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Extracellular Vesicle-based liquid biopsy in bladder cancer: establishing a protocol for the detection of urothelial carcinoma-derived EVs in the urine

Ana Margarida Barreira

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive”
Fernando Pessoa

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A.M. Barreira, M.C. Oliveira, A. Fraga, M.J. Oliveira, M.H. Vasconcelos, R. Ribeiro and H.R. Caires “Establishing a two-step protocol for the isolation of Extracellular Vesicles from urine samples of urothelial carcinoma patients: preliminary results”, 8th i3S Annual Meeting, November 2019, Póvoa de Varzim, Portugal

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BC	Bladder Cancer
BCG	Bacillus Calmette-Guérin
BTA	Bladder Tumour Antigen
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CL	Cell Lysate
CLE	Confocal Laser Endomicroscopy
CTU	Computed Tomography Urography
CIS	Carcinoma In Situ
DSC3	Desmocollin-3
EAU	European Association of Urology
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
EVs	Extracellular Vesicles
EORTC	European Organisation For Research and Treatment of Cancer
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FISH	Fluorescence In Situ Hybridization
FGFR3	Fibroblast Growth Factor Receptor 3
FOXA1	Forhead box Protein A1
GATA 3	Gata Binding Protein 3
HG	High Grade
HR	High risk
ISEV	International Society for Extracellular Vesicles

LG	Low Grade
LR	Low Risk
MIBC	Muscle Invasive Bladder Cancer
MISEV	Minimal Information for Studies of Extracellular Vesicles
MRU	Magnetic Resonance Urography
MWCO	Molecular Weight Cut Off
NMIBC	Non-muscle Invasive Bladder Cancer
NMP	Nuclear Matrix Proteins
NTA	Nanotracking Particle Analysis
NBI	Narrow-Band Imaging
OCT	Optical Coherence Tomography
PDD	Photodynamic Diagnosis
PI3CA	Phosphoinositide 3-Kinase
PUNMPL	Papillary Urothelial Neoplasm of Low Malignancy
RT-pPCR	Real-time quantitative (or semi-quantitative) polymerase chain reaction
UC	Ultracentrifugation
UCa	Urothelial Carcinoma
UF	Ultrafiltration
RB1	RB Transcriptional Corepressor 1
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Eletrophoresis
SEC	Size Exclusion Chromatography
STAG2	Stromal Antigen 2
TCC	Transitional Cell Carcinoma

TEM	Transmission Eletron Microscopy
TGM1	Transglutaminase 1
THP	Tamm-Horsfall Protein
TURBT	Trans-Urethral Resection of the Bladder Tumour
WB	Western Blot
WHO	World Health Organization

ABSTRACT

Urothelial carcinoma (UCa) represents 90% of all bladder tumours, is a frequently diagnosed malignancy across the world and in Europe, and associates with elevated mortality. Even though most (~3/4) are non-muscle invasive tumours, 35-80% of patients experience relapse whereas 10-20% will progress to muscle invasive, therefore requiring a rigorous and expensive follow-up. Patients are clinically followed with regular invasive cystoscopic procedure and low sensitivity urine cytology. Thus, innovative liquid biopsy-based biomarkers are desirable for a cost-effective follow-up of this disease.

Extracellular vesicles (EVs) are being increasingly recognized as key players in cancer progression and considered a potential source of cancer biomarkers in liquid biopsies. Nevertheless, there is currently lack of standardized protocols for the isolation of urinary EVs.

In this dissertation we aim to implement a protocol for the isolation and characterization of urinary EVs from UCa patients. We first isolated and characterized UCa-derived EVs using a 639V cell line in a 2D *in vitro* system. Then, we tested two urine sampling methods: i) spontaneous urine and (ii) cystoscopy urine on the yield of isolated urinary EVs. Upon selection of the urine sampling approach we optimized a three-step protocol involving ultracentrifugation (UC) in tandem with size-exclusion chromatography (SEC) and ultrafiltration (UF) or protein precipitation (PP) for the isolation of urinary EVs from these patients. Finally, we analysed the presence of UCa-associated protein biomarkers in patient's urinary EVs. The urinary EVs isolated from 12 UCa patients were then characterized according to particle size, concentration (NTA), morphology (TEM), protein amount (Lowry method), and presence of EV-associated and disease-associated protein markers (Western blot).

We demonstrated that UCa-derived 639V cells are able to shed EVs that carry the UCa-associated UPIII-A protein marker. Importantly, no significant differences were found in the particle concentration, size and protein concentration of urinary EVs isolated from spontaneous urine or intravesical lavage urine obtained from 3 paired UCa patients. Notably, the EVs isolated from intravesical lavage urine seemed enriched in EPS8L2 and Periplakin-1 proteins. Additionally, we have shown that ultracentrifugation (UC) allows the isolation of 30% of the urinary EVs present in the urine, which are co-pelleted with THP and albumin protein contaminants. Furthermore, we have also demonstrated that UC in tandem

with SEC allows the purification of urinary EVs, and that UF is a superior approach to concentrate the SEC-purified EVs for downstream analysis in comparison to PP. Strikingly, periplakin, mucin-1 and EPS8L2 were found to be specifically enriched in the isolated urinary EVs, compared to the original urine sample.

The results indicate that a three-step EV isolation protocol was properly implemented and validated in UCa patients-derived urinary samples. Notably, several UCa-associated biomarkers were detected in the urinary EVs isolated from these patients. Thus, the work performed in this dissertation serves as a proof-of-principle for the feasibility of using EV-based liquid biopsy for the detection of UCa protein markers, establishing the technical foundations for expanding the work to a larger UCa cohort.

Keywords: Urothelial Carcinoma, Extracellular Vesicles, Ultracentrifugation, Size Exclusion Chromatography, Ultrafiltration, Urine Biomarkers

RESUMO

O carcinoma do urotélio (CaU) representa cerca de 90% dos tumores da bexiga e tem origem nas células epiteliais uroteliais. Apresenta mais de 118 000 novos casos e 53 000 mortes anuais na Europa. Ao diagnóstico, 75% dos tumores da bexiga são não-músculo-invasivos. Nestes casos, o tratamento consiste na ressecção transuretral dos tumores. Contudo, cerca de 35-80% destes doentes recidivam e 10-20% progridem para tumor músculo-invasivo com mau prognóstico. A monitorização periódica de doentes com CaU não-músculo-invasivo requer cistoscopia, um procedimento invasivo, combinada com citologia da urina, um método com baixa sensibilidade para deteção de tumores de baixo grau. Neste contexto, a descoberta de novos biomarcadores tumorais com valor preditivo na urina destes doentes, proporcionaria o seguimento de forma não-invasiva da progressão tumoral.

Recentemente, tendo vindo a ser reconhecido o papel relevante das Vesiculas Extracelulares (VE) na progressão tumoral sendo por isso uma potencial fonte de biomarcadores em biopsias líquidas. No caso do CaU, o contacto das células neoplásicas com a urina torna as vesiculas urinárias isoladas deste fluído uma fonte promissora de biomarcadores para diagnostico desta patologia. Contudo, a comunidade cientifica debate-se com a corrente falta de estandardização do isolamento de vesiculas extracelulares urinárias a partir deste fluido corporal.

Neste contexto, esta dissertação tem por objetivo implementar um protocolo para o isolamento e caracterização de vesiculas urinárias a partir de amostras de urina de pacientes com CaU. Para isso, as vesiculas-derivadas de CaU foram inicialmente isoladas e caracterizadas a partir de sistema *in vitro* de cultura 2D de células 639V. Posteriormente, o comparámos as vesiculas urinarias isoladas a partir de dois métodos de colheita da urina dos doentes: (i) urina espontânea e (ii) lavado intravesical. Após a seleção do método de recolha da urina, um protocolo de 3 passos para isolamento de vesiculas urinarias foi otimizado. Este protocolo baseia-se na ultracentrifugação (UC) seguida por cromatografia de exclusão molecular (SEC) e ultrafiltração (UF) ou precipitação proteica (PP) da amostra de urina. Por último, analisamos a presença de biomarcadores com base em proteínas associadas ao CaU nas vesiculas urinárias isoladas destes doentes. As VEs isoladas de amostras de urina de 12 doentes com CaU foram caracterizadas de acordo com o seu tamanho e concentração (Nanoparticle Tracking Analysis), morfologia (Microscopia de

Transmissão Eletrónica), concentração proteica (método de Lowry) e presença de marcadores de VE e de doença (Western Blot).

Os nossos resultados sugerem que cada célula 639V é capaz de produzir centenas de VEs que transportam marcadores proteicos UPIII-A que estão associados ao CaU.

Relativamente ao tipo de colheita de urina, os resultados demostraram que não existem diferenças significativas entre urina espontânea e o lavado intravesical nomeadamente na concentração de partículas, no seu tamanho nem na concentração proteica de VEs urinarias isoladas de 3 doentes com CaU. É de realçar, que as VEs urinarias isoladas do lavado intravesical parecem estar mais enriquecidas em marcadores de doença como as proteínas EPS8L2 e Periplakin-1. De destacar que a ultracentrifugação apenas permite o isolamento de 30% de vesiculas urinárias presentes na urina dos doentes, sendo estas co-isoladas com contaminantes proteicos como THP e albumina. Adicionalmente, demonstramos que a UC combinada com SEC é uma abordagem eficaz para purificação de vesiculas urinárias. Para além do referido, a UF apresenta-se como o melhor método para concentrar vesiculas purificadas por SEC em comparação com PP. É de realçar que proteínas associadas ao CaU como a periplakin-1, a mucina-1 e EPS8L2 apresentaram-se especificamente enriquecidas nas VEs urinárias isoladas comparativamente à urina inicial.

No seu conjunto, os resultados indicam que o protocolo de isolamento de três passos foi corretamente implementado e validado em amostras de urina de pacientes com carcinoma do urotélio. É de salientar, que foi demonstrado que alguns marcadores proteicos associados ao CaU foram detetados nas VEs urinárias isoladas destes doentes. Deste modo, o trabalho realizado nesta dissertação confirma a viabilidade da analise do perfil de VEs urinarias em contexto de biopsia liquida para deteção de marcadores de doença em carcinoma do urotélio. Este trabalho, estabelece portanto as bases técnicas e metodologias para a realização de um futuro estudo clinico alargado em CaU.

Palavras Chave: Carcinoma do Urotélio, Vesiculas Extracelulares, Ultracentrifugação, Cromatografia de Exclusão Molecular, Ultrafiltração, Biomarcadores da Urina

INTRODUCTION

1. Urinary system

The human urinary system is composed by the upper (kidneys and ureters) and the lower (bladder and urethra) urinary tract. These organs contribute to production, storage and elimination of urine and metabolic products from the blood stream [1].

Briefly, urine is formed in the kidneys and excreted through the ureters. Ureters are bilateral tubes with the primary function of draining urine towards the bladder. The bladder is a distensible organ, comprised namely by smooth muscle, collagen and elastin, that collect and store urine from kidney [1]. Finally, the urethra has the function of eliminating urine from the bladder.

Importantly, the bladder wall is composed by different tissue layers which include the mucosa, muscle or muscularis propria, submucosa or lamina propria and perivesical fat or adventitia/serosa (Figure 1).

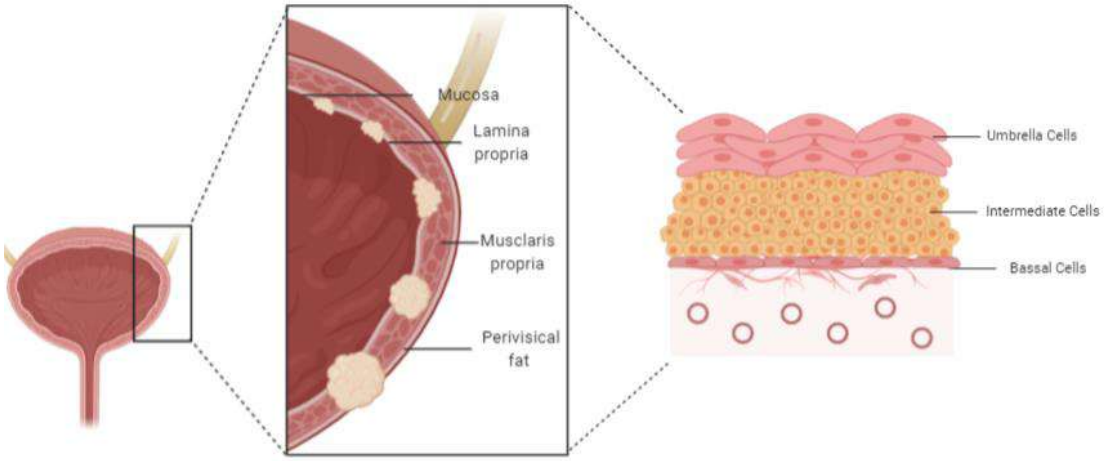


Figure 1- Bladder wall anatomy and histology. From the outside of bladder there is perivesical fat / serosa, muscularis propria / muscle, lamina propria / submucosa and epithelium. The apical cell layer is composed of umbrella cells with barrier molecules such uroplakins, mucins and glycosaminoglycans. Attached to the membrane are the intermediate and basal cells. In basal layer, afferent nerves can infiltrate the urothelium, as well as blood and lymphatic vessels for immune protection. Author: AM Barreira.

The mucosa is constituted by a specialized epithelium named urothelium, lining the urinary tract. This epithelium located between blood and urine, functions as a permeability barrier of toxic urinary substances and regulate surface area through the micturition cycle [1]. In terms of structure, the normal urothelium is a stratified epithelium, consisting of a single cell type with differences in phenotype: (i) “umbrella”, (ii)

intermediate and (iii) basal cells (Figure 1). Although the basal and intermediate cells are similar morphologically, the “umbrella” cells are distinct. These cells form a membrane specialization, the asymmetric unit membrane (AUM), constituted by four major membrane proteins: uroplakins Ia, Ib, II and III [2]. Interestingly, recent data suggests that upper urinary tract urothelium is intrinsically different from bladder urothelium due to distinct embryonic origin [3, 4]. Therefore, it was established that uroplakin proteins are lower in upper urinary tract compared to bladder urothelium [4].

Notably, the transformation of these urothelial cells due to chronic exposure to toxic substances excreted in the urine often leads to urothelial carcinoma later in life.

2. Urothelial Carcinoma

Urothelial carcinoma (UCa), or transitional cell carcinoma (TCC), originates in the epithelial cells of the urothelium (urothelial cells), lining the urinary system (both upper and lower tracts) [5-7]. Most UCa are found in the bladder (90-95% of cases) while 5-10% are located in the upper urinary tract.

Importantly, UCa also represents up to 90% of all BC. They are broadly divided into invasive (Muscle Invasive Bladder Cancer-MIBC), papillary (Non-Invasive muscle Bladder Cancer) or papilloma [8]. Histologically, the UCa can be divided into 13 distinct subtypes although the most frequent are squamous, glandular, micropapillary, nested, lymphepithelioma-like, plasmacytoid and sarcomatoid subtypes [9]. The remaining 9% includes non-urothelial adenocarcinoma, squamous cell carcinoma and small cell carcinoma [8]. Lastly, the other 1% are non-epithelial neoplasms.

2.1. Numbers & Figures

According to GLOBOCAN, bladder cancer (BC) is the 10th most frequent cancer worldwide, representing 3% of all cancers (Figure 2 A) [10, 11]. Specifically, BC is the sixth most common cancer in men and seventeenth in women, with an incidence of 9,6 and 2,4 per 100 000 individuals, respectively [12]. BC was responsible for more than 549 393 new cancer diagnosis and 199 922 cancer deaths worldwide in 2018 (Figure 2 B), making it the 13th most lethal malignant neoplasia, being responsible for 2.1% of all cancer-related deaths. [13]

A **B**

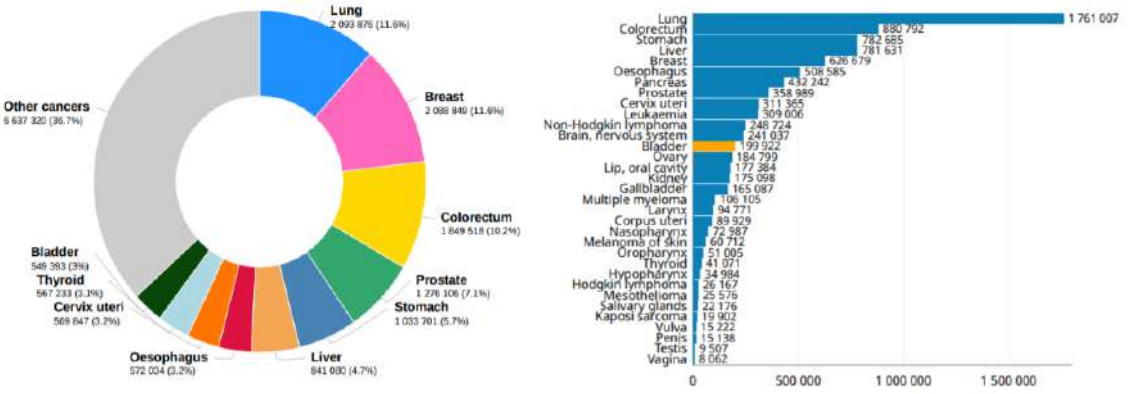
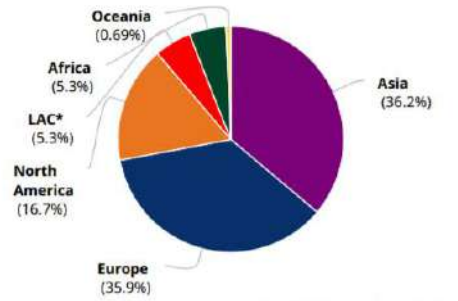


Figure 2-Bladder cancer incidence and mortality (A) Worldwide cancer incidence in 2018, for both genders and all ages. (B) number of deaths due to BC (yellow bar), in both genders and for all ages. Adapted from Globocan 2018.

Europe has one of the highest incidences of this disease, accounting for 35,9% cases of BC diagnosis (Figure 3) [13]. The 5-year survival rate in Europe for BC fluctuate between 60-80% according to the country, disease stage and location [12]. Specifically, in Portugal, BC was the fifth most incident cancer in 2018 in both genders, with 200 000 cases [13].



Population	Number
Asia	198 753
Europe	197 105
North America	91 689
*Latin America and the Caribbean	29 098
Africa	28 954
Oceania	3 794
Total	549 393

Figure 3- Incidence of BC on different continents for both genders, Adapted from GLOBOCAN 2018.

2.2. Risk factors for urothelial carcinoma

This type of cancer seems to be more frequent in older individuals. Indeed, more than 90% of BC patients are diagnosed after the age of 55 years[14]. This feature can be

partially related to the requirement for decades of exposure to toxic chemicals prior to the development of bladder cancer [13].

BC is more prevalent in men that women, which is accompanied by a mortality four times higher in men [11]. Another risk factor is occupational exposure to chemicals, such as aromatic amines and polycycles aromatic hydrocarbons, during the industrial manufacture of metals, dyes, rubber and petroleum [15-17]. Additionally, smoking seems to account for 50-65% of the new BC cases yearly. At last, obesity and pathogens (e.g. Schistosoma haematobium) are also known risk factors for UCa initiation [13, 18, 19].

2.3. Clinical Diagnosis of Urothelial Carcinoma

Painless haematuria is the most common symptom of BC [20, 21]. Irritative voiding symptoms such urinary frequency and urgency are often present at the time of BC presentation [8].

Diagnosis of BC includes cystoscopy followed by biopsies' histology and cytology of voided urine, and complemented with imaging techniques (computed tomography and magnetic resonance imaging) [22].

2.3.1. Cystoscopy

Cystoscopy consists in inserting a cystoscope - a flexible device with a small camera in the front - throughout the urethra up to the bladder for a visual inspection of bladder epithelium. If any abnormal tissue is observed, this technique allows excision and extraction of the abnormal tissue for histological analysis [23]. This procedure offers histopathological information not only for diagnosis but also for staging and grading the disease, as discussed below. Nevertheless, this method is invasive and might miss up to 25% of the tumours. Moreover, other tumours located in the upper urinary tract might be missed. In addition, it is operator-dependent and its recurrent use in BC follow-up increases the risk for urinary infections and/or haematuria [24, 25].

To overcome current limitations to cystoscopy, adjuvant techniques have emerged, such as "Photodynamic Diagnosis" (PDD) and "Narrow-Band Imaging" (NBI). These two techniques in combination with cystoscopy present advantages in clinical practice, for instance NBI reveals a better sensitivity in low grade tumors (95% vs 70% for NBI and conventional cystoscopy, respectively) [26].

Moreover “Confocal Laser Endomicroscopy “(CLE) and “Optical Coherence Tomography (OCT)” are two microscopy tools which allows real time evaluation of mucosa at cellular and subcellular level. According to some studies the OCT reveals high sensitivity and specificity in staging of Ta, T1 and T2 (90%, 75%, 100% sensitivity and 89% 97% and 90% specificity, respectively) [27].

On the other hand, CLE has demonstrated an improved detection and differentiation of BC from normal tissue. Also, CLE seems to have an improved sensitivity and specificity to detect low grade tumors when compared to cystoscopy (64% vs 55% sensitivity and 81 vs 50% specificity, respectively) [28, 29].

2.3.2. Cytology

Cytology is another diagnostic aid, which is based on the microscopical analysis of exfoliated cancer cells (or pre-cancer cells) in voided urine. This method is typically used in combination with cystoscopy. Albeit cytology is not invasive, presents low representativity of malignant cells and has low sensitivity for low grade tumours. Likewise, it is a morphological test and the results are subject of high interobserver variation. Complementary to these observations, pre-analytical issues such as poor preservation, haematuria or change in salt concentrations, may bias the results [24, 30, 31] (Table 1).

Some options as the ImmunoCyt™ and the UroVysion™ tests combined with cytology enhance diagnosis sensitivity and specificity as discussed bellow [31, 32].

2.3.3. Imaging

Macroscopic haematuria is a frequent symptom indicative of BC. To further investigate presence of urological malignancy, imaging of urinary tract (cystoscopy and computed tomography urography, CTU) might be of use [33]. It is another auxiliary diagnostic method based on imaging of kidney, ureters and bladder [34].

When CTU is not applicable due the renal dysfunction or exposure to ionizing radiation, magnetic resonance urography (MRU) is used. MRU combines anatomic imaging with quantitative evaluation of urinary tract. Although both procedures do not increase specificity and sensitivity of BC diagnosis, they are useful for disease staging [33].

Table 1- Sensitivity, specificity and associated costs for different diagnostic methods used in the urothelial carcinoma.

Diagnostic Method	Sensitivity	Specificity	Associated Costs	References
Cystoscopy	71%	72%	342€	[35]
Cytology*	86 %	71%	98€	[36, 37]
CTU	79%	94%	277€	[38, 39]

*sensitivity and specificity for high grade tumours

2.4. Urothelial Carcinoma Staging

The urothelial carcinomas can be divided into non-muscle muscle invasive (NMIBC) and muscle invasive (MIBC) bladder cancer [37]. Additionally, the World Health Organization (WHO) guidelines can further stratify the UCa into Low Grade (LG) and High Grade (HG) accordingly to the tumour aggressiveness [40]. A more detailed description on each of the classification systems will be provided below.

2.4.1. Non-Muscle-invasive Bladder Cancer (NMIBC)

Up to 75% of the diagnosed urothelial cancers are NMIBC. As the name implies, these tumours do not invade the muscle wall of the bladder and are stratified according to the TNM staging system. Thus, depending on the degree to tumour invasion the UCa can be classified as Ta, carcinoma in situ (Tis) and T1 depending on invasion or not of connective tissues of bladder wall [40, 41].

2.4.2. Muscle-invasive Bladder Cancer (MIBC)

The MIBC is characterized by the invasion of the bladder muscle. Importantly, the TMN sub-classification from T2 to T4 is used to reflect the depth of muscle invasion and extension extra-bladder or towards adjacent organs. Thus, T2, T3 and T4 classification reflect tumour invasion into deep muscle, perivesical tissue or adjacent organs, respectively [42].

Most patients with MIBC present with haematuria as the most relevant symptom. Several studies reported that gross haematuria was frequently associated with advanced stages of the disease and poorer prognosis [43-45].

2.5. Tumour grading

The WHO currently graded UCa into three distinct categories: papillary urothelial neoplasm of low malignancy (PUNLMP), low grade carcinoma and high grade carcinoma [42, 46, 47]. This classification added a new category, PUNLMP to describe tumours that although not completely benign have a low probability of progression [40, 48].

