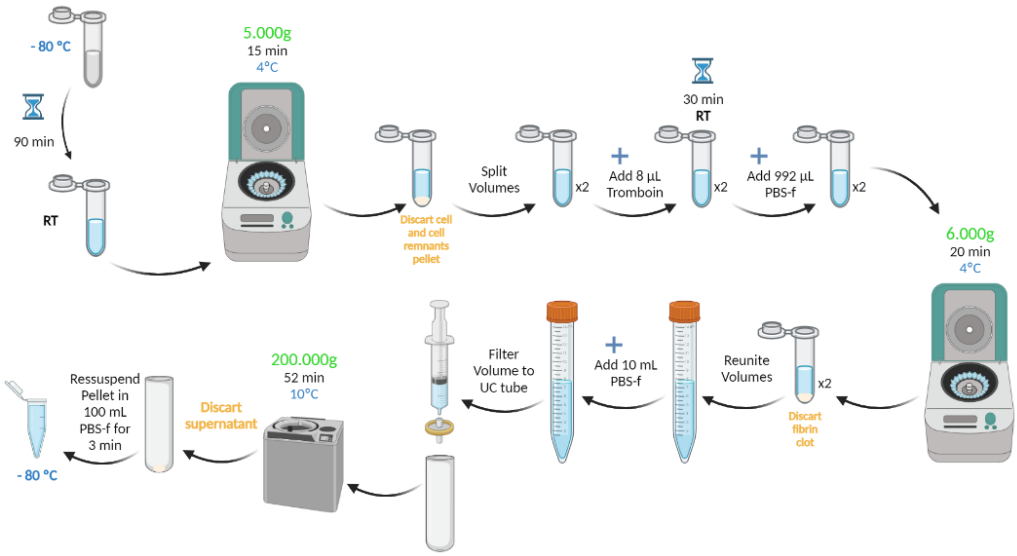
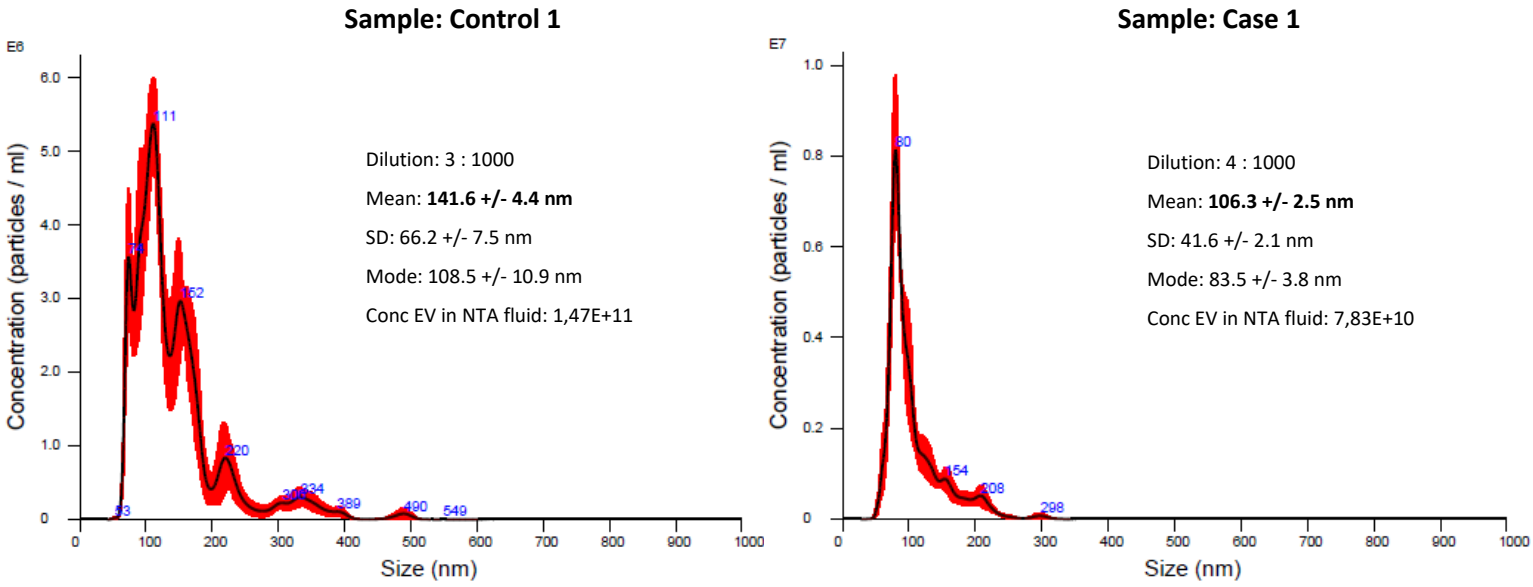
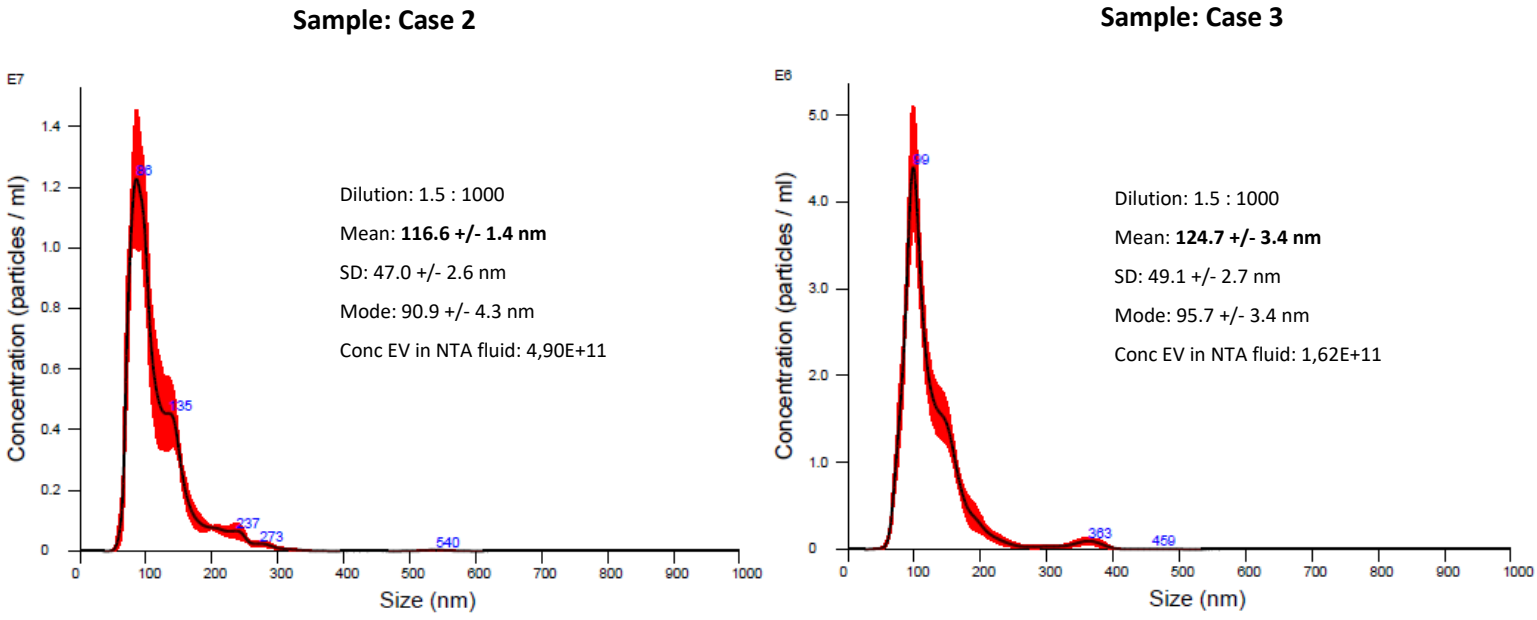


