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Diagnostic potential of extracellular vesicles in prostate cancer

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

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1. INTRODUCTION

1.1 Prostate gland

1.1.1 Prostate anatomical structure and function

The prostate is a small, chestnut-sized, inverted cone-shaped gland of the male reproductive system located in the pelvis above the urogenital diaphragm. From above, it adheres to the urinary bladder, from the back to the rectum and from below to the seminal vesicles. Across the prostate runs the prostatic part of the urethra, into which surrounding muscles inject the prostate's secretion during ejaculation [1, 2]. The prostate might be divided into four zones: the peripheral zone, the central zone, the transition zone and the anterior fibromuscular stroma zone. The peripheral zone is the most common site of malignancy occurrence, and the transition zone is the most common place where benign prostate hyperplasia originates [3]. The prostate is surrounded by a fibrous capsule that supports and protects the gland from injury. The prostate's primary role is producing secretion, which makes up 30% of semen. The prostate secretion contains high glucose levels, thus providing the necessary energy material for the sperm. It also consists of many proteins, enzymes, polyamines and kallikreins (e.g. Prostate Specific Antigen, PSA) [4].

1.2 Prostate cancer

1.2.1 Epidemiology and aetiology

Prostate Cancer (PCa) is a global problem, as it is the second most common type of cancer among males, according to data from the newest GLOBOCAN study [5]. Results of this study have shown that just in 2020, over 1 414 256 new cases of PCa were noted, with 375 304 deaths related to PCa, making PCa the fourth most deadly type of cancer (Figure 1). This data shows a rise in the new cases detected compared to the results from 2018, where 1 276 106 new PCa cases were noted, as well as a rise in the total number of deaths related to the PCa – 358 989 [6]. However, a slight improvement is noted if we compare the relative percentage of PCa-caused deaths to the new discovered cases - 26,53% in 2020 vs 28,13% in 2018. However, these results confirm that PCa is a global problem that touches many people and still requires efforts to reduce related deaths.

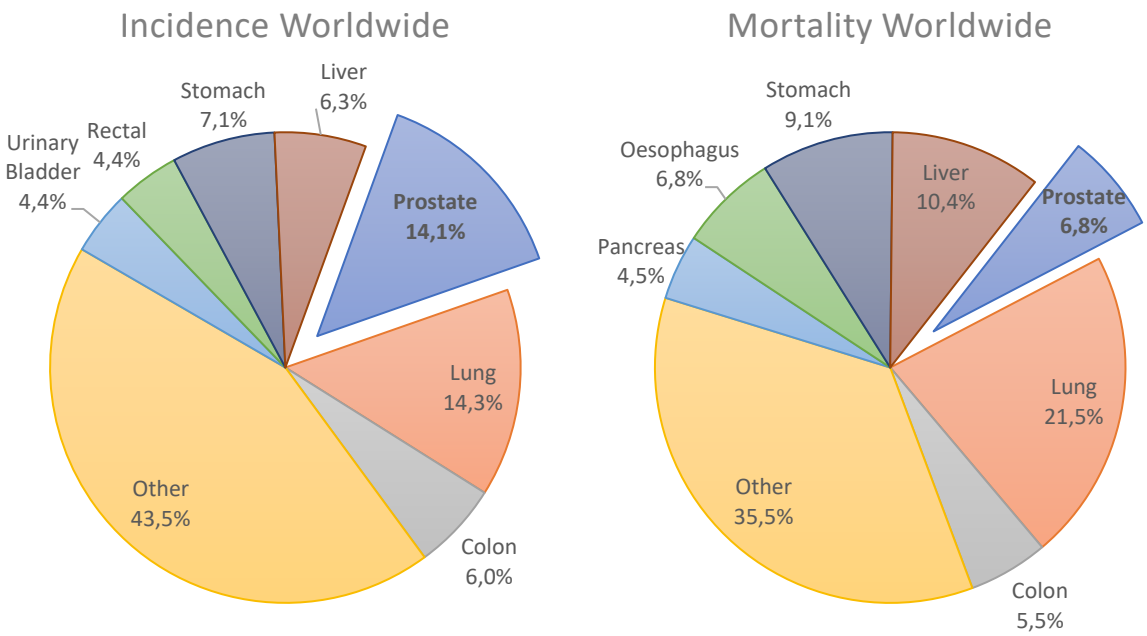


Figure 1 – Incidence and mortality rates of different types of cancer among males worldwide in 2020, based on GLOBOCAN study data [7].

The situation in Poland concerning the relative frequency of PCa among other types of cancer reflects global trends, as PCa is also the second most common type of cancer among males, according to the latest 2020 epidemiology data [8]. In 2020 new 14 244 PCa cases were noted, with 5 748 deaths related to PCa (Figure 2). This data, when compared to 2018 results shows a higher number (16 414) of new PCa cases and slightly fewer (5574) PCa-related deaths. However, comparing PCa-related deaths to new cases ratio (40,35% in 2020 and 33,95% in 2018), the situation shows different trends than the worldwide ones. The relative deaths/new cases ratio in Poland has risen in opposition to the mean world results, but the values are also almost twice as high, showing that the situation in Poland is much more severe and requires specific attention. However, what needs to be noted, is that in 2020, there was a SARS-CoV-2 pandemic which might have skewed the relative view of the situation, as access to healthcare was severely affected, and thus more cases might have been omitted and not diagnosed.

The most common type of cancer of the prostate gland is adenocarcinoma. Other less common types of prostate cancers include: transitional cell carcinoma of the prostatic urethra, squamous cell carcinoma, small cell carcinoma, sarcomas and lymphomas [9-11].

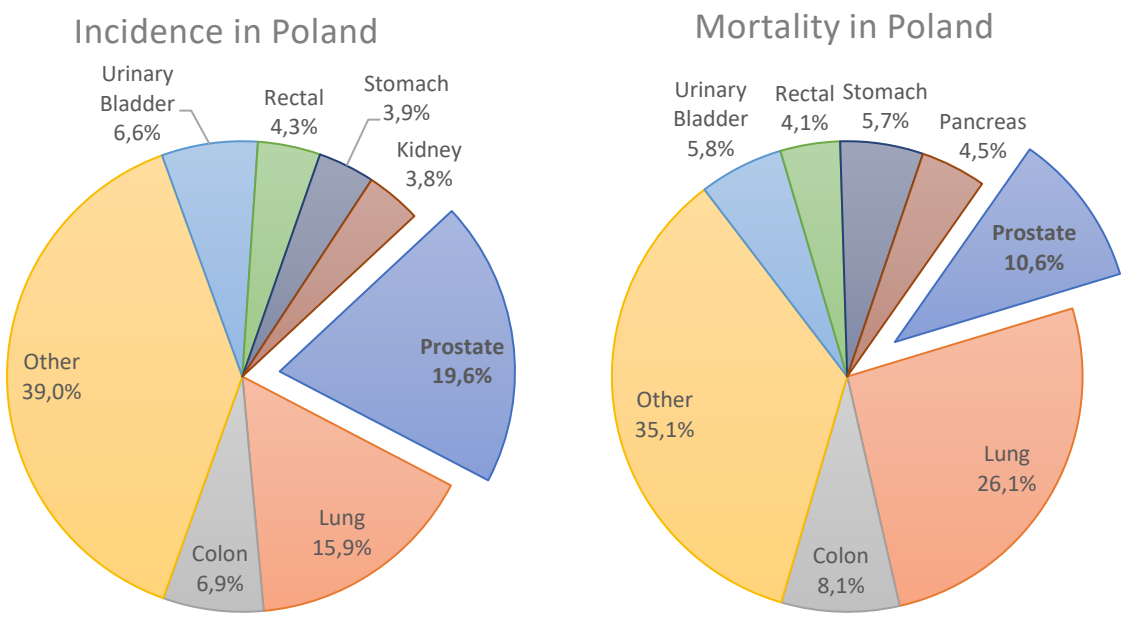


Figure 2 – Incidence and mortality rates of different types of cancer among males in Poland in 2020, based on Polish National Cancer Registry data [8].

One of the most significant risk factors of death related to PCa is African American race, as it was found that African American men have a much higher risk of dying from low-grade PCa than non-African American men [12]. Among many possible factors influencing PCa incidence only in a minor group of patients (10-15%), an actual hereditary background of the disease is found [13]. Another potential susceptibility to PCa might come from germline mutations, as they are found in up to 17% of cases independent of stage [14]. Among environmental factors, various dietary factors have been proven to influence the probability of developing a PCa [15]. Among them, high intake and total abstinence from alcohol have been found to correlate with PCa risk [16]. Interesting results have been found for vitamin D influence as both low and high levels of this vitamin are associated with the increased risk of PCa, especially a high-grade [17]. Moreover, it was shown that a higher intake of phytoestrogens, lycopene and prolonged proper consumption of selenium and vitamin E might lower PCa risk [18-20]. However, in light of the recent meta-analyses, many previously thought factors, like high intake of unsaturated fats and red or processed meat, do not contribute to risk of PCa development [21, 22].

1.2.2 Prostate cancer and androgens dependency

The co-dependency of androgens and prostate function is undisputable. Androgens - testosterone and its derivative 5α-dihydrotestosterone play a vital role in the development and physiology of a prostate gland, especially in the pubertal and postpubertal period when a relative prostate volume rises up to 10 times [23]. Dihydrotestosterone has been shown to play a role in benign prostatic hypertrophy, as it also affects the continued growth of the prostate in a postpubertal period [24]. Thus the influence of testosterone and other androgen levels on PCa cells should also be considered. A set of *in vitro* studies have shown that testosterone and dihydrotestosterone positively affect the proliferation of PCa cells. However, a correlation between rising androgens concentration and increased proliferation was found to flatten out at a certain level of hormones [25]. Androgens deprivation therapy (ADT) on non-malignant patients has significantly reduced prostate volume and PSA levels during the admission of the drugs, confirming androgens' influence on prostate functioning [26]. A similar study on PCa patients showed that ADT caused significant, up to 90% lowering of the PSA levels, confirming the influence of androgens on prostate cells, also in malignant glands [27].

1.2.3 Prostate cancer screening and diagnosis

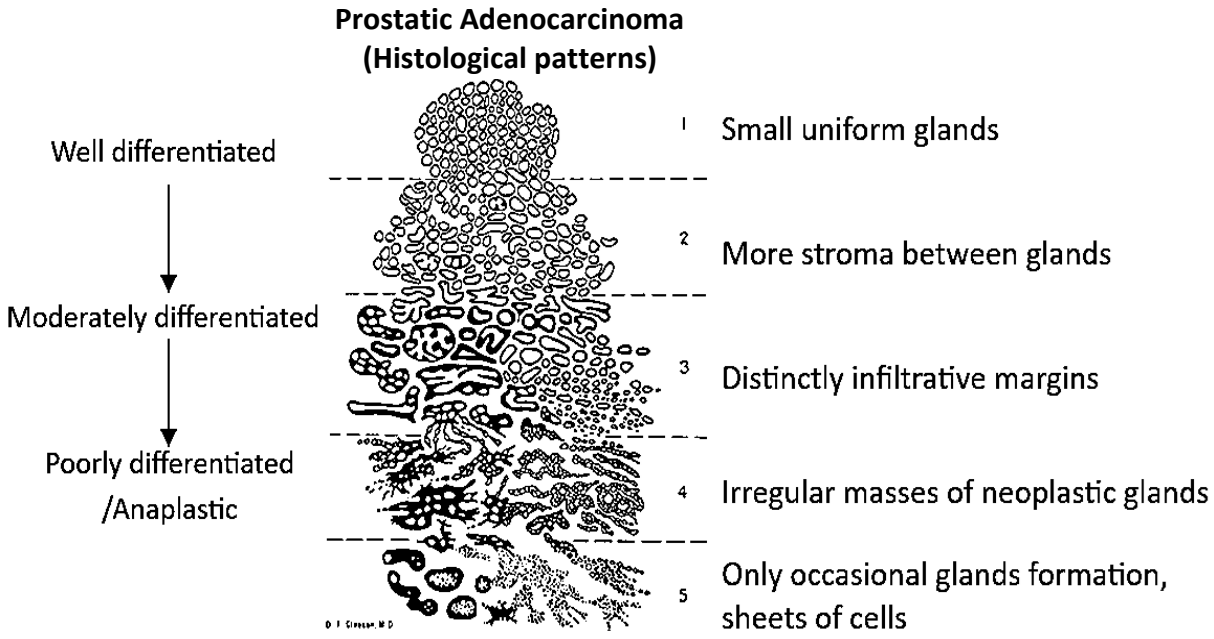
PSA – ups and downs of a controversial gold standard for PCa screening

PSA is a protein released only by prostate cells, and its level analysis in the blood is up to date, the easiest and widest-used PCa screening tool in clinical practice [28]. The initial results showed a significant decrease in mortality rates since introduction, as more early-stage tumours were diagnosed [29]. However, further analysis of the performance and outcomes of treatment by the US Preventive Services Task Force in 2012 indicated a significant increase in overdiagnosis and overtreatment, with no significant positive influence on cancer-specific mortality, recommending a reduction of the use of PSA in early diagnosis [30]. However, more recent studies and meta-analyses provide contradictory evidence of a long-term positive effect of population PSA screening on cancer-specific mortality [31]. The critical fact is that not only PCa might be responsible for the change in PSA level as it was proven that prostatitis, benign prostate hyperplasia (BPH) or any prostate trauma might increase the PSA level. On the other hand, an extensive patient interview should never be underestimated,

as numerous drugs (e.g. aspirin) have been reported to decrease PSA levels [32, 33]. Thus, as contradictory results and recommendations arise, PSA still might be considered a controversial-gold standard for early PCa diagnosis, emphasising the necessity of finding better, non-invasive tools [34, 35].

Gleason Score and International Society of Urological Pathology grade

Additional to clinical Tumour, Node, Metastasis (TNM) staging, histologic pattern of carcinoma cells is significant for the final diagnosis of PCa and the further choice of therapy. For this reason, a scale for histopathological grading of the changes was proposed in 1966 by dr Donald Gleason and further modified several times in later years into Gleason Score [36, 37]. The Gleason Score (GS) represents the sum of Gleason Grades of the most extensive pattern and a secondary pattern. The Gleason Grade is a scale of a biological advancement of the PCa, based on the dedifferentiation state of the gland tissue, as presented in Figure 3. Based on the GS, a five-grade system was proposed, representing the potential risk of advancement of the malignancy, with significant differentiation of the two intermediate risk possible outcomes for GS 7 – favourable (3+4) and unfavourable (4+3) [38]. Based on the Gleason Score International Society of Urological Pathology (ISUP) proposed a scale to limit the insignificantly different risk groups and stratify the patients for further decision-making [37] (Figure 3).



Gleason Score	2-6	7 (3+4)	7(4+3)	8 (4+4, 3+5, 5+3)	9-10
ISUP grade	**1**	**2**	**3**	**4**	**5**

Figure 3 – Original Gleason Grade scale and International Society of Urological Pathology 2014 grade (group) system for histopathological evaluation of biological advancement of PCa, based on [36, 37].

Clinical classification and risk staging

Among classification systems used for predicting clinical outcome and recurrence risk: Tumour, Node, Metastasis (TNM) and complex European Association of Urology (EAU) risk group classifications are most commonly used [39, 40]. Within the TNM classification Tumour - characterize the size and local advancement of a primary cancer site, Nodes – describe changes in local lymph nodes and Metastases – describe the presence and location of distant metastasis sites (Tab.1)

Table 1 – Tumour, Node, Metastasis clinical classification of PCa [39].

T - Primary Tumour		
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
T1	Clinically inapparent tumour that is not palpable	
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen [PSA])
T2	Tumour that is palpable and confined within the prostate	
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule	
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall	
N - Regional (pelvic) Lymph Nodes		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
M - Distant Metastasis		
M0	No distant metastasis	
M1	Distant metastasis	
	M1a Non-regional lymph node(s)	
	M1b Bone(s)	
	M1c Other site(s)	

However the TNM classification provides some basic information about clinical advancement of the malignancy of a PCa patient, a more complex classification, partially basing on TNM was proposed by EAU, for risk stratification and recommendations for further patient management. This groups classification takes together results of the aforementioned PSA analysis, TNM classification and biopsy results, into three main risk groups for local and locally advanced PCa (Tab.2).

Table 2 - EAU risk groups for biochemical recurrence of localised and locally advanced prostate cancer, based on [15].

Definition			
Low-risk	**Intermediate-risk**	**High-risk**	
PSA < 10 ng/mL and GS < 7 (ISUP grade 1) and cT1-2a	PSA 10-20 ng/mL or GS 7 (ISUP grade 2/3) or cT2b	PSA > 20 ng/mL or GS > 7 (ISUP grade 4/5) or cT2c	any PSA any GS (any ISUP grade) cT3-4 or cN+
Localised			**Locally advanced**

PHI, 4Kscore – improved, PSA-based blood tests

As the crude PSA level alone proved ambiguous performance, many efforts have been made to find potential co-indicating factors that might raise the diagnostical accuracy. Prostate Health Index (PHI) is a mathematical formula which utilizes not only total PSA level but also other forms of PSA: freePSA and proPSA, a serum isoform more closely related to PCa. This approach has provided much higher specificity and lowered the number of unnecessary biopsies [41, 42]. Additionally, a relatively minor modification to the equation of adding an ultrasound-measured prostate volume has been proposed, raising the diagnosis accuracy [43].

Another PSA-based test proposed for PCa diagnosis is 4Kscore. This four-kallikrein panel, composed of totalPSA, freePSA, intactPSA and human kallikrein-related peptide, together with patients' age, DRE (Digital Rectal Examination) results and prior biopsy history, allows calculation of high-grade PCa risk [44]. The muti-centred study confirmed that 4Kscore significantly lowered the number of unnecessary biopsies and predicted up to 20 years ahead the metastases probability [44-46]. However, as these two approaches differ, a direct comparison of their performance does not indicate significantly better results for any of them against another [47].

Urinary biomarkers for PCa diagnosis and risk assessment

Not only have blood-based tests been found to be useful for PCa diagnosis, but many studies have been done to find potential biomarkers from urine, as its content might be influenced by prostate cells. Prostate cancer gene 3, a long non-coding RNA, was found to be overexpressed and present in urine samples of patients after DRE. This finding led to the first FDA-approved lncRNA-based commercially available test – Progensa [48, 49]. Moreover, this test was proven non-inferior to previously described PHI in many studies, providing an additional alternative for proper diagnosis and risk management [50, 51]. A similar test for two mRNAs (HOXC6 and DLX1), also called SelectMDX, is proposed. The potential of SelectMDX for significantly lowering the number of unnecessary biopsies has been proven in multi-centred studies [52, 53].

However, besides those evaluated, proven, and guidelines recommended tools for PCa diagnosis, emerge of new, yet to be thoroughly evaluated, technologies is noted [15]. For example, TMPRESS2-ERG, a trans-membrane protease serine 2 (TMPRSS2) and the ETS-related gene (ERG) genes fusion is noted in many PCa cases. The product of this fusion gene can be found in urine samples of PCa patients[54]. The performance of TMPRESS2-ERG alone was unsatisfactory. However, when combined with PCA3 and serum PSA, the prediction of PCa drastically improves [55]. Another investigated approach is ExoDx. This urine-based test analyses the RNA profile of urinary exosomes from PCa patients. To date, two extensive studies proved the high Negative Prediction Values, with 92% sensitivity. However, more multi-centred and comparative studies must be performed to properly asses ExoDx's diagnostical value before it might be widely recommended [56, 57].

Imaging techniques for PCa diagnosis

Another branch for PCa diagnosis is imaging, especially with well-proven MRI and currently one of the most interesting PSMA-PET scans [58, 59]. The MRI shows good sensitivity for the detection and localisation of tumour sites and also as a supplementary technique for guiding biopsy for more accurate results [58, 60]. Prostate Specific Membrane Antigen (PSMA) is a protein found to be overexpressed on the surface of PCa cells. Based on that fact, the approach utilizing PET-sensitive PSMA-targeted radioactive ligands provides more targeted analysis than traditional PET scans had been proposed [61]. This approach has been

proven safe and possesses superior specificity reaching up to 100% [59]. Moreover, allowing for precise localisation of not only the primary site of the tumour but also metastatic sites [62].

1.2.4 Prostate cancer treatment possibilities

Active Surveillance and Watchful Waiting

In localized PCa, one of the most critical factors in deciding therapy management, besides the clinical advancement of the tumour, is life expectancy (LE), with the border set around ten years of the expected life of a patient. The observational study results have shown that patients with well-, moderately- and poorly differentiated tumours had 10-year cancer-specific survival rates of 91%, 90% and 74%, respectively [63]. However, studies with 20 years of follow-up show that those values are lowering significantly to even 32% [64]. Thus whether patients' LE exceeds ten years, current European Urology Association guidelines suggest one of the approaches: Active surveillance for patients with over ten years of LE and Watchful waiting for patients below ten years of LE. Active surveillance (AS) is a curative approach to minimize the possibility of overtreatment of low-risk changes, with thorough, low-invasive, regular control of the patient's state to start treatment if the disease becomes life-threatening. This approach is based on regular PSA-level measurements, MRI and rebiopsies if needed. However, the Active Surveillance should be applied only for patients with less aggressive stages of PCa, thus only patients with GS6 & GS7 (3+4) should be considered for Active Surveillance. Patients with GS7(4+3) and higher, other therapeutic approaches are recommended. Moreover, the high invasiveness of repetitive biopsies and unsatisfying performance of PSA measurements provides necessity of finding new, better tools, for AS patients stratification. Watchful waiting is a palliative approach to minimize unnecessary treatment, provide better life comfort, and react when syndromes get burdensome for the patient [15].

Surgical approach - radical prostatectomy

When a localized PCa is diagnosed, and an LE of a patient is beyond ten years, surgical removal of the prostate gland – radical prostatectomy, might be performed. This approach has evolved from open surgery approaches, through the laparoscopic method, to the newest robot-assisted technique [65]. Even though techniques have changed, the main aim has stayed

the same, to remove the entire prostate with the least possible damage to the surrounding tissues and to preserve the best possible life standard for the patients afterwards. However, despite the rise in the precision of the surgery technique, radical prostatectomy is still connected with many possible post-operative complications, among which the most common are urinary incontinence and erectile dysfunction [66, 67].

Radiotherapy

One of the most common non-surgical approaches for PCa treatment is radiotherapy. This therapy can be either external beam radiation therapy, proton beam therapy or brachytherapy. External beam radiation therapy utilizes high doses of x-ray radiation for damaging cancer cells and might be used for either localized PCa or locally advanced PCa. Recent studies present that an approach with rising doses of radiation provides better results with longer cancer-free time [68]. Proton beam therapy is very similar to external beam radiation therapy, where a proton beam is used rather than a photon beam. Theoretically, such an approach allows for more specific and targeted action, sparing surrounding tissues. However, studies comparing this technique to the classical approach do not show the significant superiority of proton-based therapy and thus should be considered as a potential alternative with caution [69]. Brachytherapy is a more localized radiotherapy approach, where a small portion of radioactive material is implanted into the prostate. In the case of the low dose, the implantation is permanent, while in high-dose, introducing a highly radioactive source is temporary in single or multiple fractions [70, 71].

Hormone therapy

As the PCa development is dependent on testosterone, a testosterone-lowering therapy (castration) might be applied. The primary aim of androgen deprivation therapy (ADT) is to lower the levels of androgens from PCa cell growth support. Castration level of testosterone in many cases <50ng/dl (1,7nmol/l) concentration is considered. However, recent studies show that thanks to, among others, the rise of sensitivity of current testing, a <20ng/dl (1 nmol/l) level should be applied, as better results are observed with that change [72]. Over the years, many different approaches to testosterone-lowering pharmacotherapies have been introduced: oestrogen, luteinising hormone-releasing hormone agonists and antagonists, steroidal and non-steroidal anti-androgens [15]. Oestrogens are a group of the

main female sex hormones, and their use for testosterone deprivation has been proven effective. However, despite the possible easy oral application and lack of bone density influence, the severe side effects, like thromboembolic complications, discourage using them as a first-line ADT treatment [73]. Another group of drugs for ADT are luteinizing-hormone-releasing hormone agonists, currently the main form of this therapy. They are applied as a regular injection on a time-depending basis. The first dose usually leads to a sudden increased release of testosterone, also called a "flare-up" effect, which might be connected with many deleterious, even life-threatening side effects, like hypercoagulation or obstructive renal failure [74]. Because of that, a supplemental therapy with anti-androgens must be thoughtfully conducted to manage the risk of side effects, especially with the unknown long-term impact of anti-flare-up therapy [75]. On the other hand, luteinizing-hormone-releasing hormone antagonists do not lead to a flare-up effect because of their different mechanisms of action. Despite the potential advantages over agonists, this group of drugs is also connected with less severe side effects like decreased libido, weight gain, and erectile dysfunction, and their long-term efficiency is yet to be proven [76]. Anti-androgens are oral compounds with a mechanism of action based on competition with androgens at the receptor level. The can be divided into steroidal and non-steroidal compounds. One of the most significant differences between these two sub-groups is that steroidal anti-androgens might pass the blood-brain barrier, causing their progestational properties [77]. When despite properly adapted ADT tumor is still rising it is defined as Castrate-Resistant Prostate Cancer (CRPC). Moreover, hormonal therapy might be combined with the radiotherapy mentioned above, providing better results, whether used as an adjuvant or neoadjuvant option [78].

Chemotherapy

The last possibility of currently used non-surgical therapy approaches is chemotherapy. Usually, this approach is used as a palliative treatment when hormone therapy is not working, indicating castration-resistant PCa, which usually is connected with distant metastasis formation [79]. Among the most commonly used chemotherapeutics, one might mention docetaxel, cabazitaxel, mitoxantrone and estramustine. However, many other compounds are experimentally evaluated for their potential in PCa therapy, among which special attention is brought to repurposing antibiotics, as according to the "two-hit" hypothesis, they might possess a dual mechanism of action. Not only might they directly affect the PCa cell, but they

also eradicate chronic inflammation causes, reducing the potential of new malignant cell formation [80].

1.3 Extracellular Vesicles

1.3.1 Definition and classification of extracellular vesicles

Extracellular Vesicles (EVs) are a heterogeneous group of membranous vesicles released by all types of cells. They differ in size, biogenesis, release mechanisms, cargo (mRNAs, miRNAs, lncRNAs, proteins), and subcellular markers [81]. Their presence have been confirmed in many biofluids, such as blood, urine, saliva, cerebrospinal fluid, and others [82-85]. The role of EVs in different pathomechanisms, especially by the cargo they are carrying, caused a rise in interest in their potential application as a multicomponent biomarker platform in clinical diagnosis. One of the main factors determining the classification of EVs is their biogenesis mechanism. Based on these criteria, three main EV categories can be distinguished: apoptotic bodies, microvesicles, and exosomes.