PUNMPL presents with discreet lesions, not fused and typically covered by normal urothelial cells. These lesions may have cytological atypia. Importantly, necrosis is absent and the progression rate is very low [40].Low grade (LG) tumours are characterized by fibroblastic cores covered by normal urothelium in an overall orderly appearance. The nucleus size and shape are not significantly altered, and tumour cells have minimal variability in architecture and lack cytological atypia. Mitosis are rare events and, if present, restricted to the basal layer [49].

The high grade (HG) tumours have cytonuclear and architectural cell disorganisation. Additionally, the nuclear atypia is an important feature of HG tumours which typically have a prominent and pleomorphic nuclei and frequent mitotic events. The urothelium has a considerably thickness and intraepithelial necrosis might be present. Since HG tumours are more prone to display aggressive traits, they are frequently associated with Ta or T1-4 stages of the disease [40, 50]. Noteworthy, carcinoma in situ (CIS) tumours are often considered non-invasive UCa.

2.6 Molecular characterization of UCa cells

Molecular analyses of urothelial carcinoma cells are pivotal for understanding the tumours' behaviour and subsequently to predict the clinical outcome [51].

The diagnosis and staging of UCa relies mostly on anatomo-pathological features of the tumour. However, claims exist for increasing molecular characterization of tumours to improve disease prediction and prognosis [51].UCa frequently present with several driving mutations. The burden of mutational load in UCa has been proposed to determine tumour aggressiveness [52, 53]. A large cohort including 460 NMIBC allowed molecular profiling of tumours into three different groups [54]. Importantly, these molecular groups associated with different pathological features and with progression free survival [55].

For patients with MIBC, tumours have been categorized into luminal and basal subtypes. The basal subtype had expression of cytokeratin 5 and 6 (CK5/6), CD44, TP53 and EGFR, whereas luminal type are characterized by FGFR3 mutations, with upregulated

expression of KRT20, GATA 3, uroplakins and ERBB2, and without expression of CK5, CD44 or EGFR [51, 56].

TCGA data analysis allowed identification of five molecular signatures according to tumour mRNA expression. The five subtypes, luminal-papillary, luminal-infiltrated, luminal, basal-squamous and neuronal had different clinical outcomes and can be used to guide treatment. The common FGFR3 and CDKN2A deletions of luminal papillary subtype have better overall survival [51, 56], supporting a target therapeutic opportunity for FGFR3 inhibitors. Otherwise, the neuronal subtype present with the worst clinical outcome, having high proliferation rate and expression of TP53 and RB1. Patients bearing tumours with this subtype are then eligible for chemotherapy [51, 57].

Taken together, these studies illustrate the importance of proteomic profiling of UCa cells for a better understanding of tumour aggressiveness and predict disease progression. According to landmark proteomic studies, the plakin protein family seems to be a potential candidate for UCa tumor biomarker. Periplakin-1 is essential for maintaining epithelial cell barriers and was described that their normal expression could suppress tumor progression [58]. Periplakin-1 molecular weight is 195 KDa and is involved in cellular attachment [59, 60]. Conversely, a loss of expression of Periplakin-1 in BC has been reported when compared to non-cancerous tissues using immunohistochemistry [59]. This alteration in expression, facilitate cancer cells detachment, becoming invasive and accessing other organs or systems. Additionally, loss of periplakin expression was associated with pathological stage due to the inactivation of cell adhesion for development of bladder cancer (vascular and lymphatic) [58, 59]. However, RNA analyses suggest that despite Periplakin-1 is mostly expressed in genitourinary organs it can also be expressed in the epithelial component of other tissues [61].The protein mucin-1 (MUC-1) is a member of the mucin family. It is a large and highly glycosylated protein (120-224kDa) typically located at the surface of the cell membrane in urothelial cells [62]. This protein is involved in protection and hydration of epithelia, with an important role in cell-cell adhesion and interaction. Moreover, MUC-1 regulates receptor tyrosine kinase signaling and consequently cell growth, being frequently overexpressed in many malignancies, particularly BC, facilitating tumour progression [62]. MUC-1 expression is observed in basal and intermediate layers of urothelium, with the pattern and intensity of the expression being correlated with worst prognosis of BC (high grade) [62, 63]. Interestingly, the concomitant high expression of MUC1 and HER3 has been associated with favorable prognosis [64].

Cytokeratins comprises a diverse group of intermediate filamentous proteins that integrate the cytoskeleton of epithelia tissue. Cytokeratin 7 is a type II cytokeratin, membrane-bound, that aggregates normal cells with affinity for simple sugars. It is expressed in the female genital tract and adenocarcinomas, namely of thelung, breast and urothelial [65, 66]. In fact, cytokeratin 7 was found to be highly expressed in UCa and is a useful diagnostic marker in the definition of cancer origin, being highly correlated with the high grade MIBC [67].

Uroplakins (UPs) belong to a glycoprotein family that form plaques on the apical surface of the urothelium. Specifically, the uroplakin III (UPKIII) has a role in the maintenance of the permeability of the bladder wall barrier, preventing the urine efflux from the urinary tract lumen [68].Importantly, Tsumara *et al.* demonstrated a decreased expression of UPKIII in tumour cells in aggressive BC [69].Noteworthy, this protein has been suggested as potential biomarker for BC. Indeed, Lai el al reported that BC patients present higher levels of UPKIII in urine compared to subjects with benign lesions and healthy subjects [68]. Likewise, Tsumara *et al*. found higher expression of UPKIII in MIBC patients when compared to levels observed in NMIBC patients [69]. Interestingly, no differences in the UPKIII urinary levels have been observed between LG and HG lesions [70].

The *EPS8L2* gene encodes a protein associated with epidermal growth factor receptor kinase substrate 8. The protein has a role in the membrane ruffling, remodeling actin activity in cells' cytoskeleton. Importantly, it was reported that BC patients have an increase amount of *EPS8L2* mRNA in urinary EVs when compared to healthy control individuals [71, 72].

2.7. UCa Prognosis

The Genito-Urinary Cancer Group of the European Organisation for Research and treatment of Cancer (EORTC) proposed to stratify UCa patients based on an integrative score system. The EORTC score system integrates several clinical and pathological features such as the tumour number, tumour diameter, previous recurrences, presence of CIS and tumour grade (Table 2). This score allows stratification of risk for tumour recurrence and progression [73]. For CIS tumours, no recommended prognostic factors are currently available, even though, some studies suggest that the response to therapy could be a prognostic factor, since a response to treatment leads to a decrease in the

progression rate (10%-20% vs 66%) [74]. Another prognostic factor reported for MIBC progression is the clinical response to intravesical treatment after 3 months [75].

The European Association of Urology Guidelines (EAU) stratify patients with BC into three risk groups. These, integrate the pathological conditions of the patients and several prognosis factors (Table 2 and 3).

Table 2- Clinical and pathological features necessary to calculate the EORTC and EAU prognosis and recurrence risk.

Factor	Recurrence	Progression
Number of tumours		
Single	0	0
2-7	3	3
>8	6	3
Tumour diameter		
≤3	0	0
≥3	3	3
Prior Recurrence Rate		
Primary	0	0
≤ 1 recurrence/year	2	2
>1 recurrence/year	4	2
Category		
Ta	0	0
T1	1	4
Concurrent CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total Score	0-17	0-23

Table 3- Pathological conditions according EAU considering for classifying the patients on group risks.

Group Risk	Clinical Features
Low-risk	- Primary Tumour; - Solitary tumour; - Ta, grade 1 - PUNLMP - Tumour less than 3 cm - No CIS
Intermediate Risk	- All tumours that cannot be defined between the two categories (Low risk or High risk)
High Risk	- T1 tumour, G3 tumour or TaG1 LG or TaG2 LG (with all characteristic present) - CIS - Recurrent Tumour, Large (>3 cm), multiple - T1G3 HG with concurrent CIS - Multiple or large T1G3 HG or recurrent with CIS in urethra - Some variants histological of urothelial carcinoma subtypes with lymphovascular invasion

2.8. UCa Treatment

The treatment of UCa should take into consideration patients' risk-stratification and prognosis [73]. The treatments for NMIBC and MIBC are different. In NMIBC, the first option is local tumour excision followed by adjuvant therapy, intravesical chemo or immunotherapy. In fact, despite TURBT can eradicate T1 tumours, they frequently recur and may progress to MIBC, making imperative to consider adjuvant therapy in all patients [23].Patients with NMIBC but staged in the most aggressive group of tumours in high risk stage or those refractory to adjuvant therapy (namely to BCG) may be eligible for removal of the bladder (radical cystectomy). Conversely, MIBC usually implies radical cystectomy preceded or not by platin-based neoadjuvant chemotherapy [8]. Chemotherapy in the

adjuvant setting is recommended only after radical cystectomy when no neoadjuvant chemotherapy was given [76, 77].Here, we will focus in NMIBC since these patients can benefit the most from novel urine-based liquid biopsies for improved prediction of disease recurrence in follow-up.

During cystoscopy, suspected lesions are resected using TURBT to allow histopathological confirmation. The TURBT consists in the insertion of a cystoscope into the urethra. This cystoscope has an incorporated camera to observe any possible tumours in the bladder wall. Urologists can then excise suspected lesions using the same instrument. To minimize the risk of tumour recurrence and progression the patients may undergo subsequent adjuvant therapy, BCG or even intravesical chemotherapy [8, 78].

Low risk tumours are treated with TURBT and immediate instillation of chemotherapy (mitomycin C, epirubicin or doxorubicin) [20, 79].A recent meta-analysis reported that a single instillation of intravesical chemotherapy after TURBT can decrease the recurrence rate at 5 years to up to 14% when compared to TURBT alone. Importantly, the efficacy of adjuvant intravesical chemotherapy upon TURBT seems to be relevant to patient prognosis, reducing the recurrence risk by 35% [80].

In the intermediate risk group of patients, those with low recurrence rate (one or less recurrence per year) and with an EORTC score lower than 5 are eligible for treatment similar to low risk. For the other intermediate risk patients, the treatment is based in TURBT followed by intravesical chemotherapy or instillation of Bacillus Calmette-Guérin (BCG) immunotherapy, throughout 1-year duration[23].

For high risk tumours (T1, high grade or CIS) the treatment consists of intravesical BCG (1-3 years) or radical cystectomy. The subgroup of highest-risk tumours (T1 high grade with concomitant CIS, or multiple and/or large T1G3 and/or recurrent T1G3, T1G3 with CIS in the prostatic urethra and variant histology of UCa), radical cystectomy should be considered as first option, with intravesical BCG (1-3 years) instillation for refusals or unfit subjects [8, 77, 81]. The benefit of RC must consider the associated risks, morbidity and impact on quality of life, a decision being made after patient´s agreement [23].

2.9. UCa patient follow-up

In the first year after NMIBC treatment, up to 61% relapse and 17% progress to invasive disease with a dismal prognosis (Figure 4 A and B).Therefore, patients harboring NMIBC

require extensive surveillance after initial therapy to avoid disease recurrency and progression. Follow-up usually relies on cystoscopy and cytology in combination. The frequency and duration of cystoscopy/cytology follow up heavily depends on patients' risk stratification.

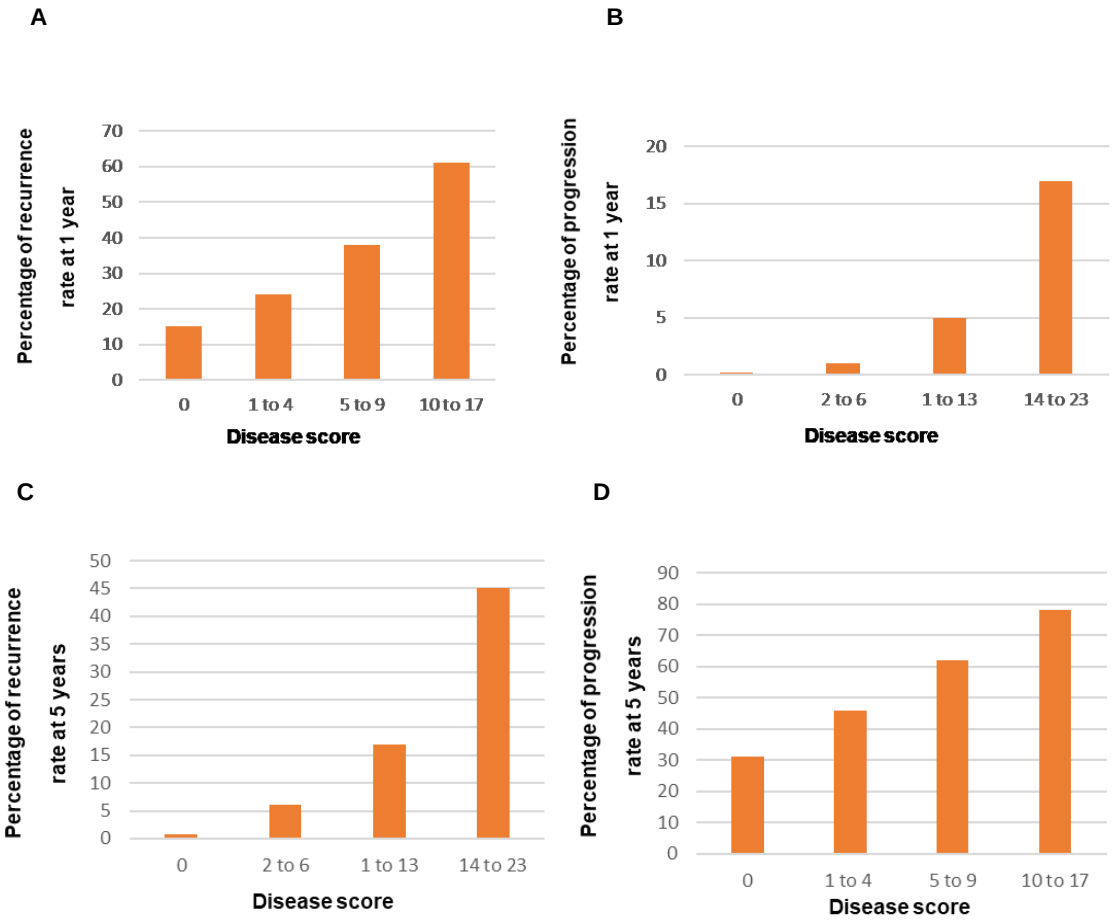


Figure 4- Probability of Recurrence and progression at 1 year and 5 years (A) Percentage of progression at 1 year according score: score=0= 15% of recurrence, score=1 to 4=24% score=5 to 9= 38% and score 10 to 17= 61% (B) percentage of recurrence at 1 year according recurrence score:: score=0= 0,2 % of recurrence, score=2 to 6=1% score=7 to 13= 5% and score 14 to 23= 17%. (C) Percentage of progression at 5 years according score: score=0= 31% of recurrence, score=1 to 4=46 % score=5 to 9= 62% and score 10 to 17= 78% (D) percentage of recurrence at 5 years according recurrence score: score=0= 0,8 % of recurrence, score=2 to 6=6% score=7 to 13= 17% and score 14 to 23= 45%.

Accordingly to Holmang *et al*., negativity of first cystoscopy performed at 3 months after the initial treatment determined a decreased tumour recurrence and progression for all grades in stage Ta and T1 [82]. Therefore, all patients with Ta and T1 should performed an initial cystoscopy at 3 months after treatment [76]. For Low risk UCa (primary, solitary, Ta and/or T1, low grade, <3 cm, without CIS), if no suspected lesions are found at this point, patients will only be monitored at 9 months and then 5 years after treatment. Since the risk of recurrence upon 5 years is rather low for low-risk UCa the follow up is discontinued 5 years after treatment. On the other hand, intermediate and high risk UCa patients follow a closer follow-up schedule. These patients have a recurrence rate of 54% and 73%, respectively [83, 84]. Therefore, it is recommended to perform cystoscopy and cytlogy every 3 months during 2 years after treatment start . If no lesions are detected, this follow-up continues every 6 months up to 5 years after initial UCa treatment (Figure 5).

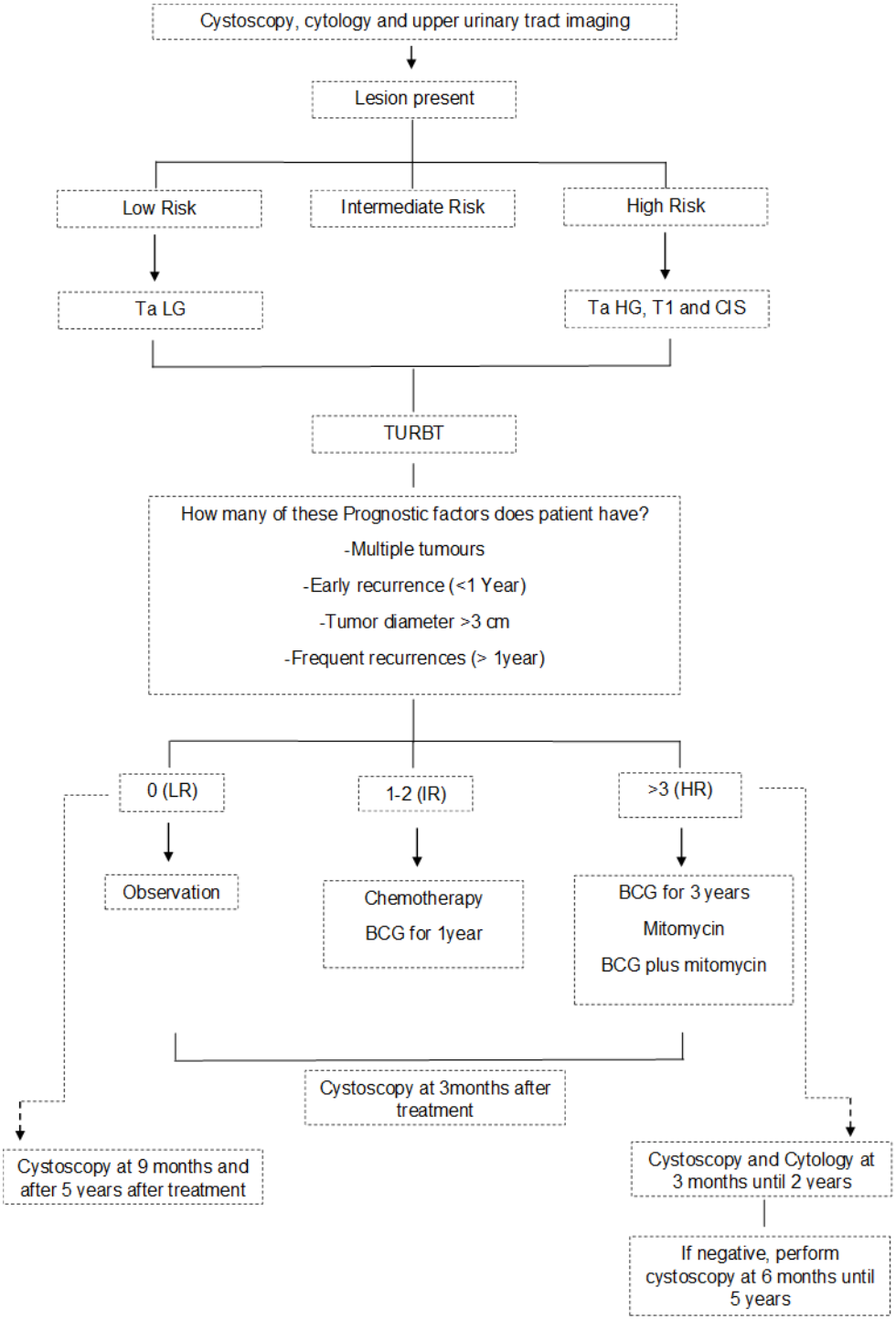


Figure 5- Diagnosis, treatment, and follow-up algorithm for NMIBC. LR=Low risk, IR= Intermediate Risk and HR= High risk [78, 79].

3. Need for innovative Liquid biopsy methods for improved clinical management of UCa

Cystoscopy is a highly invasive and operator-dependent technique which might miss up to 25 % of UCa tumours [85]. Moreover, cystoscopy cannot detect the presence of UCa tumours that develop in the upper urinary tract, which accounts for 5-10% of UCa [5]. On the other hand, urine cytology is frequently performed in combination with follow-up cystoscopy, even though it has a recognizably low sensitivity issue for low grade UCa tumours [86].In face of these issues, the elevated rate of progression and recurrence associated with NMBIC tumours require innovative less-invasive liquid biopsy tests that can be used to monitor in real-time recurrence of disease. The development of such method would replace the need for urinary cytology and potentially decrease the frequency of invasive cystoscopy used for patient follow-up [87].

The term “liquid biopsy” refers to the analysis of the presence of tumour biomarkers in biologic fluids as blood, plasma, urine, saliva or pleural fluid. Noteworthy, a tumour biomarker refers to a measurable indicator of a pathological condition or response to a treatment. Importantly, in liquid biopsies, these body fluids are typically collected in a minimally invasive way allowing the frequent monitoring of tumour progression via analysis of circulating tumour cells, circulating tumour DNA/RNA, proteins, metabolites and more recently tumour derived extracellular vesicles (EVs) [86, 88].Over the past few decades, liquid biopsies have been used to profile the tumor’s genetic profile in many cancer types, with increased predictive and prognostic value [86, 89-91].

The use of urine-based liquid biopsies seems a good source of tumour biomarkers in UCa. Indeed, the close contact of urine with urothelium has the advantage of better representing the tumor environment and clonal heterogeneity when compared to traditional tissue biopsies. Moreover, urine collection is a cost-effective, non-invasive and patient-friendly method to obtain tumour biomarkers. Notably, some urine cancer biomarkers have shown equivalent (or even better) sensitivity and specificity when compared to traditional cystoscopy [25, 92].In agreement, there are already urine-based biomarkers approved by the Food and Drug Administration (FDA) for clinical use in UCa. These methods can be performed in combination with the standard cystoscopy techniques for both diagnosis and follow-up of UCa patients. These FDA-approved tests and also other Laboratory Development Tests (used in clinics yet not approved by the FDA) for UCa monitoring will be discussed bellow [25].

3.1. Marker analysis for UCa diagnosis and follow-up (all technologies)

3.1.1 Biomarkers approved by FDA

Currently, four urine-based cancer biomarkers are approved by the FDA for diagnosis and follow-up of UCa. These include BTA, NMP22, ImmunoCyt™ and Urovysion™ [25, 93].

The bladder tumour antigen (BTA) is a protein related with H factor of the human complement, produced in malignant cells. The over-expression of this protein by cancer cells confers a selective survival advantage, supporting cancer progression [94].Currently, there are two BTA tests available: the (i) BTA TRAK and the (ii) BTA stat which are quantitative or qualitative tests, respectively. Both tests use monoclonal antibodies directed at H factor of human complement protein derived from cancer cells to detect the antigen [95]. The BTA TRAK requires laboratory processing and analysis within a few hours while the qualitative BTA stat test can be performed as a bedside point-of-care by the urologist in 5 minutes. According to published reports, the sensitivity and specificity of BTA STAT is 64% and 77% whereas for BTA TRAK is 65% and 74%, respectively [95, 96].Nevertheless, an unacceptable rate of false positive results with BTA tests have been reported in UCa. This might be related with the presence of unspecific factor H related protein present in the patient's blood, observed in patients with hematuria and/or bladder inflammation [97].

The nuclear matrix proteins (NMPs) integrate the internal structures of cell nucleus. NMPs have a role in DNA replication, transcription and gene expression [98]. Specifically, one member of this family, NMP22 (nuclear mitotic apparatus protein) was found to be overexpressed by approximately 25-fold in urothelial cancer cells compared to normal cells [25, 98-100].In practice, there are two type of tests for detecting NMP22: (i) the NMP22 Bladder Cancer Test, a quantitative test and (ii) NMP22 BladderCheck test, a qualitative test [99]. The reported sensitivity and specificity for each test is of 69% vs 58% and 77% vs 88%, respectively. As for BTA, these tests are prone to yield false positive results due to the presence of apoptotic cells in relation with bladder inflammation or hematuria [25].

While other biomarkers detect tumour-derived molecules in patients' urine, other assays identify the presence of UCa via detection of urine exfoliated tumour cells. One case of such examples is the ImmunoCyt™ test. This assay is an immunocytological test

performed by trained cytopathologists [101]. It uses monoclonal antibodies labelled with green fluorochrome to detect BCa antigens, such as M344, LDQ10 and 19A211 in the urinary exfoliated cells. The M344 and LDQ10 antibodies recognize mucins while 19A211 antibody binds to the carcinoembryonic antigen (CEA) in the surface of UCa cells [102]. Importantly, the sensitivity ranges between 83-85% and specificity between 75-87% [41].Despite this test is FDA approved, it is only recommended for BCa follow-up in combination with cytology [101].The overall sensitivity of Immunocyt™ assay combined with cytology is higher when compared with cytology alone (81-90% vs 61-78%, respectively) [101, 103]. Moreover, when BC was diagnosed by histological type, the combined sensitivity was higher than either technique alone (cytology or ImmunoCyt™). Indeed, the combination of cytology and Immunocyt™ granted sensitivities of 79.3%, 90.3% and 98.9% for UCa grade 1, 2 and 3, respectively [104].