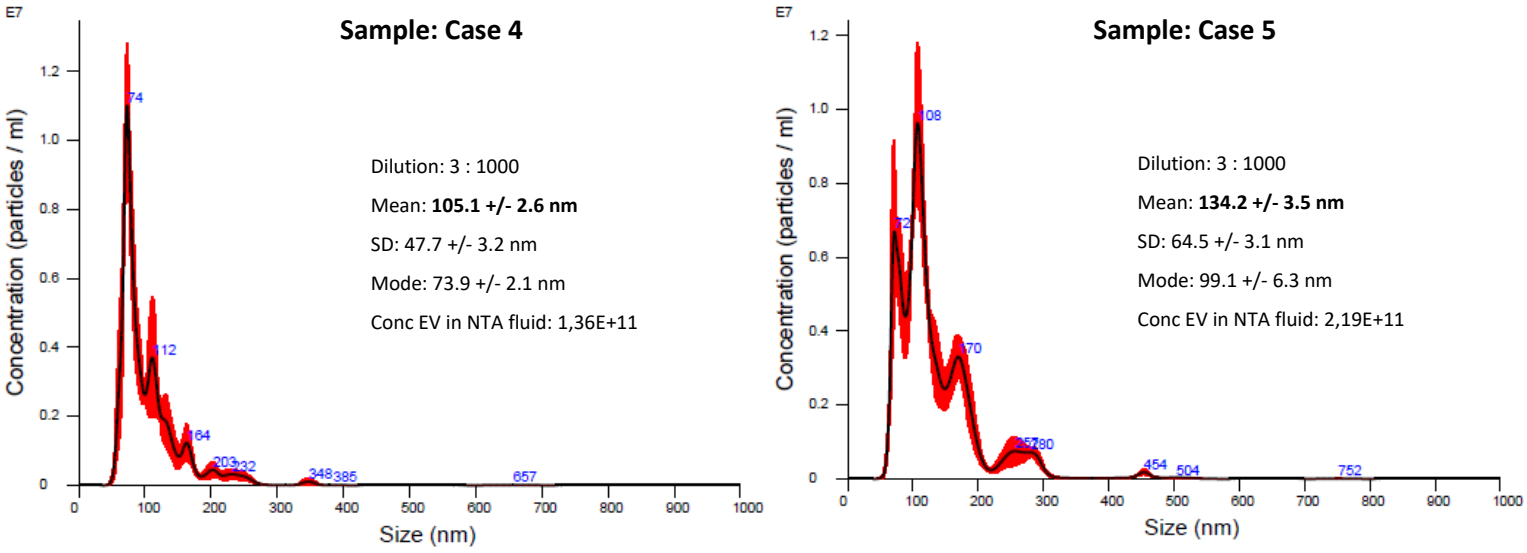
Figure 14 – Overview of EV separation and concentration protocol. EV – Extracellular vesicle; min – Minute; RT – Room temperature; ºC – Degree(s) Celsius; µL – Microliter; g – Relative centrifugal force units; mL – Milliliter; PBS-f – Filtered Phosphate-Buffered Saline. UC – Ultracentrifuge.