Apoptotic Bodies (ApoBDs) are generated within programmed cell death and are characterized by the largest size among EVs, ranging from ~50-5000nm [86]. The mechanism of ApoBDs generation is connected with one of the final steps of the apoptosis process. The generation process might be divided into three main steps: plasma membrane blebbing, apoptotic membrane protrusion formation and ApoBDs fragmentation [87]. The membrane blebbing process, as the first stage, is regulated by many kinases which cooperate, including, but not limited to PAK2, LIMK1 and ROCK1, which are activated by caspase-3 [88-90]. The next step is apoptotic membrane protrusion forming, during which apoptopodia or beaded apoptopodia are formed. Such phenomenon might be observed for T cells and thymocytes, epithelial cells, monocytes, and fibroblasts [91]. The last stage of the generation of ApoBDs is their disassembly by fragmentation. This stage is the least studied and explored, as it has been shown that both intrinsic and extrinsic signals might be responsible for the release of ApoBDs from apoptopodia [92].

Microvesicles (MVs) are class smaller EVs than ApoBDs ranging within ~100-1000nm. Their primary generation mechanism is based on outward budding and fission of the plasma membrane. Many cell-dependent factors, including membrane composition, are incorporated in this mechanism, as repositioning of phosphatidylserine's outer and inner parts and

redistribution of phospholipids are observed. The pathways responsible for MVs generation involve Endosome Sorting Complex Required for Transport (ESCRT), including Alix, Tsg101, Vps22, Chmp1/3 and Vsp4 [93]. Another mechanism involved in MVs generation in a cell is the hypoxia-inducible factor (HIF) signalling pathway. During hypoxia, HIF-α degradation is suppressed, thus leading to the upregulation of RAB22A, which is required for the budding of the plasma membrane [94, 95]. Another mechanism of plasma membrane shedding is the ARF6–dependent pathway. This small GTP-binding protein activates PLD and thus ERK and MLCK cascade. The most exciting part is that this mechanism was first found in tumour cells where ARF6 was upregulated, providing potential cancer therapies target [96, 97].

Based on their biogenesis, the third and the smallest class of EVs are exosomes (Exo) with the smallest range of size ~30-150nm. Their biogenesis is mostly explored among EVs subtypes and is based on Intraluminal Vesicles (ILVs) formation within Multivesicular Bodies (MVB). The formation of ILVs is associated with endosome membrane reorganization and tetraspanins enrichment. During this process, cargo is packed within newly formed vesicles with ESCRT-dependent and ESCRT-independent pathways. The ESCRT-dependent pathway is based on detecting specifically ubiquitinated proteins and selection via interaction with syndecan and ALIX [98]. The ESCRT-independent pathway is associated with raft-based microdomains enriched in sphingomyelinases and ceramides formation. In this mechanism, tetraspanins, enriched during the formation of ILVs and other specialized mechanisms, are also involved [97, 99]. The final step of Exo generation is a fusion of MVBs with the plasma membrane. Rab GTPases are crucial in this process, as they are responsible for intracellular vesicle trafficking [100]. Exact Rab GTPase activity and influence are cell-dependent. However, commonly, they interact with SNARE proteins inducing Exo release.

However, consensus about specific markers of different subtypes of EVs has not yet emerged, as they might be highly cell dependent. For example, Red Blood Cells derived exosomes do not present characteristically for this class of EVs tetraspanins on their surface [101]. A similar problem is met with purification methods, which at this moment are thought to never fully separate just one pure subtype of EVs [102]. As the use of nomenclature grouping EVs by their size is recommended, in this paper as small EVs (sEVs) EVs of size ≤150nm are considered, and as medium-sized EVs (mEVs) with size of ≥120nm and ≤1000nm.

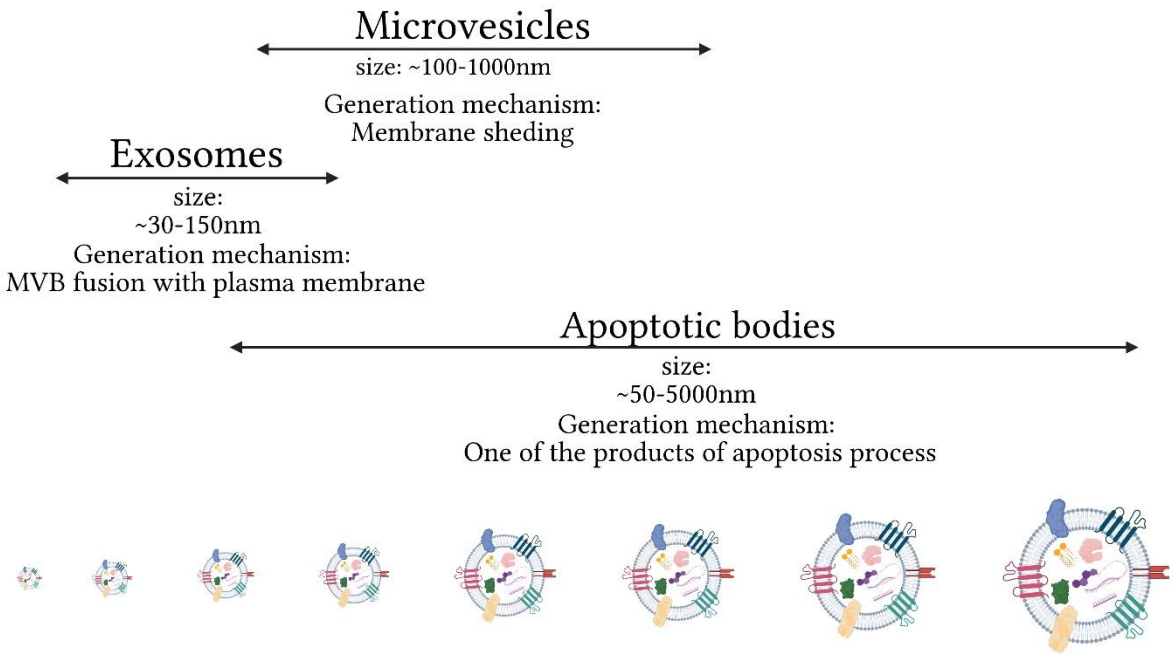


Figure 4 - Classical extracellular vesicles types, based on their size and biogenesis mechanisms. [103].

Besides classically distinguished EVs classes, recent discoveries of new nanoparticle classes: supermeres and exomeres, with an average size <50nm, confirm that there might be even more classes of EVs which are currently below the resolution of possible analytical methods, yet still to be discovered. [104, 105]

1.3.2 Methods used for extracellular vesicles characterization

Digital Light Scattering

Digital Light Scattering (DLS), called photon correlation spectroscopy, is the oldest optical-based method used for the size and concentration analysis of EVs. In this technique, a sample of suspended particles is illuminated by laser light, and the scattering of the light by Brownian motion moving particles is analyzed. The detector might be placed at one or multiple angles, affecting the reliability and sensitivity of the measurement [106, 107]. The method is relatively simple and robust. However, the most significant disadvantage of DLS is the lack of adequate distinguishing discrete subpopulations based on averaging the obtained results [108].

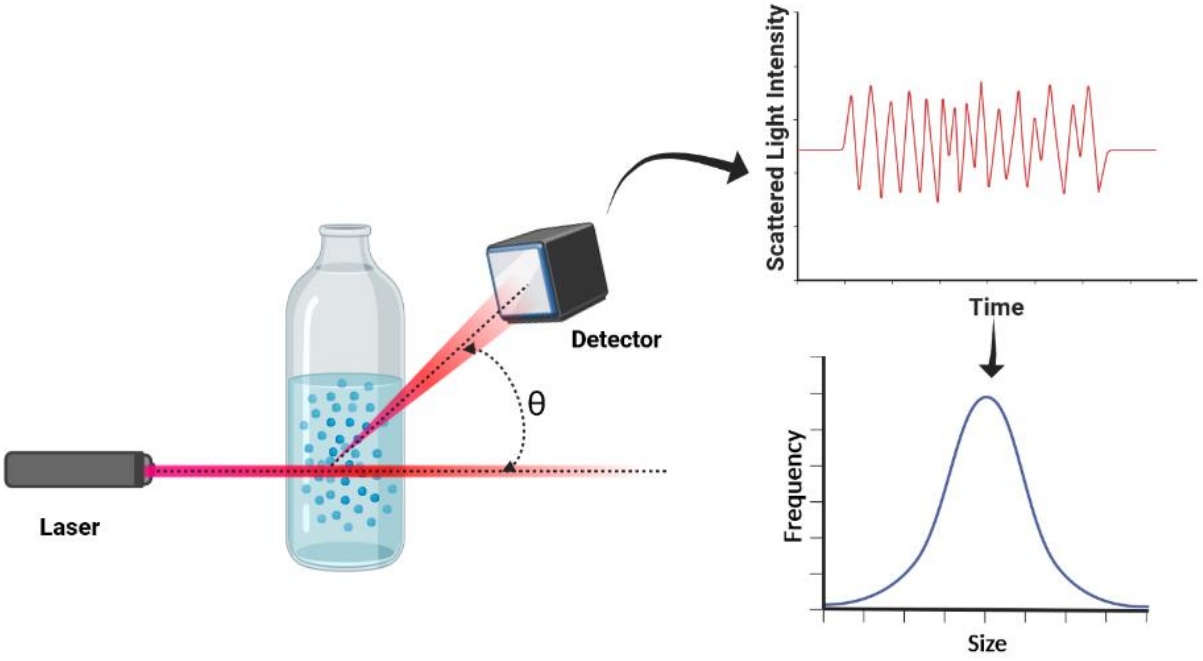


Figure 5 – Digital Light Scattering method scheme.

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) is currently one of the most commonly used methods for the size and concentration characterization of EVs. Its main principle is also based on the scattering of the laser light by the particles suspended in the liquid. However, compared to DLS, the detection method is not a detector but a camera coupled with the microscope objective. The significant advantage of such an approach is moving from averaging results to per-particle analysis. Moreover, besides concentration, a single fluorescence parameter might be analyzed with this technique, providing both physique and biological properties [109]. However, similarly to DLS, this technique relies on the Brownian motion of the stationary sample. Thus, multiple detections of the same particle are unavoidable, providing an uncertainty of the obtained results [110]. Moreover, the detection limit and advanced data analysis of NTA results provide additional difficulties for more widespread use.

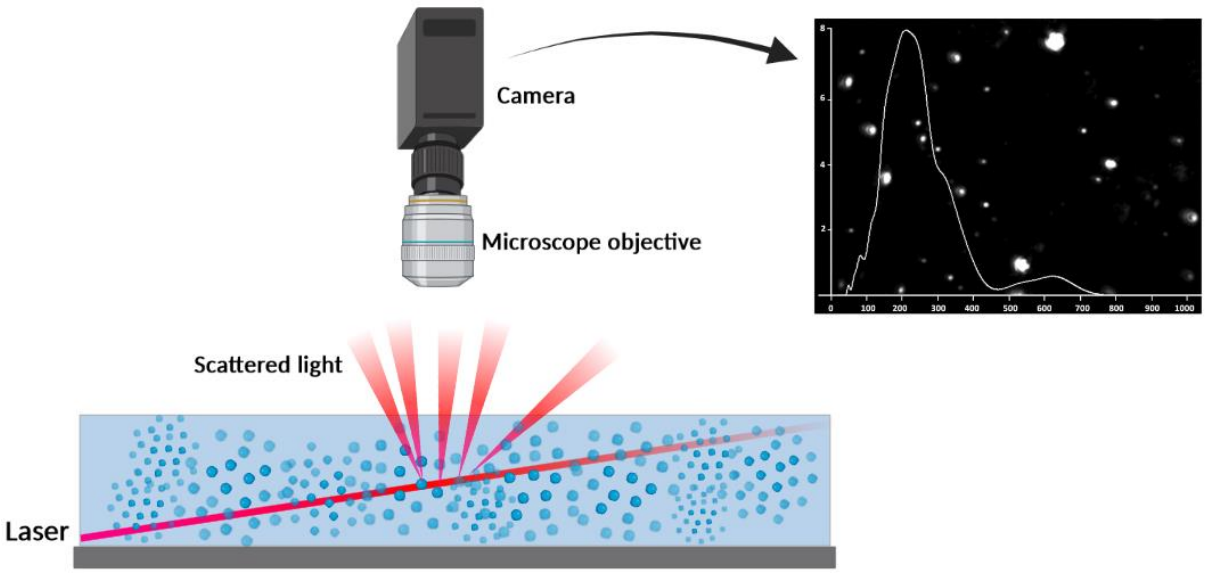


Figure 6 – Principle of the Nanoparticle Tracking Analysis.

Nano Flow Cytometry (nFCM)

Nano Flow Cytometry (nFCM) is a modification of conventional Flow Cytometry (FC) with focusing on sub-micron particles. Depending on the actual hardware unit, the lowest limit of detection might be even 40nm. However, in most cases, the lower limit is closer to 60-70nm with unmodified machines [111]. One of the main changes between conventional FC and nFCM is using the side scatter parameter as a size-defining one instead of forward scatter. Another potential change is gathering side scatter from other light sources than traditional 488nm laser, like 405nm laser, which raises both sensitivity and resolution of sub-micron particles. One of the benefits of nFCM over NTA is the flow of the sample, which solves the problem of possible multiple times measurements of the same particle, thus providing more accurate concentration-wise data [112]. An additional benefit over NTA is the possibility of multicolour analysis, allowing more complex biological properties analysis with maintaining a high-throughput typical for FC. Moreover, the rise of interest in this technique brought up techniques allowing intravesicular content analysis of the intact vesicles, providing more reliable evidence of vesicles' involvement in specific cargo carrying [113]. Additionally, the efforts to standardize results obtained with different machines by calibrating the results into standardized units provide more potential for further reliability improvement [114]. Another advantage of nFCM is the potential for further improvement of the studies possibilities by employing Fluorescence Based Sorting, which would allow for downstream

analysis on only selected EV subpopulations at costs lower than immunomagnetic separation methods [115]. To overcome an operator dependency on the gathered data, special measures, like the MIFlowCyt-EV framework, are taken by international societies and their special task forces to ensure the appropriateness of the obtained results, thus raising their scientific significance [116]. One of the downsides of the current nFCM approach is based on the optical properties, a refraction index, which at the scale of the nanometers particles raises problems with the assumption of either a solid sphere or core-shell approach for the subsequent calculations [117]. Moreover, the lack of standardized, appropriate calibration particles, other than most commonly used polystyrene beads, that possess a unified spherical shape and different refractive index than membranous EVs is a challenge that still needs to be faced [118].

Tuneable resistive pulse sensing (TRPS)

In opposition to previously described methods, Tuneable Resistive Pulse Sensing (TRPS) is not based on optics reading but on the electric current analysis. The primary mechanism behind this technique is the modified Coulter Counter principle. The device for TRPS is composed of two chambers filled with an electrolyte between which a membrane with a single pore is found. As the particles pass through the single pore, they disrupt the current flow between the chambers. These blockades are sensed, analyzed and used for the calculation of the particle size. The appliance of the known pressure on the upper chamber and proper calibration allows analysis of the concentration of the particles in the sample based on the frequency of the blockades. The control over pore size allows for changing the range of the measurements. One of the most significant advantages of the TRPS method is the single particle resolution, which provides both high resolution and accuracy of the measurement [119]. One of the most significant drawbacks of the TRPS is relatively low throughput possibilities and high dependency on the proper sample preparation step, as particles bigger than set pore size might result in permanent pore blockade, making it impossible to continue analysis and forcing repetition of whole machine setup and calibration process.

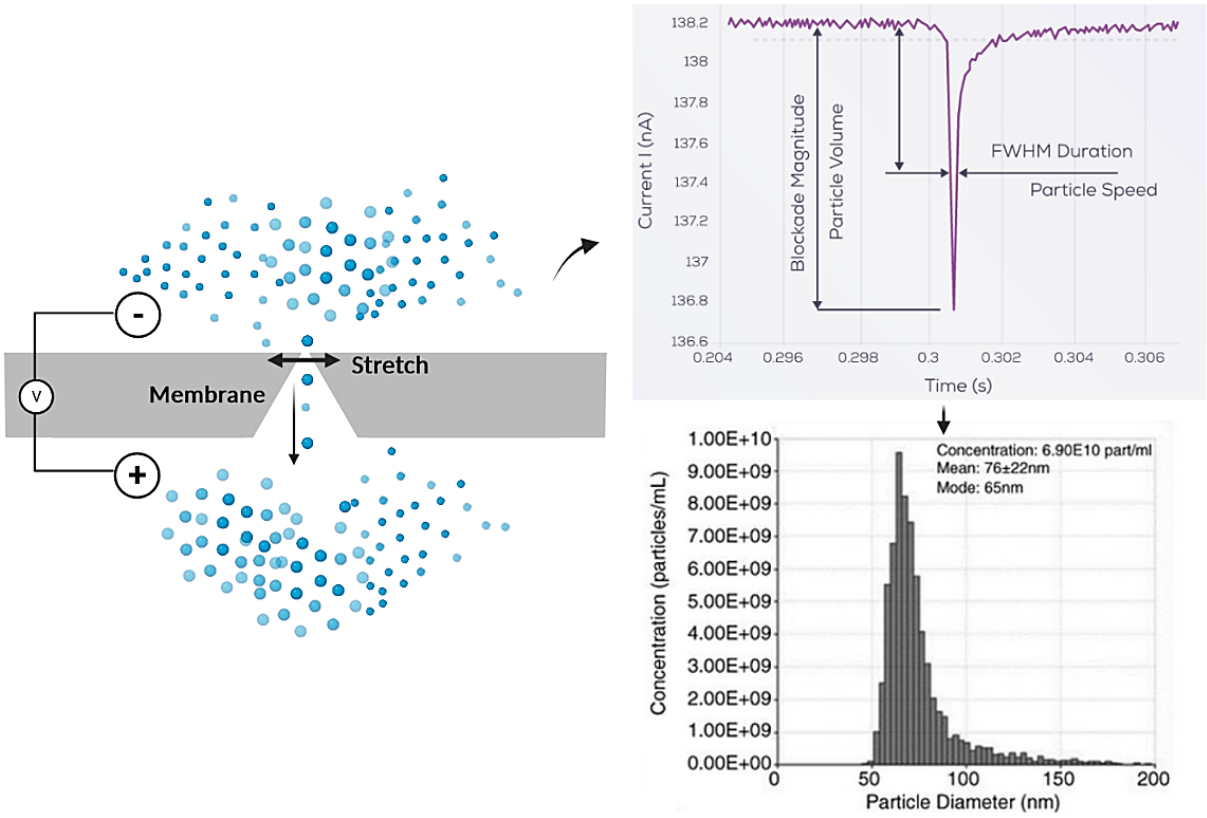


Figure 7 – Principle of Tuneable Resistive Pulse Sensing.

1.3.3 Roles of extracellular vesicles in physiological and pathophysiological processes

An essential part of EVs' importance is their relatively easy but distinct mechanisms of uptake by recipient cells and, thus, involvement in cell-to-cell communication. The primary mechanisms of EVs uptake are direct interaction, fusion with the plasma membrane and the most sophisticated internalization. Direct interaction is the most straightforward mechanism utilizing signalling molecules present on the EVs surface by a ligand-receptor pathway, inducing further downstream signalling in the recipient cell. This route mainly transmits immunomodulatory and apoptotic function signals [120, 121]. Another route of EVs-cell interaction is membrane fusion. In this mechanism, SNARE and Rab proteins, the same that are responsible for MVs and Exo generation, are involved [122, 123]. An additional factor that might influence the EVs uptake is related to the pH of the surrounding microenvironment by increased sphingomyelin and rigidity [124]. Finally, the most common way of EVs-cell interaction regarding Exo is internalization [125]. Several pathways might facilitate this mechanism: clathrin-mediated endocytosis, lipid raft-mediated endocytosis, caveolin-mediated endocytosis, phagocytosis and macropinocytosis [126-130]. The most

common pathway of internalization for most types of cells is clathrin-mediated endocytosis, as it was shown in the case of both healthy and malignant cells [131]. Moreover, what needs to be noted is that this process can be influenced by the composition of EV and the carried cargo [126]. Lipid raft-associated membrane invagination, connected with microdomains of cholesterol, on the other hand, is one of the primary mechanisms to influence the shift of EVs cargo uptake into early endosomes [127]. Caveolin-dependent endocytosis is a very similar process to the clathrin-mediated path, as they even share some of the proteins involved in the process, like dynamin-2, which makes it challenging to distinguish between one another [126]. However, internalization pathways might have different routes, they all mostly lead to the fusion of EVs with the early endosome. Phagocytosis and pinocytosis are two internalization pathways that may lead to the degradation of the received EVs by connecting them with lysosomes. The phagocytosis route is mainly characteristic for the immune cells, as they predominantly use this mechanism to engulf large particles like bacteria or dead cells [129]. Macropinocytosis is a mechanism utilizing actin lamellopodia to form macropinosomes intracellular compartments. This mechanism also might lead to degradation by fusion with lysosomes or transfer to early endosomes and further intracellular transfer [132, 133].

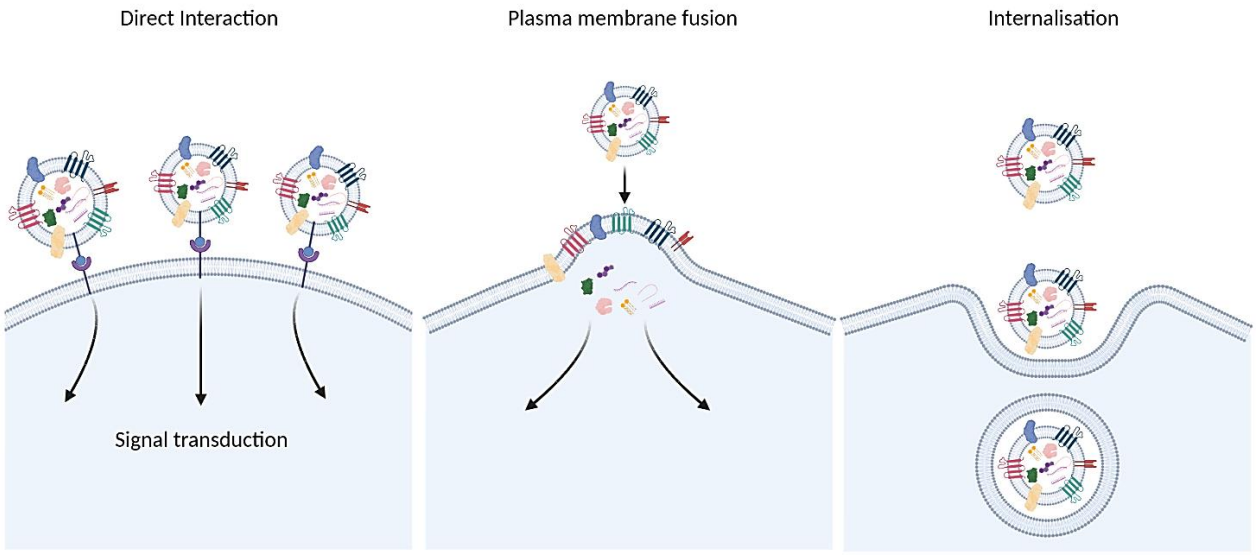


Figure 8 - EVs and recipient cell possible interaction for carried signal.

The role of EVs in intercellular communication in physiological processes has been widely studied. However, a particular focus on the immune response and functionality of the

Central Nervous System (CNS) is especially noted [134]. The involvement of EVs in immune response has been found on many levels, regarding most of the cell types taking part in this process. It was proven that EVs might be involved in the antigen presentation process, bearing major histocompatibility complexes (MHC) to T cells and MHC interaction between B cells and dendritic cells [135, 136]. However, not only have the transmembrane proteins carried by EVs been found to play roles in immune response regulation. Depending on their maturity stage, EVs-mediated transport of miRNAs between dendritic cells affects their differentiation and cytokine synthesis [137]. EVs are also involved in building and maintaining immune synapses between antigen-presenting cells and T cells; moreover, the whole process has been found to be unidirectional from T cells to antigen-presenting cells [138]. Another process where EVs-mediated cellular communication has been found is an immunosuppressing effect, where the signal affects T lymphocytes and natural killer cells and induces T regulatory cells and myeloid cells to inhibit immune response [139, 140]. The role of EVs in CNS is based on a similar mechanism as in immune response. For example, EVs play a vital role in microglial response to damage and further tissue repair by transferring similar proteins as EVs secreted by B cells and dendritic cells, like MHC complexes [141]. EVs also play a role in transporting crucial components for trophic support of axons, e.g. myelin proteolipid protein, myelin basic protein and myelin oligodendrocyte glycoprotein [142].

Many researchers have also evaluated the role of EVs in pathomechanisms, especially with a focus on carcinomas. One of the leading hypotheses is that EVs, mainly small ones like exosomes released by cancer cells, contain genetic material sufficient to cause metastasis niche when integrated with normal cells [143]. Another important aspect is the involvement of EVs in the promotion of angiogenesis. As sEVs are already confirmed to carry angiogenesis-inducing vascular endothelial growth factor, which might promote vascularisation in the tumour site and activate Epithelial-Mesenchymal Transition (EMT) [144, 145]. Another significant involvement of EVs in malignancies development is their role in immunomodulation and immune evasion. Different studies have shown that cancer-derived EVs may carry higher levels of immunosuppressing molecules like macrophage migration inhibitory factor or PD-L1 forming metastatic niche [146-148]. This mechanism could be responsible for local transmission and relapses and considering the ease of EVs migration to the main blood circuit and distant metastasis (Figure 9). These facts encourage the analysis of EVs in cancer patients for diagnostic procedures and finding new diagnostic targets.

However, many technical difficulties are met considering one of the first steps - the isolation procedure. Several studies show that the choice of method plays a vital role in further downstream analysis [102]. The difficulty of repetitive, time- and cost-efficient isolation procedures is one of the most significant drawbacks of the possible clinical application of EVs. Nevertheless, it is a challenge to face before their potential application in routine diagnostic procedures.

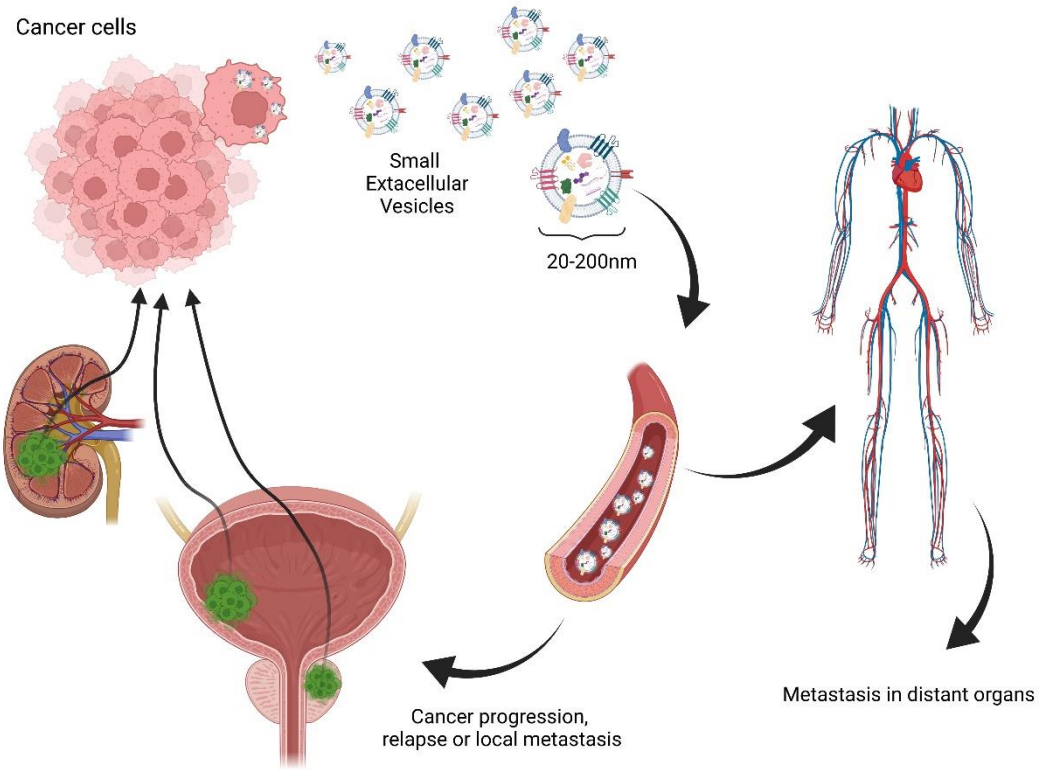


Figure 9 – Potential hypotheses of extracellular vesicles involvement in cancer progression and metastasis [103].