Urovysion™ is an assay that uses fluorescence in situ hybridization (FISH) to detect chromosomal aberrations in the urinary exfoliated cells [101] . It detects the presence of aneuploidies of chromosomes 3, 7 and 17 and/or loss of the region p21 of chromosome 9[105]. These chromosomal alterations are frequently associated with the pathogenesis of BCa. Recent studies reported that the sensitivity of Urovysion™ is 76% (0,95 [CI, 0.65 to 0.84]) while specificity is 85% (0.95 [CI, 0.78 to 0.92]).

3.1.2.Laboratory Development Tests (LDTs)

Given the limitations of the FDA approved diagnostic biomarkers, many research groups have been proposing novel biomarkers for the diagnosis and follow up of BC patients [31]. The laboratory Development Tests (LDTs) are *in vitro* diagnostic tests produced in house and used in laboratory for the detection/measurement of several analytes, such as proteins, metabolites or nucleic acids. Nevertheless, these tests were not submitted to the rigorous clinical validation required for approval by the certified entities. Nevertheless, many of these innovative tests can still be needed by urologists to aid the clinical diagnosis and/or follow up of BC patients. Some of the commonly used LDTs in UCa may include Cxbladder ™, AssureMDx™ and Uromonitor tests.

The Cxbladder™ test measures five mRNAs that are highly expressed in BC using qRT-PCR. These mRNAs include *IGFBP5*, *MDK*, *HOXA13*, *CDK1* and *CXCR2*. The first four mRNA are related with growth of tumour tissue while *CXCR2* is associated with inflammatory conditions [91, 106].Currently, there are 3 types of tests: (i) Cxbladder

Triage, (ii) Cxbladder Detect and (iii) Cxbladder Monitor. Each assay is recommended for different stages of the disease. The Triage is used for the exclusion of non-UCa related pathologies and early screening of symptomatic population. The Cx bladderDetect™ and Monitor™ are useful for UCa diagnosis and follow-up, respectively [107].They have different specificity and sensitivity. The Cxbladder™ monitor reveals an overall sensitivity of 93% increasing up to 95% in high-risk BC patients [107]. Moreover, Lotan *et al*. showed that Cxbladder Monitor has a higher sensitivity compared to other approaches (91%), being able to surpass the cytology by 22% and the NMP22 test by 11% [108]. The Cxbladder™ Detect has a sensitivity of 80% and a specificity of 83% [109]. Importantly, some authors claim that the Detect might be a potential tool to delay the necessity of recurrent cystoscopies in low risk patients [93, 108].

The Assure MDx™ test identifies both BC-associated mutations in *FGFR3*, *TERT* and *HRAS* genes, combined with the methylation profile of *OTX1*, *ONECUT2* and *TWIST1* for detection of BC in subjects presenting with haematuria. The methylation of these genes leads to a decrease tumour suppressor activity [110, 111]. A study demonstrated that Assure MDx™ has a specificity of 86% and sensitivity of 93% of for the diagnosis of UCa [112]. Accordingly, Kessel *t al.* have suggested that the clinical use of Assure MDx™ can diminish up to 77% the number of cystoscopies required for UCa follow-up [112].

The Uromonitor™ is a non-invasive and ultrasensitive test based in qRT-PCR of hotspot mutations in *TERT*p and *FGFR3*. This test is indicated for patients with haematuria and can be used in combination with cystoscopy [113, 114].Authors report that when there is a negative result it is advisable to repeat the test every 3 months until one year after treatment. On the other hand, if there is a positive result for these hotspot mutations, confirmatory cystoscopy and TURBT are recommended [113].According to authors, this test has a sensitivity of 73,5% and specificity of 73,2% for the detection of BCa. Interestingly, it seems to yield better results than cytology and comparable to cystoscopy, and that when performed with cystoscopy the combined sensitivity can increased up to 100% while the specificity is of 88,6% [113].

3.2. Emerging EV-based liquid-biopsy biomarkers

3.2.1. Extracellular vesicles

Recently, Extracellular Vesicles (EVs) have been reported to be a potential source of biomarkers in many diseases, including cancer [115]. Accordingly, the International

Society for Extracellular Vesicles (ISEV), defined EVs as nanosized particles with a lipid bilayer that are shed by cells and that are unable to replicate [116]. These EVs can present sizes ranging from 30 to 5000 nm depending on their biogenesis [116], and apparently all cells of the body are able to shed EVs for inter-cellular communication purposes. Indeed, EVs can be found in many biological fluids including blood, urine, saliva, milk and others. Thus, cells can communicate, eventually changing the behaviour of target cells and tissue function [117].

3.2.1.1. EV biogenesis & function

Extracellular vesicles can be broadly divided into 3 different subpopulations depending on their biogenesis mechanism: (i) exosomes, (ii) microvesicles and (iii) apoptotic bodies [118].Exosomes are particles released by all cells with size ranging between 30-120 nm. They originate from the budding of intracellular endosomes. Then, these endosomes mature into intraluminal vesicle (ILVs) within multivesicular bodies (MVBs) [93]. After maturation, the multivesicular bodies can either fuse with plasma membrane, releasing the exosomes for the extracellular environment, or be degraded into cells' lysosomes [117]. Interestingly, the formation of ILVs typically involves two different processes. The first requires tetraspanins, a class of membrane proteins, involved in the organization of endosome. They cluster the required proteins for ILV formation, with CD63 and CD9 further involved in the formation of exosomes by this mechanism . The second process relies on formation of multi-protein complexes, the Sorting Complex Required for Transport (ESCRTs 0, I, II and III). Several authors report that ESCRT I and II proteins are responsible for the membrane budding and that ESCRT III proteins with ALIX and TSG101 proteins complete the budding of the MVB with the plasma membrane [119].

Microvesicles also known as cellular microparticles or ectosomes, includes vesicles with higher heterogeneity in size, that can range from 50 nm to up to 1000 nm [117]. The microvesicles originate from the cells' plasma membrane and their biogenesis can rely on distinct mechanisms. The formation of microvesicles is a result of asymmetric phospholipid redistribution and cytoskeletal protein contraction. This asymmetric distribution is regulated by aminophospolipid translocases. The membrane budding induced by translocation of phoshatidylserine is typically completed through actin-myosin cytoskeletal contraction [119].

The apoptotic bodies are originated during the apoptotic process of programmed cell death. In this case, the dying cell will disintegrate their cellular components into vesicles

designated apoptotic bodies or apoptosomes. The apoptotic bodies formed during the programmed cell death process have a highly heterogeneous size range that can go from 50 nm to up to 2000 nm [119, 120].

Throughout these processes, EVs seem to have a selective packaging of several proteins, nucleic acids and lipids according to the cell of origin. It was reported that several proteins, lipids and nucleic acids associated to EVs can modulate the function of target cell upon EV internalization. Despite this, the specific biological function of each EV subpopulation for the tissue homeostasis remains largely unknown. Nevertheless, robust evidence exists supporting that tumour-derived EVs might have a key role in promoting cancer hallmarks, including promotion of cell proliferation, contribution of cell invasion and metastasis, sustained angiogenesis, and modulation of the tumour microenvironment through inflammation and immune response evasion [121] [122]. Due to the fine-tuned EV biology it is accepted that analysis of the EV cargo can be used to interrogate the pathological status of the cell of origin.

3.2.1.1.1. Urinary EVs: a source of UCa biomarkers?

Attending to their intimate relationship with tumour progression, urinary EVs have been perceived as a potential source of cancer biomarkers in bladder cancer [123]. Indeed, several authors report that proteins and RNAs that originate from the kidney, prostate and urothelial cells can be found into urinary EVs (uEVs) [124-129].

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3.2.1.1.1.1 Urinary EV composition and function in homeostasis

Urinary EVs are composed by an heterogeneous group of vesicles, that include mainly microvesicles and exosomes [130]. They seem to be shed primarily by cells from the genitourinary system, namely from kidney and urothelium [131]. Nevertheless, EVs shed by other cell types are also observed in the urine. Indeed, most of circulating EVs present in the blood plasma can be filtrated at the glomerular level and excreted into the urine [132]. Arguably, this complex mixture of urinary EVs from different origins can hamper a straightforward analysis of the UCa-derived EVs in the patients' urine [131].

Several authors report a key role of urinary EVs for maintaining tissue homeostasis. Indeed, urinary EVs seem to play a role in maintenance of extracellular body fluid volume, in the innate immune system at urinary tract level [133] and at the intra-nephron

communication [124]. Moreover, EVs can have a role in tubular interstitial cell communication [124]. Indeed, the presence of proteases and glycosidase at the EVs surface enables the degradation of extracellular matrix. Several authors have demonstrated that this EV-mediated mechanism can facilitate cell adhesion, invasion and tissue remodelling [134, 135].

3.2.1.1.1.2. EVs and cancer hallmarks: focus on genitourinary tumours

Remarkably, the conservation of the physiological EV-mediated cell-to-cell communication seems to be severely disturbed in cancer. Here, we will discuss how this EV communication network can be hijacked by cancer cells to favour the bladder cancer growth and progression, but also in other urologic neoplasms as kidney and prostate cancer [93, 122].

In bladder cancer, EVs were shown to influence the tumour microenvironment by (i) modulating the migration and invasion features of tumour cells, (ii) promoting local angiogenesis to support tumour growth and by (iii) inhibiting apoptosis in tumour cells. Interestingly, high grade BC cell lines were shown to secrete EVs that carry high levels of EGF-like and discoidin 1 like domain containing protein 3 (EDIL-3). Subsequent knock-down of EDIL-3 in BC cells led to an inability of the shed EVs to promote angiogenesis and tumour cell migration *in vitro* [136]. Importantly, these pro-angiogenesis and pro-migration proteins were also found in higher levels in the urinary EVs of BC patients compared to heathy controls [136]. Additionally, Silvers et al. reported that EVs shed by MIBC cells carry periostin protein that is involved in the maintenance of the MIBC aggressive traits via ERK pathway [137]. Moreover, studies suggested that BC progression can be supported by EV-derived miRNAs. Ostenfeld et al. have shown that vesicular miR-23b is involved in the invasion, angiogenesis and metastasis in EVs from BCa patients [138]. Perez *et al*. reported that *LASS2* and *GALNT1* were expressed in urinary EVs from BC patients and have been associated with disease progression.

In renal cancer, EVs were described as vehicles used for the re-education of the immune cells by the tumour cells. Indeed, several authors have shown that in renal cancer the EVs have a powerful immunosuppressive activity in several immune cell populations including T cell lymphocytes, tumor-infiltrating macrophages and neutrophils[122, 139, 140]. For instance, renal cancer cells can activate the pro-apoptotic caspase pathway on T lymphocytes via renal cancer-derived EVs [122, 141].In prostate cancer (PC),

Castellana *et al.* have shown that the EVs shed by a PC3 prostate cancer cell line carry several proteins able to modulate the tumour microenvironment [142]. Indeed, among these the vesicular CX3CL1, metalloproteases and TGFβ1 were shown to induce the transformation of underlying fibroblasts into cancer-associated fibroblasts, an essential step for PC progression [142, 143]. Moreover, the PC-derived EVs were also involved in tumour initiation via upregulation of pro-survival protein STAT3 in cancer cells [144, 145]. Additionally, Bijnsdorp *et al.* described that PC-derived EVs carry high-levels of UTGA3 and ITGB1 proteins that increase the migration and invasion of cancer cells [146].

3.2.1.2. EV isolation methods

Several studies have reported numerous methodologies for isolating EVs from different biological fluids. Regarding to EV separation from urine samples some of conventional techniques used include differential ultracentrifugation (dUC) [72], size exclusion chromatography (SEC) [147], density gradient ultracentrifugation (gUC) [148, 149] and ultrafiltration (UF) [150-152]. Most researchers use more than one method in order to have a compromise among recovery yields, EV integrity and purity [116].

3.2.1.2.1. Ultracentrifugation and density-based gradients

The Ultracentrifugation (UC) is the gold standard technique for the EV isolation. There are two distinct modatilities of UC: (i) differential ultracentrifugation (dUC) and (ii) density gradient ultracentrifugation (gUC).

Importantly, the dUC is the most common modality of UC that isolates EVs from complex biological fluids based on the particle's sedimentation speed at distinct gravitational forces. Thus, with dUC it is possible to isolate EVs from other biological components present in the urine (exfoliated cells, cell membrane debris and apoptotic bodies) which sediment at a considerably lower gravitational force [93]. Most importantly, dUC is optimal to isolate EVs diluted in large fluid volumes (as observed in urine samples) and has a low associated sample processing cost. Unfortunately, dUC requires heavy and specialized equipment to operate and is a time-consuming technique [131, 153].

The gUC is another UC modality used for the EV isolation that separates particles based on their density [154]. This approach is typically used with either (i) sucrose or (ii) OptiPrep™ as flotation media [155]. Upon initial ultracentrifugation the proteins aggregates and lipoproteins are separated from EVs due to their different flotation density [156]. Importantly, this methodology has a higher recovery yield than dUC,

however is also a time-consuming procedure with an extremely low throughput which limits its translation into clinical application [93, 131, 153].

3.2.1.2.2. Size Exclusion Chromatography

Size Exclusion Chromatography (SEC) uses a gel filtration matrix approach to separate particles with distinct sizes. The type of matrix that compose the SEC column (stationary phase) typically defines the resolution of EV separation from other particles. The most widely used SEC matrix for the isolation of EVs is Sepharose 2B. Sepharose 2B matrix is composed by a 2% agarose-based beads with a mean diameter of 60-200 μm. Importantly, the Sepharose 2B beads have an internal micro porosity with a mean pore size of 75nm [157]. Thus, upon sample loading, larger particles will cross the macropores of SEC matrix faster being eluted in the earlier SEC fractions while smaller particles will infiltrate through the micropores of the Sepharose beads, travel a longer path through the matrix and eluted in the later SEC fractions [154, 157, 158].

Importantly, compared to UC-based techniques the SEC is a faster, technically more accessible and cost-efficient approach to isolate EVs from complex biological samples. Unfortunately, SEC requires the input of small volumes of sample (approximately 1-10% of the total volume of the SEC column). Moreover, due to its intrinsic features, SEC typically dilutes the original sample throughout the distinct SEC fractions [131, 153].

3.2.1.2.3. Ultrafiltration

Ultrafiltration (UF) is designed to isolate EVs through a membrane filter with a suitable pore size. This methodology use nanomembranes inside a filter, with specific molecular weight cut off (MWCO) to isolated and/or concentrate particles with diverse molecular weights [159, 160].

Although it is a widely used technology for many applications, from analyte concentration to the isolation of EVs, the UF is reported as a highly reproducible and time-efficient methodology [122, 161].

New separation methods have recently emerged as promising approaches based in (i) microfluid or (ii) nanofiltration. These methods are mostly based on microfluid devices and chips. These technologies separate EVs according to their size, immunoaffinity, acoustic trapping and nanowires anchored with microfluid substrate [127, 162-164].

These new methodologies can rapidly process small volumes of sample compared with others isolation methods[165]. Nevertheless, these devices are still in an early stage of development and have some disadvantages, such as the lower overall applicability, price per sample and issues related with EVs purity[166]. Therefore, these problems should be resolved before these tools can became implemented in clinical practice [153].

3.2.1.2.4. Precipitation Method

This method uses a precipitation reagent to precipitate EVs out of the complex urine mixture. This reagent could be a hydrophilic polymer such polyethylene glycols, sodium acetate or other organic solvent [167-169]. Based on solubility of proteins and EVs, these reagents precipitate the EV proteins (and possibly other proteins). Despite this technique is considerably faster than ultracentrifugation it is reported to also co-precipitate a large amount of contaminant proteins that exist in the initial sample[170].More importantly, several authors report that this technique can severely disrupt the isolated EVs morphology and function. Attending to the features of EV protein precipitation-based methods this method has a high yield for the isolation of EVs at the expenses of EV purity and integrity which limits their use in clinical application [131, 153].

3.2.1.2.5. Affinity Interactions

The immunocapture of EVs via antibodies targeted at EV antigens is the most widely used format of EV isolation by affinity interaction. In this process, specific antibodies are typically coupled at magnetic/latex beads and used to capture specific proteins or lipids exposed on the surface of the EVs. When applied to biological fluids, EVs would be captured by binding of specific membrane markers (CD9, CD81 or CD63) to beads functionalized with the respective antibodies [131, 153]. This affinity binding makes this technique highly specific, being able to deplete most of the protein contaminants. Moreover, it is a faster methodology when compared to the most widely used UC-based approach. On the other hand, the high specificity could become a disadvantage since this methodology is not the most appropriate to study all EVs subpopulations [171]. Indeed, the use of common biomarkers such CD63, CD81 and CD9 that are widely used for EV capturing are not the “universal” markers for all EVs [172]. Moreover, the efficacy of this isolation method is highly reliant on the quality of the antibody used for immunocapture.

Interestingly, this technique could be coupled with other methods to improve isolation technique. Indeed, Mariantonia *et al*. reported that this technique could be associated with UC for the isolation and analysis of EVs from a PC cell line [173]. Similarly, Zhao *et al*. associated the magnetic beads with microfluidic chip for isolation of EVs from ovarian, reporting that this protocol could be efficiently applied to the diagnosis of ovarian cancer [174].

SCOPE OF THE THESIS

1. Rationale & thesis aims

Urothelial carcinoma (UCa) is a malignant cancer that affects the urothelial cells and represents 90% of all bladder tumours [175]. Importantly, each year there are nearly 118,000 new cases and 52,000 deaths attributed to UCa in Europe [175]. Strikingly, since the European UCa is strongly related to age, its incidence is projected to increase to 219,000 new cases per year by 2030 [176].

Currently, clinical presentation, urine cytology and cystoscopy are the standard of care for BC diagnosis and follow-up. Despite cystoscopy is the current gold standard, it is a highly invasive and expensive procedure that has been reported to neglect nearly 25% of bladder tumour cases. In addition to cystoscopy, urine cytology is a method routinely performed in the clinical setting, despite its low sensitivity for cancer detection (particularly for low grade tumours) has been recognised and reported [177-180]. Therefore, innovative liquid biopsy-based biomarkers that might circumvent the invasiveness and low sensitivity of cystoscopy and the low sensitivity of urine cytology, are highly desirable for improving diagnosis and follow-up of UCa patients [181, 182].

2. Hypothesis and goal

With this project we propose to develop a minimally-invasive method for the diagnosis and follow-up of UCa patients, by analyzing the EVs shed from UCa cells in patients' urine. For that, the main objective of this work is to establish a protocol to isolate and characterize UCa-derived EVs in patients' urinary samples (Figure 6). The specific aims are:

2.1. Perform an *in vitro* characterization of UCa-derived EVs

In order to develop an EV-based liquid biopsy for diagnosis and follow-up of UCa patients, we first interrogated whether the EVs shed by UCa cells carry a representative immunophenotypic/proteomic profile of the cells of origin. Thus, the UCa-derived EV size, abundance and proteome were characterized using an *in vitro* model with the bladder cancer cell line 639V. This approach will allow us to identify UCa-associated EV protein markers for downstream analysis in UCa patient urine samples.

2.2. Select the most advantageous urine sampling method for downstream EV analysis

Typically four pre-analytical factors should be carefully considered prior the development of urine-based biomarkers as these may hamper clinical applicability: i) sampling method, ii) transport, iii) preservation and iv) sample dilution of urine. Thus, here we aimed to compare two sampling methods for the analysis of UCa-derived urinary EVs. For that, we expect to include paired spontaneous voided urine and cystoscopy-derived urine lavage from 3 UCa patients, and compare for the isolation of urinary EVs. The most suitable urine sampling for UCa-derived EV analysis will be selected for downstream experiments.

2.3. Implement an ultracentrifugation-based protocol for the isolation of EVs from the urine of UCa patients

After the identification of the molecular profile of pure UCa-derived EVs, we aim to detect the presence of these EVs in complex urine samples obtained from UCa patients at diagnosis. To do that, we will first optimize and validate the isolation of urinary EVs from urine using an ultracentrifugation-based (UC) protocol. This profile of UC-isolated EVs will be then compared to the EVs present in the original crude urine samples. The EVs will be isolated by UC and characterized by size, concentration, abundance of EV protein markers and depletion of common non-vesicular urine contaminants. The yield of urinary EV recovery by UC approach will also be estimated.

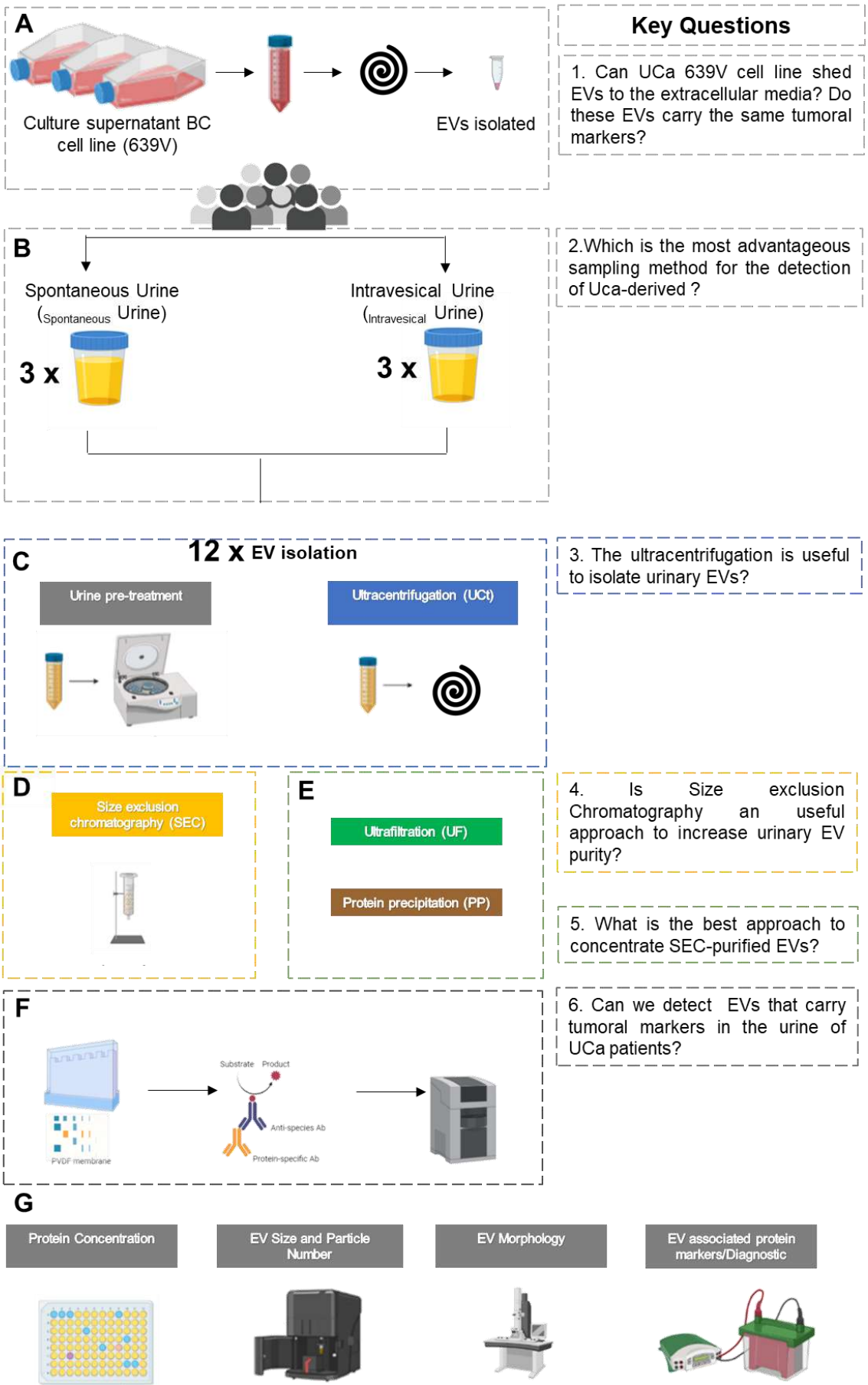
2.4. To develop a two-step protocol for the purification and concentration of urinary EVs isolated by UC

Bench-to-bedside implementation of EV-based liquid biopsies may be compromised by urinary non-vesicular protein contaminants and/or inappropriate isolation of urinary EVs. To avoid this, the fourth aim was to develop a two-step protocol for the isolation of highly pure urinary EVs using a size-exclusion chromatography approach combined with either Ultrafiltration (UF) or Protein Precipitation (PP) to concentrate these SEC-eluted EVs for downstream analysis. The two approaches will be compared, and the optimal concentration protocol selected for downstream EV analysis.