Figures 15 - NTA results, from Control 1 and Case 1. mL – Milliliter; nm – nanometer; EV – Extracellular vesicle; SD – Standard deviation; NTA – Nanoparticle tracking analysis; E – Exponential number.



Figures 16 - NTA results, from Case 2 and Case 3. ml – Milliliter; nm – Nanometer; EV – Extracellular vesicle; SD – Standard deviation; NTA – Nanoparticle tracking analysis; E – Exponential number.



Figures 17 - Nanoparticle Tracking Analysis results, from Case 4 and Case 5. ml – Milliliter; nm – nanometer; EV – Extracellular vesicle; SD – Standard deviation; NTA – Nanoparticle tracking analysis; E – Exponential number.

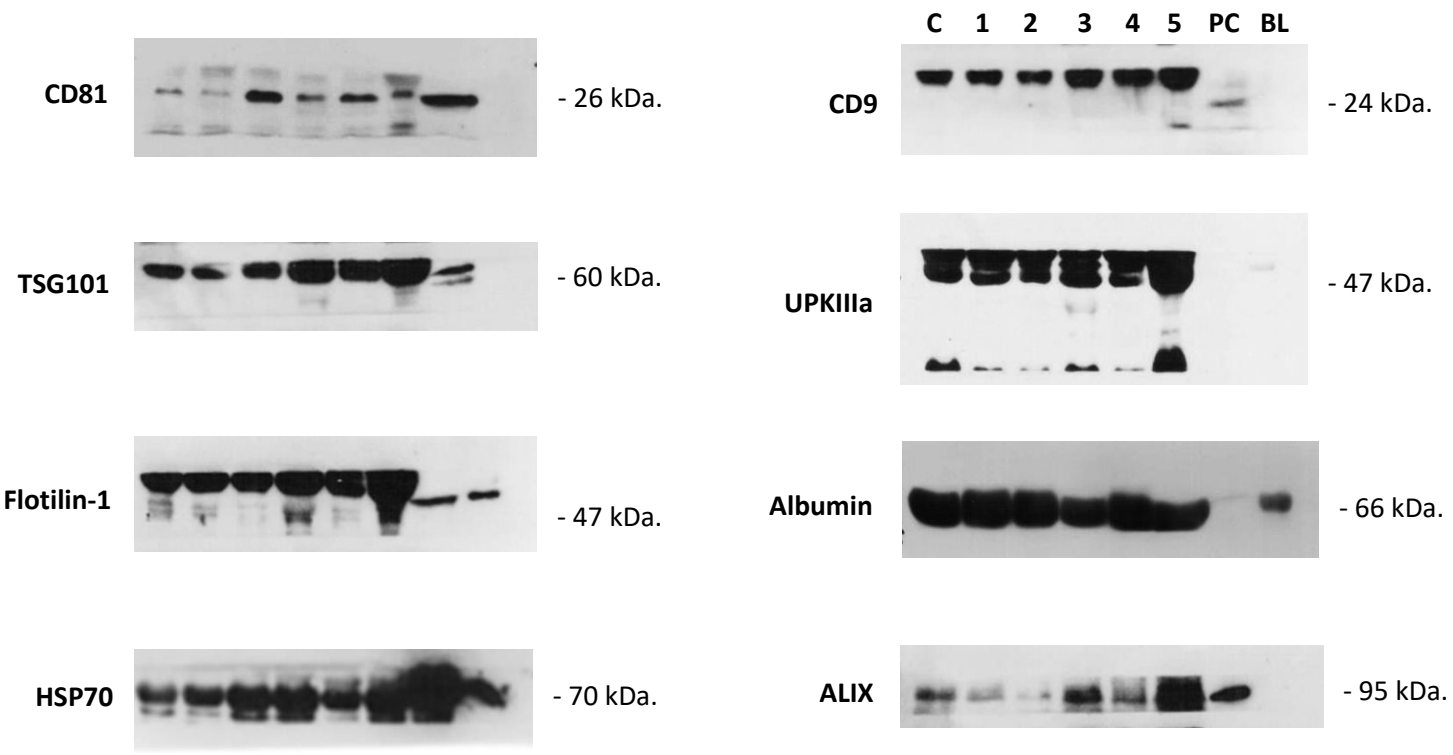
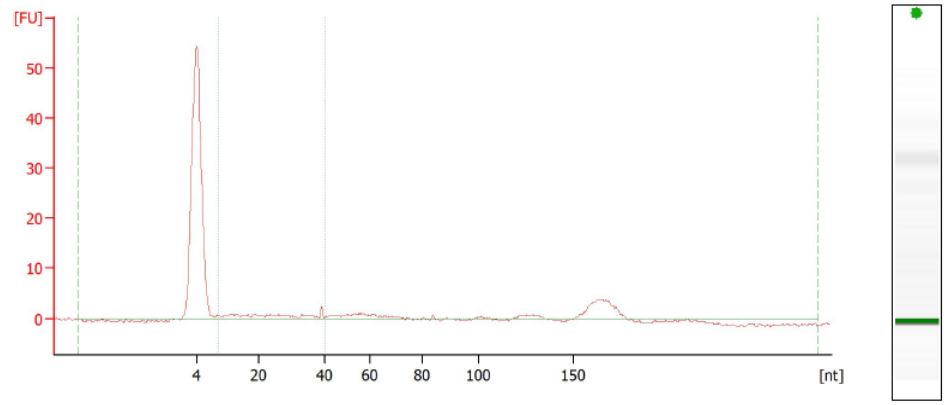


