Review

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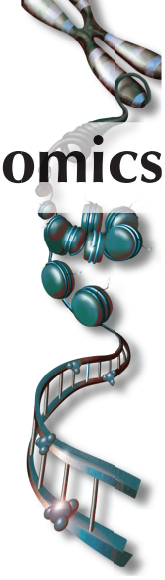
Epigenomics



miRNA in prostate cancer: challenges toward translation

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Prostate cancer (PCa) represents the most commonly diagnosed neoplasm among men. miRNAs, as biomarkers, could further improve reliability in distinguishing malignant versus nonmalignant, and aggressive versus nonaggressive PCa. However, conflicting data was reported for certain miRNAs, and there was a lack of consistency and reproducibility, which has been attributed to diverse (pre)analytical factors. In order to address current challenges in miRNA clinical research on PCa, a PubMed-based literature search was conducted with the last update in May 2019. After identifying critical variations in designs and protocols that undermined clear-cut evidence acquisition, and reliable translation into clinical practice, we propose guidelines for most critical steps that should be considered in future research of miRNA as biomarkers, especially in PCa.

First draft submitted: 20 September 2019; Accepted for publication: 14 February 2020; Published online: 8 April 2020

Keywords: cancer biomarker • liquid biopsy • miRNA • preanalytical variables • prostate cancer

Prostate cancer (PCa) is the most commonly diagnosed neoplasm among men. It is the third-leading cause of cancer-related deaths in the world (Globocan) [1]. With the rise of the average life expectancy and Western-like living, epidemiological predictions do not look promising. According to WHOs' (Globocan) projections for 2040, the number of deaths caused by PCa is expected to nearly double, while the number of newly diagnosed men in the world could reach 2,300,000 per year [2].

Screening for PCa involves the prostate-specific antigen (PSA) test and the digital rectal examination (DRE), while the diagnosis is based on histopathological analysis of prostate biopsy cores. Specimens from transurethral resection of the prostate or prostatectomy for benign prostatic hyperplasia (BPH) are also used. Biopsies are analyzed for histologic aggressiveness using the Gleason grading system where tumor tissue gets a Gleason score (GS) from two to ten based on the differentiation of the glandular structure. The International Society of Urological Pathology (ISUP) introduced a new grading system a few years ago, which modifies GS into five groups, in other words, ISUP grades 1–5, aiming to simplify the grading system while allowing for more accurate grade stratification and better correlation to prognosis [3]. Management of the disease depends on various factors: tumor staging, PSA levels, the GS, ISUP grade and the man's age. Due to disease heterogeneity, it is a challenge to predict a tumor's course. The utmost challenge remains in distinguishing between a patient who may benefit from some type of treatment (surgery, hormone therapy, radiotherapy and chemotherapy) and a patient who may benefit from monitoring the disease until disease progression is evident. Monitoring procedures (active surveillance and watchful waiting) are often sufficient as a first-line treatment, as PCa is an indolent and slow growing disease in a notable population of men. However, monitoring procedures commonly include repeat biopsies, which add to healthcare cost and increase the risk of serious complications [4].

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Table 1. Overview of proposed prostate cancer biomarkers. Clinical value of prostate cancer biomarkers varies across the literature. Indeed, to date the US FDA has only approved the Prostate Health Index and the prostate cancer antigen-3 for clinical use.