2.5. To detect the presence of urothelial carcinoma biomarkers in the patients´ urinary EVs

Upon characterization of the UCa-derived EV profile, selection of the most adequate urine sampling method and the development of a two-step protocol for the purification of urinary EVs, we will explore the feasibility of using an EV-based liquid biopsy for cancer detection in UCa patients. Thus, the fifth aim will be to confirm whether UCa-related protein biomarkers are expressed in EVs isolated from the urine of 12 UCa patients.

It is expected that the work performed in this dissertation serves as a proof-of-principle for the feasibility of using EV-based liquid biopsy for diagnosis and follow-up in UCa patients, thus establishing the foundations for a future project in a larger UCa cohort.



Key Questions

1. Can UCa 639V cell line shed EVs to the extracellular media? Do these EVs carry the same tumoral markers?

2.Which is the most advantageous sampling method for the detection of Uca-derived ?

3. The ultracentrifugation is useful to isolate urinary EVs?

4. Is Size exclusion Chromatography an useful approach to increase urinary EV purity?

5. What is the best approach to concentrate SEC-purified EVs?

6. Can we detect EVs that carry tumoral markers in the urine of UCa patients?

Figure 6 – Schematic representation of the experimental layout of this dissertation. (A) To understand whether the EVs shed by Urothelial Carcinoma cells carry the same tumour-associated proteins present in the cells of origin we have set up a 2D TCPS *in vitro* system for the production of UCa-derived EVs using the 639V cell line as a donor cells. The 639V-derived EVs shed to the conditioned media were then isolated by ultracentrifugation. (B) Comparison of spontaneous voided urine (spontaneous urine) and cystoscopically-collected intravesical lavage urine for the isolation of urinary EVs and selection of the most advantageous urine sampling method for downstream analysis of UCa-derived EVs; (C) Implementation of an UC-based protocol for the isolation of urinary EVs from the urine of urothelial carcinoma patients.; (D) Implementation of a size-exclusion chromatography (SEC) protocol for the purification of urinary EVs from UC pellets. (E) Comparison of ultrafiltration (UF) and TCA-based protein precipitation (PP) technologies for the concentration of SEC-purified urinary EVs. (F) Relative quantification of the abundance of UCa-associated protein markers in the urinary EVs isolated from the urine of UCa patients. (G) Methodological approaches to characterize the isolated EVs: (i) protein concentration (Lowry method), (ii) EV particle concentration and size (NTA), (iii) EV morphology and size (TEM) and (iv) profile of EV-associated proteins markers (Western Blot).

MATERIAL & METHODS

1. Isolation of UCa-derived EVs

1.1. Cell Culture

The adherent cell line 639V derived from a urothelial carcinoma grade III was cultured in Dulbecco´s Minimal Essential Medium (DMEM, ThermoFisher) supplemented with 10% Fetal Bovine Serum (FBS, BioWest). Cells were grown at 37ºC in a 5% CO_2 humidified incubator.

Cells were passaged every 2-3 days, when confluence reached approximately 80%. For sub-culture, cells were washed with PBS and detached upon incubation for 3-5 minutes at 37ºC with trypsin (Tryple Express Enzyme, Thermo Fisher Scientific). Upon trypsin blockade, the cell suspension was transferred into a sterile 50 mL Falcon tube and centrifuged at 300x g for 5 min at RT. Then, the supernatant was discarded, and the cell pellet resuspended in fresh DMEM supplemented with 10% FBS. The cell number and viability were determined using trypan blue exclusion assay. Cells were plated at 80 400 cells per cm^2 in T175 culture flasks corresponding to a seeding density of approximately 35%. Cell concentration (cells/mL) and viability were calculated according to the following equations:

(1) $\text{Cell concentration (mL)} = \text{average number of cells} \times 10^4 \times \text{dilution factor}$

(2) Cell viability (%) = $\frac{number\ of\ living\ cells}{number\ of\ total\ cells} \times 100$

All procedures involving cell cultures were performed under clean and sterile conditions, in biological laminar flow hoods. Aseptic technique was used for handing sterile reagents and materials. Cell cultures were routinely observed in a Leica Microscope (Leica DMi1) for signs of abnormal cell morphology, growth, or contamination. Cells were genotyped, to confirm the ID, and routinely tested for mycoplasma contamination.

1.2. Preparation of EV-depleted FBS

EV-depleted FBS was obtained by ultracentrifugation of FBS at 100 000x g at 4ºC for 16 hours (Ultracentrifuges Beckman Optima L80-XP or Beckman Optima XE100, sterilized 26,9 mL Quickseal tubes). Then, supernatant was collected and filtered with 0,22 μm filter (Merck Millipore) to avoid biological contamination of the EV-depleted FBS. The DMEM supplementation with EV-depleted FBS was designated as EV-depleted culture medium.

1.3. *In vitro* collection of 639V-derived EVs

UCa-derived EVs were obtained by allowing the 639V cell line to grow and shed EVs in a 2D Tissue Culture Polystyrene (TCPS) *in vitro* cell culture system.
For that, 639V cells were seeded at a density of 457 142 cells per cm^2 in 150 cm^2 TCPS (Sarstedt). They were cultured in DMEM supplemented with 10% of EV-depleted FBS and maintained at 37ºC in a 5% CO_2 humidified incubator for 72 hrs.
Upon 72 hrs of incubation, the number and viability of 639V cells were determined using a haemocytometer. The conditioned media was then collected to isolate 639V culture-derived EVs by differential centrifugation.

1.4. Isolation of 639V-derived EVs by Ultracentrifugation

To isolate 639V-derived EVs the conditioned media was collected to sterile 50 mL falcon tubes and centrifuged for 300x g for 10 minutes at 4ºC (Centrifuge 5810R, Eppendorf). Then, supernatant was transferred to new 50 mL falcon tubes and centrifugated at 2000x g for 30 min at 4ºC to eliminate cell debris or apoptotic bodies. Subsequently, the supernatant was transferred to 26,9 mL Quickseal tubes (Beckman) and ultracentrifuged at 100 000x g during 75 minutes (Type 70 Ti Rotor, Fixed Angle) at 4ºC. The, UC supernatant was then discarded by decantation and the EV pellets dissolved in 200 uL of sterile filtered 0.32% PBS citrate. The isolated 639V-derived EVs were then pooled together, transferred to sterile 1.5 mL Eppendorf tubes and stored at -80ºC until further analysis.

At the end of the procedure, UC Quickseal tubes were extensively washed with alkaline detergent for 24 hours, washed with deionized water and air dried to avoid sample cross-contaminations between experiments.

2. Clinical Samples

2.1. Clinical Study

Twenty urine samples were obtained from patients diagnosed with urothelial carcinoma at the Urology Department of Centro Hospitalar e Universitário do Porto - Hospital de Santo Antonio (Porto, Portugal), between September 2019 and February 2020. This study was designed according to the tenets of the Declaration of Helsinki approved by the institutional Ethics Committee. All participants signed the informed consent before enrolment.

2.2. Urine Collection, Storage and Transport

Spontaneous void urine and bladder washing urine specimen were collected from 3 paired UCa patients: 2 men (71 and 75 years) and 1 women (77 years), to compare the urine sampling approach. Then, bladder washing urine specimen was collected from 12 UCa patients at diagnosis (age range from 54-93 years). Approximately 80 mL of urine was collected into sterile 100 mL urine collection containers and transported at RT to the Lab for processing. The time between urine sampling and sample pre-processing was always bellow 4 hours.

2.3. Pre-processing of Urine Samples

In the Lab, all samples were pre-processed using a standard protocol. Patients' urines were transferred to sterile 50 mL falcon tubes (Thermofisher) and centrifuged at 300x g for 10 minutes at RT. The, the supernatant was transferred to a new sterile 50 mL falcon tube (Thermofisher) and centrifugated at 2000x g for 30 minutes at RT.

3. EV Isolation

3.1. Ultracentrifugation (UC)

After urine pre-processing, the supernatant was transferred to 26,9 mL Quickseal tubes (Beckman) and ultra-centrifugated at 100 000x g for 75 minutes (Ultracentrifuge Beckman Optima L80-XP/ XE100, 70 Ti Rotor). The supernatant was discarded by decantation and the resulting urinary EV pellet was re-suspended in 200 uL of PBS citrate, aliquoted, and stored at -80ºC until further analysis. The obtained ultracentrifuged urinary EV pellet is described as UC.

3.2. Size Exclusion Chromatography (SEC)

3.2.1. Sepharose 2B column packing

The EV isolation was performed by SEC as previously described [157]. Briefly, approximately 22 mL of Sepharose-CL-2B (Sigma, CL2B300-100 mL) was washed twice with 0,32% sodium citrate phosphate buffered saline and packed in 10 mL syringe (10 mL SOFT JECT, HSW). A 3 MM paper filter was used on top of the matrix to prevent disturbance of the packed Sepharose during sample loading. The column was then washed with PBS-Citrate. Finally, the column was submerged in 20% ethanol solution (bacteriostatic solution) and stored at 4ºC for 24 hours to allow the packing of the matrix.

3.2.2. Separation of the UC EV pellet components via SEC

Prior to sample loading, the SEC column was washed twice with citrate-PBS to remove the bacteriostatic solution. Then, 500 uL of UC EV pellet was loaded in the SEC column for further purification of the urinary EVs. The size-resolved components of the UC EV pellet were then collected in 10 SEC fractions of 1 mL each into sterile 1.5 mL Eppendorf tubes and immediately stored at 4ºC. The quality control of the SEC column was routinely performed for each experiment. For that, there was a visual inspection for the existence of air bubbles entrapped in the Sepharose matrix or any unconformities of the SEC column packing (e.g. uneven matrix distribution, presence of macroscopic contaminants, etc.). Moreover, the corresponding SEC column flowrate was registered for each experiment and maintained constant at 3,1±0,7 mL per minute.

Upon sample processing by SEC, the SEC column was washed twice with PBS-citrate and with 0,01% Triton (to dissolve any biological material attached to the Sepharose beads). Then the SEC column was washed twice with PBS citrate and then with 20% ethanol. Finally, the column was submerged in 20% ethanol (bacteriostatic agent) in a 50 mL falcon and stored at 4ºC until further use.

3.3. Ultrafiltration (UF)

In order to concentrate SEC-purified EVs we used ultrafiltration. Approximately 900 µL of each SEC fractions F3 to F6 were polled together and transferred to a centrifugal filter unit with a 100 KDa a cut-off (Amicon Ultra-4 Centrifugal Filter Unit, MilliPore). Then, the centrifugal filter units were centrifugated 5-10 minutes at 4ºC at 4000 rpm (Centrifuge Eppendorf 5810R) to enable the ultrafiltration of SEC-purified EVs. Finally, up to 150µL of the SEC-purified ultrafiltrate was collected from the centrifugal filter units and transferred to a sterile 1.5 mL Eppendorf tube. The designated SEC-UF EVs ultrafiltrate sample was then stored at 4ºC for immediate analysis or at -80ºC for protein analysis.

3.4. Protein Precipitation (PP)

Trichloroacetic acid (TCA) was used to precipitate proteins from SEC-purified EV samples. Thus, to concentrate the EV-associated proteins, TCA was added to the pooled F3 to F6 SEC fractions at a final concentration of 50%. Then, this mixture was incubated for 30 min at 4ºC and centrifugated at 10 000 g (Centrifuge MicroStar 17R) for 5-15 min at 4ºC. The EV precipitate was then washed twice with 500µL of cold acetone and centrifuged at 10 000 g for 5 min at 4ºC. The obtained EV pellet was allowed to evaporate

the acetone at RT, and dissolved with 4X SDS loading buffer with a heating cycle at 95ºC for 5 min, followed by vigorous vortex.

4. EV Characterization

4.1. Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was used to determine particle size distribution and particle concentration in all samples. To do this, samples (i) pre-processed urine upon 300g centrifugation (Urine 300G), (ii) urinary EVs recovered by ultracentrifugation (UC), (iii) SEC-purified EVs obtained in SEC fractions 3 to 6 (SEC) and (iv) concentrated SEC-purified EVs by ultrafiltration with a 100KDa cut-off membrane (SEC-UF) were pre-diluted in filtered 0,32% PBS citrate (between 1:100 to 1:10000, final volume 1 mL). Importantly, this sample pre-dilution was required to obtain a particle concentration in the optimal NTA reading range (10^8 to 10^9 particles/mL). The EVs from distinct samples were separately vortexed and immediately loaded in a NanoSight NS300 (Malvern Instruments) with a 1 mL syringe (Omnifix® 100 Solo, B|BRAUM). For sample loading, a NanoSight syringe pump (Malvern Instruments) was used to infuse the samples at constant flow rate at RT. Then, three separate 30-seconds videos were recorded with the following specifications: sCMOS camera type; laser Type Blue488; Camera Level 15; Slide Shutter 1206; Slider Gain 366; FPS 25; Temperature 25.3ºC; Viscosity 0,883-0,884 cp; Syringe Pump Speed 40.

4.2. Transmission Electron Microscopy

Negative staining transmission electron microscopy (TEM) was used for the morphological assessment of the isolated EVs. Briefly, 12 µL of freshly isolated EVs from pooled SEC fractions 3 to 6, before and after ultrafiltration, were mounted in Formvar-Carbon coated electron microscopy grids (FCF300-Ni, EMS) for 2 min and dried with a filter paper. Then, TEM grids were counter-stained with uranyl acetate and observed with a Jeol JEM 1400 at 80 keV with amplifications between 25 000x and 120 000x. Pooled SEC fractions F3 to F6 (SEC) and the same sample concentrated via ultrafiltration (SEC-UF) were pre-diluted with 0.32% Sodium Citrate in PBS (1:2), prior to loading in TEM grids, to allow a proper visualization of EVs. Representative TEM photographs were acquired and the size of EVs present in the pooled SEC fractions F3-F6, before and after ultrafiltration, was measured by image analysis using the ImageJ software. A minimum of 200 EVs were counted in both samples. The size profile of EVs from those samples was plotted as frequency curves.

4.3. Protein Quantification

The amount of protein in each sample (either EV-bounded or urine protein contaminants) was quantified in a 96 well plate using a Lowry-based protein detection method, accordingly to the manufacturer protocol (DC™ Protein assay, Bio-Rad). After 30 min. incubation in a dark chamber, the absorbance of the samples present in a 96 well-plate was analysed in a microplate reader (Synergy™ Mx, BioTek) with 488 nm wavelength excitation and read at the 655 nm. The data was obtained with the Gen5 Software and exported to the Microsoft Excel Software for further analysis. Data was plotted as protein concentration in µg/mL per sample type.

4.4. Protein analysis by Western Blot

4.4.1. Sample Preparation

Patients' urine exfoliated cells (pellet collect at the initial 300x g urine centrifugation) and the UCa 639V cell line (used as positive control in the WB experiments) were lysed at 4ºC during 30 minutes in Wyman´s buffer (500 µL EDTA 50 mM (RT), 150 µL NaCL 5M (RT), 50µl NP-40 (100%), 500 µL Tris-HCl pH 8 (4ºC) and 3800 µL deionized water) supplemented with 200 µL of protease inhibitors per 5 mL of Wynman´s Lysis Buffer. Then, cell lysates were centrifuged at 300x g for 5 min and the supernatant's proteins stored at -20ºC until further analysis. Noteworthy, the EV containing samples were lysed in loading buffer (10% SDS, Tris-HCL 1M (pH 6,8), 50% glicerol, β-mercaptoethanol and 1%bromophenol blue) and denatured for 5 minutes at 95ºC.

4.4.2. Sample Concentration

Occasionally, the EVs concentrated by UF or PP were further concentrated by Speed Vacuum technology (SPD121P Digital Series SpeedVac™, SystemsThermo Fisher Scientific™) before SDS-PAGE loading. The procedure was performed at RT until the required sample volume was achieved.

4.4.3. Analysis of Protein Abundance by Western Blot (WB)

The quantity of several EV-associated and urine protein contaminants in the urine-derived samples were analysed using western blot (WB). Importantly, similar protein amounts were loaded in each lane of the sodium dodecyl surface polyacrylamide gel electrophoresis (SDS-PAGE) to allow comparison of the relative abundance of each protein in the distinct samples. The electrophoresis was performed on 10% SDS-PAGE gel and transferred into nitrocellulose membrane for immunoblotting of target proteins.

After this, nitrocellulose membranes were incubated in Ponceau staining to confirm protein loading and WB transfer efficiency. Photographs of Ponceau stained nitrocellulose membranes were acquired in ChemiDOC™ XR+ (BioRad) and used latter on for protein lane normalization. Then, nitrocellulose membranes were cut in stripes and labelled accordingly to the analysed panel of proteins (Supplementary Figure 10). Then the nitrocellulose membranes were blocked during 2 hours with 5% milk (Molico) in TBS-T (Tris Buffered Saline (TBS) with 0,1% Tween-20 (Promega) at RT. The blocked nitrocellulose membranes were probed with primary antibodies during 1 hour and 30 minutes: anti-albumin (1:2000, Santa Cruz Biotechnology, sc-271605), anti-CYT-C (1:200 and 1:1000,Santa Cruz Biotechnology, sc-13560), anti-actini-4 (1:200,GeneTex ,GTX113116 or 1:2000, Santa Cruz Biotechnology, sc-1616), anti-CD63 (1:200, System Biosciences, ExoAB-KIT1), anti-CD81 (1:500, Santa Cruz Biotechnology-7637), anti-CD9 (1:200,Santa Cruz Biotechnology, sc-131185), anti-HSP70 (1:500, System Biosciences, ExoAb-KIT1), anti-mitofilin(1:500, sc-390707), anti-GATA-3 (1:200, Santa Cruz Biotechnology,sc-268), anti-UPIII-A (1:100,Santa Cruz Biotechnology, sc-166808), Annexin A10 (1:100, Santa Cruz Biotechnology,sc-398736), anti-S-100P (1:10000, Santa Cruz Biotechnology, sc-374547), anti-cytokeratin-7 (1:100,Santa Cruz Biotechnology, sc-23876), anti-Kiss-1 (1:200, Santa Cruz Biotechnology, sc-101246), anti-EHBP1 (1:100, Santa Cruz Biotechnology, sc-515619), anti-Annexin 1 (1:100, Santa Cruz Biotechnology, sc-12740), anti-THP (1:100, Santa Cruz Biotechnology, sc-271022), anti-Mucin-1 (1:200, Santa Cruz Biotechnology, sc-7313) anti-Ezrin (1:100, Santa Cruz Biotechnology, sc-58758) and anti-CD36 (1:100, Santa Cruz Biotechnology, sc-7309). Then, nitrocellulose membranes were washed 3 times with TBST-T for 10 minutes at room temperature under orbital shaker agitation. Depending on the species reactivity of each primary antibody, nitrocellulose membranes were subsequently incubated with peroxidase coupled secondary antibodies during 1 hour: anti-mouse (1:2000,Amersham ECL HRP-Conjugated Antibodies, Na931V) and anti-rabbit (1:2000,Na 934V).

Protein bands in the nitrocellulose membranes were detected by chemiluminescence. For that, nitrocellulose membranes were incubated with a chemiluminescence solution (ECL Western Blotting Detection Reagent, GE Healthcare) and, in a dark room, a chemiluminescence film (Amersham Hyperfilm™ ECL, GE Healthcare) was placed on top of membranes. The signal from the protein bands is then transmitted from the nitrocellulose membranes to the chemiluminescence films. The signal was detected by

Fuji Medical Film Processor (FUJI PHOTO FILM CO, LTD). The complete process of isolation procedure and characterization of EVs is represented in Figure 7.

4.4.4. Blot Imaging and Densitometric Analysis

For comparing the band intensities among all the lanes of the same blot a normalization method was implemented. For that, the films were scanned in a GS800 Calibrated Densitometer (Bio-Rad) and analysed with Quantity One 4.6.3 software (Bio-Rad). The obtained scanned blot images were converted into 8-bit and inverted using Image J software (version 1.8). Then, the loaded protein amounts in each SDS-PAGE lane and the corresponding protein band intensity were quantified by Image Lab 6.01. (Bio-Rad). For that, the Band Analysis Tool of Image Lab was used to select and determine the mean pixel intensity of the lanes and bands of proteins in all the Ponceau stained membranes and blots, respectively. Values were then normalized using loaded protein in the corresponding lane.

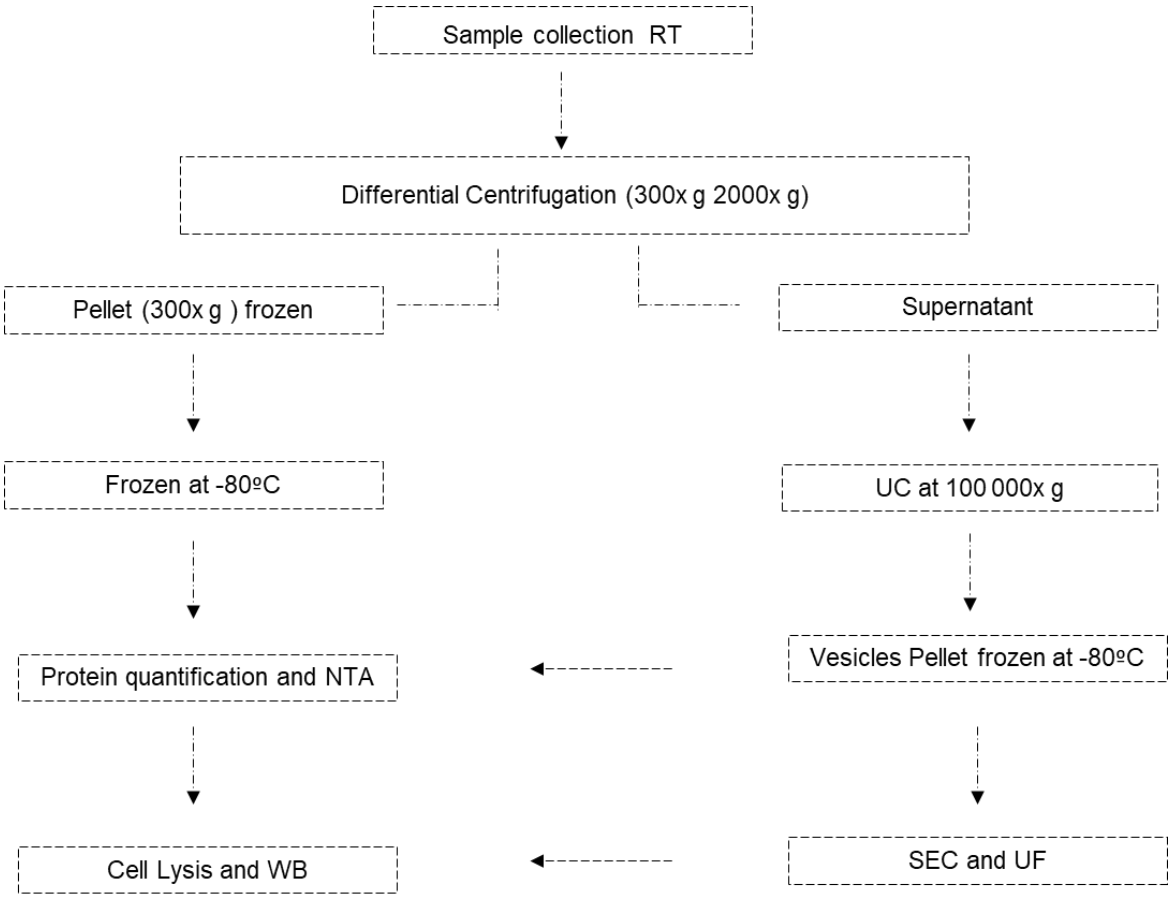


Figure 7- Representative scheme of the isolation processing and characterization for urine EVs.

5. Statistical Analysis

Data was summarized using as descriptive statistics the (i) mean and standard error of the means for continued variables and (ii) frequency curves and percentages for categorical variables. Statistical analysis was performed in GraphPad (Prism 8.0.2). The data normality was analysing using Shapiro-Wilk Test. Statistical results were performed using Wilcoxon-Mann-Whitney test. Statistical significance was considered at a p value < 0.05.

RESULTS

The contents of this chapter are based on:

Extracellular Vesicles as a Source of Bladder Cancer biomarkers: characterization of a three-step protocol for the isolation of urinary EVs from Urothelial Carcinoma patients

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1. Urothelial carcinoma cells shed EVs that carry UCa-associated proteins

EVs have been recognized as a potential source of disease biomarkers, particularly in cancer. In most tumours, the EV-based biomarker research is focused in identifying disease markers in the cargo of the tumour cell-derived EVs that are relevant for tumour progression. In bladder cancer, such molecular markers are still not used in the clinical for diagnosis or disease follow-up.