2. AIM OF THE STUDY

This study was based on two main aims:

1. Comparison of different small extracellular vesicles (sEVs) purification methods from peripheral blood serum and urine of prostate cancer patients for further analysis.

2. Analysis of medium-sized extracellular vesicles (mEVs) surface markers and carried miRNA from peripheral blood plasma of prostate cancer patients for their potential diagnostical value in Active Surveillance risk stratification.

3. MATERIALS AND METHODS

The whole study has been divided into two subsection: comparison of small extracellular vesicles purification methods from peripheral blood serum and urine; and analysis of diagnostic potential of medium-sized peripheral blood plasma extracellular vesicles for prostate cancer.

3.1 Gathering of the blood and urine samples

Blood and urine samples were collected from patients with diagnosed prostate cancer, administered to radical prostatectomy at General and Oncological Urology Clinic, Academic Hospital no.1 in Bydgoszcz, Poland. Blood samples were continuously collected, as the second tube after fresh injection, into BD SST II Advanced Tubes (BD, USA) – for serum analysis and BD Vacutainer ACD Tubes (BD, USA) for plasma samples. The blood samples were collected from patients on the fasting regime, at a very similar time of a day, to minimize the influence of circadian cycle. Spontaneous urine samples were collected into sterile urine IVD containers (EL-COMP, Poland) only from patients without active catheterization. A total of 39 patients were recruited for the studies. Among those, 15 patients were recruited for the comparison of sEVs purification methods and 24 for analysis of medium-sized plasma EVs (mEVs) diagnostic potential. The patients were divided into two sub-groups, based on the confirmed, post radical prostatectomy histopathological GS results. The groups were patients who were candidates for active surveillance (GS6 & GS7(3+4) - AS group, and who were not candidates for active surveillane (GS7(4+3), GS8 & GS9) - non-AS group.

The study was voluntary and required the informed consent of the patients in accordance with the consents of the local Bioethics Committee (KB 239/2019 and KB 183/2019).

All samples were delivered to the laboratory within 1 hour from collection time. After delivery, both urine and blood samples were centrifuged at 2000*xg* for 10 min at room temperature (RT) for serum/plasma separation and cell removal. After centrifugation, obtained serum/plasma and urinary supernatant were moved to the fresh 15ml tubes (Corning, USA) and centrifuged again at 2000*xg* for 10 min at RT, for purification from dead cell residues. Finally, the obtained supernatant was transferred to the fresh 2ml Eppendorf low protein binding tubes (Eppendorf, Germany), with at least 0,5ml residue left in the origin

tube, to lower the possibility of moving the contamination from precipitant. For final purification of bigger vesicles and particles, samples were centrifuged at 14 000*xg* for 45 min at 4ºC, with the slowest brake settings. For serum sEVs analysis supernatant from this procedure was taken for further purification. In case of plasma samples, the precipitate obtained from 14 000*xg* was resuspended in 0,2ml of freshly filtered PBS (Corning, USA), and frozen at -80°C until analysis. Additionally, 12ml of urine samples were concentrated with Amicon Ultra-0.5 ml Centrifugal Filters 10k (Merck Millipore, USA), centrifuged at 10 000*xg* for 10min at RT. For the subsequent purification methods, 0,5ml of matrix samples were taken.

3.2 Comparison of purification of the small extracellular vesicles protocols

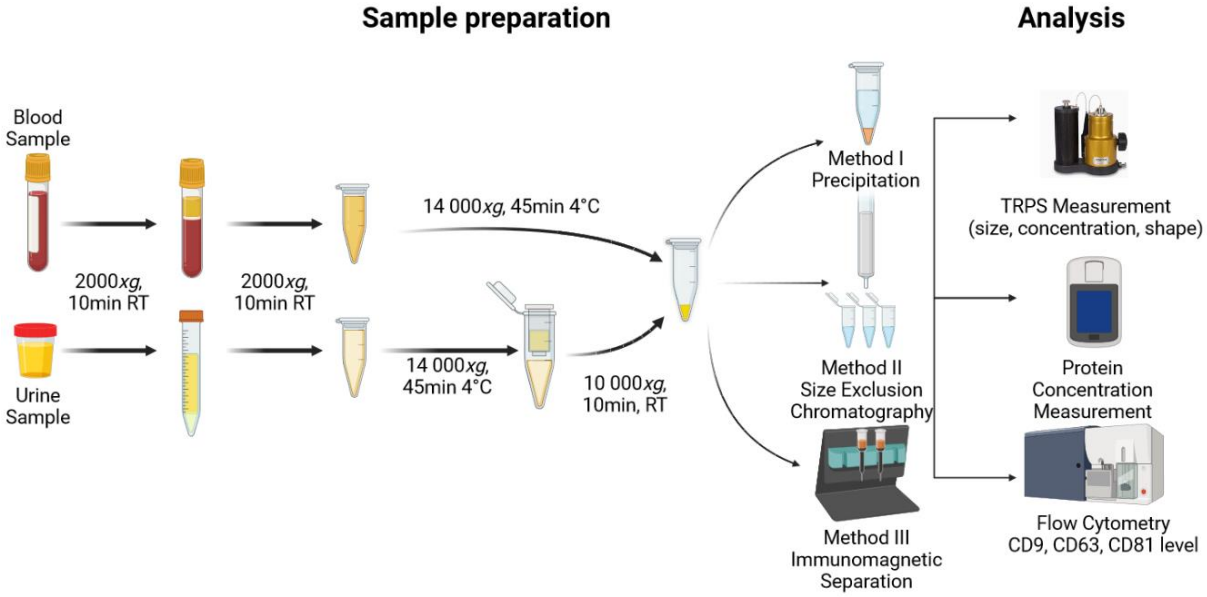


Figure 10 – Graphical summary of the comparison of small extracellular vesicles purification method part of the study.

3.2.1 Precipitation

The main principle of the precipitation of the small EVs from the liquid sample is utilization of polymers tying up water, and with use of relatively low centrifugation forces to precipitate the non-soluble components from the sample. Such an approach is not very specific, but provides easiness and quickness of the procedure.

The pre-purified serum samples were mixed with Total Exosome Isolation Reagent dedicated for serum samples (ThermoFisher Scientific, USA) in 1:5 ratio, incubated for 30 min at 4°C, and centrifuged at 10 000*xg* for 10 min at RT. After centrifugation, the supernatant was

removed, tubes were quick-spined to gather the remaining supernatant residues, and the obtained pellet was resuspended in 200µl fresh filtered PBS (Corning, USA).

The concentrated, purified urine samples were mixed with Total Exosome Isolation Reagent dedicated for urine samples (ThermoFisher, USA) in 1:1 ratio, incubated for 60 min at RT, and centrifuged at 10.000*xg*, 60 min at 4°C. After centrifugation, the supernatant was removed, tubes were quick-spined to collect the remaining supernatant residues, and the obtained pellet was resuspended in 200µl fresh filtered PBS.

After vesicles reconstitution, filtration through a 0,22µm syringe filter (Millipore, USA) was performed in both serum and urine samples to obtain a single vesicles suspension.

3.2.2 Size exclusion chromatography

Size Exclusion Chromatography (SEC) is a method utilizing special chromatographic columns filled with modified agarose resin. As the sample pass through the column, the molecules and particles suspended in the sample are separated by the size and affinity to the resin particles. Such an approach provides separation basing mostly on the size of the particles suspended in the sample.

Purified serum and urine samples were subjected to SEC with commercially available columns – Izon qEV original/70nm, gen. 1 (Izon, France). For this purpouse 500µl of samples were applied onto the column. Freshly filtered PBS was used as a mobile phase. Fractions 4,5,6 were pulled together, as fractions with vesicles of the size of interest with the lowest protein impurities level, into 1,5ml of the sample.

3.2.3 Immunomagnetic separation

Immunomagnetic separation (ImSep) is a method of separation basing on the presence of specific molecules on the surface of the analyzed particles. With use of the magnetic beads coupled with antibody against specific molecule, only particles which possess that molecule on their surface are separated from the rest of the sample.

Purified serum and urine samples were subdued to immunomagnetic separation with Exosome Isolation Kit Pan (Miltenyi Biotec, Germany). This reagent kit separate sEVs from the sample basing on the presence of CD9, CD63 and CD81, tetraspanins characteristically

enriched on the surface of sEVs. Prepared samples were incubated with microbeads coated with anti-CD9, -CD63, and -CD81 antibodies for 60 min at RT. After incubation, samples were applied onto the µColumns (Miltenyi Biotec, Germany) and magnetically separated. After elution with 100µl of isolation buffer, samples were applied onto the 0,22µm syringe filter (Millipore, USA) with an additional 100µl of freshly filtered PBS for obtaining the single vesicle suspension.

3.3 Small Extracellular Vesicles size and concentration analysis

All measurements were performed with a qNano (Izon, France) TRPS device. NP100 size nanopores and CPC100 calibration particles were chosen, with the measured size range calibrated to 50-200nm (Izon, France). A supplied Reagent kit (Izon, France) was used for calibration and measurements. Depending on the necessity, samples were diluted in the Measurement Electrolyte within the 2 - 10x range. Additionally, calibration particles were run as the first and the last sample during the measurement series and reagents-only samples for internal control. To analyze the flow characteristics of the obtained vesicles and aggregates presence, Blockade Time to Full Width at Height Maximum (FWHM) duration ratio to Particle Diameter were compared.

$$Flow\ characteristic = \frac{Blockade\ duration}{FWHM\ duration} / Particle\ diameter$$

3.4 Small Extracellular Vesicles surface markers analysis

3.4.1 Immobilization of extracellular vesicles on latex aldehyde/sulfate beads

For analysis of different characteristic tetraspanins levels, flow cytometry was used. To overcome the limitation, of sEVs size being below the resolution of the available flow cytometer (BD FACSCanto II, Becton Dickinson, USA), immobilization on 3µm aldehyde/sulfate latex beads (ThermoFisher Scientific, USA) was performed based on the modified protocol of Suarez et al. [149]. Samples of 5.0E+8 of sEVs in 300µl, diluted with freshly filtered PBS, were incubated with 1µl of beads suspension overnight at 4ºC on a thermoshaker (Biosan, Latvia). After incubation, 1ml of 2% albumin was added, and the subsequent incubation for 2h was performed at RT. Samples were centrifuged (2000*xg*,5min, RT), beads with immobilized

sEVs were resuspended with 500µl of 15mM Glycine (POCH, Poland) in PBS solution, incubated for 15min, RT, and centrifuged once more (2000xg 5min, RT).

3.4.2 Staining and flow cytometry analysis

The immobilized sEVs samples were stained for the characteristically enriched tetraspanins on their surface: CD9, CD63 and CD81. After centrifugation, samples were resuspended in 50µl of PBS and stained with fluorochrome-conjugated antibodies: anti-CD9-FITC (Exbio, Clone: MEM-61, Czechia), anti-CD63-PE (Exbio, Clone: MEM-259, Czechia), and anti-CD81-APC (Exbio, Clone: M38, Czechia) for 30 min in RT. After staining, samples were washed and resuspended in PBS for analysis. The beads without immobilized sEVs, and unstained samples were used as controls. The results are presented as Staining Index in accordance with the formula:

$$SI = \frac{MFI_{pos} - MFI_{neg}}{2xSD}$$

SI – Stain Index, MFI_{pos} – Mean Fluorescence Intensity in positive population, MFI_{neg} - Mean Fluorescence Intensity in negative population, SD – Standard Deviation

3.5 Protein contamination analysis

For analysis of protein contaminations, the total protein concentration was analyzed from each sample with Qubit protein Assay (Thermofisher Scientific, USA). To minimize the effect of vesicles concentration on the obtained results, all of the analyzed samples were diluted to 5.0E+08 vesicles within the same 100µl volume.

3.6 Analysis of plasma medium-sized EVs

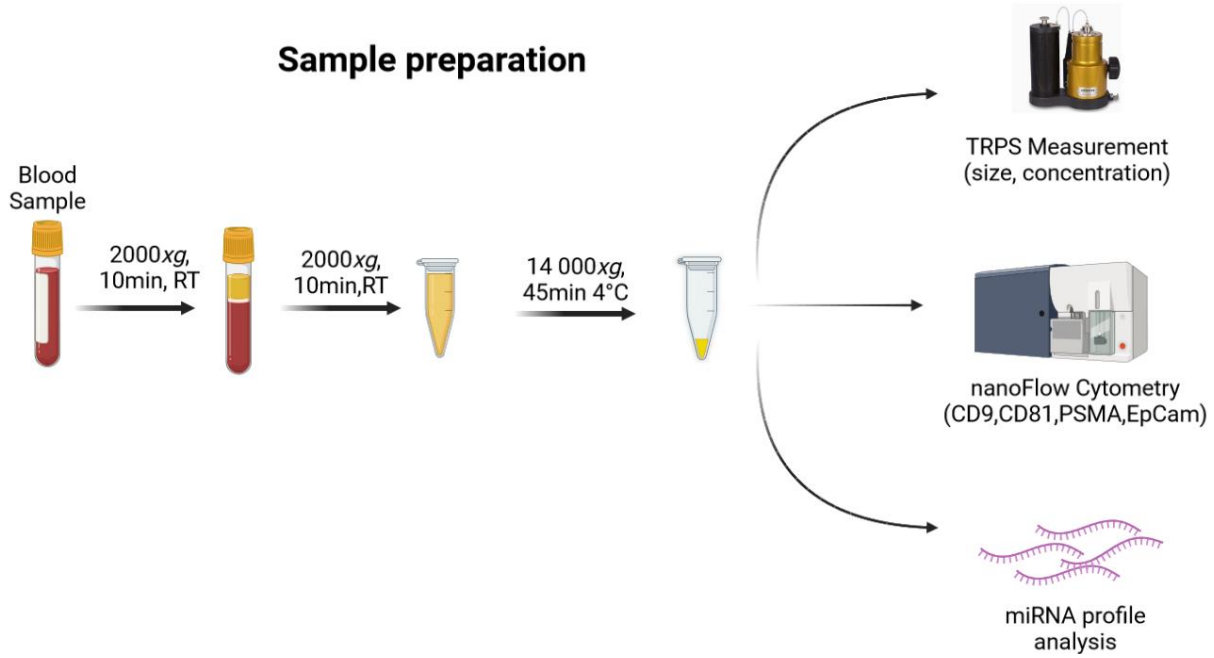


Figure 11 – Graphical summary of the plasma medium-sized extracellular vesicles analysis part of the study.

3.6.1 Plasma mEVs size and concentration analysis

All measurements were performed with a qNano (Izon, France) TRPS device. NP2500 size nanopores and CPC400 calibration particles were chosen, with the measured size range calibrated to 120-500nm (Izon, France). A supplied Reagent kit (Izon, France) was used for calibration and measurements. All of the samples were diluted in the Measurement Electrolyte in 1:5 ratio. Additionally, calibration particles were run as the first and the last sample during the measurement series, for stability of measurement analysis and reagents-only samples for internal control.

3.6.2 Flow cytometer setup for nanoparticle scale analysis

As the majority of EVs are below the resolution limit of standard cytometers setup, a modification for the BD Canto II machine optical setup has been made. As according to Mie Theory and Raleigh Light scattering, the amount of light scattered by any particle is directly proportional to the diameter of the particle and inversely proportional to the wavelength, a shorter wavelength 405nm, instead of 488nm laser was chosen for side scatter analysis [150]. For enabling such an approach a stock 450/20 Bandpass filter (BP) for the 405nm line, was exchanged for 405/10 BP filter (Chroma, USA) for Violet Side Scatter (VSSC) gathering.

Additionally as the triggering parameter a VSSC was chosen. To properly asses the detection limit, and setup of the VSSC threshold and voltage settings, 100nm polystyrene NIST traceable CPC100 beads were used (Izon, France). Additionally for the setup of the range of detection for bigger particles, a Megamix SSC (Biocytex, France) beads mixture, containing various sized FITC fluorescent polystyrene beads (160nm, 200nm, 240nm and 500nm) were used. For fluorescence channels voltage setup and acquired signals stability analysis, a Rainbow Calibration 8-peak beads were used (Thermofisher Scientific, USA).

3.6.3 nanoFlow cytometry (nFCM) analysis of mEVs surface markers

Plasma mEVs were stained for an analysis of set of surface markers with potential for diagnostical application in PCa: PSMA - highly expressed enzymatic protein on PCa cells surface, EpCam – a protein overexpressed on surface of many cancer types cells, CD9,CD81 – tetraspanins involved in processes of cell activation and transduction, typically enriched, but not exclusively, on surface of small extracellular vesicles. For detection of these markers on mEVs surface a set of antibodies was used: anti-EpCam-AF647 (Exbio, Clone: VU-1D9, Czechia), anti-PSMA-PE (Thermofisher Scientific, Clone: GCP-05, USA), anti-CD9-FITC (Exbio, Clone: MEM-61, Czechia), anti-CD81-APC (Exbio, Clone: M38, Czechia). Based on that a panel, for proper setup, compensation and analysis, was set for every patient sample: 1.Nonstained; 2. CD9+; 3. CD81+; 4.PSMA+; 5.EpCam+, 6.PSMA+,EpCam+ 7.PSMA+,CD9+,CD81+. The samples were combined of 5µl of mEVs, antibodies solutions and PBS up to 50µl volume. All of the samples were stained at 4^{o}C for 60 min. After staining 1ml of freshly filtered PBS was added to every sample to stop the staining. Additionally to prevent the swarming effect, every sample was further diluted (if needed) so the sample acquisition event rate was up to 7000 events/s and electronic abort rate <5% of acquisition event rate [151]. The samples were acquired for at least 1min before recording the results, to allow stabilization of the sample flow.

Because of the risk of self-quenching and non-specific signal generation, caused by transfer of energy between fluorophores, resulted from small size of analyzed particles, no more that 3-color staining was chosen.

3.6.4 FCM_{Pass} results calibration and data analysis

As every flow cytometry machine differs in exact method of collecting scattered and fluorescent light, a standardization from arbitrary units to standardized ones, enabling proper interpretation of the results across different machines was proposed. For that reason results of VSSC scatter were calibrated with data obtained with size-calibration polystyrene beads and FCM_{PASS} software [114]. After calibration results were analysed with FlowJo v10.4 software (Becton Dickinson, USA). The following gating strategy was chosen for every sample: stability of flow with: Time x VSSC -> aggregates discrimination with VSSC-Width x VSSC-Height -> appropriate fluorescence-Height channels. For gating of FITC and PE signals FITC-H x PE-H plots were used, which allowed omitting problem of spillover influence without necessity of calculating compensation. APC signal was gated with VSSC-H x APC-H plot. When APC was analyzed in combination with another fluorophore, a VSSC-H x APC-H gate was used on population from PE-H x FITC-H gated plot.

3.7 Plasma mEVs miRNA content analysis

3.7.1 miRNA isolation

For miRNA isolation from plasma mEVs miRNeasy serum/plasma advanced kit was used (Qiagen, USA). 100µl of mEVs from every sample was subjected to the miRNA isolation process. Additionally for isolation process efficiency analysis, and inter-sample standardization of Real-Time PCR results a RNA Spike-In Kit, For RT (Qiagen, USA) containing synthetic spike-in resembling miRNAs in structure but lacking close sequence similarities to known miRNAs (UniSp2, UniSp4, UniSp5). Finally a reverse transcription was performed with use of miRCURY LNA RT Kit (Qiagen, USA). Into every sample UniSp6 and exogenous cel-miR-39-3p miRNA spike-ins for reverse transcription process control, were used. The reverse transcription was performed with Mastercycler Nexus Gradient thermocycler (Eppendorf, Germany) with reverse transcription step at 42^{o}C for 60 min, and inactivation at 95^{o}C for 5 min.

3.7.2 Real-Time PCR miRNA analysis

For analysis of the miRNA profile a prepared plate with set of miRNAs was chosen, allowing analysis of 82 miRNAs connected with prostate cancer: hsa-let-7a-5p, hsa-let-7b-5p, hsa-let-7c-5p, hsa-let-7f-5p, hsa-miR-100-5p, hsa-miR-101-3p, hsa-miR-106b-5p, hsa-miR-

125a-5p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-126-5p, hsa-miR-128-3p, hsa-miR-133a-3p, hsa-miR-135a-5p, hsa-miR-135b-5p, hsa-miR-141-3p, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-146a-5p, hsa-miR-146b-5p, hsa-miR-148a-3p, hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-17-3p, hsa-miR-17-5p, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-182-5p, hsa-miR-183-5p, hsa-miR-184, hsa-miR-194-5p, hsa-miR-195-5p, hsa-miR-196a-5p, hsa-miR-19b-3p, hsa-miR-200b-3p, hsa-miR-200c-3p, hsa-miR-203a-3p, hsa-miR-205-5p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-21-5p, hsa-miR-218-5p, hsa-miR-22-3p, hsa-miR-221-3p, hsa-miR-222-3p, hsa-miR-223-3p, hsa-miR-224-5p, hsa-miR-23b-3p, hsa-miR-24-3p, hsa-miR-25-3p, hsa-miR-26a-5p, hsa-miR-26b-5p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-296-5p, hsa-miR-29b-3p, hsa-miR-30c-5p, hsa-miR-31-5p, hsa-miR-3163, hsa-miR-32-5p, hsa-miR-330-3p, hsa-miR-331-3p, hsa-miR-34a-5p, hsa-miR-34c-5p, hsa-miR-361-5p, hsa-miR-365a-3p, hsa-miR-3662, hsa-miR-3666, hsa-miR-374b-5p, hsa-miR-375, hsa-miR-425-5p, hsa-miR-449a, hsa-miR-455-5p, hsa-miR-494-3p, hsa-miR-616-3p, hsa-miR-7-5p, hsa-miR-9-3p, hsa-miR-92a-3p, hsa-miR-93-5p, hsa-miR-96-5p, hsa-miR-99a-5p, hsa-miR-99b-5p (the full reference sequences of analyzed miRNAs are presented in Attachment 2). For this reason commercially available plate Human Prostate Cancer Focus, miRCURY LNA miRNA Focus PCR Panel (YAHS-212Z; Qiagen, USA) with primers for aforementioned miRNAs, and proper controls was used. 10µl of cDNA obtained from reverse transcription step from every sample was mixed with 2x miRCURY SYBR Green Master Mix and nuclease-free water from miRCURY LNA SYBR Green PCR Kit (Qiagen, USA). After addition of sample and master mix, plates were spined and left for incubation 5 min at RT, to allow dissolution of primers located on the plate wells. The plates were later run on Roche LightCycler 480 (Roche, Switzerland). The protocol for run was: initial heat activation – 2 min, 95^{o}C; 2-step cycling – denaturation (10 s, 95^{o}C) and annealing (60 s, 56^{o}C) for 45 cycles; and Melting Curve analysis. All of the steps were performed with maximal ramp rate, according to manufacturer's recommendations. After the run results were analysed with GeneGlobe analysis software (Qiagen, USA). The normalization method chosen for the analysis was Global Ct mean of Expressed miRNAs. The lower limit of detection Ct value was set to 40, and the statistical analysis was set to un-paired 2-tail t-test.

3.8 Statistical analysis

For the analysis of comparison of the sEVs purification protocols from serum and urine, 2-way ANOVA with Sidak correction for multiple comparison was used. For the analysis of size and concentration of mEVs from PCa patients, and nFCM results analysis multiple-t test with Sidak-Bonferroni correction for multiple comparisons, and alpha set for 0,05 was used, for every parameter independently. The statistical analysis has been performed with GraphPad Prism 7 software (GraphPad Software, USA).

4. Results

4.2 Comparison of Small Extracellular Vesicles purification protocols

4.2.1 Analysis of size and concentration of purified small extracellular vesicles

sEVs size

Analysis of size comparison revealed a mean size of obtained sEVs, lining in proper range of <150nm. A significantly bigger mean size of sEVs purified with precipitation in serum samples (107,33±30,2 nm) than with SEC (80,5±9,8 nm) or ImSep (77±9,96nm) was noticed. However, in the case of urine samples, no significant differences in the size of sEVs from precipitation (87±7,51nm), SEC (91,67±8,66 nm), and ImSep (84,5±12,27nm) were observed (Fig.12). For reagents only samples, no signals were detected. The exemplar results of size distribution of the obtained sEVs samples were presented in Fig. 13 and Fig.14, for serum and urine samples respectively.

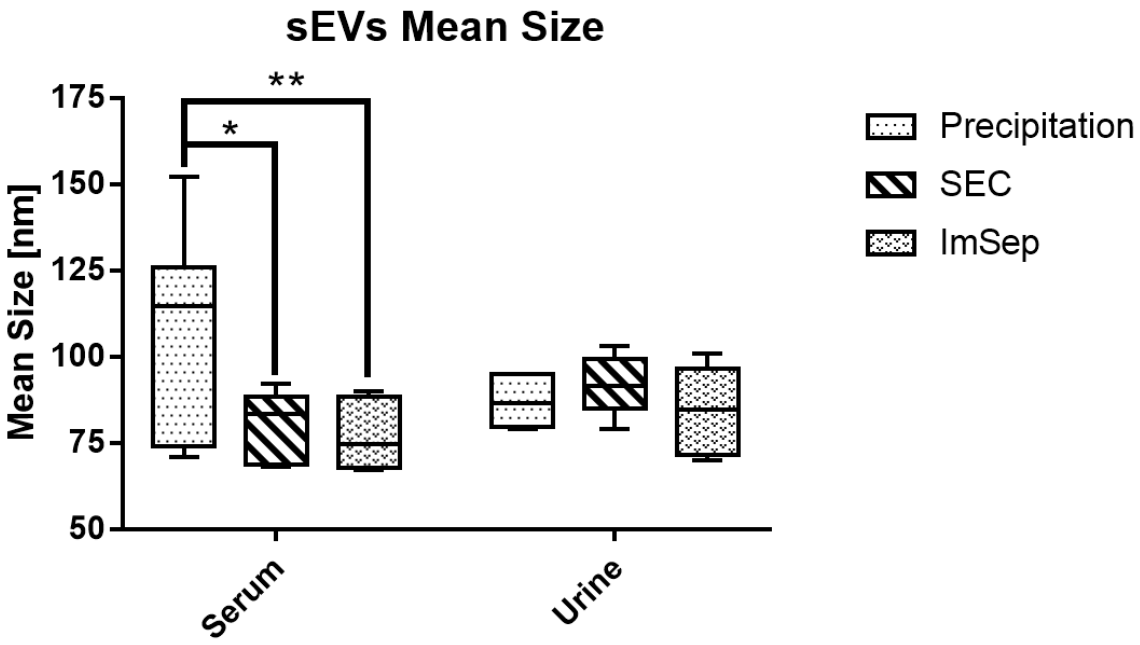


Figure 12 - Results of analysis of size analysis for sEVs samples obtained from serum and urine with Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation (ImSep).* p<0,05; ** p<0,01; *** p<0,001.