Test/assay	Components	Sample type	Indication/clinical utility
PCA3 (progensa)	*PCA3* ncRNA	Urine	Diagnosis, rebiopsy (history of negative biopsy)
PCA3 score	*PCA3*/PSA mRNA ratio × 1000	Urine	Rebiopsy
Mi-prostate score	Serum PSA, urinary PCA3 and *T2-ERG* mRNA	Blood (serum), urine	Diagnosis, prognosis, initial biopsy
4K score	fPSA, tPSA, intact PSA, kallikrein-like peptidase 2 (hk2)	Blood (plasma)	Diagnosis, initial biopsy or rebiopsy (history of negative biopsy)
PHI	proPSA, fPSA, tPSA	Blood (serum)	Diagnosis, prognosis, initial biopsy
ExoDx prostate intelliscore	Exosomal mRNA (*T2-ERG, PCA3, SPDEF*)	Urine	Diagnosis, initial biopsy
Confirm MDx	Methylation of *RASSF1*, *GSTP1* and *APC* genes	Tissue (biopsy)	Diagnosis, rebiopsy (history of negative biopsy)
SelectMDx	*HOXC6, DLX1, KLK3* mRNA levels	Urine	Diagnosis, prognosis, initial biopsy
Decipher GC (GenomeDx)	22-gene panel	Tissue (RP)	Radical prostatectomy, cancer risk stratification for patients received therapy
Oncotype Dx GPS	12 cancer-related genes + five reference genes	Tissue (biopsy)	Cancer risk stratification, men on active surveillance
Prolaris CCP score	31 cancer-related genes involved in cell cycle (CCP) + 15 reference genes	Tissue (biopsy or RP)	Cancer risk stratification, men on active surveillance
Prostarix risk score	PCa-related metabolites: sarcosine, alanine, glycine, glutamate	Urine	Cancer risk stratification in the 'gray zone' – suspicious DRE and PSA 2.5–10 ng/ml
ProMark	Proteomics platform of 12 biomarkers	Tissue (RP)	Radical prostatectomy, cancer risk stratification
TMPRSS2:ERG fusion gene	*TMPRSS2:ERG* mRNA in relation to PSA mRNA	Urine	Rebiopsy

CCP: Cell cycle progression; DRE: Digital rectal examination; GC: Genomic classifier; GPS: Genomic prostate score; PCa: Prostate cancer; PCA3: Prostate cancer antigen-3; PHI: Prostate health index; PSA: Prostate-specific antigen; RP: Radical prostatectomy.

Although PSA is still a gold-standard biomarker, it has limited value in pretreatment decisions since it is not very helpful in distinguishing aggressive from nonaggressive disease. Its limited specificity results in a high rate of false positives, since PSA is not cancer specific. Elevated levels are found in nonmalignant prostate pathology such as benign prostate hyperplasia or prostatitis and can be caused by prostate manipulations like DRE, cycling or ejaculation. PSA testing has also been generating an increasing number of low-risk PCa that are being diagnosed and overtreated. The traditional cutoff of 4.0 ng/ml was severely questioned since up to 15% men with PCa have PSA levels below the cutoff and up to 70% of men with PSA in the range of 4–10 ng/ml have a negative prostate biopsy [5,6]. There is a great clinical need for more specific, sensitive and reliable biomarkers, which would help the pretreatment decision-making, especially to better distinguish malignant versus nonmalignant conditions, as well as aggressive versus nonaggressive tumors.

Research on PCa has expanded the palette of new potential biomarkers in body fluids and tissue. A summary of newly developed assays is presented in Table 1.

Much emphasis has been placed on noninvasive liquid biomarkers due to minimal risk compared with tissue-based assays. Liquid biomarkers allow for more sophisticated and specific screening, diagnosis, prognosis and monitoring. In that category are miRNAs; endogenous, small-noncoding RNAs that post-transcriptionally regulate gene expression. According to miRBase, 2.654 mature miRNA sequences were identified in the human genome [7]. miRNAs are involved in the pathogenesis of different tumors through regulation of tumor suppressors and oncogenic factors. miRNA profiling of solid tumor tissues suggested specific patterns of upregulated and downregulated miRNAs. That discovery paired with their stability and ability to be detected by different techniques (microarray, real-time quantitative reverse transcription PCR [RT-qPCR], next-generation sequencing) led to the investigation of unique miRNA signatures in biofluids of cancer patients. miRNAs in biofluids can exist as cell-free molecules, bundled into cellular-like extracellular structures such as exosomes or microvesicles, or associated with proteins and lipoproteins.

Mitchell *et al.* in 2008 were first to show the presence of miRNAs in plasma of PCa patients [8]. Since then a great number of studies were published on miRNAs as potential biomarkers in PCa, both in liquid biopsies and prostate tissue. However, all sorts of conclusions were put forward, ranging from statistically insignificant results to those showing great diagnostic performance of certain miRNAs. Different miRNAs were shown to be dysregulated

Table 2. Most frequently reported dysregulated miRNAs in prostate cancer.