Therefore, our first aim was to understand whether UCa cells are able to shed EVs into the extracellular media and whether these UCa-derived EVs carry some of the tumour-associated proteins from the cells of origin.

Thus, we set up a 2D *in vitro* system to produce pure UCa-derived EVs. This system consisted in culturing 639V cells in EV-depleted cell culture media. The 639V cells were allowed to grow for 72 hrs until 80-90% confluence was reached (Figure 8A). Then, the conditioned media was harvested to isolate 639V-derived EVs using ultracentrifugation (UC).

To confirm the isolation of 639V-derived EVs by UC, the abundance, particle size-range and presence of EV-associated proteins were analyzed (Figure 8B-D). We demonstrated that EVs were highly abundant in the conditioned media of 639V cells. Indeed, more than 10^{11} particles with a mean size of 157 nm were isolated by UC. Interestingly, more than 80% of the isolated particles were larger than 100 nm, and were positive for the presence of HSP-70, an EV protein marker. Taken together, these findings suggest the vesicular nature of the UC-isolated particles.

Additionally, we observed that both 639V cells and the isolated 639V-derived EVs express UPIII-A, a UCa-related protein marker (Figure 8D). Interestingly, after normalization, we showed that both UPIII-A and HSP-70 proteins were highly enriched in the 639V-derived EVs when compared to the cell of origin (Figure 8E and F).

These results support that EVs are secreted by and have at some extent a protein mimicry similar to UCa cells.

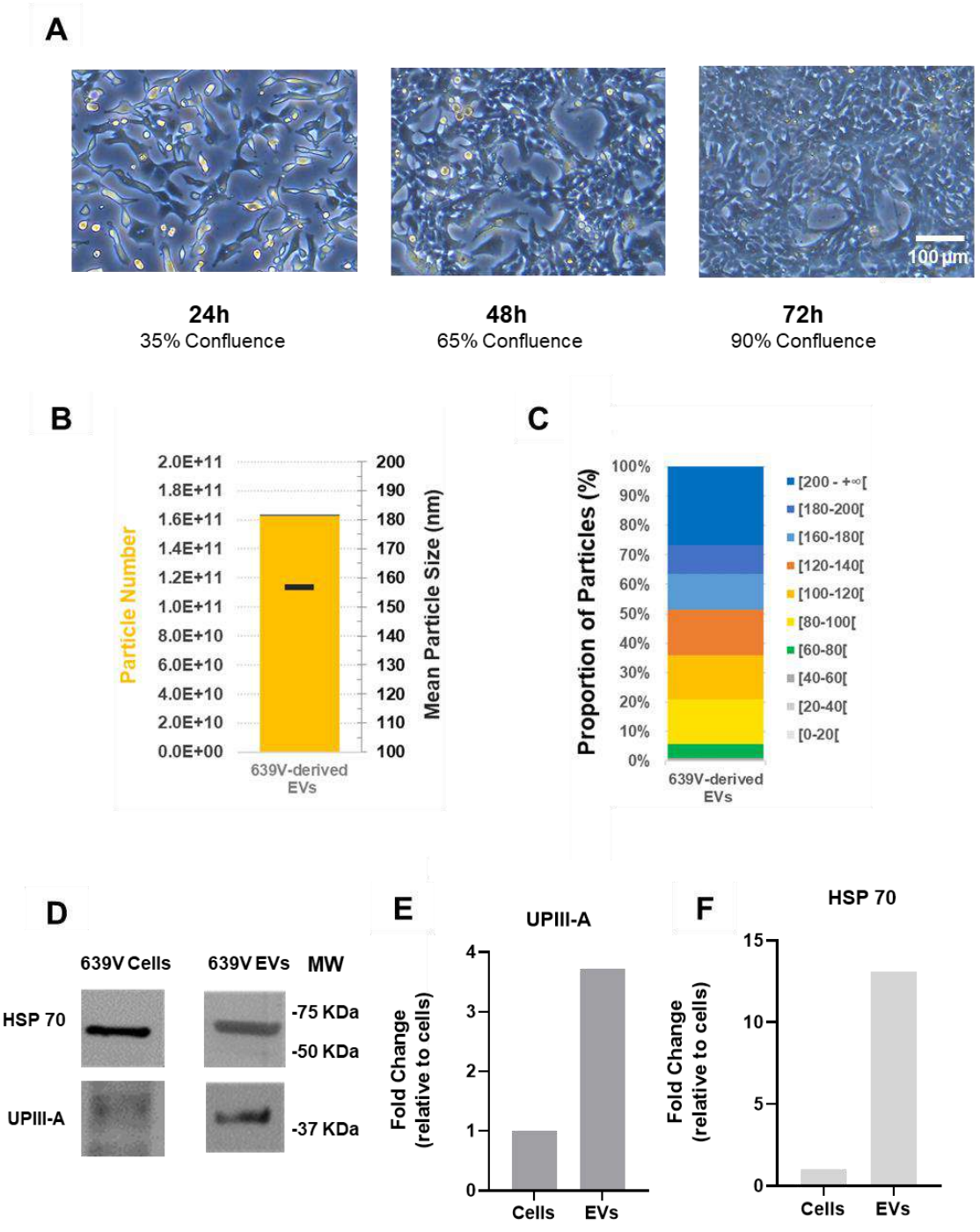


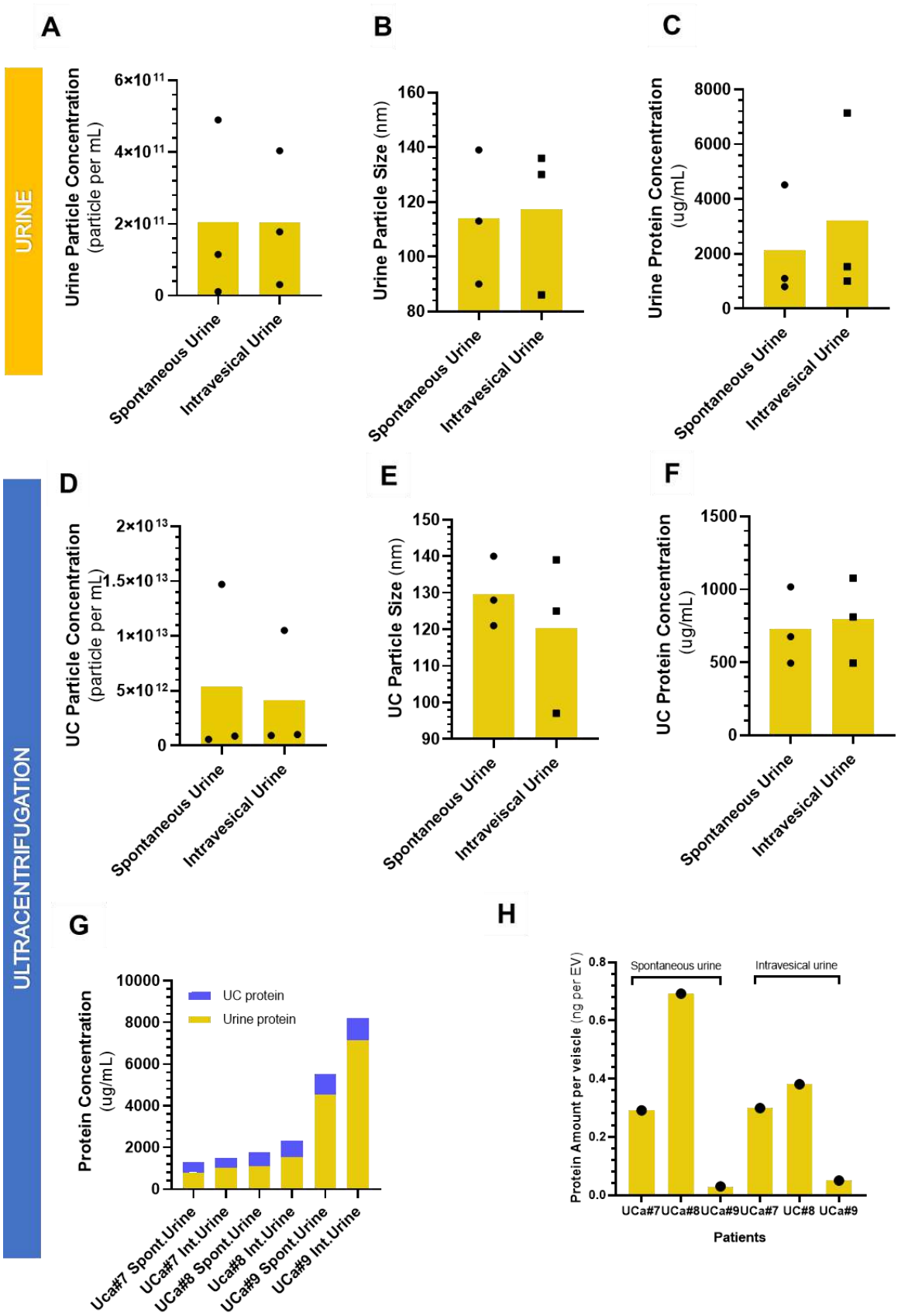
Figure 8 - Isolation and characterization of UCa-derived EVs produced by the 639V cell line. (A) Representative images of the 639V cell confluence at 24, 48 and 72h of culture before the collection of the EVs. (B) Particle number plotted against the mean particle size (nm) of the EVs isolated from 639V cell culture media by UC. (C) Proportion of the 639V-derived EVs particle size measured by Nanoparticle Tracking Analysis. (D) Western Blots representing the relative abundance of EV-associated and UCa-related protein markers (HSP-70 and UPIII-A, respectively) in 639V cells and 639V-derived EVs. Fold increase of

(E) HSP-70 and (F) UPIII-A proteins present in cells and EVs. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. Data represents one preliminary experiment (n=1). All values were normalized by the corresponding cells' protein marker intensity. All the presented protein bands are derived from the same blot. MW: Molecular Weight, HSP-70: Heat Shock Protein 70, UPIII-A: Uroplakin 3A.

2. Exploring the impact of urine sampling in the profile of urinary EVs

EV-based liquid biopsies could be either used as a stand-alone technique or in combination with the cystoscopy for the follow-up of UCa patients. Several analytical factors should be considered when addressing urine-based biomarkers. The first is related with the urine sampling procedure. To explore whether distinct urine sampling methods might influence the type and yield of urinary EVs, we compared 2 urine sampling procedures. We have compared the EVs isolated when urine was collected (i) from spontaneous micturition, with (ii) cystoscopically-collected intravesical urine from 3 UCa subjects. The evaluation of urinary EVs included particle concentration and size, protein concentration, and relative enrichment in EVs carrying UCa-associated protein markers (Figures 9 and 10) .

NTA revealed that both spontaneous and intravesical collected urine had a similar mean particle concentration in order of 10^{11} particles per mL (Figure 9A). Indeed, spontaneous urine had a mean of 2,5 x10^{11} particles against the 2,04 x10^{11} particles of the intravesical urine. Moreover, the urinary EVs in both samples do not seem to differ significantly in size (Figure 9B). The EV particles present a mean size of 114 ± 17 nm vs 117 ± 19 nm for spontaneous and intravesical urine, respectively. Similarly, the urine protein concentration is not significantly different between the paired spontaneous and intravesical urine (Figure 9C). Importantly, the similarity of the urinary EVs between the spontaneous and intravesical urine samples was maintained upon isolation by UC (Figure 9D, E and F). Moreover, we confirmed that the yield of EV protein recovery by UC was similar in paired samples (Figure 9G). No significant differences were found in the amount of protein per urinary EVs in paired urine samples (Figure 9H).



Extracellular Vesicle-based liquid biopsy in bladder cancer: establishing a protocol for the detection of urothelial carcinoma-derived EVs in the urine

Figure 9 – Comparison of the urinary EVs isolated from spontaneous or intravesical lavage urine sampling methods (A) Urine particle concentration (particle per mL). (B) Urine particle size (nm) and (C) Urine protein concentration (ug/mL) measured by NTA and Lowry method, respectively. (D) UC particle concentration (particle per mL), (E) UC mean particle size (nm) and (F) UC protein concentration (ug/mL). based on average of 3 urothelial carcinoma patients EV pellet, measured by NTA and Lowry method, respectively. (G) Urine Protein (mg) of 3 urothelial carcinoma patients' urine (yellow bar) compared with the obtained UC pellet protein (mg) (blue bar). (H) Estimative of the protein amount per vesicle (ng per EV). Data represents the average of 3 independent urine donors paired for each condition.

To further confirm whether different sampling methods have an impact in the proportion of EVs carrying UCa-associated biomarkers we have profiled the abundance of Periplakin-1 and EPS8L2 protein markers in the urinary EVs isolated from paired spontaneous and intravesical lavage urines from 3 UC patients (Figure 10).

We found that UCa-associated proteins were highly enriched in the UC-pelleted urinary EVs, whereas not detected in the corresponding original urine samples from UCa patients (Figures 10A and B). Interestingly, although no statistical significance was reached, both protein markers were more abundant in the EVs isolated from intravesical urine samples. Noteworthy, we confirmed that Periplakin-1 and EPS8L2 proteins were also expressed by 639V UCa cell line lysates. Thus, since the EVs isolated from intravesical urine lavages have a higher abundance of Periplakin-1 and EPS8L2 proteins in their cargo this sampling method was selected for the remaining experiments.

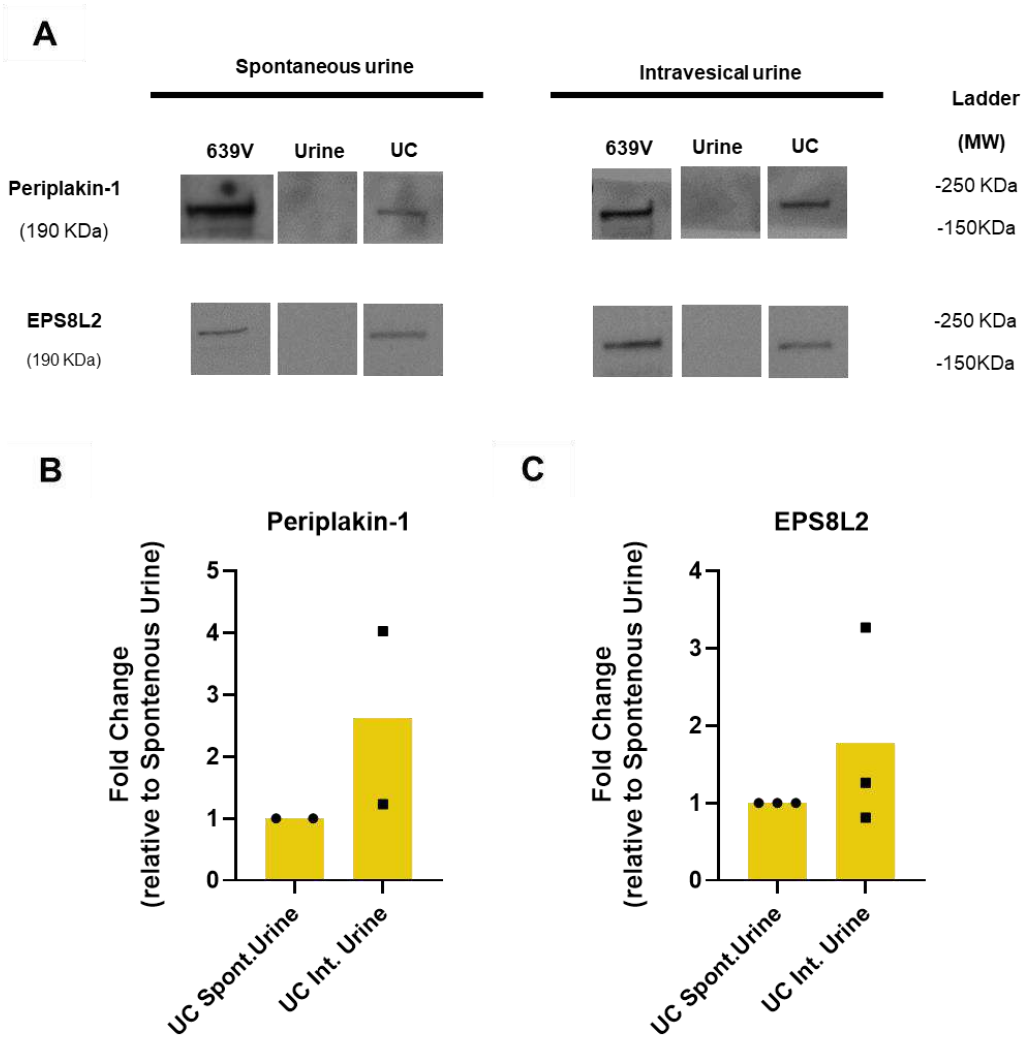


Figure 10- Enrichment of diagnostic marker in intravesical urine (A) Western blots representing the relative abundance of Periplakin-1 and EPS8L2 in UCa#8 patients' urine, in EVs isolated by UC and in the 639V cell lysate (positive control) between spontaneous and intravesical urine. Fold change of (B) Periplakin-1 and (C) EPS8L2 in intravesical urine relative to spontaneous urine. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. The quantification of the fold change of THP and Albumin represents the mean of at lest 2 independent UCa urine donors. The breaks int the images all correspond to same blot. The original blot can be found in the supplementary image 7. MW: Molecular Weight, EPS8L2: Epidermal growth factor receptor kinase substrate 8-like protein 2.

3. Ultracentrifugation recovers up to 30% of EVs present in the urine of UCa patients

Upon *in vitro* demonstration that 639V cells shed hundreds of EVs carrying tumour-associated proteins we sought to understand whether these tumour-derived EV subsets

could be isolated from the urine of UCa patients. Thus, we implemented an ultracentrifugation-based (UC) method for the isolation of urinary EVs from patients diagnosed with UCa.

The urine of UCa patients was collected and pre-processed by differential centrifugation at 300x g and at 2000x g to remove exfoliated cells and apoptotic bodies present in patients' urine, respectively. Finally, the urine supernatant was ultracentrifuged at 100 000x g to pellet the urinary EVs. To validate the isolation of urinary EVs by ultracentrifugation (UC) we analysed the abundance, size range, protein amount and cargo of the UC-isolated EVs (Figure 11). Noteworthy, we did observe that patients' urine has on average $1x10^{11}$ EV-like particles per mL of urine (Figure 11A). Foremost, we demonstrate that UC was able to concentrate these EV-like particles up to 30 fold into a pellet of urinary EVs. No major differences in the mean EV size of the urinary EVs was observed upon isolation by UC compared to the original urine sample (Figure 11B). Additionally, we determined the efficiency of UC to recover the urinary EVs from patients' urine. As expected, we did observe a significant decrease in the number of EV-like particles isolated by UC compared to the ones present in the original urine sample (Figure 10C). Indeed, approximately 30% of the urine EV-like particles are recovered using UC-based isolation. Notably, we confirmed that the UC pellet is highly enriched in EV-associated protein markers such as Actinin-4, HSP-70 and CD63 (Figure 11D). Indeed, these vesicular proteins were enriched up to 10-fold in the UC pellet when compared to the original urine sample. Collectively, these results validate the UC isolation method to isolate of urinary EVs from the patients' urine.

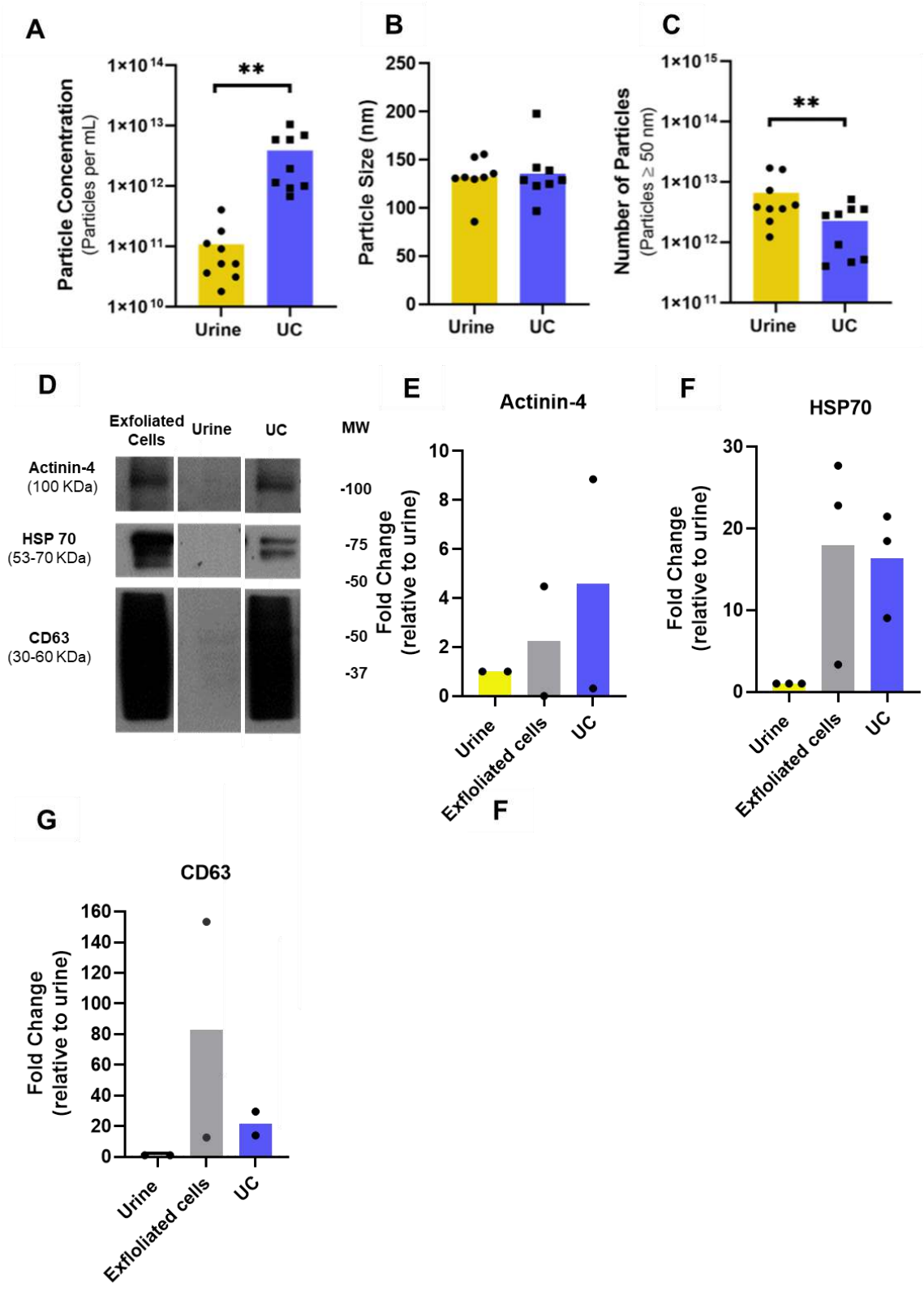


Figure 11 - Characterization of the urinary EVs isolated by UC. NTA analysis of the (A) particle concentration, (B) mean particle size and (C) total number of EV particles present in the UCa patients' urine compared with the particles isolated by UC. Data represents the average of 9 UCa patients. (D) Western

blots representing the relative abundance of actinin-4, HSP70 and CD63 EV-associated protein markers in UCa#2 patients' urine, urine exfoliated cells and urinary EVs isolated by UC. Fold increase of (E) Actinin-4 (F) HSP-70 and (G) CD63 EV-associated proteins in the urinary EVs isolated by UC compared to the original urine sample. All the presented protein bands are derived from the same blot. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. The gel loading was confirmed by Ponceau (Supplementary Figure 1). Data was obtained from at lest 2 independent UCa urine donors. MW: Molecular Weight, HSP-70: heat shock protein 70. ** p<0.01, Wilcoxon Test.

4. Non-vesicular protein contaminants are co-isolated with urinary EVs by UC

The discovery of EV biomarkers has been severely hampered due to co-isolation of EVs with contaminants, particularly in complex biological fluids such as blood and urine. Indeed, this is an important confounding factor preventing a clearer analysis of clinically relevant tumour derived EVs. In this framework, the Tamm-Horsfall (THP) and albumin are two of the most abundant urine protein contaminants that might difficult the isolation and analysis of urinary EVs. Therefore, the purity of isolated urinary EVs was evaluated by WB analysis of THP and Albumin proteins. Consistent with previous results, the WB analysis revealed that the procedure we have implemented beyond the enrichment EV markers, there is also decrease of THP and Albumin in the EV pellet, up to two-fold, when compared to the original urine sample (Figure 12). Globally, these results show that although urinary EVs are successfully isolated by UC there is also the co-isolation of other non-vesicular urine proteins that may hamper the analysis of the cargo of tumour-derived EVs.