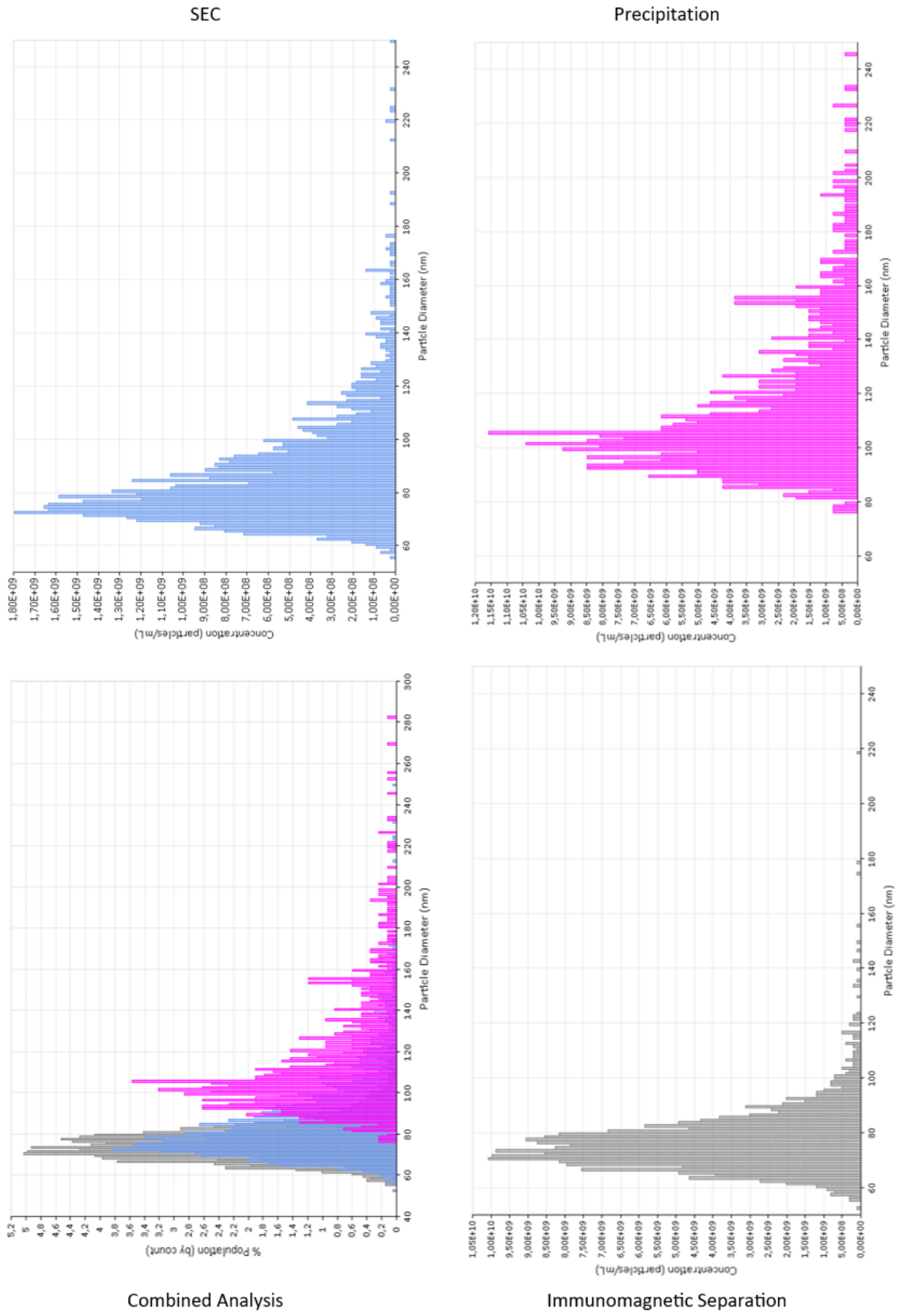


Figure 13 - Exemplar results of TRPS size distribution analysis of sEVs from serum purified with Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation.

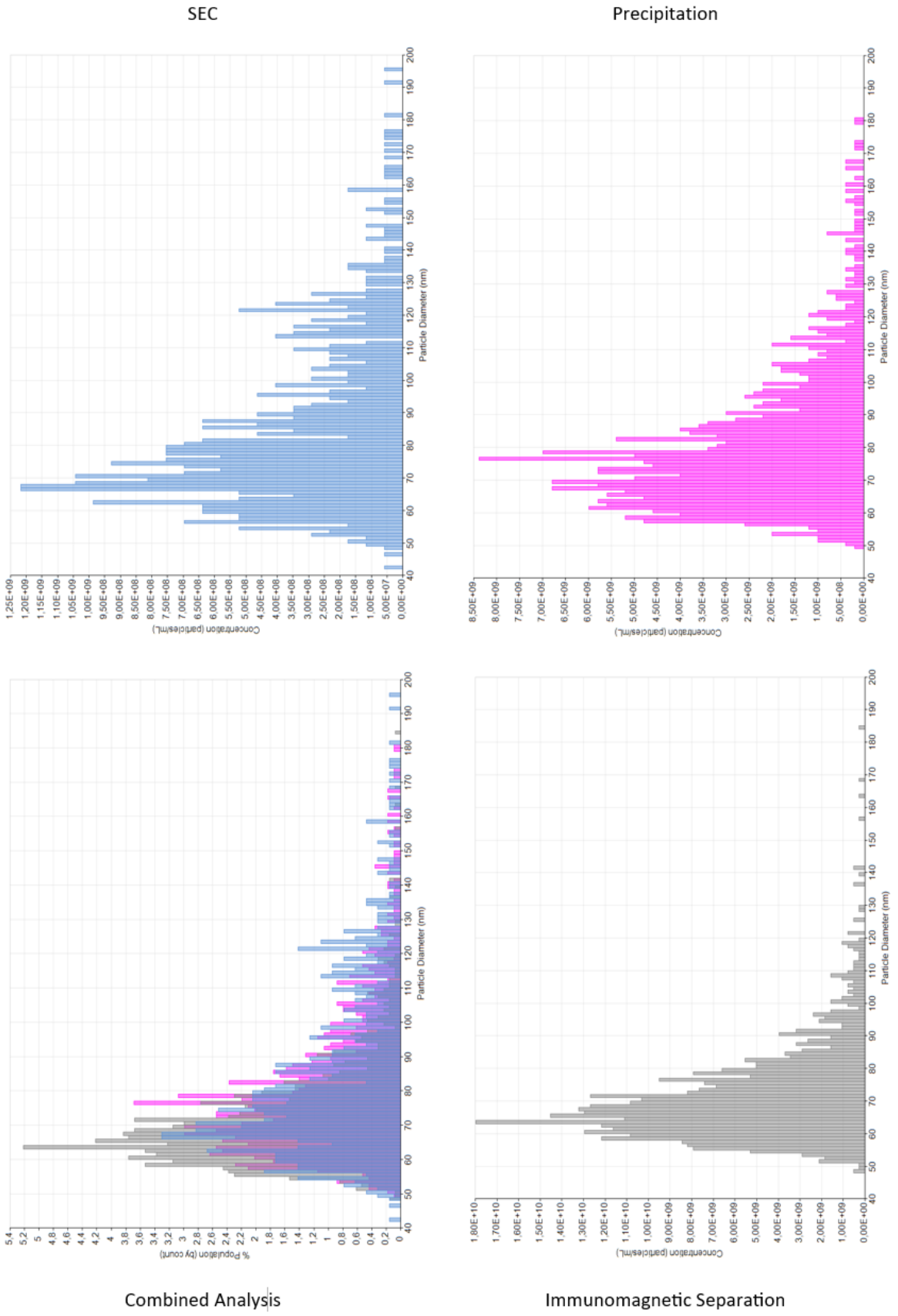


Figure 14 - Exemplar results of TRPS size distribution analysis of sEVs from urine purified with Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation.

The number of sEVs

Analysis of the total number of obtained sEVs revealed no significant differences between serum (7,82E+09 ± 6,2E+09; 2,01E+10 ± 1,23E+10; 1,73E+10 ± 1,8E+10) and urine (4,76E+09 ± 4,57E+10; 1,01E+10 ± 7,24E+09; 8,03E+09 ± 6,04E+09) samples from precipitation, SEC, and ImSep respectively. However, the lowest range of obtained sEVs was observed for the precipitation method in serum and urine samples. Moreover, in the case of immunomagnetic separation from both serum and urine samples, the highest variation was observed (Fig.15).

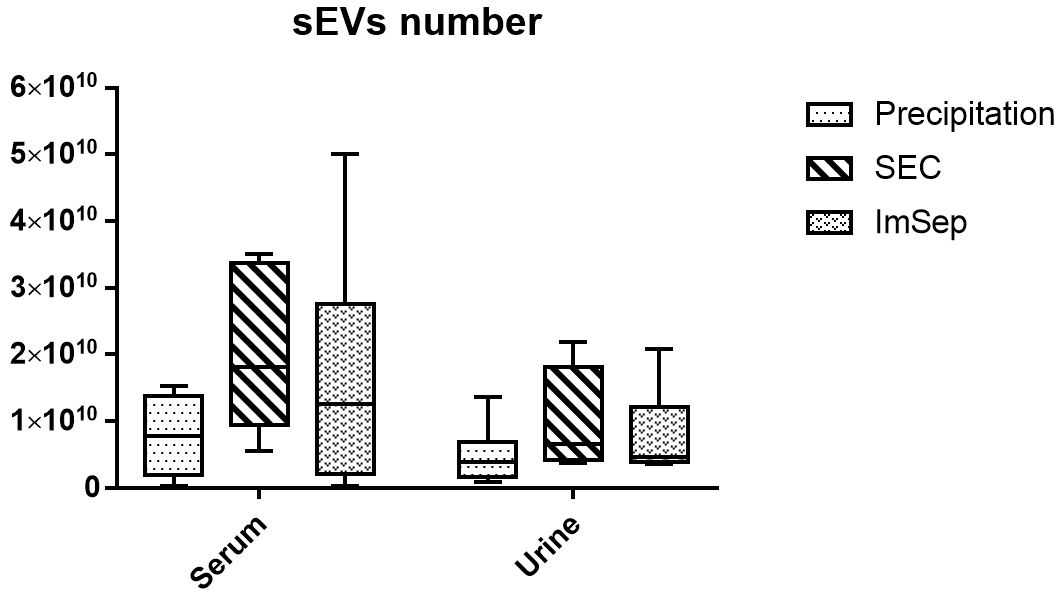


Figure 15 - Results of analysis of particles number for sEVs samples obtained from serum and urine with the Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation (ImSep).* p<0,05; ** p<0,01; *** p<0,001.

Flow characteristics of sEVs

The analysis of blockade time characteristics compared to calculated particle size revealed significant difference in case of precipitation and SEC for serum samples. For the rest of the samples no significant changes between the flow properties of the analyzed methods were found (Fig.16). However, in the case of precipitation samples from serum and urine, more frequent, temporary, or permanent full blockades of the nanopore during measurements were observed than in SEC and ImSep samples, indicating the presence of aggregates of vesicles above the measurement range (data not shown).

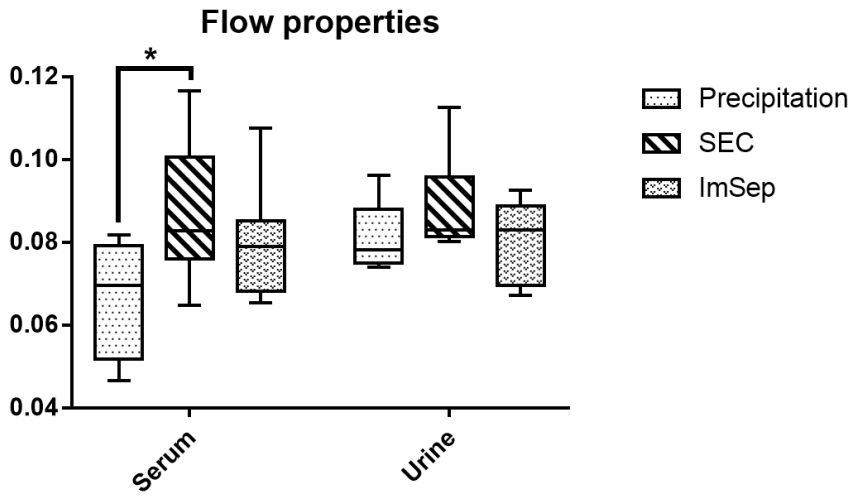


Figure 16 - Results of analysis of flow characteristic for sEVs samples obtained from serum and urine with Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation (ImSep).* p<0,05.

4.2.2 Analysis of surface protein markers of purified extracellular vesicles

The results of flow cytometry analysis of CD9, CD63, and CD81 revealed the presence of all three of the analyzed tetraspanins on sEVs surface. Moreover, there were no significant differences between the Stain Index of samples isolated by any of the analyzed methods in serum samples. In case of urine samples, significantly lower Stain Index of CD9 from ImSep was observed (Fig.17).

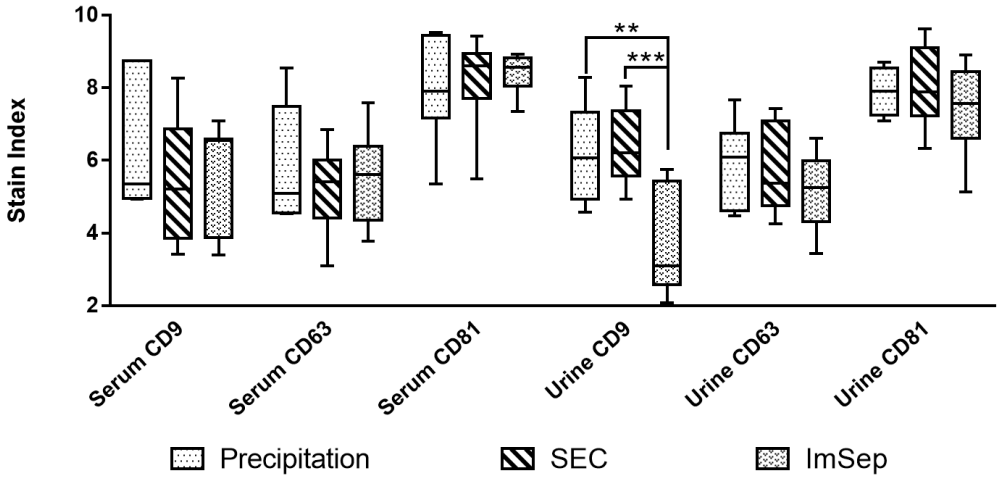


Figure 17 - Results of analysis of stain index for characteristic tetraspanins for sEVs samples obtained from serum and urine with the Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation (ImSep).* p<0,05; ** p<0,01; *** p<0,001.

4.2.3 Analysis of protein contamination in purified extracellular vesicles

Protein to vesicles ratio

Analysis of protein concentration in purified sEVs revealed the higher level of protein contamination in samples isolated with the precipitation method for serum samples than SEC ($p<0,001$) or ImSep ($p<0,001$). In the case of Urine samples, no significant differences were found. Overall the lowest protein concentration / 1E+08 of sEVs for both methods was obtained with the SEC method, with 3±3 and 1±1 µg of protein per 1E+08 of vesicles for serum and urine samples respectively. The highest protein concentration was found in the precipitation method samples, with 2007±900 and 174±167 µg of protein per 1E+08 of vesicles for serum and urine samples, respectively (Fig.18).

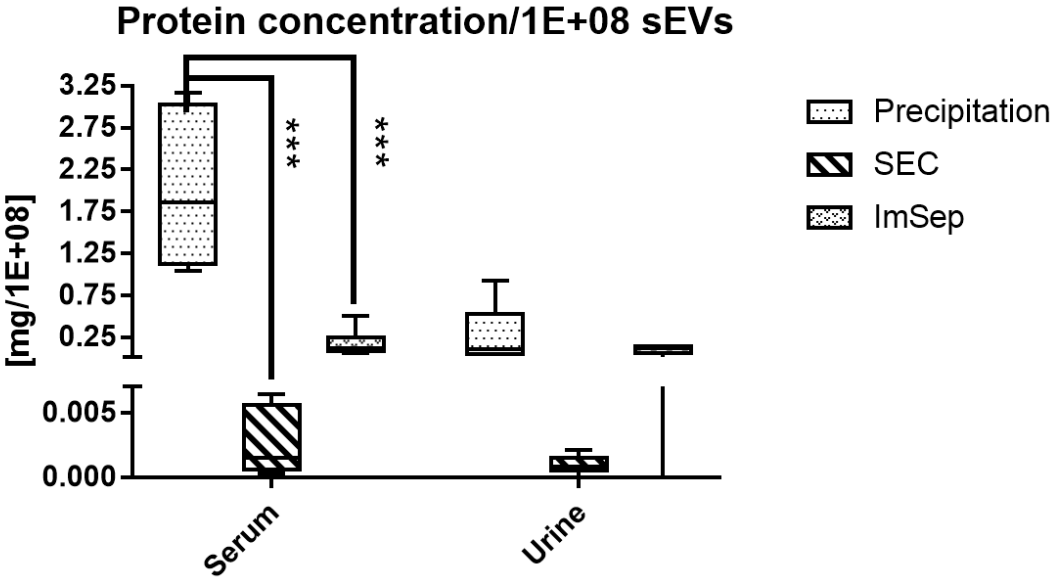


Figure 18 - Results of analysis of protein concentration for sEVs samples obtained from serum and urine with the Precipitation, Size Exclusion Chromatography (SEC) and Immunomagnetic Separation (ImSep).* $p<0,05$; ** $p<0,01$; *** $p<0,001$.

4.3 Patients characteristics

For analysis of potential diagnostic value of mEVs 24 patients were divided into two sub-groups, based on the confirmed, post radical prostatectomy histopathological GS results. The groups were patients who were candidates for active surveillance (GS6 & GS7(3+4) - AS group, and who were not candidates for active surveillance (GS7(4+3), GS8 & GS9) - non-AS

group. No significant difference were found between the groups of patients in case of: age, BMI, and PSA level before surgery.

Table 3 – Patients characteristics for mEVs diagnostical performance analysis.

	AS group GS ≥7(3+4)	**Non-AS group** GS≤7(4+3)	**p-value**
Number of patients	12	12	-
Age	68,9(±6,0)	68,5(±5,6)	0,8619
BMI	28,2(±4,5)	29,7(±2,4)	0,3186
PSA before surgery	8,3(±5,0)	20,7(±46,0)	0,3603
% of GS lower than pre-surgery biopsy	33,3%	0%	-
% of GS higher than pre-surgery biopsy	16,7%	75%	-

4.4 Size and concentration of plasma mEVs from AS and non-AS prostate cancer patients

The TRPS analysis of the size and concentration of the obtained plasma EVs did not reveal any significant differences between AS and non-AS patients' samples in either mean size (239,33 ± 8,28nm vs 231,67 ± 12,05nm), mode size (163,58 ± 12,78nm vs 162,33±5,19nm) nor concentration (4,45E+09 ± 3,02E+09 EVs/ml vs 7,05E+09 ± 1,14E+10 EVs/ml) (Fig.19). The exemplar results of size distribution of the mEVs samples are presented in Figure 20.

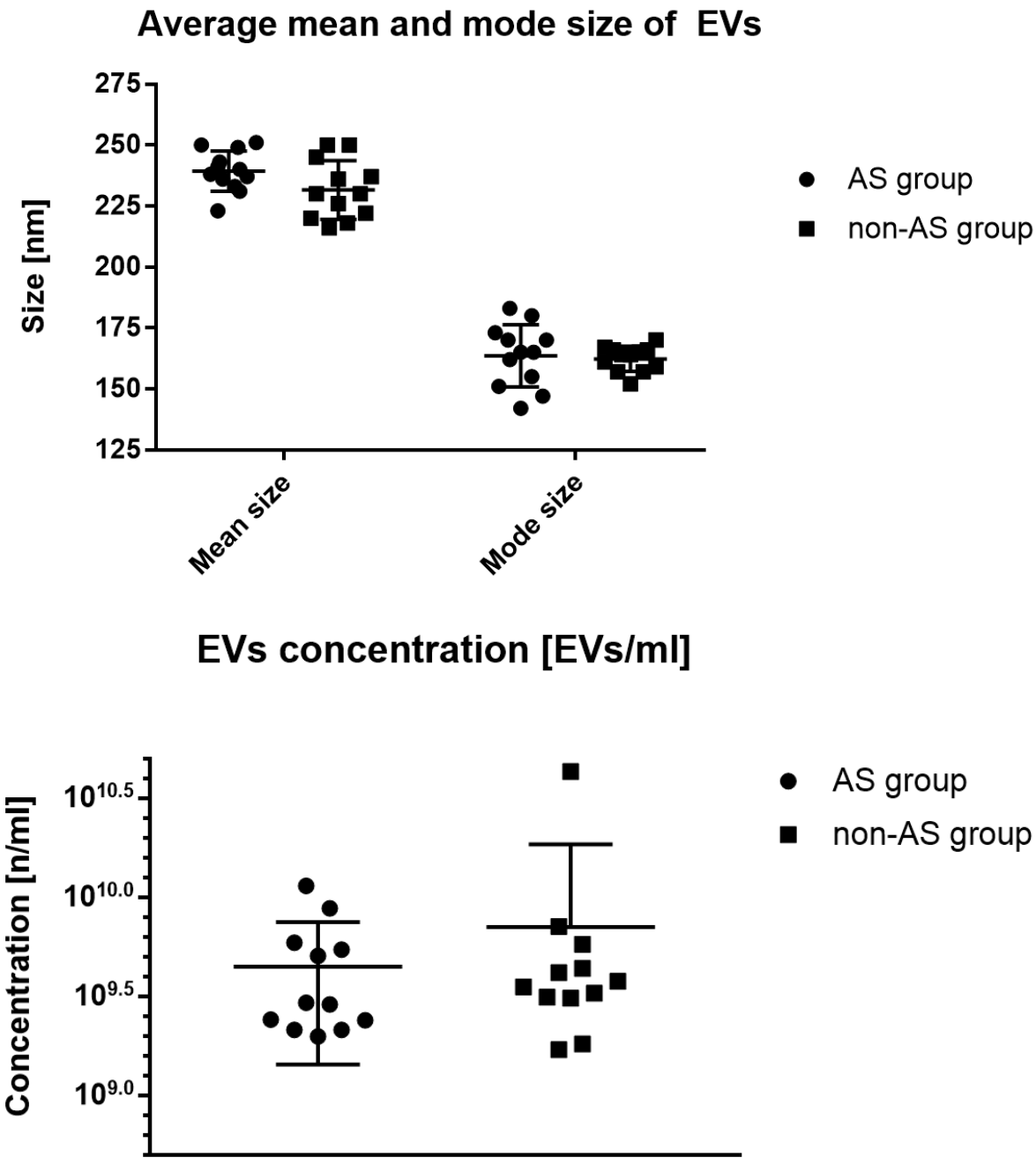


Figure 19 – Average concentration, mean and mode size of the plasma EVs from Active Surveillance (AS) and non-Active Surveillance (non-AS) prostate cancer patients.

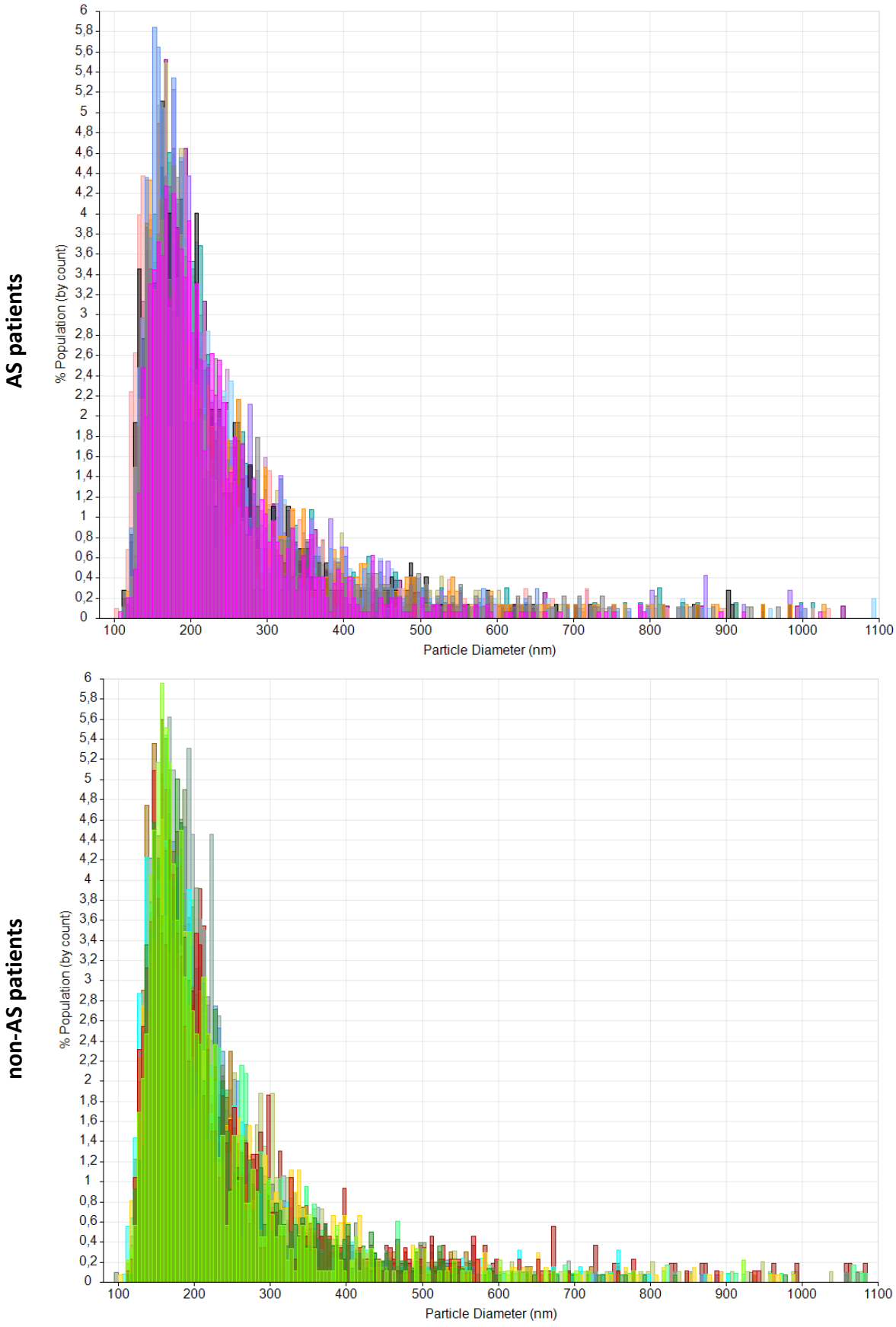


Figure 20 – Size distribution of plasma mEVs samples from Active Surveillance (AS) and non-Active Surveillance (non-AS) prostate patients groups.