Figure 18 - Western-Blot results, from Control 1, Cases 1 – 5 using PC3 cell extract and Whole Blood as controls. C – Control; 1 – Case 1; 2 – Case 2; 3 – Case 3; 4 – Case 4; 5 – Case 5; PC – PC 3 cell extract; BL – Whole Blood; CD – Cluster of differentiation; HSP – Heat shock protein; TSG – Tumor susceptibility; UPK – Uroplakin; ALIX – ALG-2 Interaction protein; kDa – kilodalton.

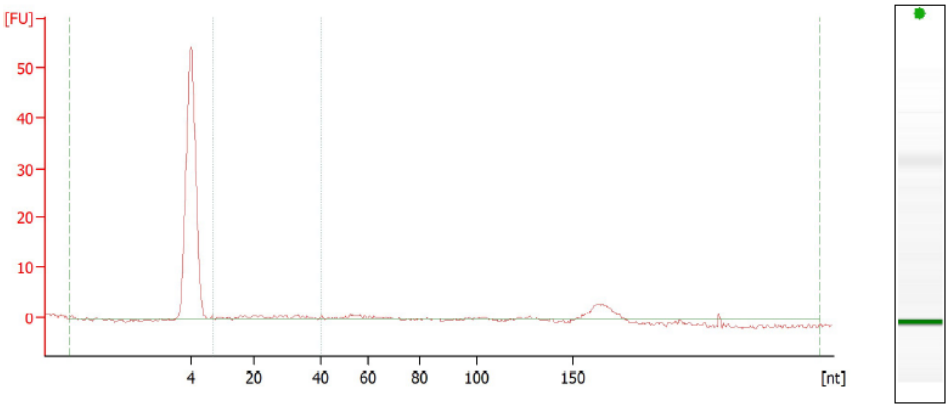
Sample: Control 1



Overall Results for sample 6 : **Sample 6**

Small RNA Concentration [pg/µl]:	152,4	Result Flagging Color:	
miRNA Concentration [pg/µl]:	92,9	Result Flagging Label:	61 % miRNA; Concentration: 92.90 pg/µl
miRNA / Small RNA Ratio [%]:	61		

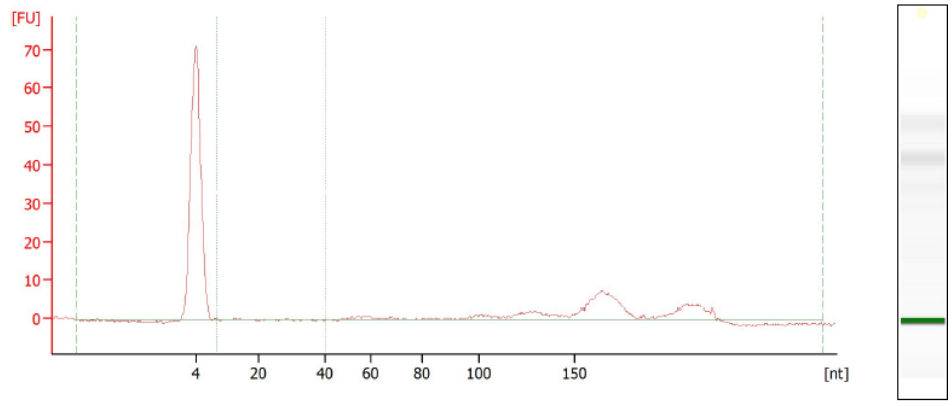
Sample: Control 2



Overall Results for sample 7 : **Sample 7**

Small RNA Concentration [pg/µl]:	78,9	Result Flagging Color:	
miRNA Concentration [pg/µl]:	45,0	Result Flagging Label:	57 % miRNA; Concentration: 45 pg/µl
miRNA / Small RNA Ratio [%]:	57		

Sample: Case 1

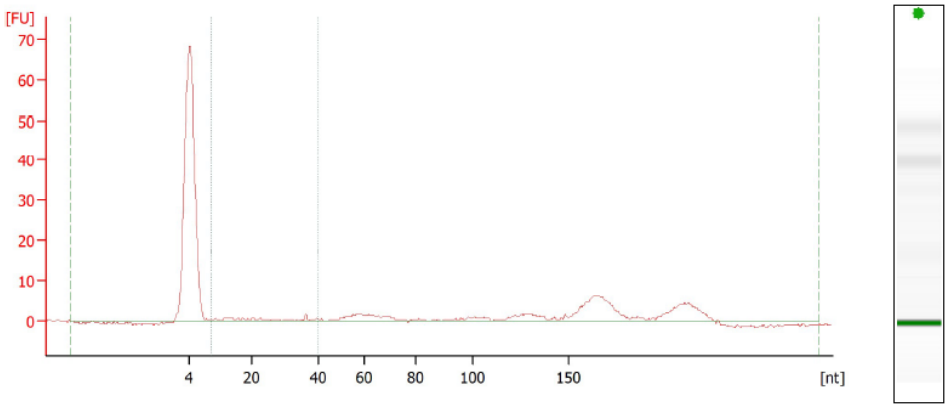


Overall Results for sample 8 : **Sample 8**

Small RNA Concentration [pg/µl]:	102,6	Result Flagging Color:	
miRNA Concentration [pg/µl]:	0,8	Result Flagging Label:	1 % miRNA; Concentration: 0.80 pg/µl
miRNA / Small RNA Ratio [%]:	1		