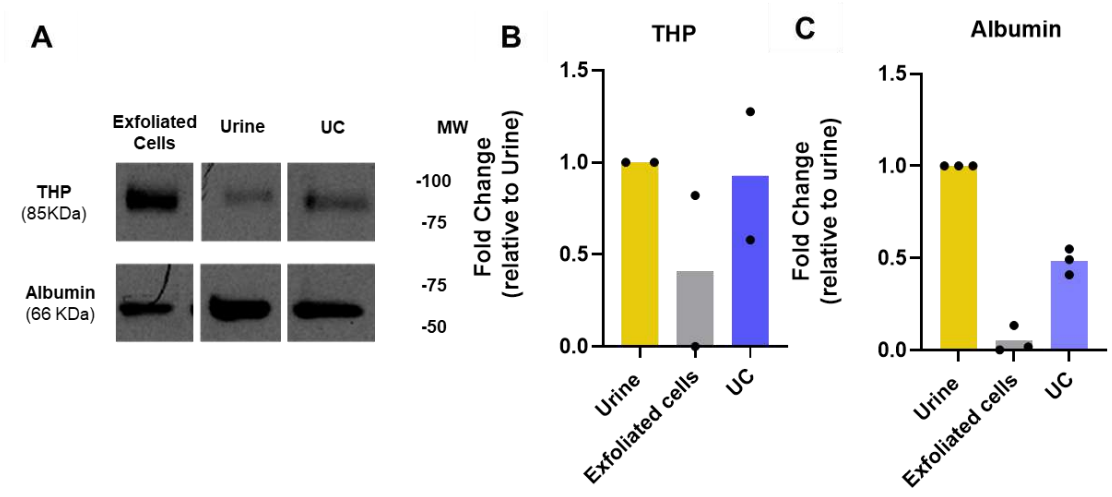


Figure 12 – Analysis of the co-isolation of urine protein contaminants in the EV pellet obtained by UC. (A) Representative Western blots representing the relative abundance of THP and Albumin in UC EV pellet of UCa#2 patient. All the protein bands are derived from the same blot. The original blot can be found

in the supplementary image 6. Semi-quantitative analysis of the fold change of (B) THP and (C) Albumin present in the UC EV pellet compared to the original urine sample. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. The quantification of the fold change of THP and Albumin represents the mean of at least 2 independent UCa urine donors. MW: Molecular Weight, THP: Tamm-Horsfall Protein.

5. Purification of Urinary EVs by Size Exclusion Chromatography

As previously shown, the UC was able to recover up to 30% of urinary EVs from crude urine. Interestingly, we showed that less than 1% of the total urine protein is likely to correspond to the UC-isolated EVs (Figure 13A). Nevertheless, as previously shown, these UC-pelleted EVs maintain significant amounts of non-vesicular protein contaminants, which may impar their diagnostic potential. Therefore, we sought to purify the UC-derived EVs using Size-Exclusion Chromatography technology to resolve urinary EVs from the co-isolated urine protein contaminants. The constituents of the urine UC pellet were resolved according to size, into 10 SEC fractions of 1 mL each.

To confirm the successful purification of urinary EVs from the remaining protein contaminants, the particle/protein concentration, particle size, and morphology were analyzed in corresponding SEC fractions. Importantly, the combined NTA and total protein analysis readouts showed that most EV-like particles (particles larger than 50 nm) were predominantly eluted in SEC fractions F3 to F6. This coincides with the first peak of protein amount (Figure 13B). Interestingly, from SEC fraction F7 onwards there was a drastic reduction in the number of eluted EV-like particles coupled with an exponential increase in the abundance of contaminant proteins. Indeed, NTA analysis revealed that larger EV-like particles were predominantly eluted in the earlier SEC fractions F3 to F6 while smaller protein contaminant particles (smaller than 50 nm) were observed in the later SEC fractions (Figure 13C). As confirmation, TEM imaging showed that the particles isolated within SEC fractions F3 to F6 have the typical lipid nature of urinary EVs. Indeed, the imaged urinary EVs were undamaged and displayed a size range between 50 to 230 nm (Figure 13D).

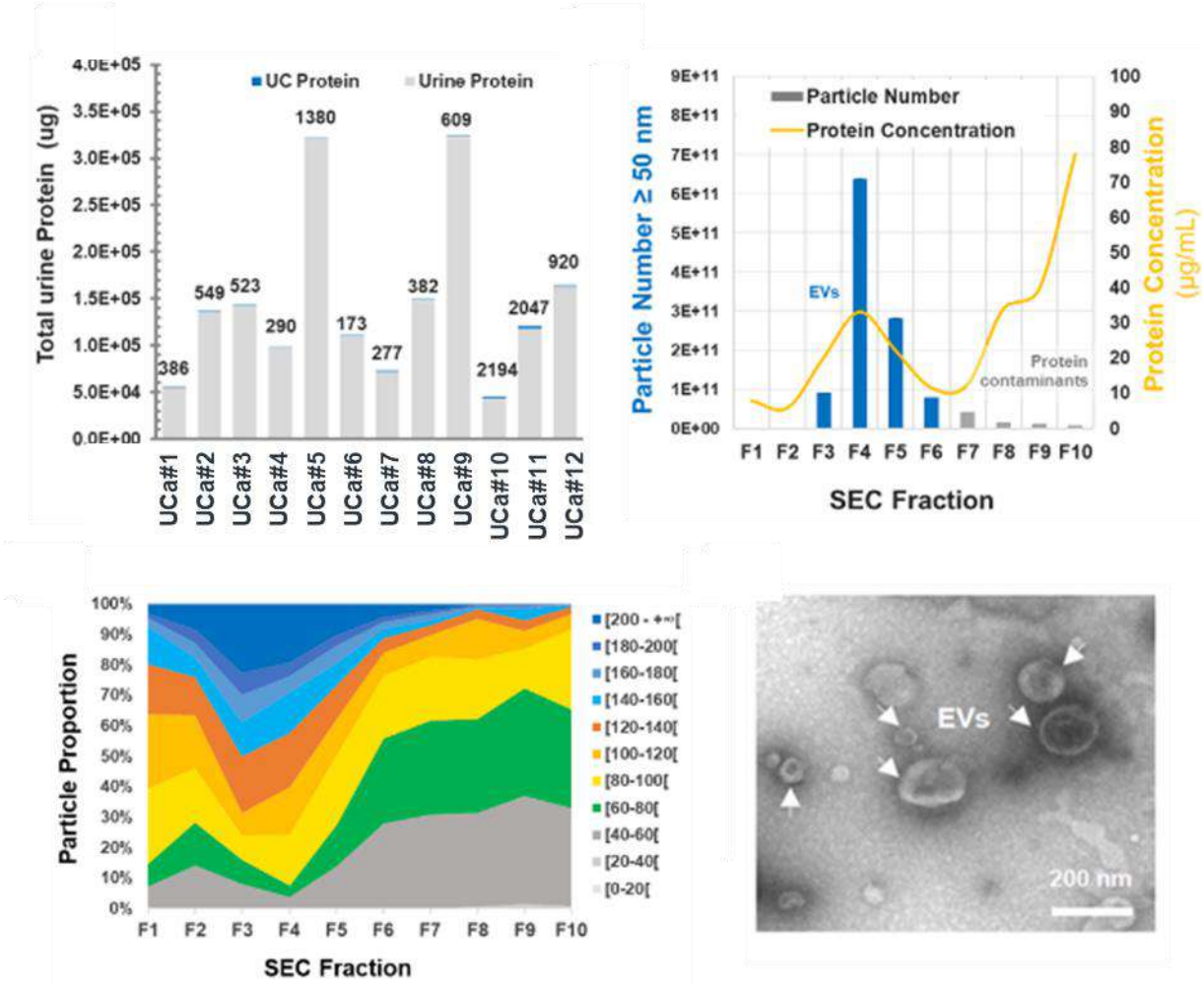


Figure 13 - Size exclusion chromatography (SEC) allows the isolation of intact and size-resolved EVs from the UC pellet obtained from the urine of UCa patients. (A) Protein quantification of 12 urothelial carcinoma patients' urine (grey bar) compared with protein associated EVs (blue bars) pelleted by UC. (B) Particle number per SEC fraction (grey /blue bars; left axis) plotted against total protein concentration (yellow line; right axis) measured by nanoparticle tracking analysis (NTA) and Lowry method, respectively, for each SEC fraction. (C) Representative proportion of EVs size distribution collected in each SEC fraction by Nanoparticle Tracking Analysis (NTA). Data corresponds to UC sample obtained from the urine of patient UCa#2. (D) Representative Transmission Electron Microscopy (TEM) photographs of EVs (arrowheads) isolated from sample UCa#4 in pool of SEC fractions (F3 to F6). Scale bar 200 nm. EV size was determined using Image J software.

6. Ultrafiltration is superior to the TCA-based protein precipitation method for the concentration of SEC-purified EVs

Even though SEC is able to further purify the urinary EVs present in the UC-isolated pellet, it also dilutes the sample up to 10-fold. This dilution difficult the subsequent

downstream EV analysis. Thus, to concentrate SEC-purified EVs we compared 2 distinct concentration methods: (i) ultrafiltration with a 100KDa cut-off membrane (UF) or (ii) Trichloroacetic Acid (TCA)-based protein precipitation (PP). The SEC-purified urinary EVs eluted in SEC fractions F3 to F6 were pooled together and exposed to UF or PP methods. Then, the concentration efficiency and sample recovery yield provided by each method was analyzed by measuring particle concentration and size, and the protein amount in pooled SEC fractions F3 to F6 and in the corresponding SEC-UF or SEC-PP concentrated samples.

Regarding to the concentration of EV-like particles and total protein, NTA and protein quantification analysis revealed that UF was able to increase sample concentration up to 15- and 8-fold, respectively, compared to the initial SEC sample (Figure 14A and B). Nevertheless, only 33% of the urinary EVs seem to be recovered in the ultrafiltrate with 67% of them remaining entrapped in the UF filter membrane (Figure 14C). Conversely, with the PP approach we only observed a 1.6- and 1.8-fold increase in the concentration of EV-like particles and protein concentration, respectively (Figure 14D and E). Importantly, the modest EV concentration provided by the PP technology comes at the expenses of a 82% loss of the EV-like particles from the original SEC sample (Figure 14F). Based on these results we have proceeded with the SEC-UF approach for the purification and concentration of the UC-pelleted urinary EVs.

Notably, we observed that the UF approach does not damage isolated urinary EVs. In fact, TEM imaging reveals that the concentrated urinary EVs have an intact round shape with a diameter from 50-286 nm which is similar to the original SEC sample (Figure 14G). Interestingly, TEM images showed less co-eluted free contaminating protein (represented as black areas) in the SEC-UF compared to the original SEC sample.

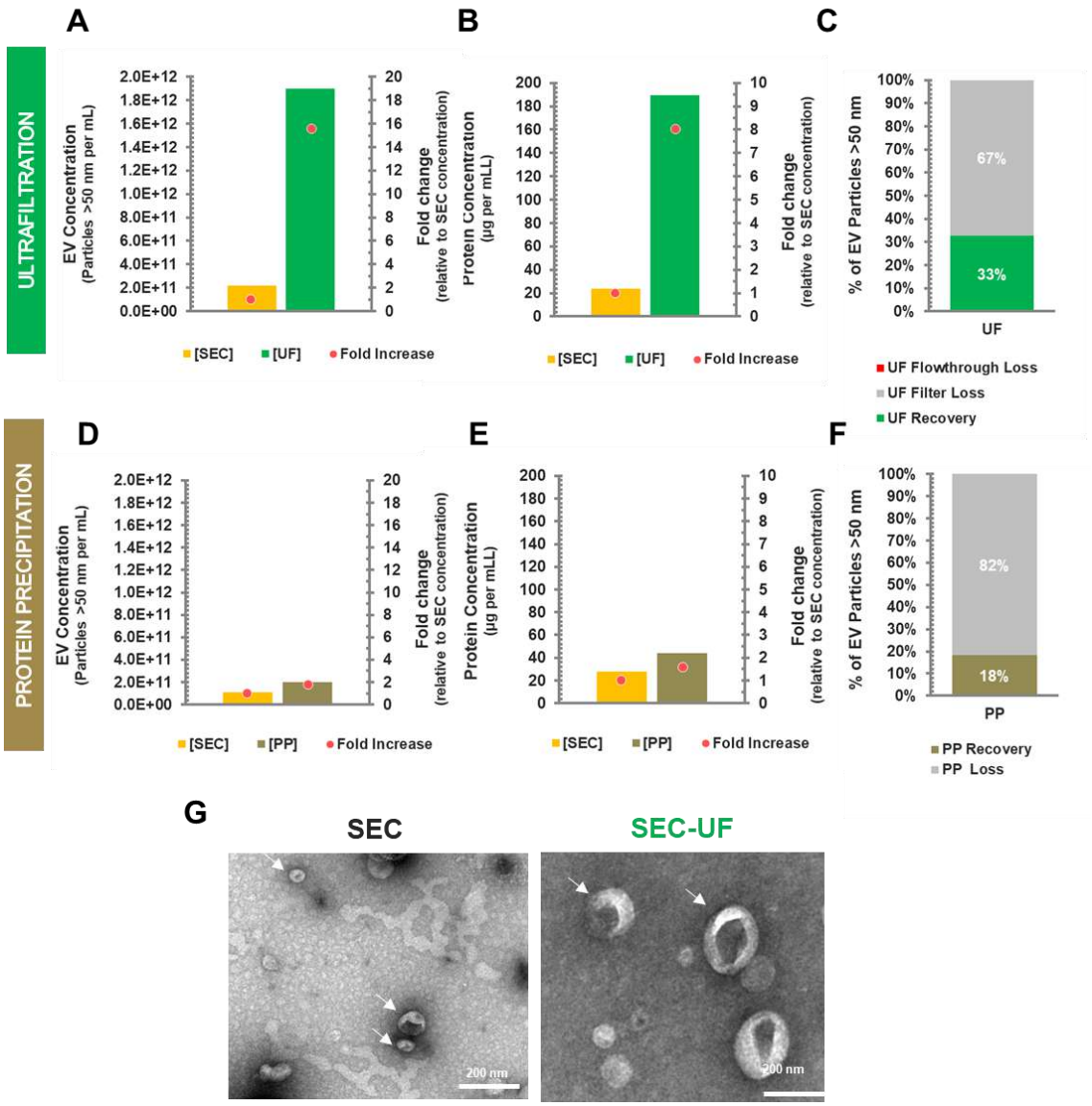


Figure 14 - Ultrafiltration allows the enrichment of SEC purified EVs. (A) Fold increase of EV concentration and (B) Protein Concentration after SEC and ultrafiltration. (C) NTA readouts of the percentage of EV particles recovered upon ultrafiltration (green) and % of urinary EVs lost due to adsorption to membrane filter (grey) or elution in the UF flowthrough (red). Data represents the average of 9 UCa patients. (D) Fold increase of EV concentration and (E) Protein Concentration upon protein precipitation relative to the original SEC sample. (F) NTA readouts of the percentage of EV particles/protein recovered upon concentration by PP (brown) and the % of urinary EVs/protein lost throughout the PP process (grey). Data represents the average of 3 UCa patients. (G) Representative transmission electron microscopy (TEM) photographs of EVs (arrowheads) purified by SEC (polled SEC fractions F3 to F6) before (left) and after ultrafiltration (right). Scale bar, 200 um. The mean EV size was determined using Image J software.

7. Combined SEC-UF approach has a higher depletion of non-vesicular urine protein contaminants

To understand whether the two-step approaches, SEC-UF or SEC-PP, also had a meaningful impact on the purity of the isolated EVs we compared them by analysing the depletion of THP and albumin protein contaminants. The relative abundance of THP and albumin proteins in the urinary EVs purified by SEC-UF or SEC-PP was evaluated by WB. Importantly, SEC-UF was able to deplete almost 99% of THP and Albumin present in the UC EV pellet (Figures 15A, B and C) while SEC-PP had a modest 40% and 26% depletion of these proteins (Figures 15D, E and F). This data supports the use of SEC-UF for the purification and concentration of urinary EVs obtained by UC.

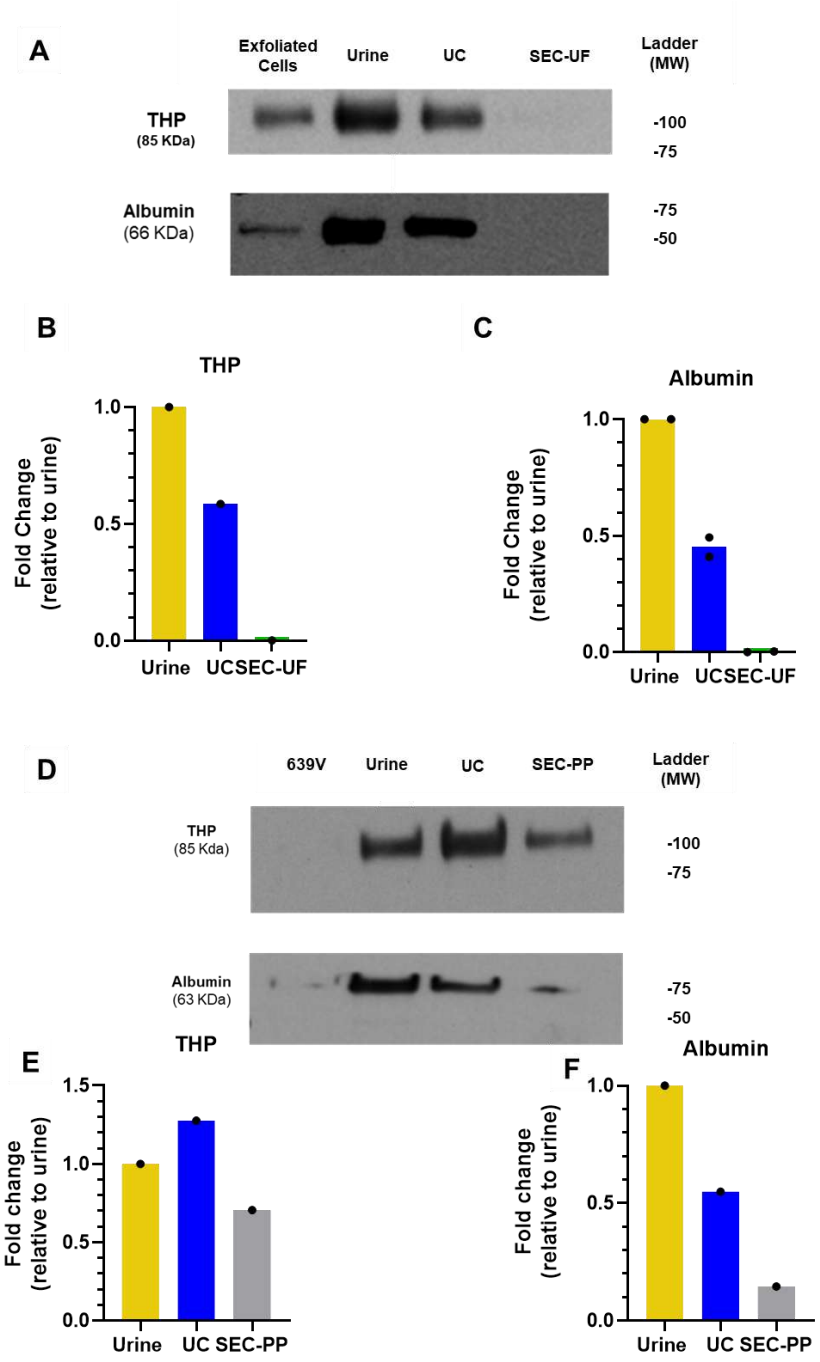


Figure 15 - Depletion of non-vesicular urine protein contaminants throughout the EV isolation process. (A) Western blots representing the relative abundance of THP and Albumin in UCa#2 and UCa#3 patients' original urine sample, EVs pelleted by UC and upon SEC-UF or SEC-PP purification/concentration approaches. Fold change of (B) THP and (C) Albumin relative to the original urine sample upon UC and upon SEC-UF or SEC-PP isolation approaches. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. The quantification of the fold change of THP and Albumin represents at least 1 independent UCa urine donor. The gel loading was confirmed by Ponceau (Supplementary Figure 1, 2 and 4*).* MW: Molecular Weight, THP: Tamm-Horsfall protein.

8. Urinary EVs isolated from UCa patients are highly enriched in tumour-associated protein markers

After the selection of the most effective urine sampling procedure and optimization of the 3-step protocol for isolating highly concentrated urinary EVs with increased purity, we sought to understand whether UCa-associated protein markers were detected in the urinary EVs isolated from patients. In order to achieve this, the relative abundance of Periplakin-1, ESP8L2 and Mucin-1 proteins in the isolated urinary EVs was determined by WB analysis (Figure 16A). Strikingly, we showed that these bladder tumour-associated proteins were highly enriched in the urinary EVs of most UCa patients as opposed to the original urine sample (Figures 16B, C and D). Noteworthy, vesicular Periplakin-1 and ESP8L2 proteins were detected in 4 and 3 out of 12 patients. Conversely, the EV-associated Mucin-1 was only detected in 2 out of 12 patients. Interestingly, the amount of Periplakin-1, ESP8L2 and Mucin-1 proteins was higher in urinary EVs of patients when compared to the 639V cell line lysates. Taken together, these results illustrate the potential of urinary EVs to diagnose/follow-up bladder cancer patients via detection of UCa-derived EVs subsets shed into the patients' urine.

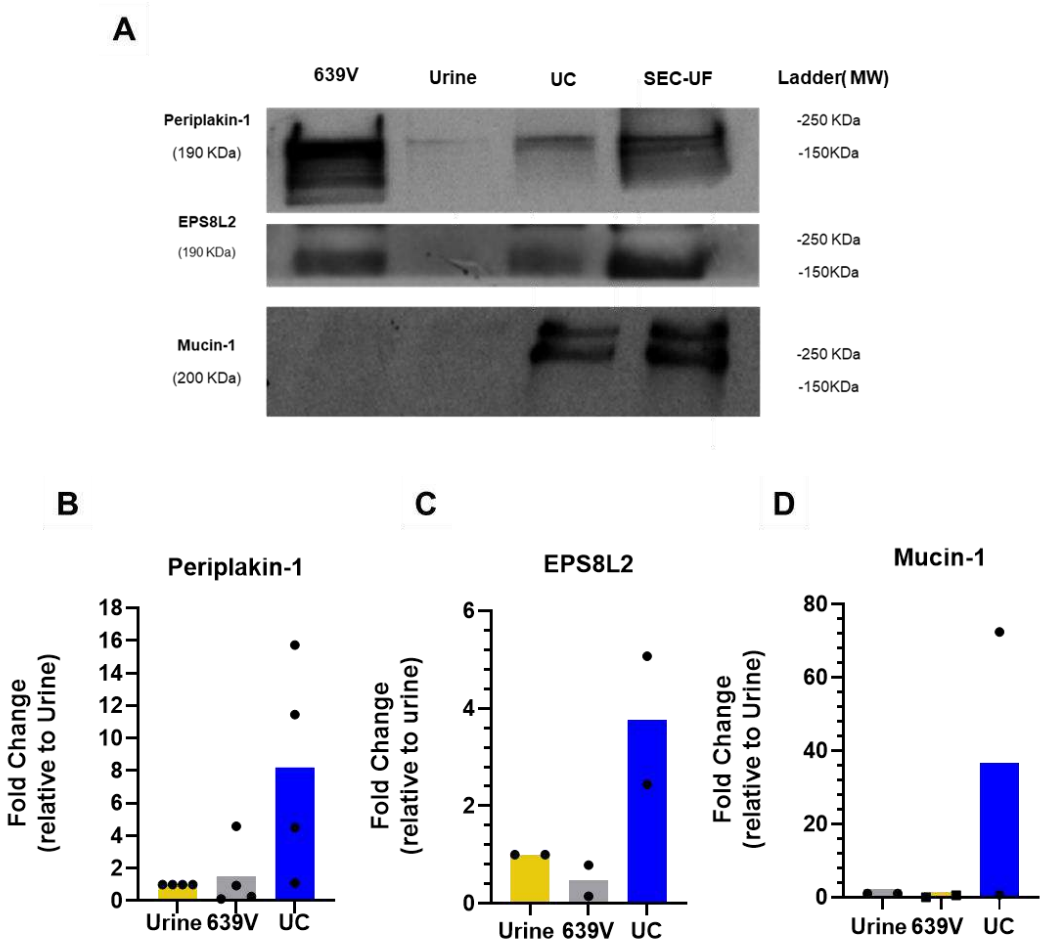


Figure 16 - Urothelial carcinoma-associated biomarkers are highly enriched in the urinary EVs isolated from UCa patients. Western Blots representing the relative abundance of (A) Periplakin-1 (B) EPS8L2 and (C) Mucin-1 in the positive control (639V cell lysate), urine, EVs isolated by UC and EVs purified by SEC-UF in patients UCa#5, UCa#8 and UCa#3, respectively. Fold increase of (B) Periplakin-1, (C) EPS8L2 and (D) Mucin-1 proteins present in the urinary EVs isolated throughout the different methodological approaches. Quantification of the blots was performed using the Image Lab software as described in the Material and Methods section. All values were normalized by the corresponding band intensity of the original urine sample. The quantification of the fold change of THP and Albumin represents the mean of at least 2 independent UCa urine donors. Western Blots are representative for periplakin-1, EPS8L2 and Mucin-1 data and blots quantification is expressed as the mean from five, four and two independent donors, respectively. The gel loading was confirmed by Ponceau (Supplementary Figure 2, 3 and 7). MW: Molecular Weight, EPS8L2:Epidermal growth factor receptor kinase substrate 8-like protein 2.