4.4 nanoFlow cytometry analysis of mEVs surface markers PSMA, EpCAM and CD9 & CD81 tetraspanins

The results of calibration showed a proper good fit of the obtained scatter calibration controls, to the theoretical model. Obtained low detection limit for the polystyrene beads was ~72nm, and for the EVs with high refraction index, to which plasma mEVs might be considered, ~121nm (Fig.21, Fig.22).

The analysis of surface markers of plasma EVs of AS and non-AS prostate cancer patients did not reveal any statistically significant differences of percentage of the following populations: CD9+ (6,939±2,514 vs 3,636±2,827); CD81+ (0,290±0,150 vs 0,159±0,166); PSMA+ (0,110±0,063 vs 0,119±0,095); EpCam+ (0,328±0,197 vs 0,152±0,135); PSMA+CD9+ (0,084±0,081 vs 0,040±0,037); CD9+CD81+ (0,070±0,040 vs 0,043±0,042). In the case of PSMA+/EpCam+, PSMA+/CD81+, and PSMA+CD9+CD81+ no proper signal within the range of analysis were detected (Fig.23). However when a ratio of PSMA+ and PSMA+CD9+ EVs is considered a significant difference is observed between AS and non-AS prostate cancer patients ($P<0,0001$) (Fig.24).

Analysis of the average mean and median size of the aforementioned positive populations revealed that in most of the cases the median size was lower than the mean size, thus, a skewness of the data distribution was found, revealing potential limitation, by the limit of detection. Statistical analysis of the results showed no significant differences, except for average median size of CD9+CD81+ population, where a significantly higher average median size was found for non-AS than in AS prostate cancer patients (Fig.25).

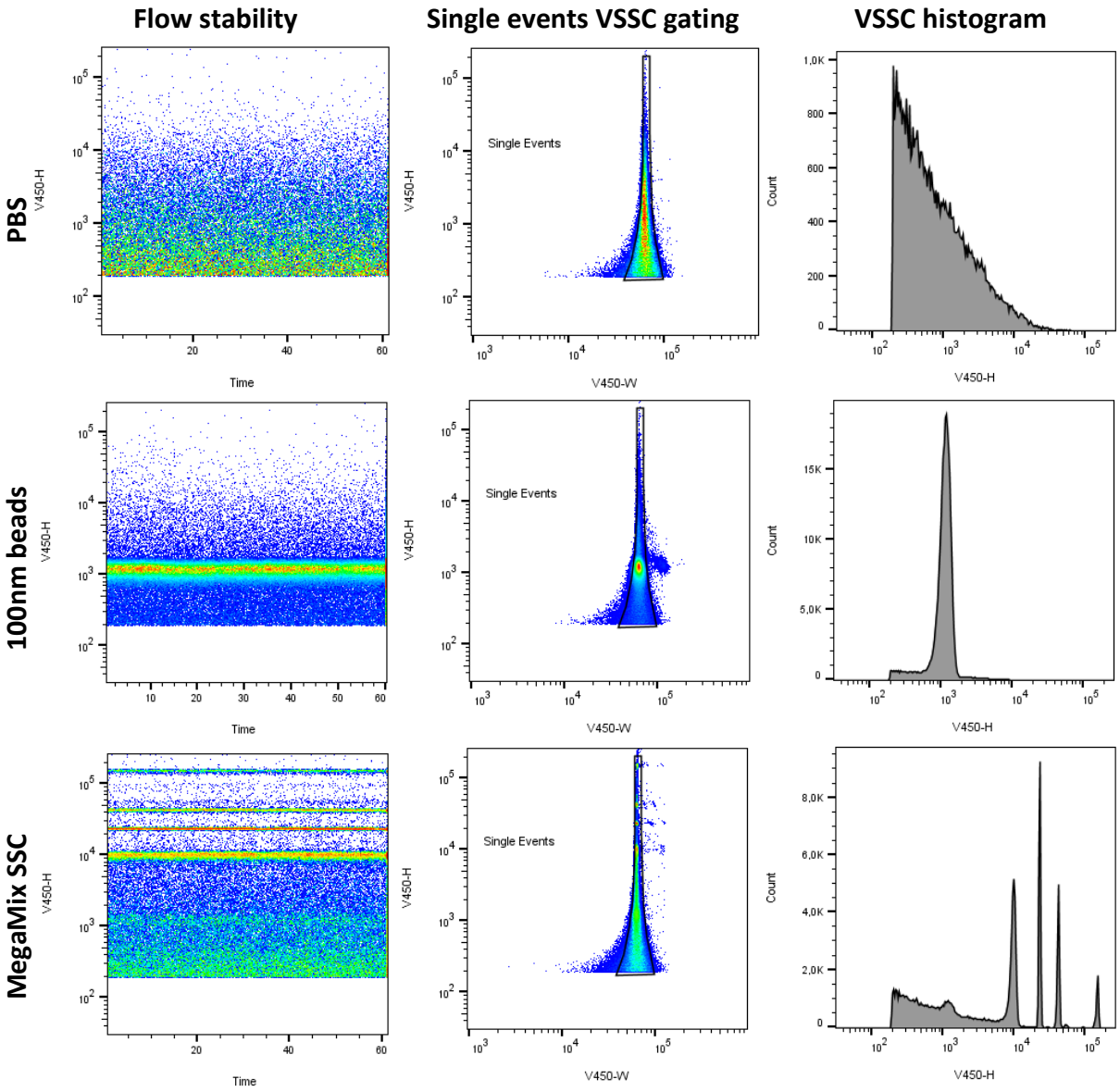


Figure 21 - Representative results of Violet Side Scatter calibration samples.

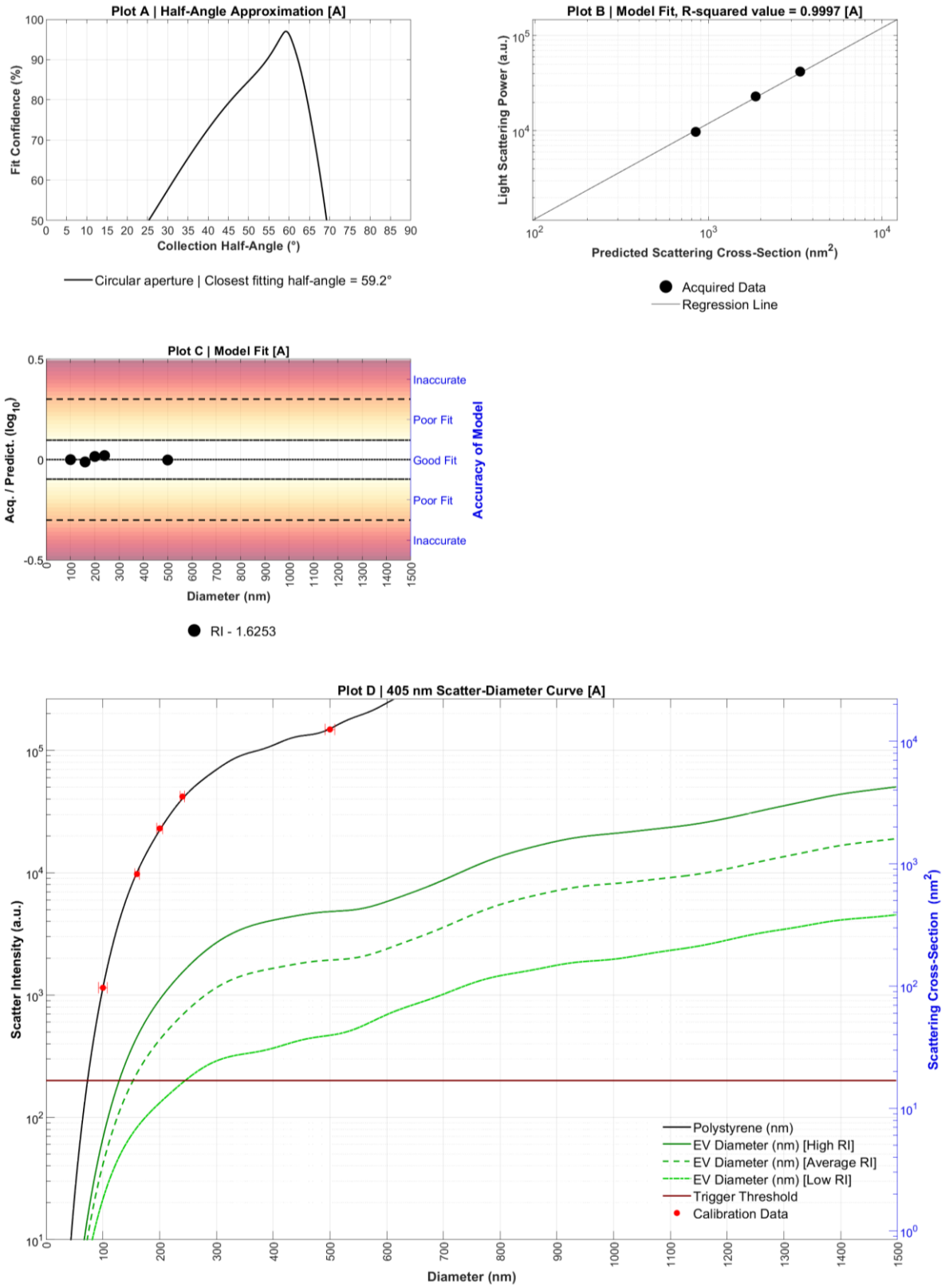


Figure 22 – FCMPass Scatter calibration report for the nFCM anaylysis of size of EVs and theoretical limit of detection.

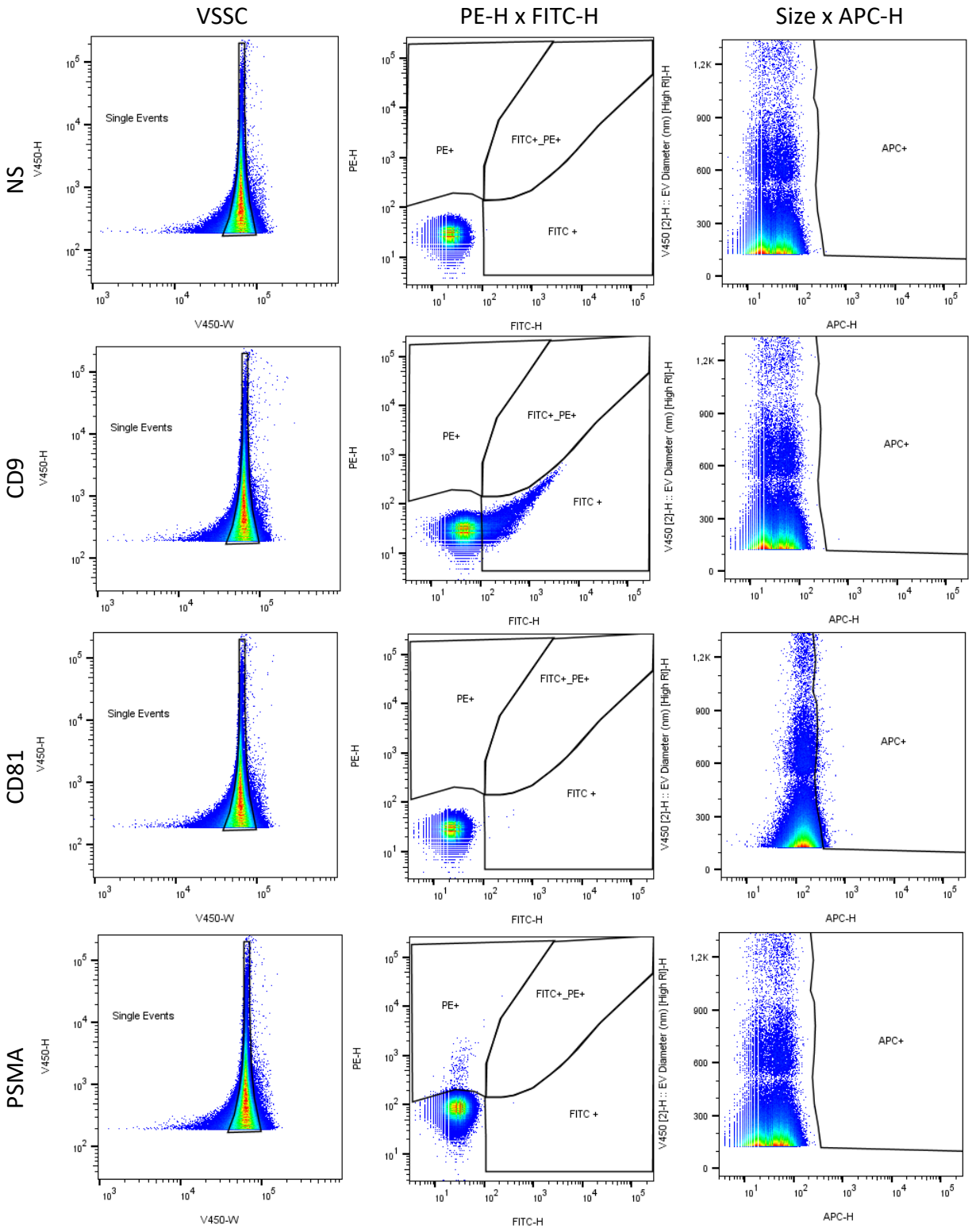
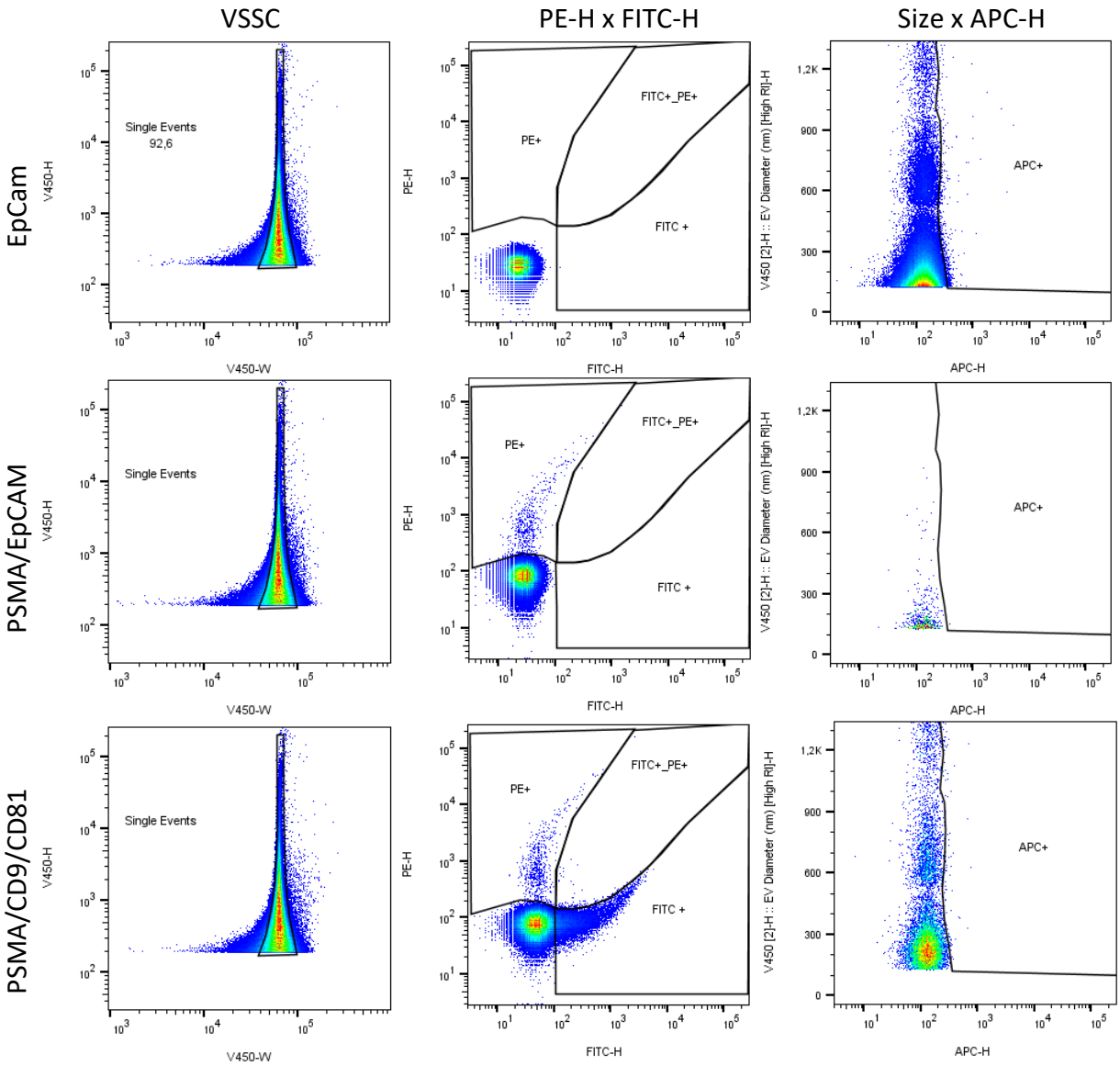


Figure 23 – Representative data with gating, for the nFCM analysis, for every sample type: nonstained (NS), CD9+, CD81+, PSMA+, EpCam+, PSMA+EpCam+, PSMA+CD9+CD81+.



cont. **Figure 23 –** Representative data with gating, for the nFCM analysis, for every sample type: nonstained (NS), CD9+, CD81+, PSMA+, EpCam+, PSMA+EpCam+, PSMA+CD9+CD81+.

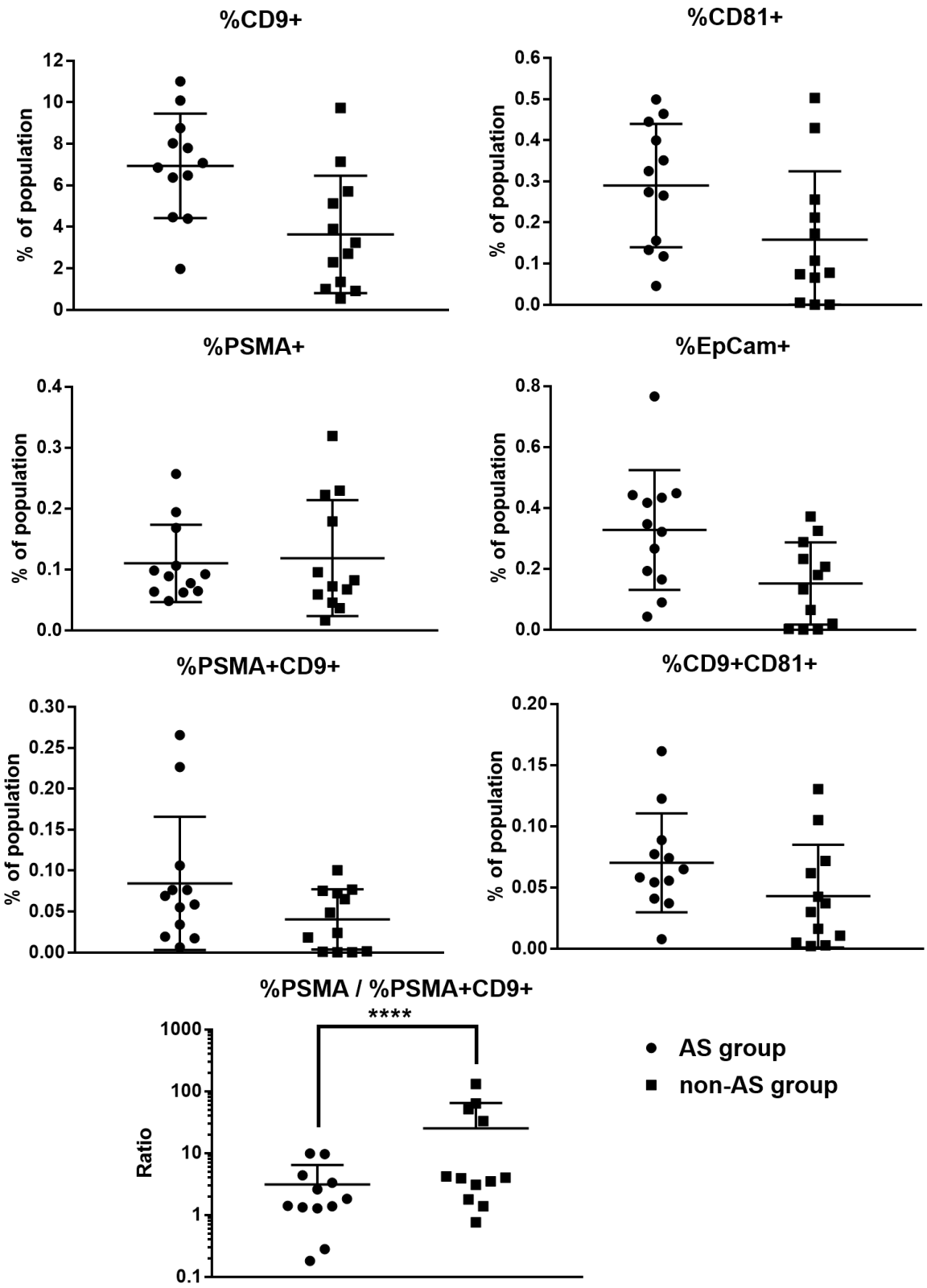


Figure 24 - Summarized results of nFCM analysis of plasma EVs from Active Surveillance (AS) and non-Active Surveillance (non-AS) prostate patients groups. **** p<0,0001

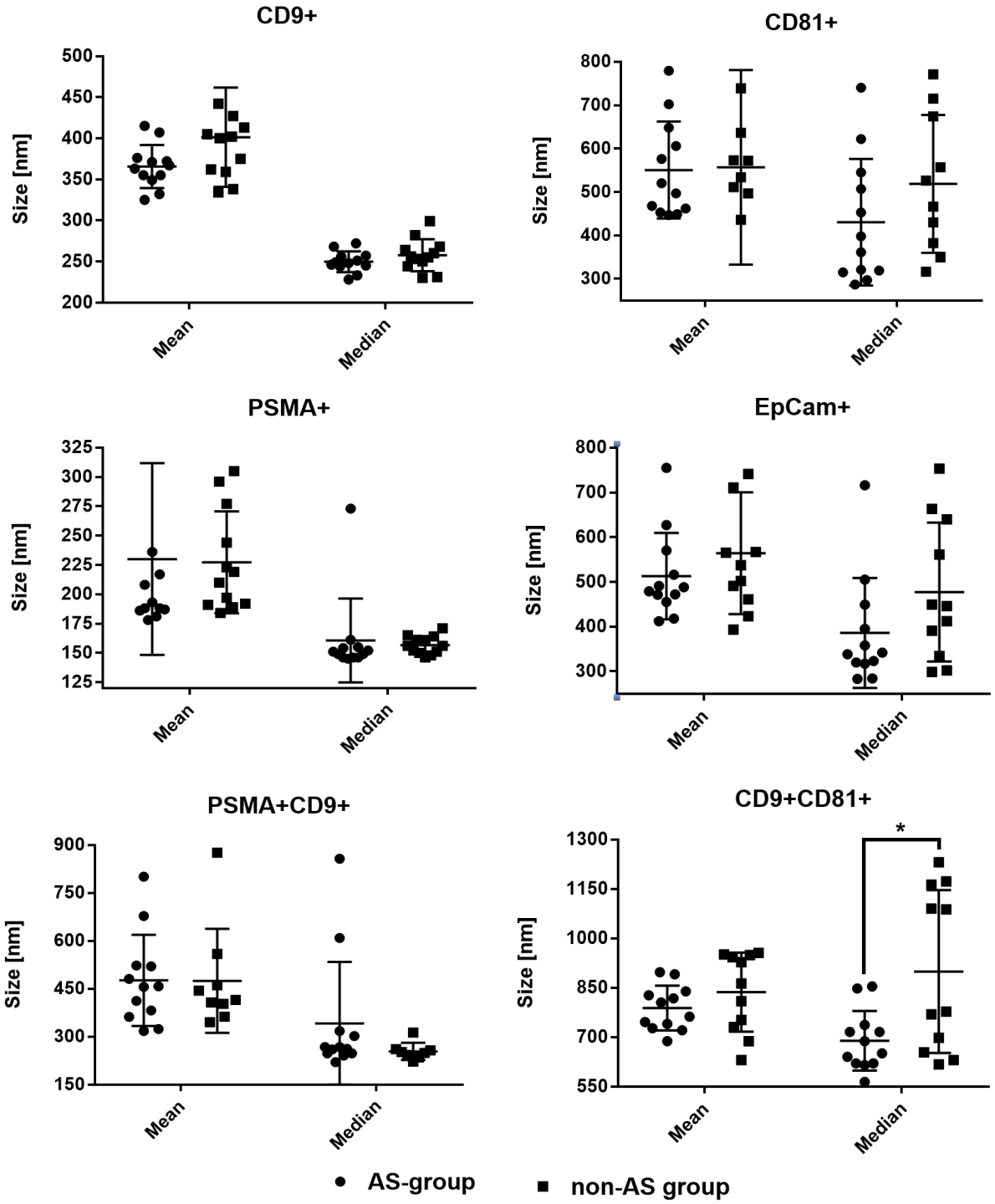


Figure 25 - Summary of size analysis of CD9+, CD81+, PSMA+, EpCam+, PSMA+CD9+, CD9+CD81+ positive populations of plasma mEVs identified with nFCM from Active Surveillance (AS) and non-Active Surveillance (non-AS) prostate patients groups. * p<0,05

4.5 miRNA profile of mEVs from prostate cancer patients

The data obtained from the qPCR analysis of miRNA profile of mEVs was performed. The Quality Control of the data revealed that among used Spike-in controls UniSP2, UniSP4 and UniSP5 had the biggest differences in Ct values among plates, indicating some level of variability on the isolation procedure level. However, cel-miR-39-3p and UniSP6 which were added at the reverse transcription step, showed that no significant variability at the reverse transcription process was not found. Moreover, consistency between two separate wells with cel-miR-39-3p shows consistent and repeatable sample application on the qPCR assay plates (Fig.27).