Figures 19 - Small RNA quantification results, from Controls 1 & 2 and Case 1. RNA – Ribonucleic acid; miRNA - Micro Ribonucleic acid; pg – Picogram; µL – Microliter; nt – Nucleotide; FU' – Fluorescence units.

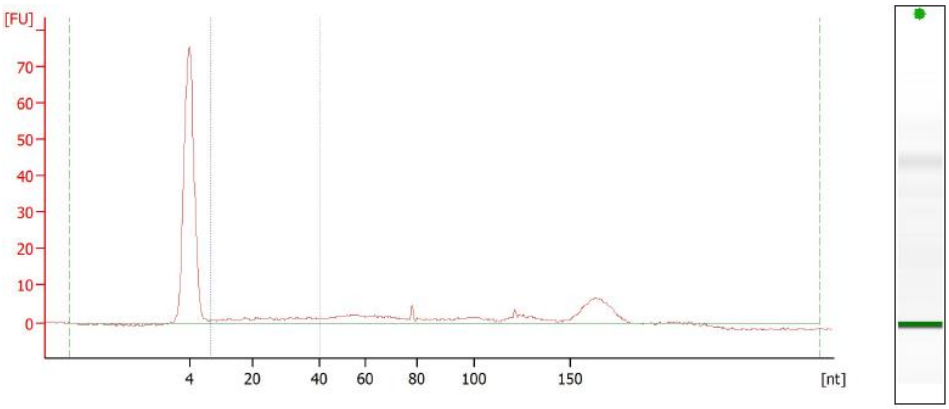
Sample: Case 2



Overall Results for sample 9 : **Sample 9**

Small RNA Concentration [pg/µl]:	166,7	Result Flagging Color:	
miRNA Concentration [pg/µl]:	44,7	Result Flagging Label:	27 % miRNA; Concentration: 44.70 pg/µl
miRNA / Small RNA Ratio [%]:	27		

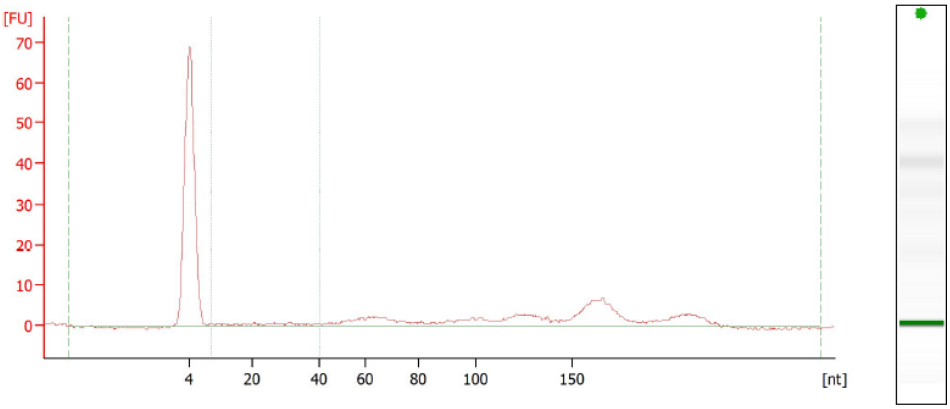
Sample: Case 3



Overall Results for sample 10 : **Sample 10**

Small RNA Concentration [pg/µl]:	250,9	Result Flagging Color:	
miRNA Concentration [pg/µl]:	114,4	Result Flagging Label:	46 % miRNA; Concentration: 114.40 pg/µl
miRNA / Small RNA Ratio [%]:	46		

Sample: Case 4



Overall Results for sample 11 : **Sample 11**

Small RNA Concentration [pg/µl]:	287,0	Result Flagging Color:	
miRNA Concentration [pg/µl]:	77,6	Result Flagging Label:	27 % miRNA; Concentration: 77.60 pg/µl
miRNA / Small RNA Ratio [%]:	27		

Figures 20 - Small RNA quantification results, from Cases 2 – 4. RNA – Ribonucleic acid; miRNA - Micro Ribonucleic acid; pg – Picogram; µL – Microliter; nt – Nucleotide; FU' – Fluorescence units.