GENERAL DISCUSSION

Discussion

Bladder cancer is one of the 10 most frequent tumours worldwide [183]. The follow-up of UCa patients relies on cystoscopical inspection of the bladder wall followed by urine cytology. However, cystoscopy is an invasive procedure, misses up to 25% of tumours, and when performed routinely in the follow-up setting associates with urethral inflammation and infection [184, 185]. Likewise, although urine cytology is a minimally invasive approach, it lacks sensitivity to detect low grade tumours [24]. Moreover, the recurrent use of cystoscopy and urinary cytology in the follow-up of these patients, increases the related costs of BC monitoring [37]. Thus, within this framework, there is a need for the development of novel liquid biopsy-based biomarkers for monitoring UCa recurrence.

As mentioned in previous chapters, the analysis of urinary EVs in liquid biopsies is justified, as these EVs may serve as biomarkers for diagnosis and surveillance of UCa. Indeed, it has been shown in other malignancies that EVs share some of the cargo of their donor cancer cells providing important clinical information on the tumour landscape [129, 186]. Nevertheless, currently there is incomplete consensus on the standard procedures and variables that influence the urinary EV-based research [116, 187]. These standards procedures and variables include: (i) standardization of urine sampling and collection, (ii) urine storage, (iii) transport conditions and (iv) the adopted methodology for the isolation of pure urinary EVs from urine samples.

In this dissertation, we aimed to address some of these issues by establishing a protocol for the isolation and characterization of putative UCa-derived EVs in urinary samples from bladder cancer subjects.

1. Urothelial carcinoma cells shed EVs that carry UCa-associated proteins

It is well established that tumour-derived EVs have an important role in enabling several cancer hallmarks, facilitating cancer progression [93, 121]. Indeed, several authors highlighted the tumour dependence on aberrant EV signalling networks [188, 189]. In line with this, Green *et al*. showed that cancer cell lines shed significantly more EVs than their non-cancer cell lines counterparts [190]. A concept that if maintained *in vivo* could be exploited for EV-based diagnostics in oncology. Here, we explored the feasibility of using UCa-derived EVs in the urine of bladder cancer patients for diagnosis and

monitoring of disease recurrence. Thus, we have first characterized the profile of EVs shed by a UCa-derived 639V cancer cell line in a 2D TCPS *in vitro* culture system.

Importantly, we demonstrated that 639V cells shed significant amounts of EVs towards the extracellular media which are highly enriched in UPIII-A proteins, similar to the cell of origin. Uroplakins are glycoproteins that compose the main structural external layer of urothelium and have been recently reported as potential biomarker for the identification of UCa [191]. In agreement, Olsburgh *et al*. showed that UPIII-A were elevated in plasma and urine of BC patients and loss of expression of this protein was associated with poor prognosis and pathologically aggressive BC [70]. Regarding to the presence of this marker in EVs, one study reported that EVs were enriched in multiple uroplakins, including UPIII-A, and that it was frequently overexpressed in BC [192]. Interestingly, most studies correlating alterations of this protein in the cargo of EVs do not confirm experimentally whether these EVs are actually derived from the patients' tumor cells. Thus, in this work we demonstrate in an *in vitro* model that urothelial carcinoma cells are able to shed EVs to the extracellular media that are highly enriched in UPIII-A proteins..

2. Exploring the impact of urine sampling in the profile of urinary EVs

The timing of urine collection could have a direct impact on the urinary EVs isolation [93]. For instance, spontaneous urine vs intravesical urine collected by cystoscopy may influence abundance of tumour-derived and overall urinary EV concentration, EV size and proportion of EV subsets [116].

Thus, our aim was to compare two sampling methods for the isolation of urinary EVs: (i) first voided urine (spontaneous urine) and (ii) cystoscopy-derived intravesical urine (intravesical urine).

Interestingly, our data did not show statistical differences in the urines' particle concentration or in the mean particle size derived from these two sampling methods. Similarly, no differences were found in the protein concentration of pelleted urinary EVs by UC.

Since there was no differences in quantitative measures among sampling method, we sought to understand whether there were differences on the abundance of EV subsets which carry UCa-associated protein markers. Considering this, although the expression in UCa biomarkers were not statistically different between samples, EPS8L2 and Peripalkin-1 seemed to be enriched in the intravesical urine. Moreover, results suggest

that there is a minimal protein degradation in the urinary tract/bladder given that there were no significant differences in the protein amount and particle concentration between the two sampling methods. Importantly, our results corroborate findings from *Yen et al*. that the difference in protein recovery and expression of EVs markers between spontaneous and intravesical urine was minimal [193]. Interestingly, some WB studies reported that EPS8L2 was overexpressed in the urinary EVs isolated from BC patients [72]. Moreover, Matsumoto *et al.* showed that serum uroplakin could be a potential biomarker for BC patients.

3. Ultracentrifugation recovers up to 30% of EVs present in the urine UCa patients

Ultracentrifugation, based in separation of particles according to density, is the most used technique for EV isolation [116]. According to studies, the UC allows the correct isolation of EVs from several biological fluids including urine, plasma and cerebrospinal fluid [170, 194]. This technique involves a centrifugation at very high g forces, where the rotation speed and procedure time are not yet standardized for all biologic fluids. Importantly, these settings can have a great impact in the obtained EV pellet [195]. Indeed, several factors can be modified in the protocol, such as acceleration, rotor-associated characteristic, centrifugation time, and taking into account sample-related variables, such as viscosity. These features lead to inconsistencies in the EV sedimentation speed and subsequently in isolation efficiency [196]. For instance, swinging bucket rotor (SW) allows a better resolution, being suitable for particle separation with similar sedimentation coefficients. Importantly, we showed that UC was able to recover up to 30% of the EV-like particles present in the original urine sample. This result is in accordance with the study reported by Geeurickx *et al.* where the authors use “spike-in” fluorescent EVs to screen the efficiency of several EV separation techniques including UC [197].

Moreover, Deun *et al.* studied the impact of different isolation methods in downstream analysis and have demonstrated that different isolation techniques could affect protein amount per EV particle [198]. Similarly, we did observed that the EV recovery yield was followed by the co-isolation of non-vesicular proteins contaminants with a great impact in the downstream analysis of urinary EVs. Despite these results, the biological confirmation of effective urinary EV isolation by UC was demonstrated by the presence

of 50-300 nm particles that were positive for the presence of CD63, HSP-70 and Actinin-4, *bona fide* EV markers by WB analysis.

4. Several non-vesicular protein contaminants are co-isolated with the urinary EVs by UC

The EV-associated proteins are several orders of magnitude less abundant than free proteins present in most biological fluids [199]. According to MISEV (Minimal information for studies of extracellular vesicles), some contaminants may aggregate with EVs and be co-isolated, decreasing sample purity and limiting biomarker discovery [116]. Therefore, the use of EVs as a potential source of biomarkers requires the isolation of highly pure EVs. MISEV guidelines for EV characterization recommends to analyze co-isolated protein contaminants accordingly to the source of EVs. In this regard, Tamm-Horsfall protein (uromodulin) and albumin are two of the most abundant non-vesicular protein contaminants present in the urine.

We demonstrated that EVs pelleted through UC were co-isolated with significant amounts of urinary protein contaminants. Indeed, we have confirmed that the majority of THP and albumin were still present in the urinary EVs pelleted by UC. Importantly, our findings are in line with the observations of Li *et al*. that showed that the EV pellet obtained after UC carries most of proteins contaminants of the original sample [200].

In this way, for an unbiased downstream analysis of urinary EVs pelleted by UC, additional EV purification steps are needed.

5. Urinary EVs are purified by Size Exclusion Chromatography

The recent trend in EV-based research relies on the combined use of additional isolation methodologies with the UC-based isolation of urinary EVs for increased sample purity. Size Exclusion Chromatography technology can further resolve complex samples by separating their components based on size. This an efficient, highly reproducible, fast and useful approach to separate larger EVs from smaller urine protein contaminants.

Here, SEC was implemented to purify the urinary EVs pelleted by UC from the urine of cancer patients. The pellet obtained after ultracentrifugation was loaded into a SEC column and their constituents were resolved by size throughout 10 fractions of 1 mL each. The resulting SEC fractions were analyzed in terms of eluted protein amount, particle number and size. It would be expected that the first SEC fractions would not

present significant amounts of particles (representing the SEC column void volume) while the last ones would elute the smaller protein contaminants. Indeed, SEC fraction 1 and 2 presented a very low protein amount and particle concentration when compared with following fractions and were excluded from downstream analysis. Similarly, the fraction 7 to 10 were not included, since the number of particles above 50 nm was rather low despite the increased protein concentration. This increased protein amount suggests that most of the protein contaminants co-pelleted with the urinary EVs by UC were eluted in these fractions. Taken together, SEC fraction 3 to 6 were selected for downstream analysis of purified urinary EVs. The confirmation that EVs had been eluted in SEC fractions F3 to F6 was further supported by TEM imaging and presence of EV-associated markers by WB. Indeed, TEM images revealed that particles eluted in fraction 3 to 6 had an intact round shape morphology.

6. Ultrafiltration is superior to the TCA-based protein precipitation method for the concentration of SEC-purified EVs

Although SEC allows the separation of EVs from smaller protein contaminants it also dilutes them up to 10-fold. The fact that some authors use more than one technique after the isolation step, led us to question whether UF or PP technique allow sample concentration. We compared the ability of ultrafiltration or protein precipitation methods to concentrate the SEC-purified EVs eluted between SEC fractions 3 to 6. Accordingly to studies, UF have a high performance and is able to concentrate EVs up to 240-fold [159, 201]. Our findings revealed that particle and protein concentration was increased by up to 16- and 8-fold upon UF, respectively. Moreover, the performance of this study and others can be discrepant. In fact, a previous study reported that UF concentrate EVs with a recovery yield of 80%, while in our work we were able to retrieve only 33% of EV-like particles. Thus, the use of UF present limitations due to the loss of sample that is probably associated with the filter during the process, despite it concentrates EVs.

On the other hand, TCA or acetone precipitation is a quick and simple protocol used for protein extraction [202]. Although there are theoretical advantages, this technique only increased up to 2-fold the EV particle and protein concentration upon SEC. Moreover, TEM photographs revealed that particles in SEC fraction 3 to 6 upon UF presented an intact round and cup- shape, confirming that there was no degradation of vesicles for downstream analysis. Nevertheless, some vesicles presented an irregular shape, an cup shape. This may be due to sample preparation, since it was reported that vesicles with

this morphology are due to dehydration caused by the technique, and therefore may be artefacts.[201]

7. Combined SEC-UF approach has a higher depletion of non-vesicular urine protein contaminants

Regarding to SEC-UF purity, according to Calabro *et al.*, UF might be used to separate and clarify solutions from low molecular weight contaminants [203]. On the other hand, Valero *et al.* reported that acetone interferes with functional proprieties of vesicular membranes resulting in co-isolation with proteins contaminants [204].

Here we observed that UF is more efficient to concentrate the SEC-purified EVs since they eliminate most of the free proteins contaminants with a MW lower than the 100KDa cut-off filter. In fact, this was demonstrated by WB analysis of the abundance of albumin and THP. The depletion of these urinary protein contaminants was less exuberant with the protein precipitation when compared to the UF approach.

8. Urinary EVs isolated from UCa patients are highly enriched in tumour-associated protein markers

Recently, urinary EVs were reported as a promising source of cancer biomarkers, both for cancer diagnostics and prognosis [129, 205, 206]. Their potential for biomarker discovery raised the interest for analysing the EVs present in the urine of UCa patients due to the close contact of the urothelial tumours present in the urothelial wall and this body fluid. Following this trend, several studies demonstrated that urinary EVs may contain associated molecules useful for clinical diagnosis and follow-up of genitourinary tumours [207-209].

Indeed, Periplakin-1, a protein with functions in cell cytoskeleton organization has been recently reported by Zhu *et al.* to be a potential diagnostic marker for BC [58]. Similarly, other authors also reported that the EPS8L2 protein, was increased in the urinary EVs isolated from BC patients [72]. This protein, EPS8L2, who has not been extensively studied, seems to have functions partially redundant to epidermal growth factor receptor pathway substrate 8 leading to actin remodeling [210]. Additionally, Ahmad *et al*. reported that Mucin-1, a glycosylated protein present in epithelial cells, was also increased in the EVs isolated from patients with BC, as well as other malignancies [62, 211]. Similarly, we observed that urinary EVs isolated from UCa patients were enriched in Periplakin-1, EPS8L2 and Mucin-1 proteins. Our work showed that it is possible to

detect the presence of the described markers in urinary EVs. Importantly, here we demonstrate that the relative abundance of these markers increase throughout the purification process of the isolated urinary EVs. Our data suggests that these proteins may have a vesicular origin, once it is likely to correspond to tumour-derived EVs shed into the urine. However, the amount of protein loaded after SEC-UF compared to the initial sample and UC was much lower and different between techniques and patients, respectively. Thus, comparison between techniques seems inadequate. Nevertheless, it is possible to observe that although the loaded amount is inferior, the abundance of these proteins after SEC-UF is equal or of greater intensity compared to the UC.

9. Experimental pitfalls

Although this work was performed most exclusively with UCa patients urine samples, some potential limitations should be considered. The original experimental layout for this dissertation consisted in the analysis of the urinary EV profile throughout the course of the disease. Indeed, this would have included urine samples collected from NMIBC patients at UCa diagnosis, upon tumour resection and eventually at the recurrence stages of the disease. Unfortunately, although 12 UCa patients were included in this study the follow-up samples were lost due to the outbreak of the COVID-19 pandemics and the study was re-adjusted to this new reality.

At this time, there is limited agreement on the pre-analytical procedures for clinical validation of EV based diagnostics in UCa. Different variables such as urine sampling, temperature, storage duration and transportation conditions may change the integrity or the EVs composition [212]. Indeed, Cheng *et al.* showed that urine samples stored at 4ºC had a higher particle concentration and expression of EVs markers when compared to lower temperatures (-20ºc and -80ºC)[213]. During the establishment of our protocol, the urine was collected and transported at RT and the UC-pelleted EVs were stored at -80ºC. These factors have a possible impact in concentration and expression of EVs markers. Regarding urine transportation, 4 hours seems to be the maximum time to minimize EV degradation [93, 214]. Moreover, this period of time was found to be optimal to minimize the influence of bacterial growth and influence the pool of analyzed urinary EVs [214]. In this work, samples were processed up to a maximum of four hours and at RT, in order to decrease the co-precipitation of Tam Horsfall protein that is observed at lower temperatures. According to other studies, DTT has been proposed to be added to the crude urine samples to reduce the THP proteins avoiding their co-precipitation and

EV entrapment in the protein network formed at lower temperatures. However, this reducing agent may also lead to a loss of EVs by altering the urinary EVs features [215, 216].

The WB analysis was an interesting technique for EV characterization in urinary EVs. Indeed, during the concentration process (SEC-UF) the EVs had much lower amount of protein, which limited the possibility to have the same amount of protein for all conditions in some patients.

Furthermore, the small amount of purified and concentrated EVs did not allow to repeat the experiment for the same patient if needed. To circumvent this issue, the amount of protein loaded in SDS-PAGE was limited to 15 ug. For comparisons of protein quantity using WB analysis, we used densitometric analysis of the loaded protein in each lane obtained by Ponceau staining. Indeed, this approach bypassed the doubts in using common cellular housekeeping markers as actin or tubulin as unproven and most likely unsuitable normalization protein marker for EVs.

10. Future perspectives

In this work we have demonstrated that urinary EVs were effectively isolated, purified and concentrated from urine samples of UCa patients. Additionally, it was confirmed that urinary EVs could be a promising source of UCa biomarkers.

Currently there is still a lack of standardization of the urine sampling method, pre-processing approach and EV isolation techniques. In the future, it would be interesting to make a more robust comparison between distinct urine sampling approaches at different temperatures, since there are not many studies reporting the optimal temperature/pre-processing approach for urine samples [217].

A relevant clinical issue is the use of healthy urine controls, which will be needed to determine the diagnostic potential of urinary EVs. Ideally, we should also compare the presence of these markers in EVs isolated from the urine of individuals which have benign inflammatory conditions. The inclusion of these controls would allow the definition of the EV-based liquid biopsy sensitivity and specificity for the diagnosis of UCa. Finally, to prove the clinical value of this approach more samples should be analysed in larger prospective clinical studies.

Additionally, it would be interesting to perform proteomic analysis of the purified urinary EVs to explore new protein biomarkers of UCa. Moreover, it would be interesting to

compare the profile of urinary EVs in UCa patients at diagnosis, at several time-points after treatment and at disease recurrence. This approach, would allow to establish clinical correlations between the number of vesicles positive for disease markers and the time observed for the patient to relapse. Although this was the initial intention of this dissertation, all the follow-up samples were missed due to the outbreak of the first wave of COVID-19 pandemics in Portugal.

The ultrafiltration process despite concentrating the vesicles, present low recovery yield. In the future, it is necessary to pay attention to this critical step, being able to opt for a new ultrafiltration device that grants a higher recovery efficiency of the purified urinary EVs. With TEM, was observed that urine EVs shown a cup shape and other irregular shape, due to the preparation of this step. The visualization of urine EVs by eletron microscopy have a challenge of sample preservation, since dehydration can cause artefact. In this way, instead of use chemical fixation that could interfere with EVs morphology, another technique such as Cryo-electron microscopy could be used. Since this method use physical fixation when compared to TEM preserve the EVs features. Finally, it was reported that methods such as affinity interactions can be used for capture EVs via binding to the CD9, CD63 or CD81 proteins present at urinary EVs surface. This technique might be of interest for this project, since EVs can be recovered with less protein contaminants when compared with UC [218].

CONCLUSION

Several diagnostic biomarkers in urologic cancers have already been described by and/or approved by the FDA and other regulatory agencies Although, these biomarkers may have an impact in the clinical practice, the majority of them focus in specific protein or in mutations in specific genes in the tumour cells. On the other hand, the EVs have been proposed as biomarker for detection of several malignancies being ideal for cancer follow-up as these can be collected from several body fluids as the blood or urine. Indeed, the EVs have been shown to transport important proteins that regulate tumour progression and virtually all the identified cancer hallmarks. In this way, these particles seem to provide important information on the tumor microenvironment landscape.

To establish the clinical usefulness of urinary EVs for the diagnosis and follow-up of UCa a careful isolation and characterization of the urinary EVs is in order. Indeed, several key issues of the EV-biomarker discovery as the urine collection, and the EV isolation approach is not yet standardized. With this work it was possible to verify that ultracentrifugation allows the isolation of up to 30% of the EVs present in the patients urine and that the pelleted EVs carry significant amounts of non-vesicular protein contaminants which can introduce significant bias into the result analysis thus limiting biomarker discovery. Importantly, we demonstrated that the purification of these UC-pelleted EVs by SEC-UF has obvious advantages for the downstream analysis of tumour-derived urinary EVs. Indeed, here we have shown that it was possible to detect EV-subsets that carry UCa-associated protein markers whose expression increases with increasing EV purity .

Taken together, this work described the implementation and validation of a 3-step protocol for the isolation of EVs from the urine of UCa patients. We demonstrated that this combined approach increased the purity of urinary EVs, which might enable an unbiased EV biomarker discovery by high throughput proteomic. Moreover, several described UCa-related protein markers were found to be specifically enriched in the isolated urinary EVs. The analysis of the tumour-derived EV subsets in the urine of these patients may hold great promise for the diagnosis of UCa and for diminishing the number of invasive cystoscopies required in a follow-up setting. Thus, the work performed in this dissertation serves as a proof-of-principle for the feasibility of using EV-based liquid biopsy for the detection of UCa, thus establishing the technical foundations for a future project in a larger UCa cohort.

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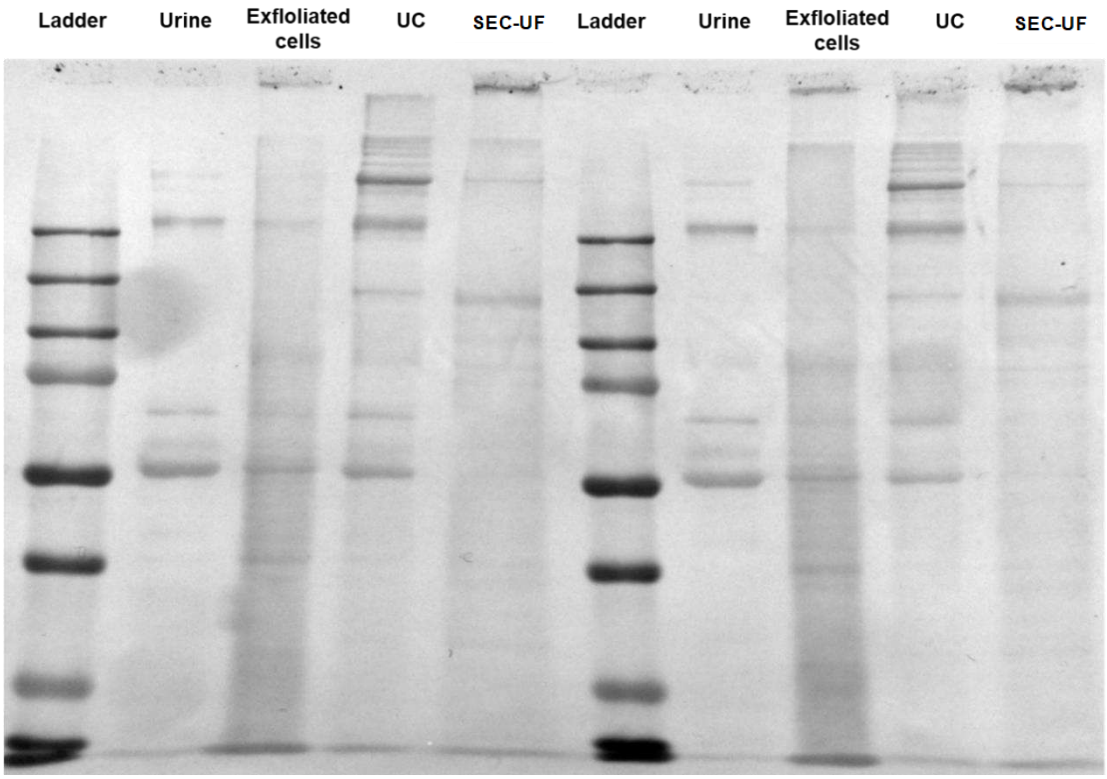
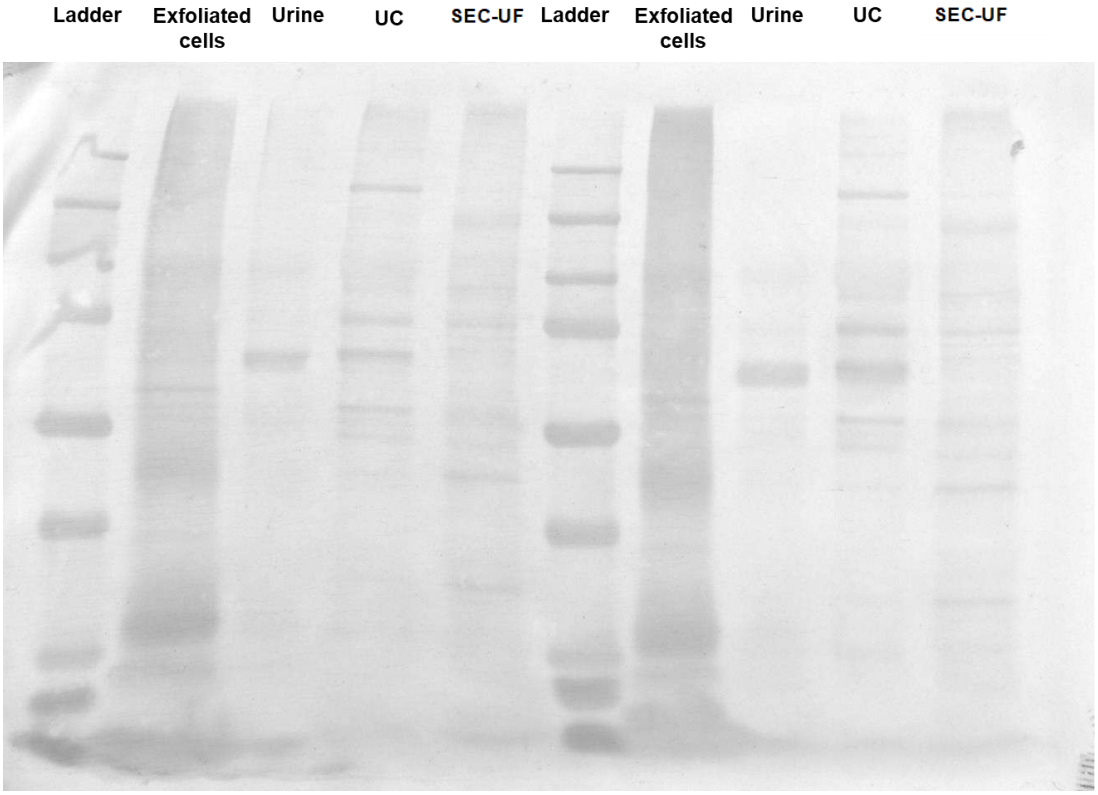
SUPPLEMENTARY MATERIAL