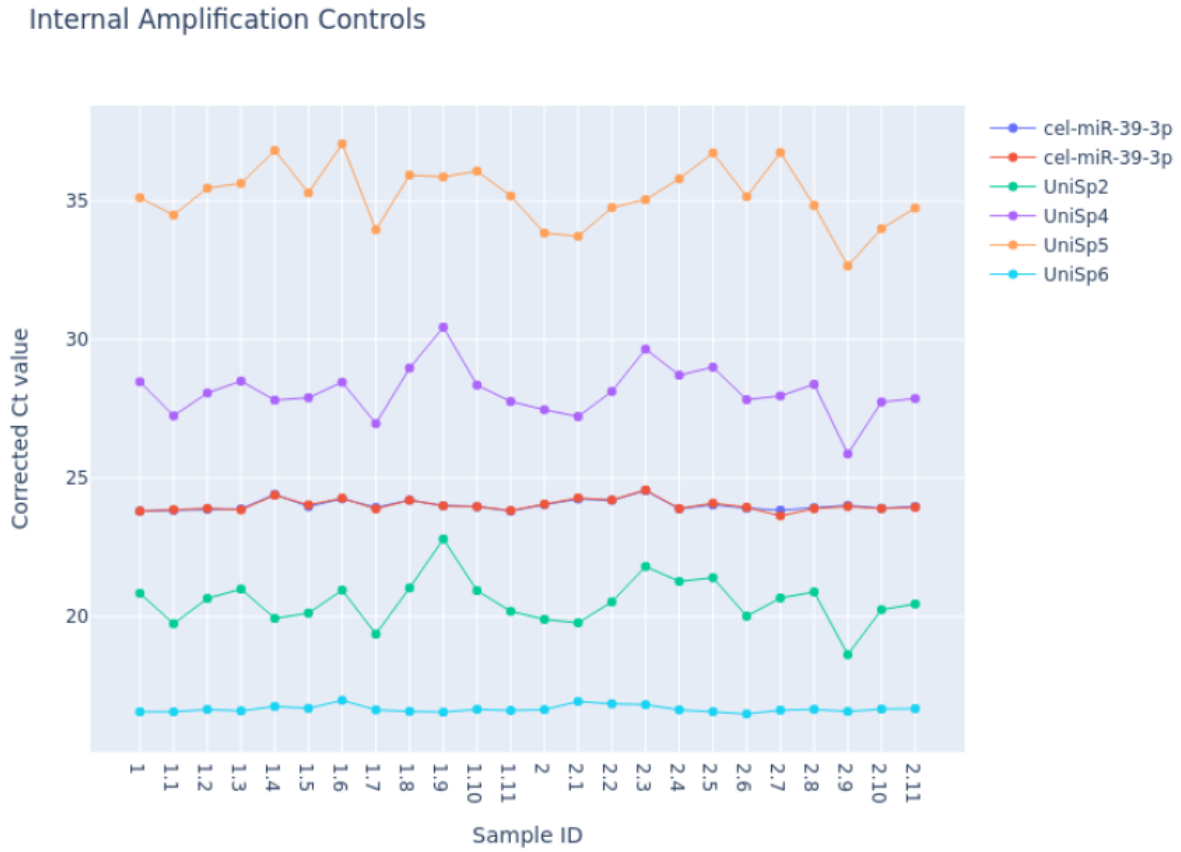


Figure 26 – Results of internal amplification controls comparison between samples, after interplate calibration. Samples 1-1.11 are patients from the Active Surveillance group, and samples from 2-2.11 are patients from the non-Active Surveillance prostate cancer group.

The results of fold change analysis, revealed some miRNA with significantly different levels between AS and non-AS prostate cancer patients. Among significantly higher levels of miRNAs found in non-AS patients samples were: miR-99a-5p (3,42 Fold Change-FC,

p=0,006), miR-125b-5p (2,37 FC, p=0,020), miR-145-5p (2,07 FC, p=0,028) and miR-365a-3p (3,55 FC, p=0,004). Among other potentially significantly higher levels of miRNAs with mean change of >1,5 was found: miR-16-5p (2,28 FC, p=0,940), miR-96-5p (2,05 FC, p=0,478), miR-100-5p (1,80 FC, p=0,289), miR-143-3p (1,52 FC, p=0,147), miR-205-5p (1,53 FC, p=0,169). No significantly lower levels of miRNA were found between compared groups, however several potentially significantly lower levels of miRNAs have been found: miR-17-5p (0,66 FC, p=0,272), miR-135a-5p (0,61 FC, p=0,918), miR-181b-5p (0,64 FC, p=0,816), miR-221-3p (0,46 FC, p=0,421) and miR-375 (0,58 FC, p=0,262) (Tab.4, Fig.28, Fig.29, Fig.30).

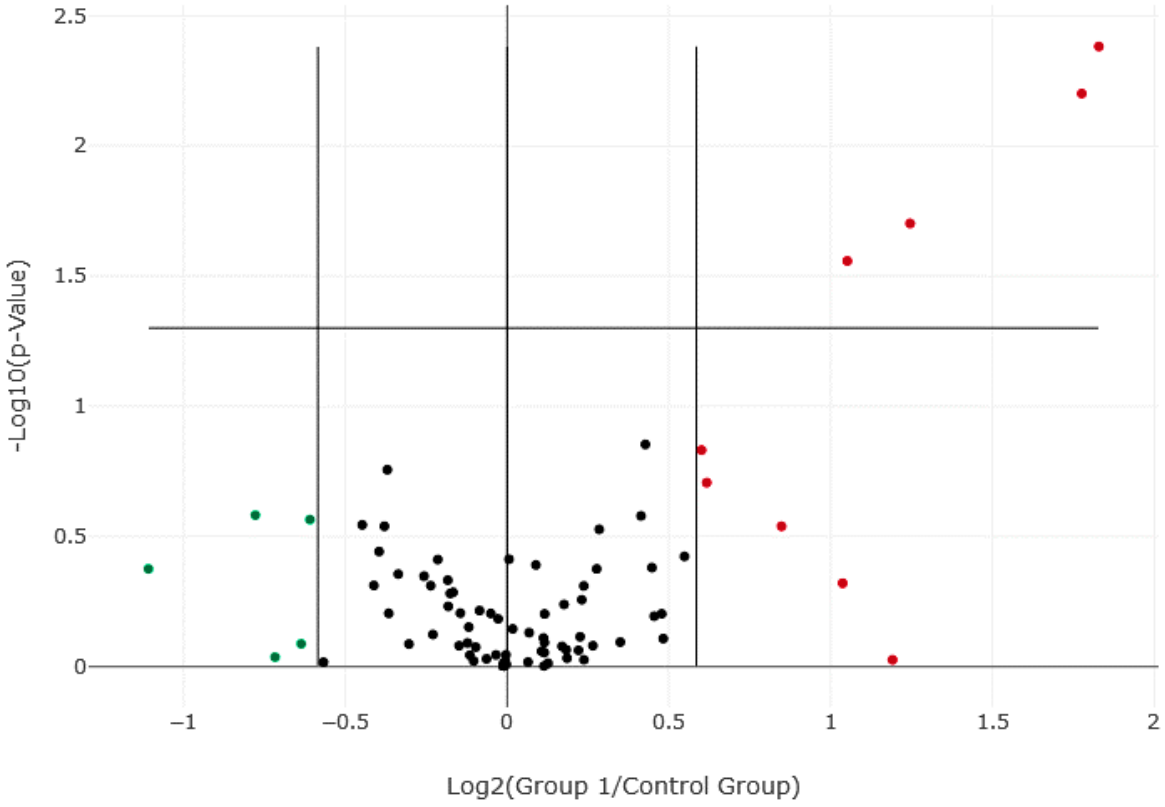


Figure 27 – Volcano plot of fold change and significance of the change in mEVs miRNA between Active Surveillance patients samples and non-Active Surveillance patients samples. The control group are Active Surveillance patients, and Group 1 are non-Active Surveillance prostate cancer patients.

Table 4 – Average fold change for all mEVs miRNA targets analysed with qPCR between Active Surveillance and non-Active Surveillance patients, with p-value of the change.

miRNA	Fold Change	p-value
hsa-let-7a-5p	1.08	0.805646
hsa-let-7b-5p	1.06	0.406172
hsa-let-7c-5p	1.05	0.739343
hsa-let-7f-5p	1.00	0.954911
hsa-miR-100-5p	**1.80**	0.288587
hsa-miR-101-3p	0.86	0.387398
hsa-miR-106b-5p	1.08	0.881903
hsa-miR-125a-5p	1.08	0.992454
hsa-miR-125b-5p	**2.37**	**0.019826**
hsa-miR-126-3p	1.22	0.296434
hsa-miR-126-5p	1.08	0.774966
hsa-miR-128-3p	1.37	0.638867
hsa-miR-133a-3p	1.13	0.575796
hsa-miR-135a-5p	**0.61**	0.917870
hsa-miR-135b-5p	1.14	0.926568
hsa-miR-141-3p	0.77	0.288662
hsa-miR-143-3p	**1.52**	0.147157
hsa-miR-145-5p	**2.07**	**0.027629**
hsa-miR-146a-5p	0.73	0.285108
hsa-miR-146b-5p	0.75	0.486740
hsa-miR-148a-3p	0.89	0.517233
hsa-miR-15a-5p	1.00	0.977190
hsa-miR-15b-5p	0.93	0.948747
hsa-miR-16-5p	**2.28**	0.940044
hsa-miR-17-5p	**0.66**	0.272034
hsa-miR-17-3p	0.67	0.959827
hsa-miR-181a-5p	1.27	0.803949
hsa-miR-181b-5p	**0.64**	0.815949
hsa-miR-182-5p	1.00	0.899546
hsa-miR-183-5p	1.39	0.625322
hsa-miR-184	0.92	0.901522
hsa-miR-194-5p	1.08	0.871256
hsa-miR-195-5p	1.05	0.958283
hsa-miR-196a-5p	1.08	0.626406
hsa-miR-19b-3p	0.85	0.751545
hsa-miR-200b-3p	1.13	0.859850
hsa-miR-200c-3p	0.76	0.360881
hsa-miR-203a-3p	1.20	0.829475
hsa-miR-205-5p	**1.53**	0.196227
hsa-miR-20a-5p	0.93	0.840268

hsa-miR-20b-5p	1.17	0.864485
hsa-miR-21-5p	0.92	0.703649
hsa-miR-218-5p	1.21	0.420399
hsa-miR-22-3p	1.01	0.714701
hsa-miR-221-3p	**0.46**	0.420578
hsa-miR-222-3p	0.98	0.899727
hsa-miR-223-3p	0.81	0.817340
hsa-miR-224-5p	0.85	0.487994
hsa-miR-23b-3p	0.99	0.966851
hsa-miR-24-3p	0.92	0.809955
hsa-miR-25-3p	0.94	0.607932
hsa-miR-26a-5p	1.12	0.834639
hsa-miR-26b-5p	1.18	0.488740
hsa-miR-27a-3p	0.89	0.522936
hsa-miR-27b-3p	0.88	0.586147
hsa-miR-296-5p	0.90	0.829919
hsa-miR-29b-3p	1.00	0.386091
hsa-miR-30c-5p	1.34	0.139948
hsa-miR-31-5p	1.40	0.779504
hsa-miR-3163	0.99	0.990567
hsa-miR-32-5p	1.17	0.766799
hsa-miR-330-3p	1.33	0.263227
hsa-miR-331-3p	0.78	0.623231
hsa-miR-34a-5p	0.88	0.464657
hsa-miR-34b-3p	0.99	0.990567
hsa-miR-34c-5p	0.99	0.990567
hsa-miR-361-5p	0.84	0.448607
hsa-miR-365a-3p	**3.55**	**0.004148**
hsa-miR-3662	0.99	0.990567
hsa-miR-3666	0.99	0.990567
hsa-miR-374b-5p	1.18	0.939169
hsa-miR-375	**0.58**	0.261561
hsa-miR-425-5p	0.90	0.622108
hsa-miR-449a	0.99	0.990567
hsa-miR-455-5p	0.99	0.990567
hsa-miR-494-3p	1.17	0.552809
hsa-miR-616-3p	0.79	0.440405
hsa-miR-7-5p	0.97	0.623861
hsa-miR-9-3p	0.77	0.175003
hsa-miR-92a-3p	0.98	0.654550
hsa-miR-93-5p	1.09	0.968269
hsa-miR-96-5p	**2.05**	0.477524
hsa-miR-99a-5p	**3.42**	**0.006291**
hsa-miR-99b-5p	1.36	0.415988

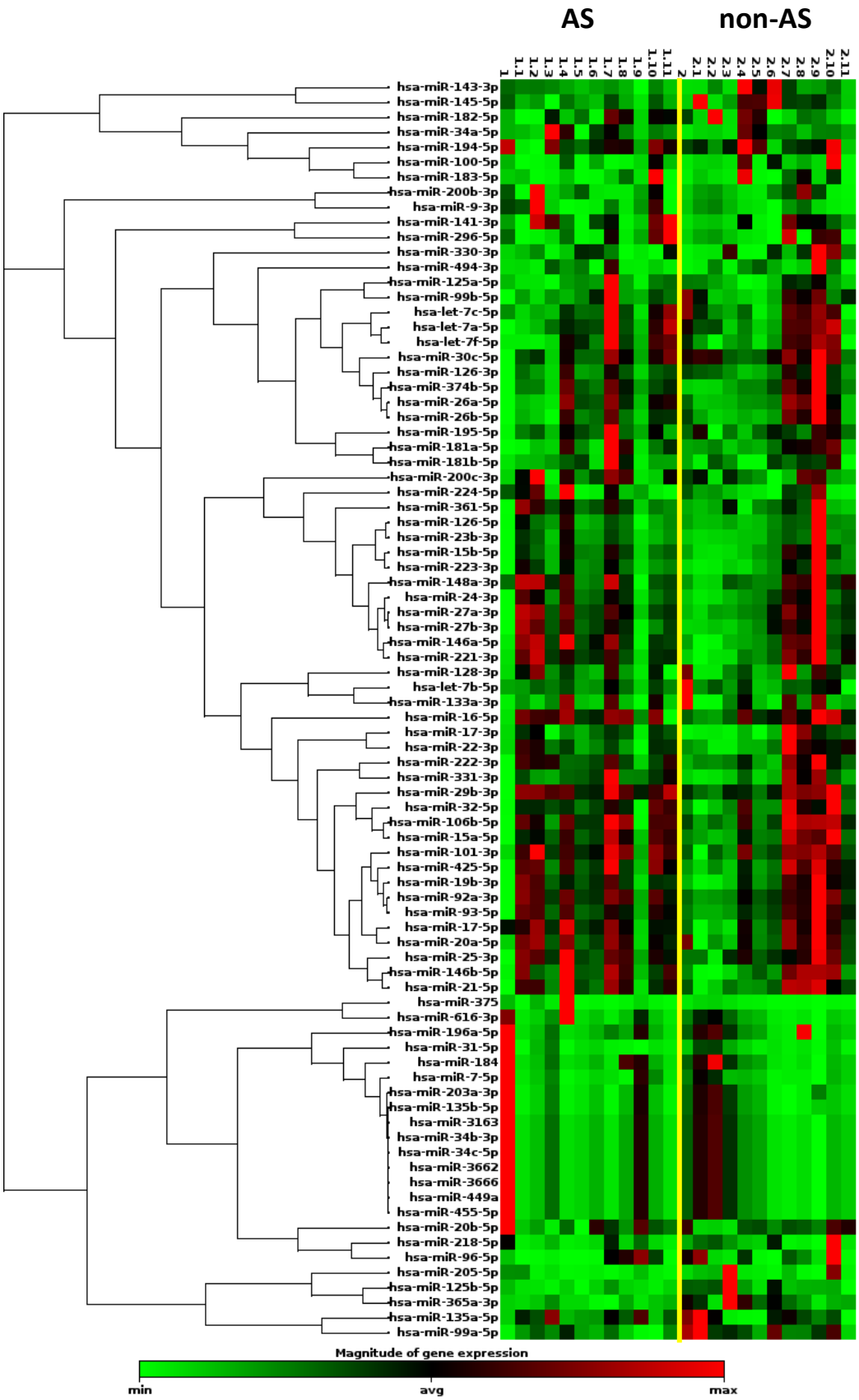


Figure 28 – Heatmap of all of the obtained results from mEVs miRNA analysis . Samples 1-1.11 are patients from Active Surveillance group, samples from 2-2.11 are patients from non-Active Surveillance prostate cancer patients group.

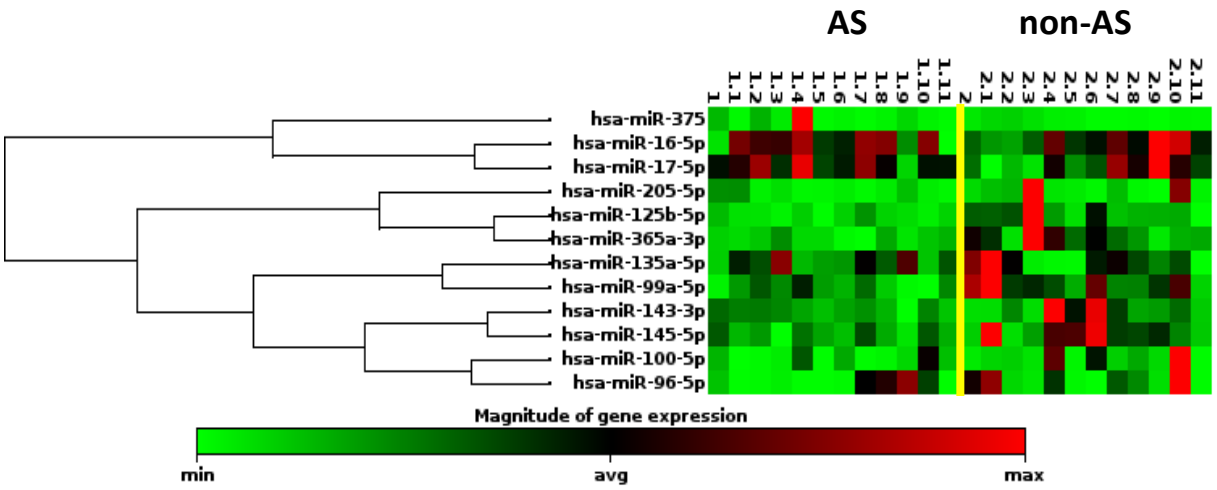


Figure 29 – Heatmap of the significantly and potentially significantly changed miRNA levels between non-Active Surveillance (non-AS) and Active Surveillance (AS) groups. Samples 1-1.11 are patients from Active Surveillance group, samples from 2-2.11 are patients from non-Active Surveillance prostate cancer patients group.

5. Discussion

Prostate Cancer is a global health problem, as it is the second most common type of cancer among men. Thus it is not surprising that much research is focused on resolving the main problems met with proper diagnosis and adoption of the treatment strategy. One of the biggest problems in diagnostical procedures is distinguishing between patients applicable to active surveillance. Currently, active surveillance is based on regular PSA-level testing, which provides unsatisfactory results, MRI scanning and burdensome rebiopsies. As inappropriate risk stratification and analysis of cancer development during active surveillance might result in a risk of life-threatening cancer progression, finding new, non-invasive markers surpassing resolving possibilities of currently used PSA-level is required.

In recent years interest in Extracellular Vesicles (EVs) has been continuously rising. An increase in the quality and quantity of performed studies is undeniable. The analysis of potential diagnostic targets of prostate cancer in sEVs is no exception in this case. The cargos of sEVs that are mainly evaluated are miRNAs and proteins. Moreover, the rise of interest combined with more complex analyses like metabolomics and protein modification patterns analysis is also observed. However, considering the recently emerging evidence indicating the involvement of micropeptides encoded by lncRNAs in the cancer development process, the rise in the analysis of this class of molecules might significantly increase [152].

Nevertheless, recent studies show that not only level miRNA should be considered, but also analysis of their posttranscriptional modifications. For example, it has been shown that methylation of miRNA may compromise the affinity to targeted sequencing, inhibiting their regulatory properties, which also might be a prognostic factor in cancers [153]. The most often sources of sEVs are urine, which has direct contact with cancer cells and serum/plasma. However, some studies show that other sources, like semen, should not be neglected [154, 155]. Regarding the most common methods for sEVs purification, ultracentrifugation and polymer precipitation are taking the lead, reflecting the maintained global trends in EVs analysis protocols [156].

Comparison of the sEVs purification methods from peripheral blood serum and urine

Until now, several studies have been conducted comparing different sEVs purification methods [157-162]. However, most of them focused only on one matrix type or compared

one method with different matrixes, not showing if the selected method may bring the same drawbacks and prerequisites for matrix purification. Furthermore, such an approach makes it harder for overall comparison, as the protocols usually differ at some point, and direct comparison is not possible. Moreover, the effect of the inter-operator differences on the results should not be neglected. The gold standard in sEVs studies is ultracentrifugation with density gradient protocols. However, the requirements for expensive and not standard diagnostic laboratory equipment - ultracentrifuge, the necessity of a well-trained operator, and the lengthy procedure duration limits their potential for translation into everyday diagnostic practice. In our study, methods that do not require extensive equipment than centrifuge with rotor compatible with 2ml tubes, reaching a relative centrifugal force of 14 000*xg* is required. Moreover, for Size Exclusion Chromatography (SEC), a rise of automated fraction collectors provides the possibility of automation and standardization by lowering the operators' influence on the obtained results by applying the sample and mobile phase and lowering the procedure's complexity.

The size of obtained vesicles (~70nm) is within a similar range as the other studies analyzing serum and urine sEVs [157, 159]. Furthermore, the differences in the size of the analyzed vesicles in the precipitation method from serum samples may be caused by the aggregation of smaller particles, as the obtained size distribution is tilted toward the bigger particles' size, with an unchanged Stain Index for the characteristic tetraspanins detection. Surprisingly, no significant differences were found in the total number of isolated vesicles between selected methods, which contradicts the results obtained in other studies but can be caused by different pre-purification protocols used [158, 163]. The lack of difference in the Stain Index for characteristic tetraspanins, except for the CD9 in Urine samples subdued to Immunomagnetic Separation, indicates that the vesicles quantification was correct in the case of tetraspanins-present vesicles. The significantly lower Stain index of CD9 from urine samples might be caused by the blocking of the binding by the remaining anti-CD9 antibody from the magnetic beads used for separation. The results obtained in this study indicate that the precipitation method significantly influences the purity of co-separated proteins and the size of the obtained sEVs. Thus, if the downstream analysis does include protein analysis or can be affected by the presence of the proteins, this method should be avoided. Moreover, if a per-particle analysis is considered, aggregation of the sEVs might also

significantly influence obtained results. However, such a phenomenon is not observed in urine samples, indicating that the choice of the sEVs separation method should also be guided by the used matrix. Summarizing, SEC provided the best results regarding the purity of the obtained samples, with non-inferior efficiency, for both of the analyzed matrices for sEVs purification. However, immunomagnetic separation should not be neglected, especially as it might provide valuable insight into a specific subpopulation of sEVs based on their surface markers, but it should be considered as a secondary technique.

However, this part of the study has several limitations, which might affect the interpretation of the results. One of the limitations of this part of the study is the low detection limit of TRPS, which was around 40nm. Especially concerning the recently discovered new class of extracellular particles – exomeres and supermeres whose average size is <30nm, no information about smaller particles and the influence of selected purification protocols on their numbers is provided. Moreover, the TRPS method used in this study does not allow discrimination of EVs from other types of particles present in the sample. Thus, the calculation might be affected by other types of particles in obtained samples. Additional analysis, including proteomics and lipidomics, for analysis of proteins aggregates, like RNA-Binding proteins (RBPs), Low-density Lipoproteins (LDL) or exomeres and supermeres markers, for proper contamination analysis might have revealed more in-depth results, especially for the precipitation samples, where high protein concentration was discovered. Another limitation is the lack of imaging results, especially in the case of the immobilization of the sEVs on latex aldehyde/sulfate beads part of the study. Imaging results might have shown exactly how the beads were covered in immobilized EVs, and to what extent the immobilisation was consistent on the beads' surface. However, for proper results interpretation, such imaging would have been done with immunoelectron microscopy, where sample preparation might have influenced the immobilization, or with super-resolution fluorescence microscopy. Another limitation is connected to the potential influence of the exact clone of the selected antibodies, as this might have affected the affinity of the antibodies to the selected markers. The final limitation of this part of the study is connected to the fact that selected markers – tetraspanins, were showed not to be present on vesicles released from one of the most numerous cells of a human organism – red blood cells. Because of that, determination of the influence of the selected method of purification

of sEVs on the obtained numbers of EVs is influenced, especially in the case of immunomagnetic separation. Moreover, the analysis of selected subpopulations based on CD9, CD63 and CD81 markers is not complete, as it does not present influence of selected methods on Red Blood Cells-derived sEVs, which might be significant for analysis of bone-metastasis PCa cases.

Considering the aforementioned limitations of these techniques, especially the high protein contaminations in the precipitation method, the results might require better validation to diminish the possibility of influence of co-purified RBP, LDL, supermeres and exomeres or other soluble protein aggregates. Furthermore, as sEVs are very challenging technically and methodologically material, because of their nano-scale size, the proper choice of method of separation and analysis can lead to different results. Especially results of more sophisticated methods like metabolomics analysis or analysis of protein modification patterns are prone to change with different methodologies [164, 165].

nFCM analysis of mEVs

The nFCM analysis of plasma mEVs did not reveal significant differences between Active Surveillance (AS) and non-Active Surveillance patient groups using the single marker or single population analysis. This contradicts the results of some of the previously reported study outcome [166].

PSMA is a cell surface protein present on most of the prostate gland cells. It was shown that PSMA is overexpressed on the surface of PCa cells, and as that might be used as a prognostic predictor [167]. Thus it might be considered a marker for the EVs released specifically by normal and malignant prostate cells. The role of PSMA overexpression in cancer cells physiology is connected with its carboxypeptidase activity, as a mutation on this section of the protein significantly affects cells' invasiveness [168]. The diagnostical potential of PSMA EVs has been evaluated in many studies. One such study was performed by Park *et al.* Their study found a significant difference between PSMA-positive EV levels in BPH, low-, intermediate-, and high-risk patients [166]. However, several differences in the studies' designs might have affected the outcome. First of all, in Park *et al.* study, no per-particle analysis was done, only a quantification of PSMA level by ELISA. This fact, connected with the precipitation method used for EVs preparation, might have altered the results

by quantification of not EVs carried PSMA. Moreover, results obtained in the study of Park *et al.* compared to this study were not limited by the low detection limit of the used techniques, thus might bring different outcomes. This possibility is also encouraged by the median size of PSMA-positive EVs, which was ~150nm, close to the theoretical low limit of detection. On the other hand, a study by Joncas *et al.* analyzed plasma sEVs PSMA-positive population, using nFCM, from localized PCa and Castrate-Resistant PCa (CRPC) patients. Their study used a more specialized machine for nFCM, with lower detection limits. However, no difference in PSMA-positive EVs number was also noted - similar to results obtained in this study, where a bigger EVs population was analysed [169]. Thus PSMA-positive mEVs percentage alone does not seem to provide meaningful information for risk determination for PCa patients. However, the results of the comparison of the ratio of CD9+PSMA+ with total PSMA+ mEVs provided some significant differences, with potential for further investigation.