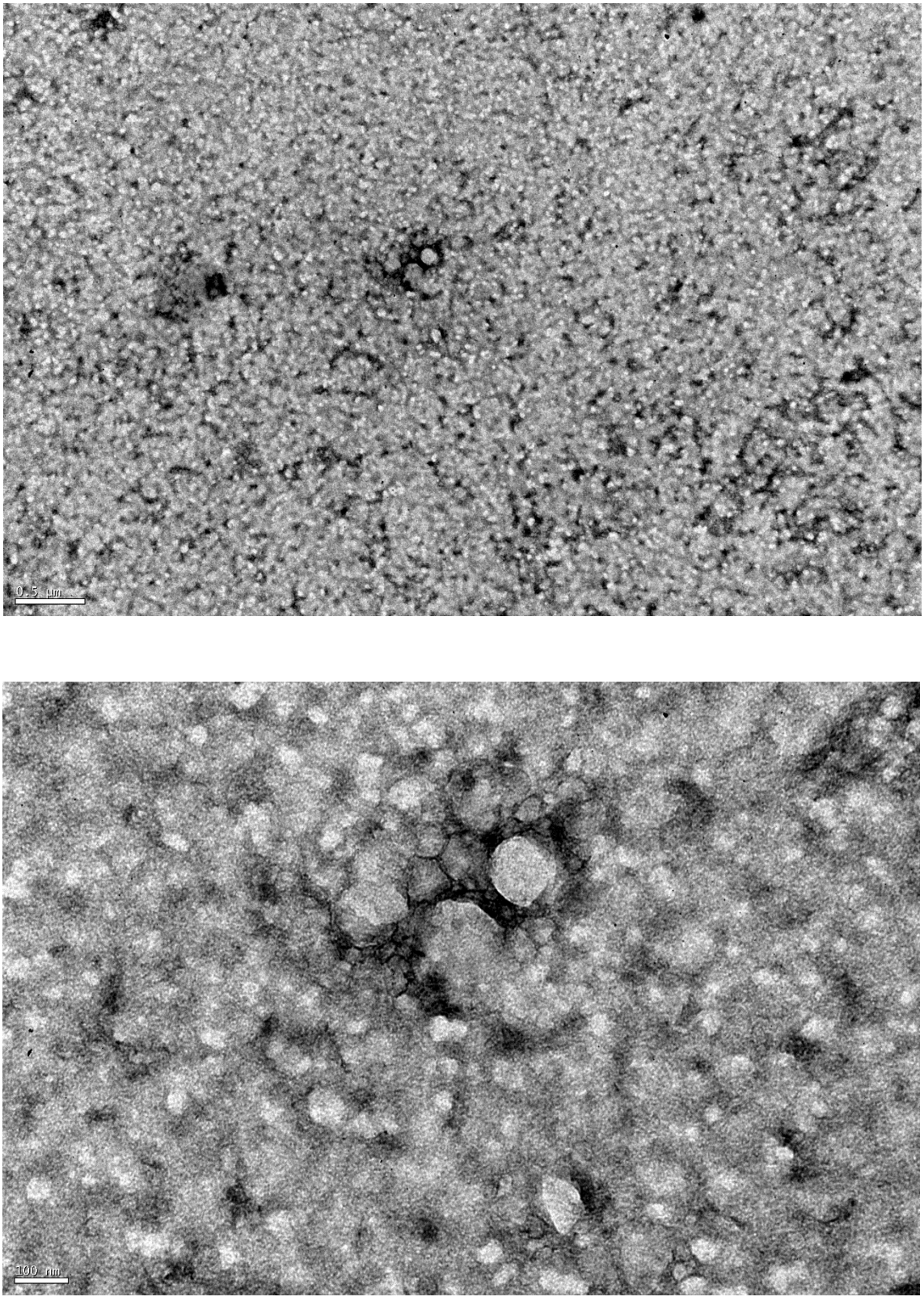


Figure 21 - TEM captures from Case 6. The isolated EVs were diluted on a 1:1 ratio with PBS (standard preparation). The above image was obtained at 25.000x magnification, and the one below is an amplified version of the central area of the former, at 100.000x magnification. nm – nanometer.