Supplementary Figure 1



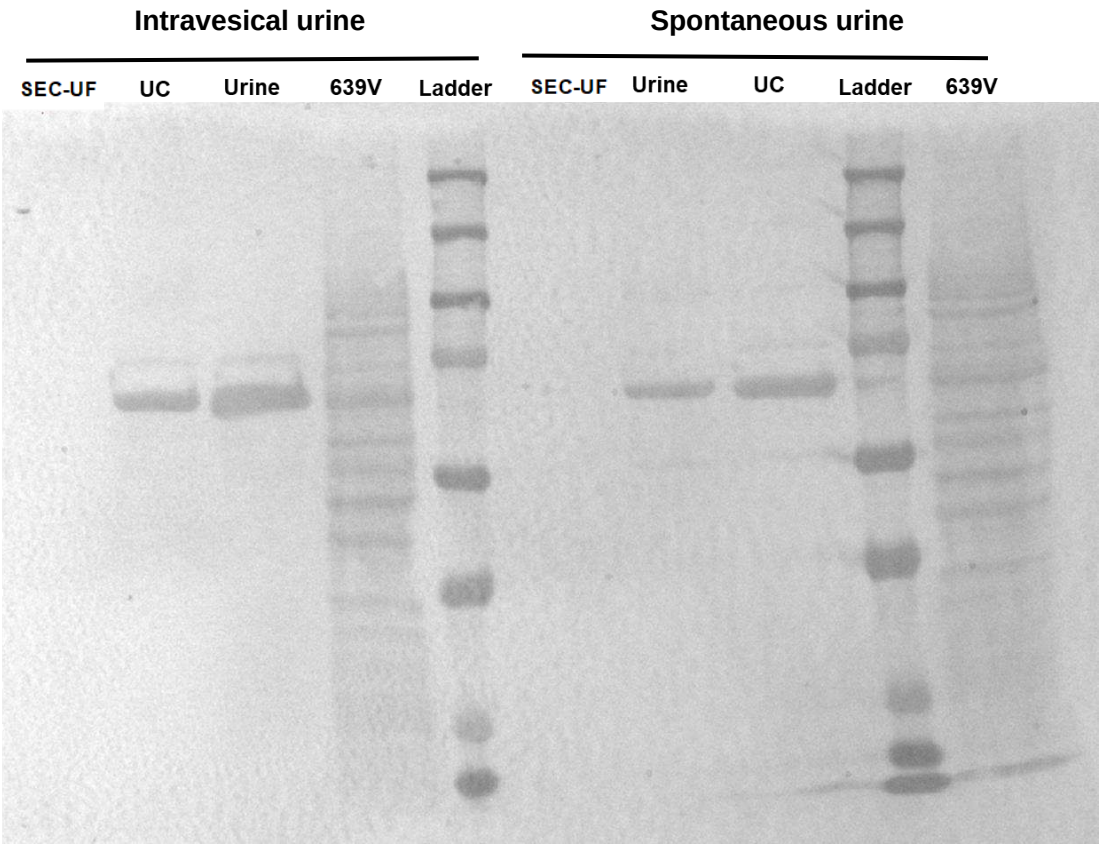
Supplementary Figure 1 – Ponceau from UCa#2 for protein staining aimed at confirmation of loading. Ponceau staining of urine, UC, exfoliated cells and SEC-UF in analysis of actinin-4, HSP70, CD63, THP and Albumin.

Supplementary Figure 2



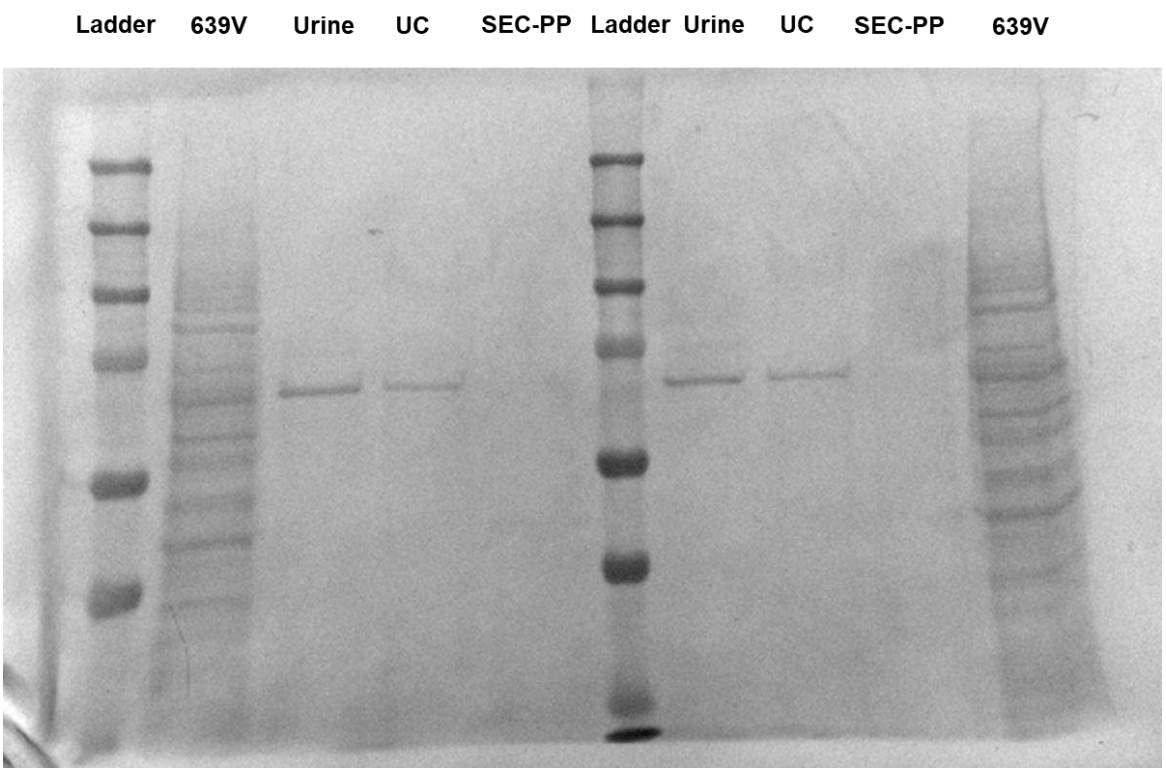
Supplementary Figure 2 – Ponceau of UCa#3 for confirmation of protein staining of loading. Ponceau staining of urine, UC, exfoliated cells and SEC-UF in analysis of Mucin and Periplakin-1.

Supplementary Figure 3



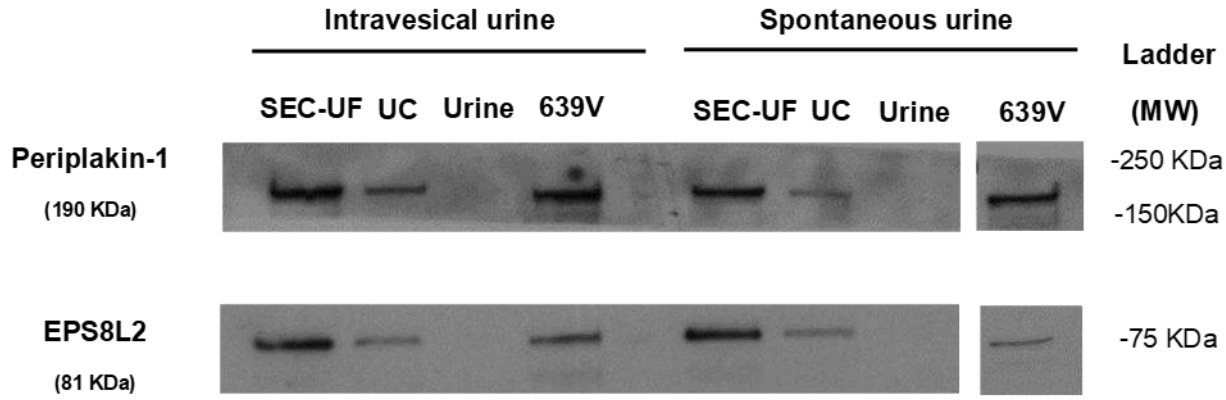
Supplementary Figure 3– UCa#8 Ponceau protein staining for confirmation of loading. Ponceau staining of 639V (positive control), urine, UC and SEC-UF for first and second urine, in analysis of EPS8L2 and Periplakin-1.

Supplementary Figure 4



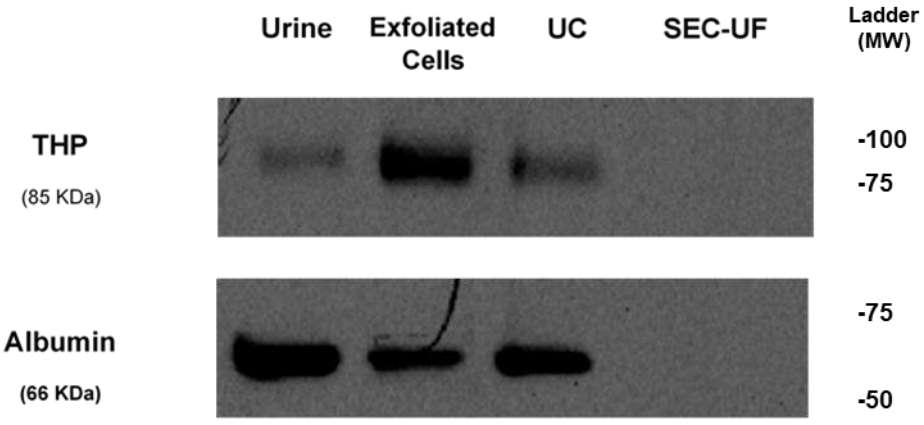
Supplementary Figure 4– UCa#10 ponceau protein staining for confirmation of loading. Ponceau staining of 639V (positive control), urine, UC and pool UF after protein precipitation in analysis of proteins contaminants (albumin and THP) and EVs markers (CD81, CD63,actinin-4 and HSP70)

Supplementary Figure 5



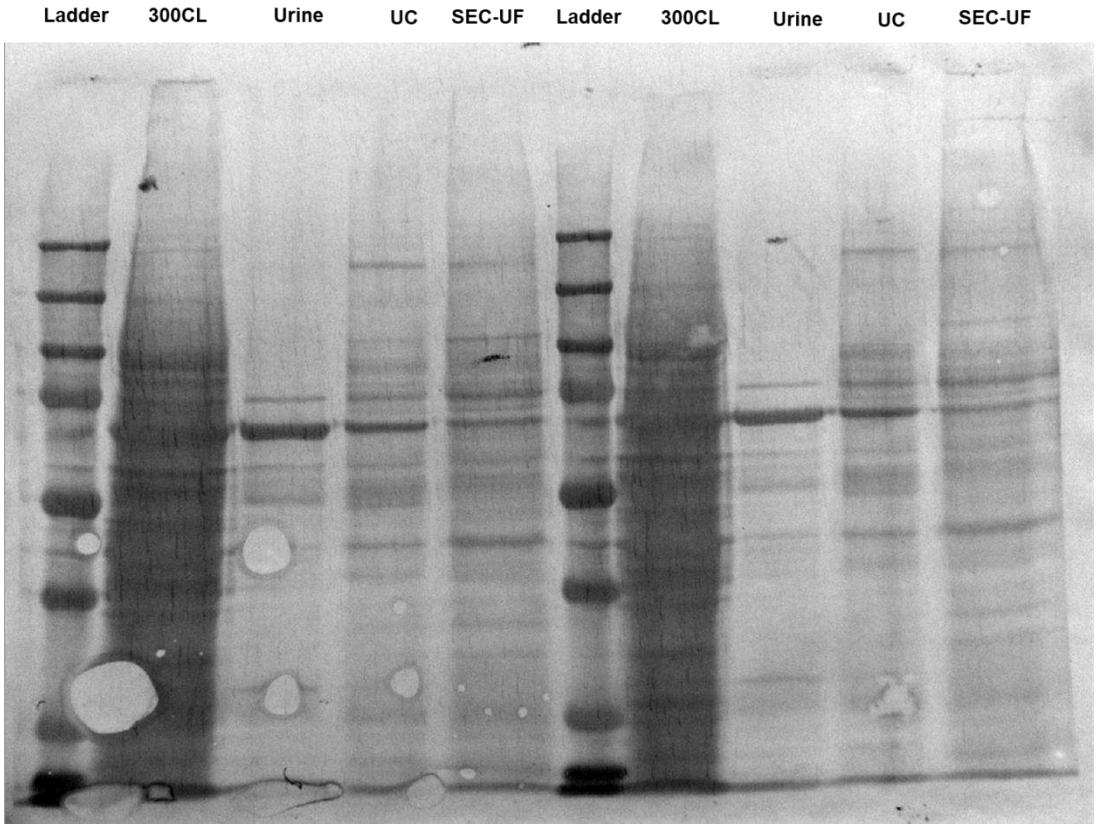
Supplementary Figure 5- UCa#8 WB spontaneous and intravesical urine for confirmation of urothelial carcinoma associated markers (periplakin-1 and EPS8L2)

Supplementary Figure 6



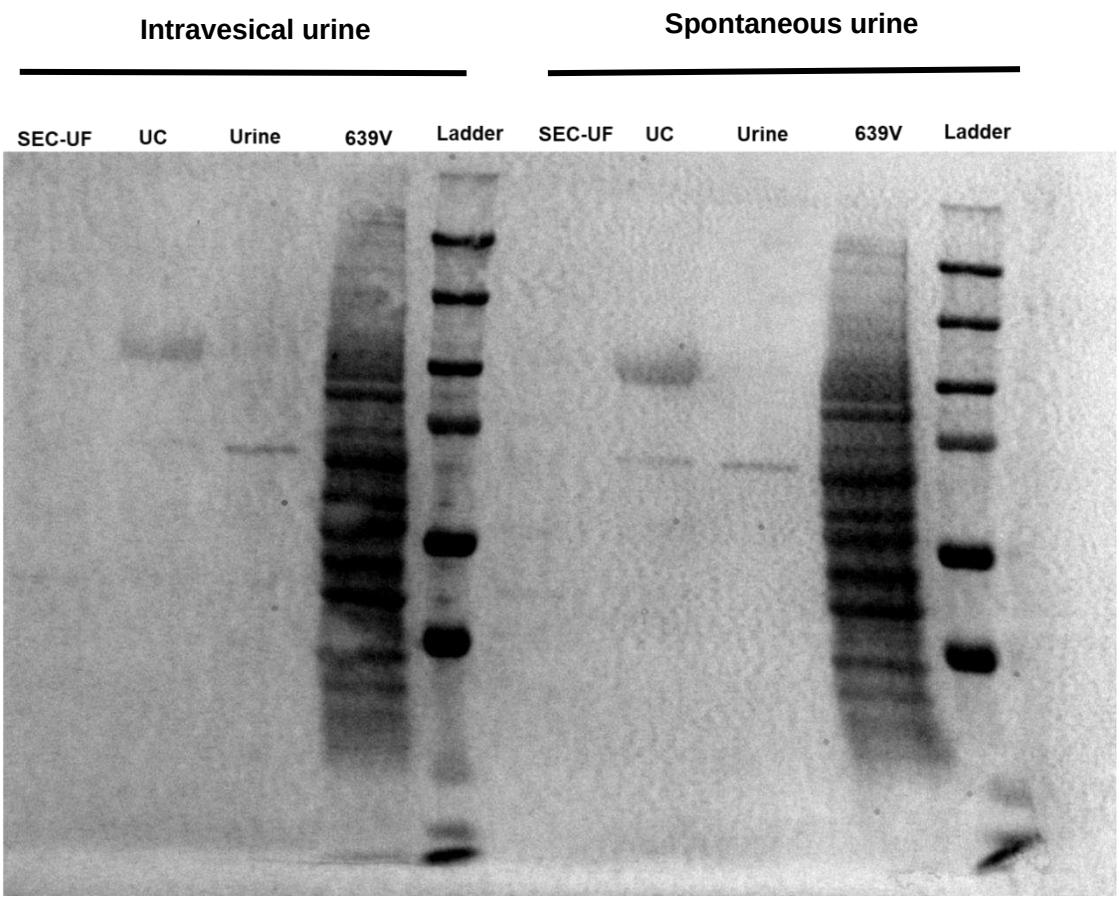
Supplementary Figure 6- UCa#2 WB for confirmation of proteins contaminants depletion (THP and Albumin)

Supplementary Figure 7



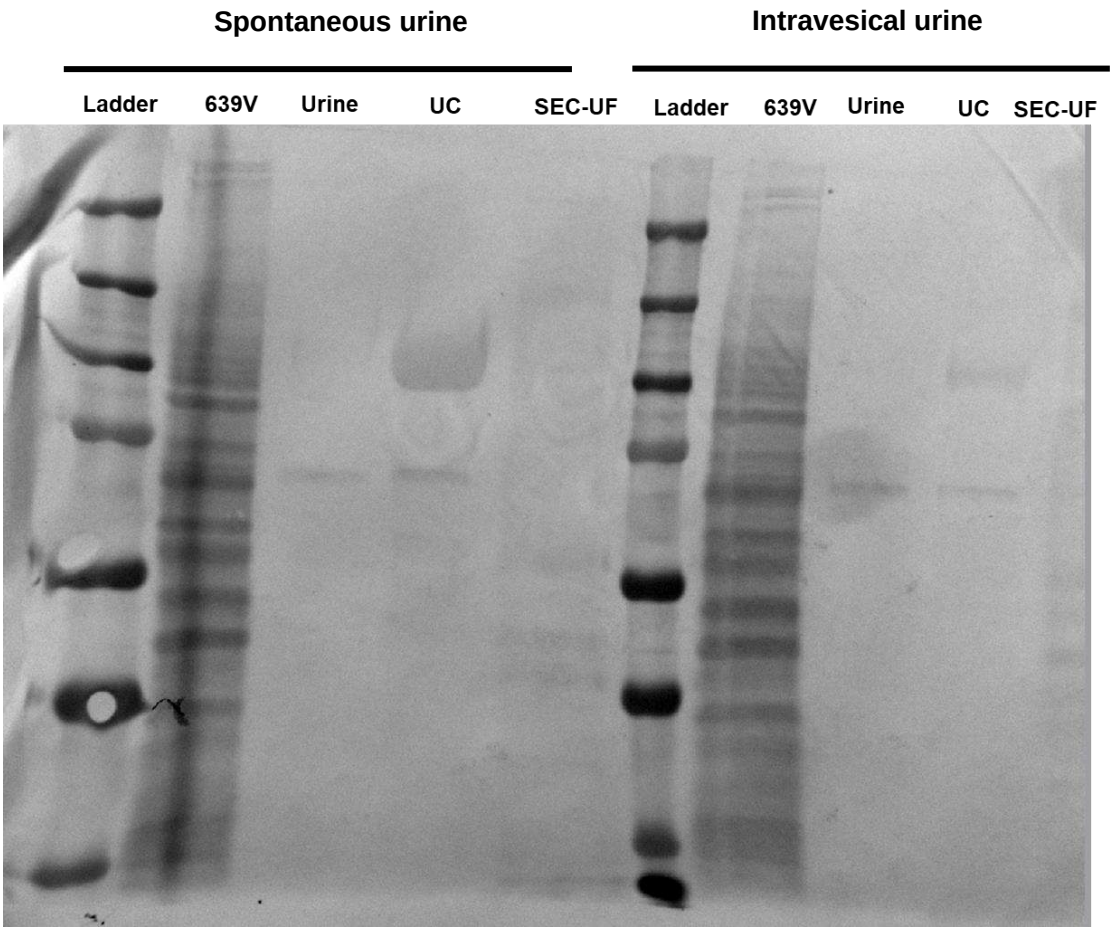
Supplementary Figure 7– UCa#5 Ponceau protein staining for confirmation of loading. Ponceau staining of 300 Cell Lysate (positive control), urine, UC and SEC UF for analysis of actini-4, THP, albumin and CD63.

Supplementary Figure 8



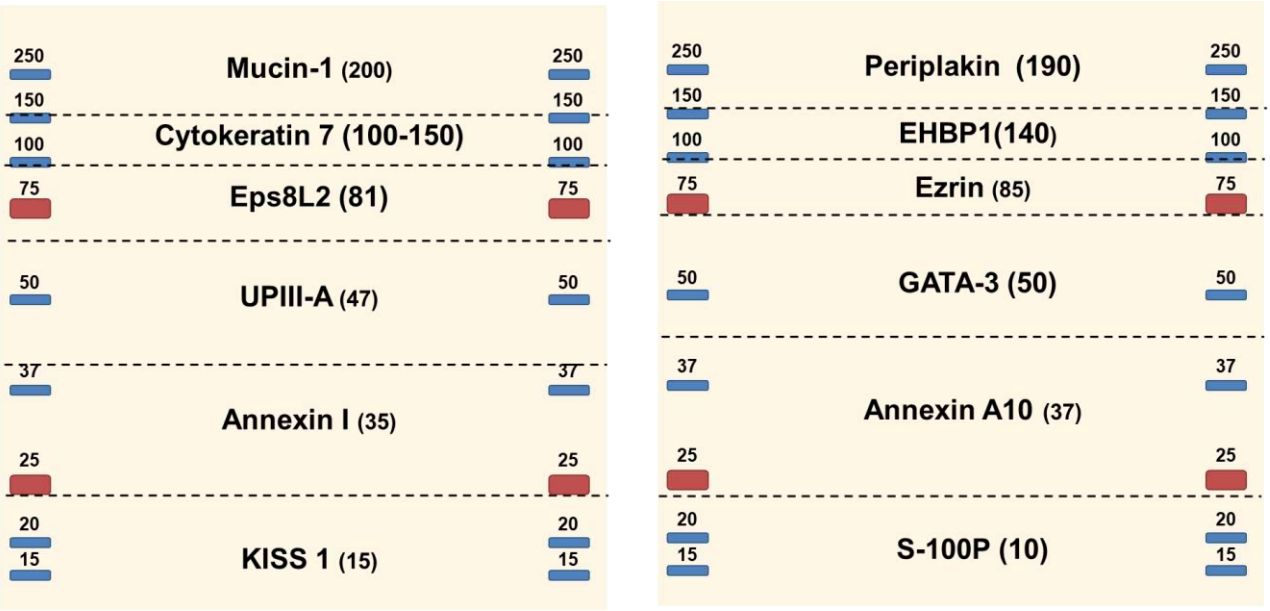
Supplementary Figure 8– UCa#7D Ponceau protein staining for confirmation of loading. Ponceau staining of 639V (positive control), urine, UC and SEC UF for spontaneous and intravesical urine, in analysis of EPS8L2 and Periplakin-1.

Supplementary Figure 9



Supplementary Figure 9– UCa#9D Ponceau protein staining for confirmation of loading. Ponceau staining of 639V (positive control), urine, UC and SEC UF for spontaneous and intravesical urine, in analysis of EPS8L2 and Periplakin

Supplementary Figure 10



Supplementary Figure 10- Membrane scheme of diagnostic antibodies and EVs markers according to the molecular weight of the band of interest.