EpCam is another cell surface marker associated with malignant cells, overexpressed in many adenocarcinomas and squamous cell carcinomas [170]. It was shown that EpCam is directly involved in Epithelial-to-Mesenchymal Transition (EMT), which affects cancer invasiveness and metastatic potential [171]. Up to date, no studies have shown the potential of EpCam-positive EVs from plasma samples of PCa patients. However, a potential of this population of EVs was found for another source of EVs – urine. In the study of Dai *et al*., a potential to distinguish PCa patients from healthy control by EpCam+ vesicles was found [172]. In their study, a higher concentration of EpCam+CD9+ EVs was a predictor of PCa. However, the analysis results for this thesis noted a trend of lower percentage of EpCam+ EVs in non-AS patients compared to AS patients. This trend might suggest a potential route for more in-depth studies of risk analysis for PCa patients.

What needs to be noted is the fact that the possibility to detect EVs in ~120nm, with the potential for further enhancement of this limit, by nFCM with a slightly modified conventional Flow Cytometer, brings possibilities to further enhance the analysis, with the incorporation of FACS sorting, which might provide a substantial alternative to costly immunomagnetic separation.

This part of the study also possesses several limitations that should be addressed. First of the limitation is the low limit of detection, which is ~120nm, where in some cases,

the majority of positive events were found in the surrounding of that level. Thus, some potentially significant EVs might not have met a threshold and were not analyzed with this approach. Another problem that might have influenced obtained results are clones of the antibodies used in this study, as there might be a difference in the affinity of different clones to the selected target proteins. Another potential problem is the limit of the detection of the fluorescence signal, as EVs are such small structures that a single copy of the protein of interest might be seen on their surface. Thus, such low-level signals might have been omitted besides properly aligned and optimized fluorescence channel detectors. One of the potential factors that might have influenced the results obtained in this study is the freezing of the samples, which theoretically might influence some of the populations of EVs or proteins on their surface. Another potential limitation might be a choice of matrix used for obtaining mEVs, as results of the similar panel from urine might have revealed different results. The last limitation is a relatively small group of patients, which might affect some of the results' significance.

miRNA profile analysis of mEVs

The analysis of the miRNA profile of plasma mEVs revealed several significantly altered molecule levels between AS and non-AS prostate cancer patients groups. One of the significantly elevated miRNAs in the non-AS group was miR-99a-5p. This miRNA is thought to be one of the cancer suppressors, as it was found to be downregulated in PCa tissue samples compared to normal tissues. Moreover, it is involved in the proliferation, migration, and invasion potential of PCa cells. Wu *et al.* have found that in DU-145 and PC3 – PCa cell lines, the addition of miR-99a mimetics significantly reduced the proliferation, migration, and colony formation of these cells. Moreover, further investigation showed a decrease in FGFR3 expression in cells exposed to miR-99a mimetics, indicating the potential mechanism of action [173]. Another study showing the potential mechanism of action of miR-99a-5p was performed by Sun *et al*. In their study, lower levels of miR-99a in cell lines that correlated with the aggressiveness of the PCa cell line were found. Moreover, they found that after transfection increasing miR-99a expression, lowering of proliferation with downregulation of PSA production was noted, showing cancer-suppressing properties [174]. Additionally, this miRNA was found to be regulated by androgens presence through IGF1R [175]. The results of this study showed a higher level of miR-99a-5p in non-AS patients EVs than in patients with

AS, which several reasons might cause. One is the active depletion of cancer-suppressing miRNA from the cancer cells using EVs. Such a phenomenon is not new and was already found in breast cancer patients, where lower levels of miR-99a were found in cancer tissue samples, with simultaneously elevated levels found in plasma [176]. Another hypothetical reason might be an attempt of defensive reaction, as organism try to compensate for the inactivating modifications of miR-99a-5p in cancer cells by increasing the expression of this molecule.

miR-125b-5p is another miRNA found significantly elevated in EVs of non-AS patients over the AS prostate cancer patients. This miRNA was also found to play a significant role in PCa. However, there are some contradictions in the reported results. From one side, a set of studies shows that miR-125b plays a pro-oncogenic role. Amir *et al.* have found that miR-125b is involved in proliferation and apoptosis in PCa cells by p53-dependent and p53-independent pathways. In their study, the rise of miR-125b was associated with increased proliferation of PCa cell *in vitro*, and suppression of miR-125b resulted in apoptosis in those cells [177]. A similar effect of miR-125b was found by Shi *et al.* with the animal xenograft model. Analysis of the tumorigenic potential of PCa cells modified for increased miR-125b expression showed significantly bigger tumour mass than non-modified cells. Moreover similar rise of apoptosis was found when miR-125b was suppressed. Moreover, the potential targets were identified, as p53, Puma and Bak1 were found to be downregulated in cells with miR-125b increased levels [178]. On the other hand, a set of studies that presents cancer-suppressing properties of miR-125b might be found. Karadag *et al.* found that induction of miR-125b-5p synthesis in DU-145 cells significantly lowered the proliferation of PCa cells. Additionally, morphological changes in those cells were found. Moreover, induction of apoptosis was found, as Caspase 3 and Caspase 8 levels increased in cells with increased miR-125b-5p. In these cells increased Bax expression with lack of Bcl-2 presence was also noted [179]. A similar PCa tumour-suppressing effect of miR-125b was found by Budd *et al*. Their study indicated a significantly lowered proliferation rate and migration potential of PCa cells transfected for higher miR-125b expression [180]. This miRNA expression also depended on androgens through IGFR1, the exact mechanism as miR-99a-5p [175]. Concerning this contradictory information, the exact reason behind detected higher levels of miR-125b-5p in non-AS prostate cancer patients' EVs is hard to explain. However, the diagnostical potential of these EVs carried miRNA was already found in urine samples. Fredsoe *et al.* have found that

the level of this miRNA in cell-free urine allowed the prediction of biochemical recurrence of PCa patients after RP [181].

Another miRNA with a significantly higher level in non-AS prostate cancer patients was miR-145-5p. This miRNA is well described as the tumour-suppressing molecule in PCa. The tumour-suppression properties were presented in the already-mentioned study of Karadag *et al*. In addition, the lowering proliferation and cell morphology altering effect was found. A potential apoptosis-inducing effect was also noted, as the rise in Caspase-3 and Bax proteins was observed [179]. The proliferation inhibitory effect was also observed in other studies, where a potential connection with SOX-2, PLD5 and WIP1 as the miR-145-5p targets in PCa cells was found [182-184]. The potential cancer suppression properties of miR-145-5p are also connected with the EMT process. In the study of Luo *et al*., the inhibitory effect of miR-145-5p on EMT in PCa cells was found through increasing E-cadherin expression and downregulating MMP-2 and MMP-9. Moreover, a decrease in PCa cells proliferation, migration and invasiveness was also confirmed. The influence on PCa cells was also connected with the potential to lower PCa bone metastasis probability [185]. The influence of miR-145-5p was also observed in neuroendocrine differentiation in PCa. Ji *et al.* have found that decreased levels of miR-145-5p in androgen-sensitive PCa cells are associated with neuroendocrine transdifferentiation. Moreover, they found that the potential signalling pathway of this process is mediated through the downregulation of SOX11/MYCN by this miRNA [186]. The diagnostical potential of miR-145-5p was also found in the analysis of the biopsy tissue samples, predicting GS rising from 6 (3+3) to higher values after confirmation of RP samples. Wang *et al.* have found that underexpression of miR-145-5p in biopsy tissue samples correlated with the possibility of GS upgrading from GS6 to GS7 after RP [187]. Thus, cancer-suppressing properties of miR-145-5p, with an elevated level of this miRNA in plasma EVs of non-AS prostate cancer patients, suggests a potentially similar mechanism as the one suggested for miR-99a-5p. Thus either increased release of EVs rich in miR-145-5p from PCa cells to lower the concentration in cells, or attempt of overcompensation of dysfunctionally modified miRNAs might explain the observed phenomenon.

The last significantly different miRNA between AS and non-AS prostate cancer patients groups is the miR-365a-3p. Not many studies have been performed about its potential

mechanism of action in PCa cells. The only study showing the potential involvement of miR-365a-3p directly in PCa cells was not directly targeted towards an analysis of this molecule but regarding the effect of LINC00641 on PCa cells. In this study, Liu *et al*. found that miR-365a-3p has been elevated in PCa cells with elevated LINC00641 expression. Further studies have shown that overexpression of miR-365a-3p in PCa cells was causing a significant rise in proliferation rate and increased migration and invasion potential. The potential target of miR-365a-3p responsible for this fact was VGLL4, as it was found to be downregulated when the expression of miR-365a-3p was artificially increased [188]. This miRNA was also already proven diagnostically useful in serum samples of PCa patients. In the study of Lyu *et al.*, miR-365a-3p, together with miR-4286, miR-424-5p, miR-27a-3p, and miR-29b-3p was proven to be upregulated in PCa patients, distinguishing between BPH and PCa patients with 78,95% sensitivity, and 92,21% specificity [189]. Analysis performed in this study also found that this miRNA is significantly elevated in EVs of non-AS compared to the AS prostate patients group.

Besides the miRNAs found significantly differentially present in plasma mEVs, some of the miRNAs levels were highly altered between AS and non-AS prostate cancer patients, but did not reach significance level, probably because of the limited group size.

One of the highly upregulated, in the non-AS prostate cancer patients' group, miRNA was miR-96-5p. This miRNA was found to possess a tumour-promoting effect and is overexpressed in malignant prostate tissues compared to a healthy gland or BPH samples [190, 191]. As for the mechanism of action, several potential pathways were found. In their study, Lian et al. found that miR-96-5p is involved in the significant downregulation of NDRG1 expression. Their *in vitro* and *in vivo* studies have shown that with the downregulation of NDRG1, a rise in migration, invasion, EMT rate and metastasis potential is observed in PCa cells [192]. Another pathway connected with EMT in which miR-96-5p is affecting PCa cells was found by Li *et al*. Their study found that miR-96-5p is actively silencing the expression of AJAP1 – a protein with the tumour suppression properties, controlling the EMT process. Suppression of miR-96-5p by lncRNA FGF14-AS2 declined the tumorigenesis potential of PCa cells in the xenograft model [193]. Other pathways identified to be altered by miR-96-5p in PCa cells were mTOR and VEGF signalling. Gujrati *et al.* have found upregulated levels of miR-96-5p in the African American population of PCa patients. Their further *in vitro* functionality study revealed that miR-96-5p was involved in significant downregulation

of MAPKAPK2, which results in higher proliferation potential of PCa cells compared to not repressed MAPKAPK2 cells [194]. Moreover, elevated tissue level of miR-96-5p was a significant indicator of lymphatic dissemination in locally advanced PCa patients [195]. Moreover, serum miR-96-5p was already proven as a potential biomarker, together with the miR-365a-3p mentioned above, allowing for sensitive a specific determination of PCa and BPH patients [189].

Another potentially significantly altered miRNA was miR-100-5p. This miRNA is another example of a molecule with contradictory roles found in PCa. On the one hand, potential cancer progression and therapy resistance role was found. Nabavi *et al.* have found that silencing miR-100-5p resulted in the promotion of apoptosis, and sensitization to androgen-deprivation therapy in PCa cells, showing the potential for promotion of CRPC development by this miRNA [196]. On the other hand, the miR-100-5p effect on the chemoresistance of PCa was also found. Samli *et al.* have found that among both androgen-dependent and independent paclitaxel-resistant PCa cells, elevated levels of miR-100-5p were found compared to wild-type cells [197]. However, the study of Damodaran *et al*. has found significantly lower levels of miR-100 in PCa tissue samples compared to BPH ones [198]. On the other hand, some of the studies show that miR-100-5p might act as a tumour-suppressing factor in PCa. Ye *et al*. found that miR-100-5p levels were higher in BPH serum samples and non-malignant prostate cell lines than in serum of PCa patients and PCa cell lines. Moreover, *in vitro* and mouse model studies have shown that miR-100-5p inhibition has raised the proliferation, migration, and tumour growth potential. As a potential pathway, downregulation of the mTOR pathway was found [199]. These results contradict the results obtained in this thesis, as elevated levels were found in the non-AS group. Considering ambiguous reports describing miR-100-5p role in PCa indicates necessity of more functional research about this miRNA.

miR-143-3p is another potentially significant miRNA with elevated plasma EVs levels in non-AS prostate cancer patients. This miRNA is thought to be one of the suppressors of PCa development. Zhang *et al.* have found that miR-143-3p is involved in CRPC cells migration, invasion and proliferation potential. They have found that expression of this miRNA is regulated by glucocorticoid receptor, which is known to be resistant to ADT. Additionally, they found that overexpression of this miRNA reduced the potential for proliferation and

migration and raised the apoptosis level of CRPC cells. Moreover, with the mouse xenograft model, an elevated level of miR-143-3p was found to lower EMT and tumorigenesis of CRPC cells significantly. As a potential pathway involved in this effect JAG1/NOTCH2 axis was found [200]. A very similar effect of the miR-143-3p expression on malignant properties of PCa cells was found by Zeng *et al.* However, another pathway was noted to be responsible for this effect, as they identified significantly downregulated TAOK2, known for its involvement in cancer aggressiveness promotion [201]. This miRNA was already found to have diagnostic potential in some cases of PCa. Guo *et al.* have found that in patients with GS7 (3+4) patients, the tissue level of miR-143-3p was able to distinguish patients with bone metastasis and those without bone metastasis, as a level of this miRNA was significantly lower in metastatic patients [202]. This miRNA was also already analysed in EVs of PCa patients, but another source was used for obtaining EVs. Rodriguez *et al.* have found that miR-143-3p was significantly downregulated in urine sEVs of PCa patients compared to healthy control. Compared to results obtained within this thesis, these results might suggest that the same miRNAs might have different distributions in EVs from different sources [203].

The last of potentially upregulated miRNA in plasma EVs of non-AS patients is miR-205-5p. That miRNA is also found to have cancer-suppression properties in PCa cells. Yamada *et al*. have found that one of the pathways of miR-205-5p in PCa suppression action is connected with its downregulating effect on HMGB3. This protein's expression was correlated with PCa cells aggressiveness, and studies of PCa tissue samples revealed overexpression of HMGB3 CRPC and non-CRPC cases [204]. Another potential pathway of miR-205-5p was found by Li *et al.*, as they found that this miRNA affects the proliferation and migration of PCa cells by involvement in the regulation of ZEB1 – one of the main promoters of the EMT process [205]. Another study also confirmed that miR-205-5p is responsible for the inhibiting proliferation, induction of apoptosis and cell cycle arrest of PCa cells, by silencing ErBB3 responsible for the regulation of p-PI3K and p-AKT proteins [206]. Diagnostic potential miR-205-5p was also already found. Ghorbanmehr *et al.* have found that this miRNA level is significantly lower in the urine of PCa patients compared to healthy controls. Interestingly a similar effect was found in the urine of bladder cancer patients, showing wide diagnostic possibilities for analysis of alterations of this miRNA [207].

In the case of miR-205-5p, similar differences in results obtained within this thesis and reported changes in urine samples are found as for the miR-143-3p mentioned above.

Not only potentially significant upregulation but also downregulation of miRNAs in plasma mEVs was found in non-AS prostate cancer patients samples. One of the potentially significantly downregulated molecules was miR-17-5p. This miRNA is thought to be one of the cancer-promoting miRNAs in PCa. The elevated level of this miRNA in the tumour epithelium was found to be a poor prognosis factor in PCa patients. Moreover, *in vitro* studies have shown that increased expression of this miRNA caused a rise in proliferation and migration potential in androgen-independent PCa cells [208]. Few potential pathways affected in PCa cells by this miRNA were found to be potentially responsible for this effect. Yang et al. found one of the potential pathways with TIMP3 as the miR-17-5p as a target. In the *in vitro* and mouse model studies, they found that the miR-17-5p effect on aggressiveness characteristics of PCa cells was caused by suppression of TIMP3, one of the metalloproteinases regulators [209]. Another potential mechanism of action of miR-17-5p was altering the expression of PCAF - p300/CBP-associated factor. Gong *et al.* have found that alteration of miR-17-5p resulted in suppression of PCAF and by that altering influence of androgens on PCa cells, like dihydrotestosterone induced increase of PSA production and cell growth promotion [210]. Another identified target of miR-17-5p in PCa cells is PTEN. Dhar *et al*. have found that this miRNA is significantly downregulating PTEN, one of the often deregulated proteins in PCa cells. Restoration of PTEN expression in miR-17-5p overexpressing PCa cells attenuated tumour growth caused by PCa cells in mouse models [211].

Another potentially downregulated miRNA in plasma mEVs of non-AS prostate cancer patients was miR-221-3p. For this miRNA, also contradictory roles in PCa were reported. On the one hand, miR-221-3p was significantly downregulated in PCa samples compared to BPH. Increasing expression of this miRNA caused a decrease in proliferation and migration with a rise in apoptosis, like in miR-125b-5p, as mentioned above, and miR-145-5p [179]. On the other hand, many studies report the pro-malignant effect of this miRNA. Krebs *et al.* have found that miR-221-3p is involved in the proangiogenic mechanism of PCa progression. Their study found direct targeting of the VEGFR-2 receptor by miR-125b-5p, inducing its downregulation. This effect was also responsible for the tyrosine kinase inhibitors-treatment escape of PCa cells [212]. Another pathway altered by miR-221-3p in PCa cells

is the Wnt pathway. Liu *et al*. found that upregulation of miR-221-3p expression increased the proliferation and invasion potential of PCa cells. Further studies have shown that this miRNA is downregulating CDKN1B resulting in C-myc level alteration and rise of stemness of PCa cells [213].

The last of potentially significantly downregulated miRNAs in non-AS prostate cancer patients' plasma mEVs is miR-375-3p. This miRNA was found to be possibly associated with a level of aforementioned miR-221-3p, as both of their levels were found to decrease in PCa tissue over time of PCa development in the TRAMP mouse model [214]. miR-375-3p was already found to have diagnostic and prognostic potential in PCa. Zedan *et al*. found that this miRNA level in plasma samples of CRPC patients correlated with response to treatment, lowering when docetaxel treatment was initiated and rising when radiological progression was observed [215]. A similar rise in the progression of PCa was found by Bidarra *et al.,* where higher levels of miR-375-3p were found in plasma samples of metastatic patients compared to less advanced PCa patients samples [216]. Another study which showed significantly higher plasma levels of mir-375-3p in PCa cancer patients was performed by Abramovic *et al.* In their study analysis of blood plasma, significantly higher miR-375-3p levels were found in PCa patients compared to BPH patients samples [217]. However, this miRNA is found to be altered not only in the bloodstream of PCa patients, as Lekchnov *et al.* have found that elevated levels of miR-375-3p was found in urine samples of PCa patients compared to healthy controls [218]. In the results obtained in this thesis, a contradictive, potential downregulation of this miRNA was found in the non-AS patients' group, which might indicate that mEVs are not a carrier in the case of miR-375-3p released from PCa cells into the bloodstream.

Summarizing the results of miRNA profiling of plasma mEVs, shows that the potential biological background behind these changes might differ, as elevated levels of both cancer-suppressing and cancer-promoting molecules were found. This effect might be caused by either attempt of PCa cells to escape the suppressing effect by depletion of miRNAs that would affect the progress of the malignancy or an attempt of the organism to compensate for the number of miRNAs that might get inactivated by functional modification inside the PCa cells. The rise of cancer-promoting molecules might suggest the risk of progression of malignancy from localized to spread disease, as they might be involved in forming a pre-metastatic niche.

The primary limitations, including this study section, are focused on its pilot-study nature. One of the reasons that, in many cases, no products were found might be caused by insufficient material to be detected. For example, the addition of a higher amount of templates might have shown different results and detection of these targets. Another potential limitation is connected with a small group of patients, as in some cases, the potential for significant differences between groups might have been affected by a lack of enough sample sizes. Finally, the potential limitation also lines in the quantitative nature of the selected method of detection, as no details about the functional state of the molecules, in terms of modifications, for example, methylation or hydroxymethylation, affecting their binding to the targets' potential were performed in this study.

Future frontiers – the therapeutic potential of EVs in PCa

The second significant branch of research about EVs, despite diagnostical purposes, is finding possible applications of EVs in cancer treatment. Research teams took several pathways. One of them is using EVs as a target of the therapy. This approach is strictly connected with hypothesized EVs involvement mechanisms in cancer progression and metastasis. Some molecules carried by EVs are thought to have dual diagnostical and treatment target potential. One such study conducted by Hasegawa *et al.* revealed that not only miR-888-5p level has been elevated in the urine of patients with high-grade PCa. The *in vitro* study showed that when miR-888-5p was artificially overexpressed in PCa cells, a significantly higher proliferation, migration and colony formation of the cells was observed. Moreover, their experiment on the mice model showed that modified PCa cells, overexpressing miR-888-5p caused the development of significantly bigger tumours compared to non-modified cells [219]. Another dual-potential biomarker carried by EVs was circ_0044516. In the study, Li *et al*. not only have they found that this circular RNA was elevated not only in prostate cancerous tissue samples but also elevated in blood sEVs of PCa patients compared to healthy control samples. Their further functional *in vitro* analysis revealed that suppression of this circular RNA in PCa cell lines inhibited proliferation and lower migration. Moreover, the potential interaction with miR-29a-3p was found to be one of the potential mechanisms of action of circ_0044516 [220]. However, such dual-potential is not exclusive to RNA molecules, as Krishn *et al*. have found αvβ3 integrin found in sEVs to have such properties. Their study found that this integrin is selectively found in plasma

PSMA sEVs from PCa patients. Moreover, their functional studies with mice models have shown the involvement of sEVs carried αvβ3 integrin in adhesion, invasion, immune escape and neovascularisation of PCa tumour cells [221].

Another branch, in its foundations, uses the exact mechanism of relatively easy integration of sEVs with recipient cells. However, the main target of this approach is to either create artificial vesicles with anticancer drugs as cargo for better, more targetable, chemotherapeutic distribution, modify cells to pack specified molecules (siRNA, lncRNA, etc.) to the targeted cells, or sensitize them to currently inefficient therapy [222, 223]. Many efforts are also taken to analyze the effect and mechanism of action of Mesenchymal Stem/Stromal Cells (MSCs) released sEVs on cancer cells. The mechanism of MSC-derived sEVs is controversial and considered to be contradictory. On the one hand, many data show enhancement of cancer cell proliferation, increased angiogenesis, and metastasis by MSC-derived sEVs. However, other studies show the tumour suppression activity of MSC-derived sEVs and great potential for manipulating the cargo they are carrying. Despite many efforts, MSC-derived sEVs are still controversial and require more knowledge before proper clinical application in cancer treatment [224].

Much work is still required to define standards and raise awareness of the proper quality of analysis methods among researchers, like the Guidelines of the International Society for Extracellular Vesicles and taskforces for specific applications. However, many studies lack at least one method for purified vesicle size and concentration analysis. Not a small number of such manuscripts show that proper methodology implementation in EVs studies is not yet established. Another often occurring problem is the lack of or feeble description of EVs purification/separation methods, which can significantly influence the results obtained by researchers. There is a disproportion between clinical and biological approaches for obtained results interpretation. From a clinical-diagnostical point of view, even if the selected method does incorporate any uncertainty, whether sEVs carry the biomarker, or another part of biofluids, like RBPs or freely dispersed proteins, it does not matter, as long as it gives essential clinical data and allows proper diagnosis. From the other point, correctly understanding markers' biogenesis is necessary to take advantage of and propose new prophylactic or therapeutic options. The EVs are an inseparable part of cancer cells. The research analyzing them will undoubtedly bring even more understanding of the

pathomechanism of cancer and new diagnostic and therapeutic targets. The relative ease of obtaining biofluids rich in sEVs (blood/urine) makes sEVs an essential part of the liquid biopsy approach for UTC diagnosis and has already led to the creation of commercially available tests like ExoDx™ Prostate EPI-CE (Exosome Diagnostics GmbH; Germany).

Summarizing, results obtained in this thesis have confirmed that choice of purification methods for small Extracellular Vesicles from peripheral blood serum and urine is a critical step, as significant differences in size and impurities levels are detected. However, as the obtained differences were not consistent between serum and urine origining samples, the method selection should also be dictated by the selected small Extracellular Vesicles source. Moreover, the diagnostic potential for utilization of nFCM analysis and miRNAs level detection of mEVs for Prostate Cancer patients was observed. For the first time, the diagnostic potential of miR-99a-5p and miR-145-5p in the blood liquid biopsy for PCa was presented. In the case of miR-125b-5p upregulation in plasma mEVs, contrary to previous results of downregulation of this miRNA in whole plasma samples of more aggressive PCa patients, indicates that differential analysis of biofluids components is critically important, as it may lead to different conclusions. As in both analyses, a set of differentially detected molecules provided better distinguishing between AS and non-AS PCa patients than currently utilized PSA, which might provide a significantly better alternative for the stratification of Active Surveillance patients.

6. Conclusions

1. Peripheral blood serum and urine of prostate cancer patients using proper purification methods can be considered feasible sources of sEVs.
2. Compared sEVs purification methods: Precipitation, Size Exclusion Chromatography, and Immunomagnetic Separation differentially affect the sEVs size, protein contamination and tetraspanins presence.
3. Size Exclusion Chromatography presents the best results in the case of protein contamination of obtained sEVs from both peripheral blood serum and urine compared to other methods.
4. Precipitation is not an optimal method for sEVs purification from peripheral blood serum, as it significantly increases the obtained EVs size and is connected with the highest concentration of protein impurities compared to other methods.
5. The Immunomagnetic Separation method should be considered cautiously for the purification of sEVs from urine for further surface marker analysis e.g. using nanoFlow Cytometry, as it can negatively affect the detection of surface markers blocked by magnetic beads.
6. The method of sEVs purification should be guided by the biofluid used as a source of sEVs and further planned analyses.
7. Peripheral blood plasma of prostate cancer patients can be a source of PSMA+ mEVs secreted by the prostate gland cells for further studies.
8. nanoFlow Cytometry analysis of peripheral blood plasma mEV surface markers and miRNA profiling provides a new, potentially better non-invasive alternative for PSA measurements to stratify the risk of prostate cancer progression during active surveillance. Among analyzed potential markers PSMA+/PSMA+CD9+ ratio and upregulation of miR-99a-5p, miR-125b-5p, miR-145-5p and miR365a-3p presented the most promising results that need to be confirmed with a bigger group of patients.

7. Summary

Prostate Cancer is the second most common type of cancer among men nowadays. One of the biggest challenges to overcome is proper diagnosis and risk stratification, as currently used PSA-level-based methods do not provide satisfactory results for Active Surveillance (AS) approach decision making. Extracellular Vesicles (EVs) are membranous nano-sized vesicles released by all types of cells. One of their most interesting features is that they can carry various classes of molecules (proteins, DNAs, RNAs, metabolites, lipids). As it was proven that EVs cargo might be successfully analyzed despite their nano-sized nature, they are a potentially interesting material for diagnostical procedures of Prostate Cancer.