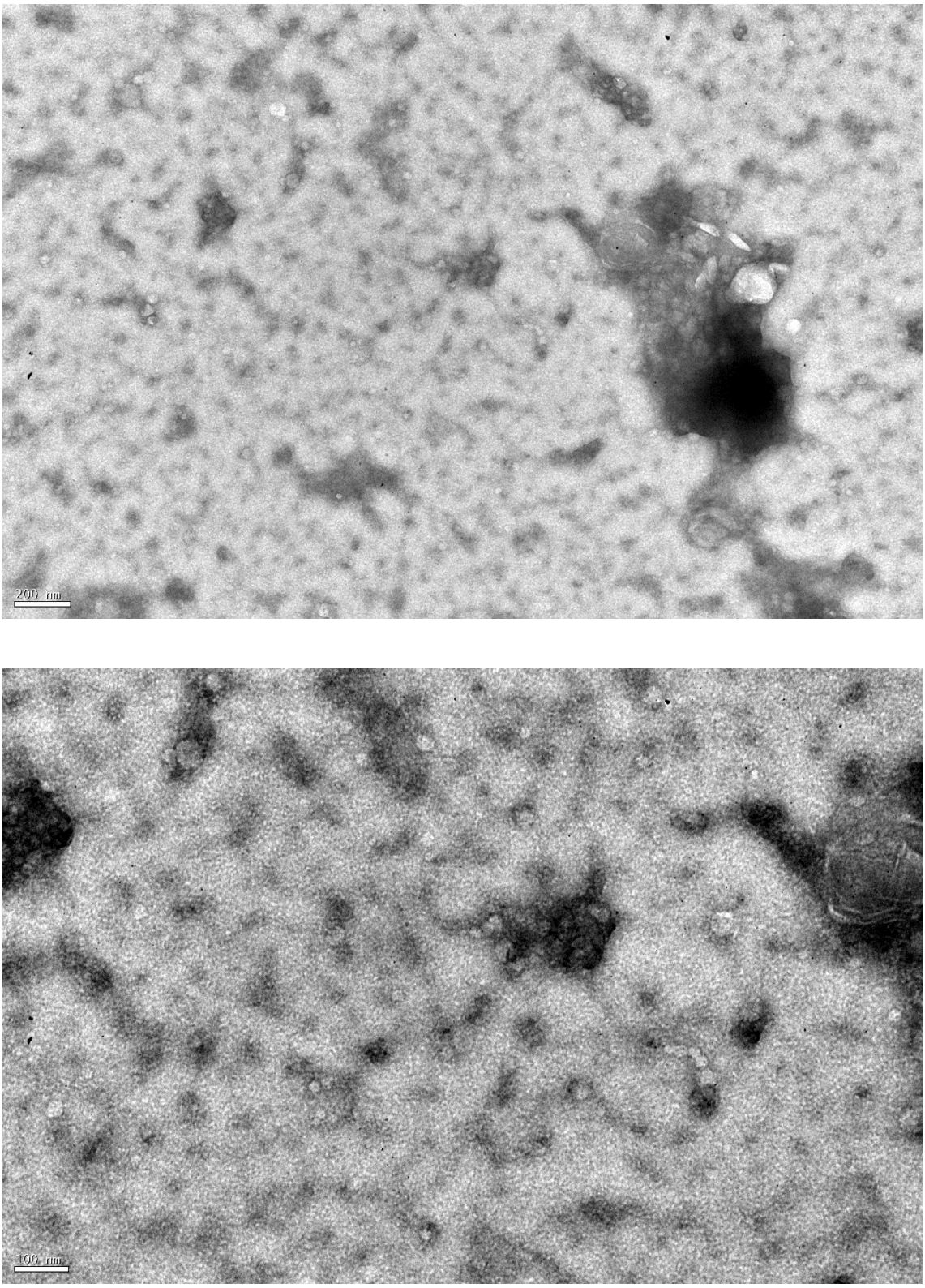


Figure 22 - TEM captures from Case 7. The isolated EVs were diluted on a 1:1 ratio with PBS (standard preparation). The above image was obtained at 25.000x magnification, and the one below is an amplified version of the central area of the former, at 100.000x magnification. nm – nanometer.

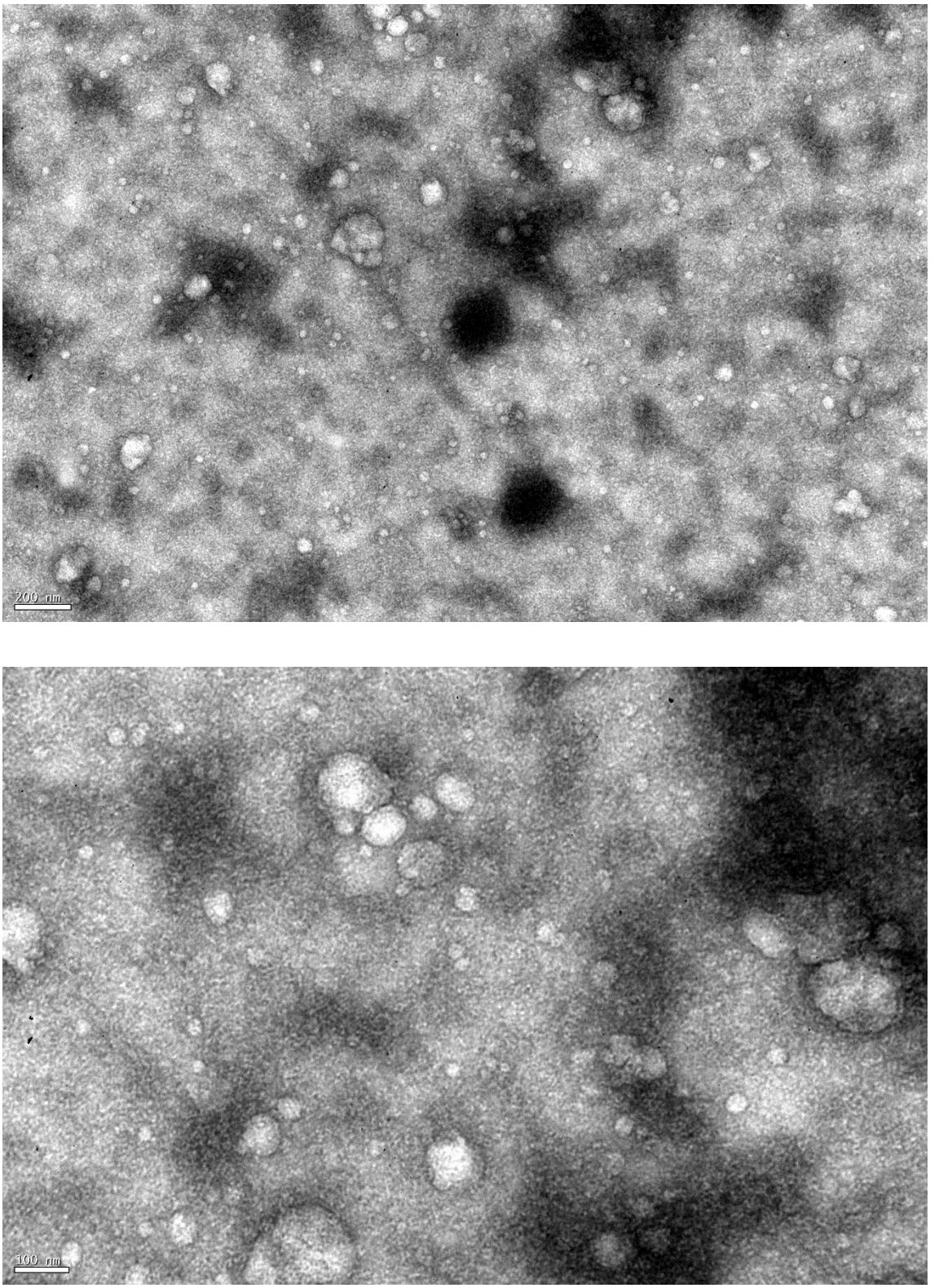


Figure 23 - TEM captures from Case 6. The isolated EVs were diluted on a 1:2 ratio with PBS. The above image was obtained at 50.000x magnification, and the one below is an amplified version of the central area of the former, at 100.000x magnification. nm – nanometer.

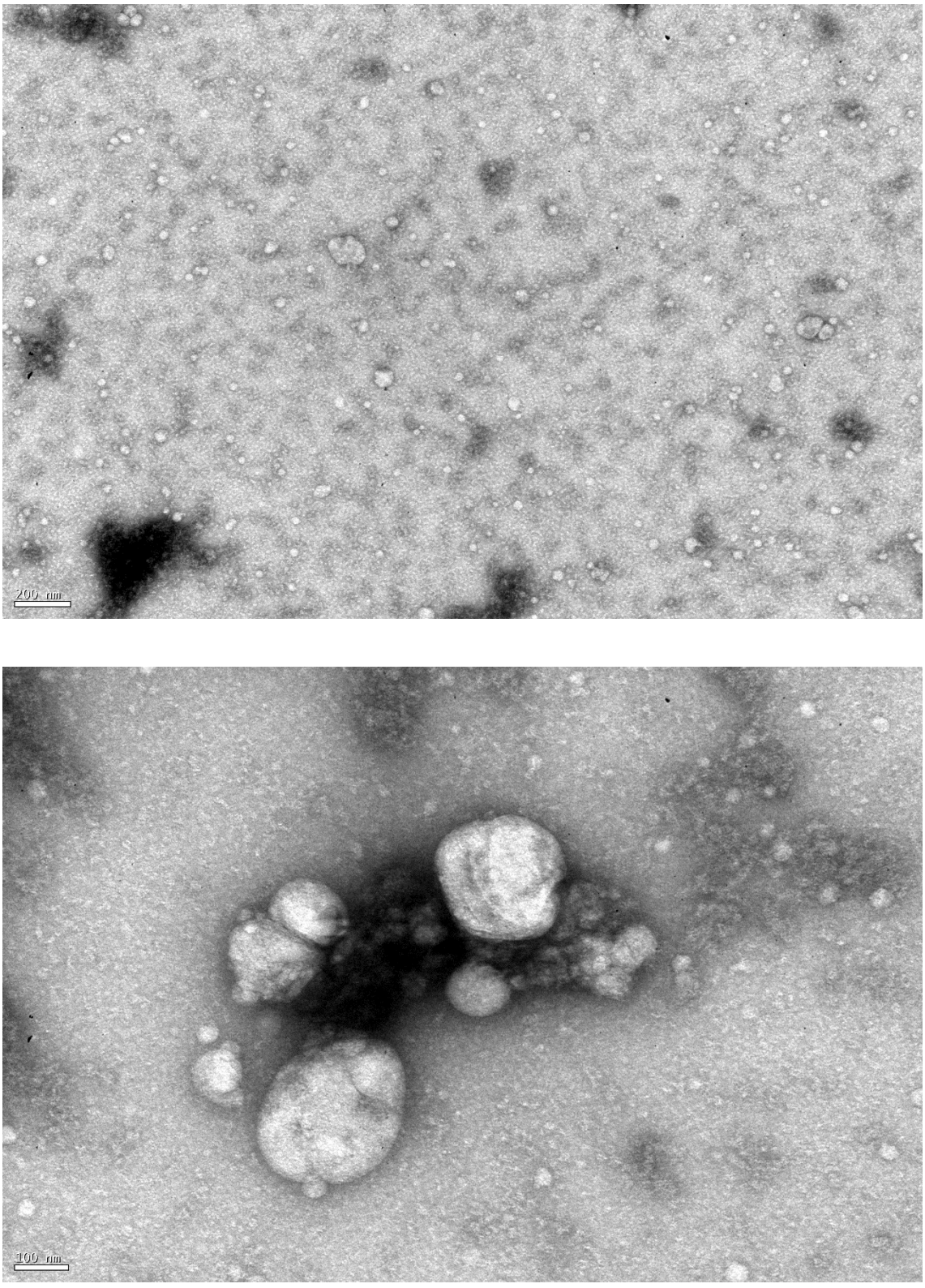


Figure 24 - TEM captures from Case 7. The isolated EVs were diluted on a 1:5 ratio with PBS. The above image was obtained at 50.000x magnification, and the below one, from a different area, was captured at 100.000x magnification. nm – nanometer.

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