The aim of this study was an analysis of the small EVs purification method from the serum and urine of the prostate cancer patients for further investigation of the potential molecules that might serve as biomarkers. In addition, another aim was an analysis of the surface markers and miRNA profile of the medium-sized plasma EVs (mEVs) for the potential of distinction between patients classified for AS and not classified for AS, because of aggressive cancer characteristics, based on the histopathological Gleason Score results.

The study included 39 Prostate Cancer patients. Among those, 15 patients were recruited for the comparison of sEVs purification methods and 24 for analysis of medium-sized plasma EVs (mEVs) diagnostic potential for AS risk stratification. For the comparison of sEVs purification methods: precipitation, size exclusion chromatography (SEC) and immunomagnetic separations (ImSep) were chosen. For the analysis of the diagnostic potential of mEVs for AS risk stratification, surface markers: CD9, CD81, PSMA and EpCam, were analyzed with nanoFlow Cytometry, and the miRNA profile of mEVs was checked.

The comparison of the purification protocols for sEVs from serum revealed that the precipitation method significantly affects the size of the obtained sEVs. Moreover, samples obtained from serum by precipitation provided much higher protein contamination than other approaches. In the case of urine, no significant differences were found besides the lower number of CD9-positive sEVs from the ImSep method. The analysis of plasma mEVs surface markers revealed that a ratio of PSMA+ EVs to PSMA+CD9+ EVs provided a significant difference ($p<0,001$) between the AS and non-AS patients. Analysis of the miRNA profile of plasma mEVs revealed that miR-99a-5p ($p<0,01$), miR-125b-5p ($p<0,05$), miR-145-5p

(p<0,05) and miR-365a-3p (p<0,01) level was significantly higher for non-AS classifying prostate cancer patients.

In conclusion peripheral blood serum and urine of prostate cancer patients using proper purification methods can be considered feasible sources of sEVs. Compared sEVs purification methods: Precipitation, SEC, and ImSep differentially affect the sEVs size, protein contamination and tetraspanins presence. SEC presents the best results in the case of protein contamination of obtained sEVs from both peripheral blood serum and urine compared to other methods. The method of sEVs purification should be guided by the biofluid used as a source of sEVs and further planned analyses. Peripheral blood plasma of prostate cancer patients can be a source of PSMA+ mEVs secreted by the prostate gland cells for further studies. nanoFlow Cytometry analysis of peripheral blood plasma mEV surface markers and miRNA profiling provides a new, potentially better non-invasive alternative for PSA measurements to stratify the risk of prostate cancer progression during active surveillance. Among analyzed potential markers PSMA+/PSMA+CD9+ ratio and upregulation of miR-99a-5p, miR-125b-5p, miR-145-5p and miR365a-3p presented the most promising results that need to be confirmed with a bigger group of patients.

8. Streszczenie

Nowotwory prostaty są obecnie drugim najczęściej występującym typem nowotworu wśród mężczyzn na świecie. Jednym z największych wyzwań jest właściwa diagnostyka i stratyfikacja ryzyka, ponieważ obecnie stosowane metody, oparte na badaniu poziomu PSA nie dają zadowalających wyników. Pęcherzyki zewnątrzkomórkowe (ang. Extracellular Vesicles - EVs) to błoniaste pęcherzyki wielkości nanometrów, uwalniane przez wszystkie typy komórek. Jedną z ich najciekawszych cech jest zdolność przenoszenia różnej klasy cząsteczek (białka, DNA, RNA, metabolity, lipidy). Ponieważ udowodniono, że ładunek zawarty wewnątrz EVs może być z powodzeniem analizowany pomimo ich niewielkiego rozmiaru, są one potencjalnie interesującym materiałem do diagnostyki nowotworów prostaty.

Celem niniejszej pracy było porównanie wybranych metod oczyszczania małych pęcherzyków zewnątrzkomórkowych (ang. Small Extracellular Vesicles – sEVs) z surowicy i moczu pacjentów z zdiagnozowanymi nowotworami prostaty, w celu doboru metody do dalszych badań nad diagnostycznym potencjałem tych cząsteczek. Kolejnym celem była analiza markerów powierzchniowych i profilu miRNA średniej wielkości EVs (ang. Medium-sized Extracellular Vesicles - mEV) pod kątem możliwości rozróżnienia pacjentów zaklasyfikowanych do aktywnego nadzoru od pacjentów nie kwalifikujących się do aktywnego nadzoru, ze względu na zbyt agresywną charakterystykę nowotworu na podstawie uzyskanego w skali Gleasona wyniku analiz histopatologicznych.

Do badania włączono 39 pacjentów z nowotworami prostaty. Spośród nich 15 pacjentów zakwalifikowano do badania dotyczącego porównania metod oczyszczania sEVs, a 24 pacjentów włączono do analizy potencjału diagnostycznego pochodzących z osocza mEVs do oceny ryzyka w strategii aktywnego nadzoru. W ramach porównania metod oczyszczania sEV przenalizowano następujące metody: wytrącanie, chromatografię wykluczania rozmiaru i separację immunomagnetyczną. W celu analizy potencjału diagnostycznego mEVs do stratyfikacji ryzyka agresywności nowotworów prostaty, przeprowadzono analizę markerów powierzchniowych za pomocą nano-cytometrii przepływowej oraz sprawdzono profil miRNA mEVs.

Surowica krwi obwodowej i mocz pacjentów z rakiem prostaty przy użyciu odpowiednich metod oczyszczania mogą stanowić łatwo dostępne źródła pęcherzyków sEVs. Porównywane metody oczyszczania sEVs: wytrącanie, chromatografia wykluczania rozmiaru i separacja immunomagnetyczna w różny sposób wpływają na wielkość sEVs, zanieczyszczenie

białkami i obecność tetraspanin. Chromatografia wykluczania rozmiaru daje najlepsze wyniki w przypadku zanieczyszczeń białkowego, zarówno z surowicy krwi obwodowej, jak i moczu w porównaniu z innymi metodami. Precypitacja nie jest optymalną metodą oczyszczania sEVs z surowicy krwi obwodowej, ponieważ znacznie zwiększa rozmiar uzyskiwanych sEVs oraz skutkuje najwyższym stężeniem zanieczyszczeń białkowych w porównaniu z innymi metodami. Przy doborze metody oczyszczania sEVs należy kierować się wybranym źródłem pęcherzyków sEVs oraz dalszymi planowanymi analizami. Osocze krwi obwodowej pacjentów z rakiem prostaty może być źródłem pęcherzyków mEVs PSMA+ wydzielanych przez komórki gruczołu krokowego, do dalszych celów badawczych. Cytometryczna analiza nanoFlow markerów powierzchniowych mEVs w osoczu krwi obwodowej i profilowanie miRNA zapewnia nową, potencjalnie lepszą, nieinwazyjną alternatywę dla pomiarów PSA w celu stratyfikacji ryzyka progresji raka prostaty podczas aktywnego nadzoru. Wśród analizowanych potencjalnych markerów stosunek PSMA+/PSMA+CD9+ oraz zwiększona zawartość miR-99a-5p, miR-125b-5p, miR-145-5p i miR365a-3p dały najbardziej obiecujące wyniki, które wymagają potwierdzenia na większej grupie pacjentów.

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10. List of abbreviations

ADT – Androgen Deprivation Therapy

AKT – AKT Serine/Threonine Kinase 1

Alix - ALG-2-interacting protein X

ApoBDs – Apoptotic Bodies

AS - Active surveillance

BAK1 - BCL2 antagonist/killer 1

BAX- BCL2 Associated X, Apoptosis Regulator

BCL2 - BCL2 Apoptosis Regulator

BPH – Benign Prostate Hyperplasia

CBP - CREB Binding Protein

CDKN1B - Cyclin Dependent Kinase Inhibitor 1B

CHMP1 - Charged Multivesicular Body Protein 1

CHMP3 - Charged Multivesicular Body Protein 3

c-Myc - MYC Proto-Oncogene, bHLH Transcription Factor

CNS – Central Nervous System

CRPC – Castrate Resistant Prostate Cancer

DLS – Digital Light Scattering

DLX1 - Distal-Less Homeobox 1

DRE – Digital Rectal Examination

EAU – European Association of Urology

EMT – Epithelial-Mesenchymal Transition

EpCam - Epithelial Cell Adhesion Molecule

ERG - ETS transcription factor

ErBB3 - erb-b2 Receptor Tyrosine Kinase 3

ESCRT - Endosomal Sorting Complex Required For Transport

EVs – Extracellular Vesicles

Exo – Exosomes

FACS – Fluorescence Activated Cell Sorting

FC – Flow Cytometry

FGF14-AS2 - FGF14 antisense RNA 2

GS – Gleason Score

HMGB3 - High Mobility Group Box 3

HOXC6 - Homeobox C6

IGF1R - Insulin Like Growth Factor 1 Receptor

ILVs – Intraluminal Vesicles

ImSep – Immunomagnetic Separation

ISUP - International Society of Urological Pathology

JAG1 - Jagged Canonical Notch Ligand 1

LDL – Low Density Lipoproteins

LE – Life Expectancy

LIMK1 - LIM domain kinase 1

MAPKAPK2 - MAPK Activated Protein Kinase 2

mEVs – Medium-sized Extracellular Vesicles

MFI – Median Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMP2 - Matrix Metallopeptidase 2

MMP9 - Matrix Metallopeptidase 9

MSCs – Mesenchymal Stem/Stromal Cells

mTOR - Mechanistic Target Of Rapamycin Kinase

MVB – Multivesicular Bodies

MVs – Microvesicles

MYCN - MYCN Proto-Oncogene, bHLH Transcription Factor

NDRG1 - N-myc Downstream Regulated 1

nFCM – nanoFlow Cytometry

NOTCH2 - Notch Receptor 2

NTA – Nano Tracking Analysis

P53 - Tumor Protein P53

PAK2 - p21 (RAC1) activated kinase 2

P300 - E1A Binding Protein p300

PBS – Phosphate-Buffered Saline

PCa – Prostate Cancer

PCA3 – Prostate Cancer Gene 3

PCAF - Lysine Acetyltransferase 2b

PD-L1 - Programmed death-ligand 1

PET - Positron Emission Tomography

PHI – Prostate Health Index

PI3K - Phosphoinositide 3-kinase

PLD5 - Phospholipase D Family Member 5

PUMA - BCL2 binding component 3

PSA – Prostate Specific Antigen

PSMA – Prostate Specific Membrane Antigen

PTEN - Phosphatase And Tensin Homolog

Rab - Ras-associated binding protein

RBPs - RNA Binding Proteins

ROCK1 - Rho associated coiled-coil containing protein kinase 1

RP -Radical Prostatectomy

RT – Room Temperature

SD – Standard Deviation

SEC – Size Exclusion Chromatography

sEVs – Small Extracellular Vesicles

SI – Stain Index

SNARE - SNAP Receptor

SOX2 - SRY-box Transcription Factor 2

SOX11 - SRY-box Transcription Factor 11

SSC - Side Scatter

TAOK2 - TAO kinase 2

TMPRSS2 - Transmembrane Serine Protease 2

TNM – Tumour, Node, Metastatis

TRAMP - Transgenic Adenocarcinoma Of The Mouse Prostate

TRPS – Tuneable Resistive Pulse Sensing

Tsg101 - Tumor Susceptibility 101

WIP1 - Protein Phosphatase, Mg2+/Mn2+ Dependent 1D

VEGF - Vascular Endothelial Growth Factor

VEGFR2 - Kinase Insert Domain Receptor

VGLL4 - Vestigial Like Family Member 4

VPS22 - SNF8 subunit of ESCRT-II

VSP4 - Vacuolar Protein Sorting 4

VSSC – Violet Side Scatter

ZEB1 - Zinc Finger E-Box Binding Homeobox 1

11. List of Tables

12. List of Figures

13. Attachment 1 – Bioethical committee approvals

Uniwersytet Mikołaja Kopernika w Toruniu
Collegium Medicum im L. Rydygiera w Bydgoszczy
KOMISJA BIOETYCZNA
Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 358/2019 Bydgoszcz, 26.03.2019 r.

Działając na podstawie art.29 Ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), zarządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U.Nr 47 poz.480) oraz Zarządzeniem Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **26.03.2019 r.** przeanalizowała wniosek, który złożyła kierownik badania:

dr hab. n. med. Marta Pokrywczyńska
Zakład Medycyny Regeneracyjnej. Bank Komórek i Tkanek
Collegium Medicum w Bydgoszczy

z zespołem w składzie

prof. dr hab. n. med. Tomasz Drewa, dr hab. n. med. Marta Pokrywczyńska, dr n. med. Jan Adamowicz, mgr Kamil Szelisk

w sprawie badania:

„Porównanie metod izolacji małych pęcherzyków zewnątrzkomórkowych z krwi obwodowej i moczu".

Po zapoznaniu się ze złożonym wnioskiem i w wyniku przeprowadzonej dyskusji oraz głosowania Komisja podjęła

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań, w zakresie określonym we wniosku pod warunkiem:

- poinformowania uczestników badania o celu oraz zakresie badań i uzyskania od każdego z nich osobnej, pisemnej, świadomej zgody na udział w badaniu, zgodnie z obowiązującymi przepisami, datowanej najpóźniej na moment rozpoczęcia badania a nie wcześniej niż data uzyskania z Komisji Bioetycznej zgody na takie badanie;
- zachowania tajemnicy wszystkich danych, w tym danych osobowych pacjentów, umożliwiających ich identyfikację w ewentualnych publikacjach;
- zapewnienia, że osoby uczestniczące w eksperymencie badawczym nie są ubezwłasnowolnione, nie są żołnierzami służby zasadniczej, nie są osobami pozbawionymi wolności, nie pozostają w zależności służbowej, dydaktycznej lub innej z prowadzącym badanie;
- sugerujemy uzyskanie podpisu uczestnika badania pod informacją o badaniu, lub sporządzenie formularza informacji i świadomej zgody na udział w badaniu na jednej kartce.

Jednocześnie informujemy, iż „Zgoda na udział w badaniu" winna zawierać m.in.: imię i nazwisko badanej osoby; Nr historii choroby pacjenta (L.ks.gł. Oddziału/Poradni) oraz datę i podpis badanej osoby, a także klauzule, że uczestnik badania wyraża zgodę na przetwarzanie danych osobowych dotyczących realizacji tematu badawczego, z wyjątkiem publikacji danych osobowych.

Kierownik badania zobowiązany jest do przechowywania wszystkich dokumentów dotyczących badania przez okres dwudziestu lat.

Zgoda obowiązuje od daty posiedzenia (26.03.2019 r.) do końca 2020 r.

Wydana opinia dotyczy tylko rozpatrywanego wniosku z uwzględnieniem przedstawionego projektu; każda zmiana i modyfikacja wymaga uzyskania odrębnej opinii. Wnioskodawca zobowiązany jest do informowania o wszelkich poprawkach, które mogłyby mieć wpływ na opinię Komisji oraz poinformowania o zakończeniu badania.

Od niniejszej uchwały podmiot zamierzający przeprowadzić eksperyment medyczny, kierownik zakładu opieki zdrowotnej, w której eksperyment medyczny ma być przeprowadzony, mogą wnieść odwołanie do Odwoławczej Komisji Bioetycznej przy Ministrze Zdrowia, za pośrednictwem Komisji Bioetycznej przy Collegium Medicum im. L. Rydygiera w Bydgoszczy, w terminie 14 dni od daty otrzymania niniejszej Uchwały.

Prof. dr hab. med. Karol Śliwka

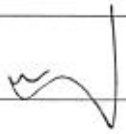
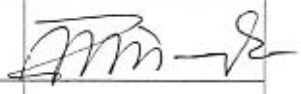
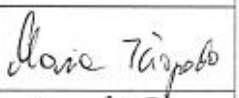
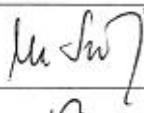
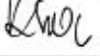
Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Marta Pokrywczyńska
Zakład Medycyny Regeneracyjnej. Bank Komórek i Tkanek
Collegium Medicum w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 26.03.2019 r.

Lp.	Imię i nazwisko	Funkcja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska		
4.	Prof. dr hab. med. Anna Balcar-Boroń		
5.	Prof. dr hab. med. Marek Grabiec		
6.	Prof. dr hab. med. Zbigniew Włodarczyk		
7.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK		
8.	Dr hab. n med. Maria Kłopocka		
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM		
10.	Dr n. med. Radosława Staszak-Kowalska		
11.	Mgr prawa Patrycja Brzezicka		
12.	Mgr farm. Aleksandra Adamczyk		
13.	Mgr Lidia Iwińska-Tarczykowska		

Uniwersytet Mikołaja Kopernika w Toruniu
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KB 358/2019

Bydgoszcz, 14.09.2021 r.

Działając na podstawie art.29 ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U. Nr 47 poz.480) oraz Zarządzenia Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(której skład podano w załączeniu) na posiedzeniu w dniu **14.09.2021 r.** przeanalizowała prośbę o:

- przedłużenie terminu ważności badań do końca **2022** roku.

którą złożył:

dr hab. Marta Pokrywczyńska, prof. UMK
Zakład Medycyny Regeneracyjnej, Banku Komórek i Tkanek
Collegium Medicum w Bydgoszczy, UMK w Toruniu

w sprawie badania:

„Porównanie metod izolacji małych pęcherzyków zewnątrzkomórkowych z krwi obwodowej i moczu."

Po zapoznaniu się ze złożonym dokumentem i w wyniku przeprowadzonej dyskusji oraz głosowania jawnego Komisja przyjęła do wiadomości podane informacje i wyraża zgodę na powyższe pod warunkami określonymi w uchwale Komisji podjętej w dniu 26.03.2019 r. oraz w ewentualnych aneksach do tejże uchwały.

Zgoda na kontynuowanie przedmiotowego badania obowiązuje do końca 2022 r.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:

dr hab. Marta Pokrywczyńska, prof. UMK
Zakład Medycyny Regeneracyjnej, Banku Komórek i Tkanek
Collegium Medicum w Bydgoszczy, UMK w Toruniu

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 14.09.2021 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. n. med. Karol Śliwka	*medycyna sądowa* przewodniczący	
2.	Mgr prawa Joanna Połetek-Żygas	*prawniczka* zastępca przewodniczącego	
3.	Prof. dr hab. n. med. Mieczysława Czerwionka-Szaflarska	*pediatra, alergologia i gastroenterologia dziecięca*	
4.	Prof. dr hab. n. med. Marek Grabiec	*położnictwo, ginekologia onkologiczna*	
5.	Prof. dr hab. n. med. Maria Kłopocka	*choroby wewnętrzne, gastroenterologia*	
6.	Prof. dr hab. n. med. Zbigniew Włodarczyk	*chirurgia ogólna, transplantologia kliniczna*	
7.	Dr hab. n. med. Maciej Słupski, prof. UMK	*chirurgia ogólna, transplantologia kliniczna*	
8.	Dr hab. n. med. Katarzyna Sierakowska, prof. UMK	*anestezjologia i intensywna terapia*	
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM	*duchowny*	
10.	Dr n. med. Radosława Staszak-Kowalska	*pediatria, choroby płuc*	
11.	Mgr prawa Patrycja Brzezicka	*prawniczka*	
12.	Mgr farm. Aleksandra Adamczyk	*farmaceutka*	
13.	Mgr Lidia Iwińska-Tarczykowska	*pielęgniarka*	

Uniwersytet Mikołaja Kopernika w Toruniu
Collegium Medicum im L. Rydygiera w Bydgoszczy
KOMISJA BIOETYCZNA
Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 183/2019 Bydgoszcz, 26.02.2019 r.

Działając na podstawie art.29 Ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), zarządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U.Nr 47 poz.480) oraz Zarządzeniem Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **26.02.2019 r.** przeanalizowała wniosek, który złożyła kierownik badania:

dr hab. n. med. Katarzyna Wawrzyniak
Klinika Anestezjologii i Intensywnej Terapii
Szpital Uniwersytecki nr 1 w Bydgoszczy

z zespołem w składzie

- dr hab. n. med. Katarzyna Wawrzyniak, prof. dr hab. n. med. Tomasz Drewa, dr hab. n. med. Marta Pokrywczyńska, dr n.med. Milena Świtońska, lek. Hanna Wajs-Kawczyńska, lek. Alicja Szyło, mgr Kamil Szeliski,

w sprawie badania:

„Ocena mikrocząsteczek komórkowych pochodzenia śródbłonkowego u pacjentów poddanych zabiegowi radykalnej laparoskopowej prostatektomii z powodu wczesnej postaci raka gruczołu krokowego."

Po zapoznaniu się ze złożonym wnioskiem i w wyniku przeprowadzonej dyskusji oraz głosowania Komisja podjęła

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań, w zakresie określonym we wniosku pod warunkiem:

- poinformowania uczestników badania w tym również uczestników stanowiących grupę kontrolną o celu oraz zakresie badań i uzyskania od każdego z nich osobnej, pisemnej, świadomej zgody na udział w badaniu, zgodnie z obowiązującymi przepisami, datowanej najpóźniej na moment rozpoczęcia badania, a nie wcześniej niż data uzyskania z Komisji Bioetycznej zgody na takie badanie;
- zapewnienia, że osoby uczestniczące w eksperymencie badawczym nie są ubezwłasnowolnione, nie są żołnierzami służby zasadniczej, nie są osobami pozbawionymi wolności, nie pozostają w zależności służbowej, dydaktycznej lub innej z prowadzącym badanie;
- UWAGA! Uczestnicy badania stanowiący grupę kontrolną nie mogą być rekrutowani spośród studentów lub pracowników podlegających zależności służbowej lub dydaktycznej z badaczami.
- zachowania tajemnicy wszystkich danych, w tym danych osobowych pacjentów, umożliwiających ich identyfikację w ewentualnych publikacjach;
- sugerujemy uzyskanie podpisu uczestnika badania pod informacją o badaniu, lub sporządzenie formularza informacji i świadomej zgody na udział w badaniu na jednej kartce.

Jednocześnie informujemy, iż „Zgoda na udział w badaniu" winna zawierać m.in.: imię i nazwisko badanej osoby; Nr historii choroby pacjenta (L.ks.gł. Oddziału/Poradni) oraz datę i podpis badanej osoby, a także klauzule, że uczestnik badania wyraża zgodę na przetwarzanie danych osobowych dotyczących realizacji tematu badawczego, z wyjątkiem publikacji danych osobowych.

Kierownik badania zobowiązany jest do przechowywania wszystkich dokumentów dotyczących badania przez okres dwudziestu lat.

Zgoda obowiązuje od daty posiedzenia (26.02.2019 r.) do końca 2021 r.

Wydana opinia dotyczy tylko rozpatrywanego wniosku z uwzględnieniem przedstawionego projektu; każda zmiana i modyfikacja wymaga uzyskania odrębnej opinii. Wnioskodawca zobowiązany jest do informowania o wszelkich poprawkach, które mogłyby mieć wpływ na opinię Komisji oraz poinformowania o zakończeniu badania.

Od niniejszej uchwały podmiot zamierzający przeprowadzić eksperyment medyczny, kierownik zakładu opieki zdrowotnej, w której eksperyment medyczny ma być przeprowadzony, mogą wnieść odwołanie do Odwoławczej Komisji Bioetycznej przy Ministrze Zdrowia, za pośrednictwem Komisji Bioetycznej przy Collegium Medicum im. L. Rydygiera w Bydgoszczy, w terminie 14 dni od daty otrzymania niniejszej Uchwały.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Katarzyna Wawrzyniak
Klinika Anestezjologii i Intensywnej Terapii
Szpital Uniwersytecki nr 1 w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 26.02.2019 r.

Lp.	Imię i nazwisko	Funkcja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska		
4.	Prof. dr hab. med. Anna Balcar-Boroń		
5.	Prof. dr hab. med. Marek Grabiec		
6.	Prof. dr hab. med. Zbigniew Włodarczyk		
7.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK		
8.	Dr hab. n med. Maria Kłopocka		
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM		
10.	Dr n. med. Radosława Staszak-Kowalska		
11.	Mgr prawa Patrycja Brzezicka		
12.	Mgr farm. Aleksandra Adamczyk		
13.	Mgr Lidia Iwińska-Tarczykowska		

14. Attachment 2 – miRNA reference sequences

miRName	microRNA target sequence
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
hsa-miR-100-5p	AACCCGUAGAUCCGAACUUGUG
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA
hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
hsa-miR-128-3p	UCACAGUGAACCGGUCUCUUU
hsa-miR-133a-3p	UUUGGUCCCCUUCAACCAGCUG
hsa-miR-135a-5p	UAUGGCUUUUUAUUCCUAUGUGA
hsa-miR-135b-5p	UAUGGCUUUUCAUUCCUAUGUGA
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-143-3p	UGAGAUGAAGCACUGUAGCUC
hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCUG
hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-17-3p	ACUGCAGUGAAGGCACUUGUAG
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181b-5p	AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-182-5p	UUUGGCAAUGGUAGAACUCACACU
hsa-miR-183-5p	UAUGGCACUGGUAGAAUUCACU
hsa-miR-184	UGGACGGAGAACUGAUAAGGGU
hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miR-196a-5p	UAGGUAGUUUCAUGUUGUUGGG
hsa-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA
hsa-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA
hsa-miR-203a-3p	GUGAAAUGUUUAGGACCACUAG
hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-218-5p	UUGUGCUUGAUCUAACCAUGU

hsa-miR-22-3p	AAGCUGCCAGUUGAAGAACUGU
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA
hsa-miR-224-5p	UCAAGUCACUAGUGGUUCCGUUUAG
hsa-miR-23b-3p	AUCACAUUGCCAGGGAUUACCAC
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU
hsa-miR-27a-3p	UUCACAGUGGCUAAGUUCCGC
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC
hsa-miR-296-5p	AGGGCCCCCCCUCAAUCCUGU
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC
hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-3163	UAUAAAAUGAGGGCAGUAAGAC
hsa-miR-32-5p	UAUUGCACAUUACUAAGUUGCA
hsa-miR-330-3p	GCAAAGCACACGGCCUGCAGAGA
hsa-miR-331-3p	GCCCCUGGGCCUAUCCUAGAA
hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU
hsa-miR-34b-3p	CAAUCACUAACUCCACUGCCAU
hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC
hsa-miR-361-5p	UUAUCAGAAUCUCCAGGGGUAC
hsa-miR-365a-3p	UAAUGCCCCUAAAAAUCCUUAU
hsa-miR-3662	GAAAAUGAUGAGUAGUGACUGAUG
hsa-miR-3666	CAGUGCAAGUGUAGAUGCCGA
hsa-miR-374b-5p	AUAUAAUACAACCUGCUAAGUG
hsa-miR-375-3p	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA
hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU
hsa-miR-455-5p	UAUGUGCCUUUGGACUACAUCG
hsa-miR-494-3p	UGAAACAUACACGGGAAACCUC
hsa-miR-616-3p	AGUCAUUGGAGGGUUUGAGCAG
hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU
hsa-miR-9-3p	AUAAAGCUAGAUAACCGAAAGU
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG
hsa-miR-96-5p	UUUGGCACUAGCACAUUUUUGCU
hsa-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG
hsa-miR-99b-5p	CACCCGUAGAACCGACCUUGCG