MiRNA	Tissue	Serum	Plasma	Urine
miR-21	↓ (Szczyrba, 2010; Wach, 2012) ↑ (Zedan, 2018; Kumar, 2018)	↑ (Porzycki, 2018; Kotb, 2014; Egidi, 2013) No diff. PCa vs controls (Sanders, 2012)	↑ (Zedan, 2018; Agaoglu, 2011; Mello-Grand, 2018; Gao, 2016), (Endzelinš, 2017) - EVs No diff. PCa vs controls (Osipov, 2016)	↑ (Ghorbanmehr, 2019; Foj, 2017)
miR-30c	↓ (Zhu, 2018; Huang, 2016) ↑ (Walter, 2013)	↓ (Moltzahn, 2011) ↑ (Mihelich, 2015) No diff. PCa vs BPH (Cochetti, 2016)	↓ (Kachakova, 2014; Chen, 2012)	↑ (Fredsoe, 2018)
miR-125b	↓ (Zedan, 2018; Schaefer, 2010) ↑ (Walter, 2013; Song, 2015)	↑ (Mitchell, 2008)	↑ (Zedan, 2018)	↑ (Fredsoe, 2018)
miR-141	↑ (Szczyrba, 2010; Kumar, 2018; Nguyen, 2013; Brase, 2014; Kelly, 2015)	↑ (Mitchell, 2008; Porzycki, 2018; Cheng, 2013; Guo, 2018), (Hao, 2016) - EVs No diff. pos. vs neg. biopsy (Westermann, 2014)	↑ (Osipov, 2016), (Bryant, 2012) - EVs ↓ (Kachakova, 2014) No diff. PCa vs controls (Agaoglu, 2011)	↑ (Ghorbanmehr, 2019; Foj, 2017) ↓ (Fredsoe, 2018) No diff. PCa vs controls (Bryant, 2012)
miR-143	↓ (Szczyrba, 2010; Wach, 2012; Zedan, 2018; Kumar, 2018; Martens-Uzunova, 2012)	↑ (Mitchel, 2008)	↑ (Zedan, 2018) No diff. PCa vs controls (De Souza, 2017)	↓ (Stuopelyte, 2016), (Rodriguez, 2017) - EVs
miR-145	↓ (Szczyrba, 2010; Wach, 2012; Porkka, 2007; Ozen, 2008; Kang, 2012; Zedan, 2018; Kurul, 2019; Schaefer, 2010; Kelly, 2015; Martens-Uzunova, 2012; Yfantis, 2008; Avgeris, 2013; Larne, 2013)	No data found	No data found	↑ (Xu, 2017) - EVs
miR-148a	↑ (Szczyrba, 2010; Martens-Uzunova, 2012; Stuopelyte, 2016; Lichner, 2015; Hart, 2013) No diff. PCa vs BPH (Dybos, 2018)	↑ (Dybos, 2018)	↑ (Al-Qatati, 2017)	↓ (Stuopelyte, 2016) No diff. PCa vs BPH (Fredsoe, 2018)
miR-182	↑ (Wach, 2012; Schaefer, 2010; Yfantis, 2008; Tsuchiyama, 2013; Costa-Pinheiro, 2015; Casanova-Salas, 2014)	No data found	No data found	No diff. pos. vs neg. biopsy (Casanova-Salas, 2014)
miR-200c	↑ (Szczyrba, 2010; Wach, 2012; Yfantis, 2008)	↑ (Cheng, 2013)	↑ (De Souza, 2017), (Endzelinš, 2017) - EVs	↓ (Fredsoe, 2018)
miR-205	↓ (Scahefer, 2010; Martens-Uzunova, 2012; Yfantis, 2008; Tsuchiyama, 2013; Verdoodt, 2013; Kalogirou, 2013) ↑ (Walter, 2013)	↓ (Guo, 2018)	↑ (Osipov, 2016)	↓ (Fredsoe, 2018) No diff. pos. vs neg. biopsy (Stephan, 2015)
miR-221	↓ (Szczyrba, 2010; Wach, 2012; Zedan, 2018; Kurul, 2019; Schaefer, 2010; Yfantis, 2008; Porkka, 2007; Tsuchiyama, 2013; Casanova-Salas, 2014; Kneitzm, 2014) ↑ (Song, 2015)	↑ (Kotb, 2014)	↑ (Agaoglu, 2011)	↓ (Fredsoe, 2018)
miR-222	↓ (Wach, 2012; Schaefer, 2010; Martens-Uzunova, 2012; Porkka 2007; Tsuchiyama, 2013)	No data found	No data found	↓ (Fredose, 2018)
miR-375	↑ (Szczyrba, 2010; Wach, 2012; Schaefer, 2010; Nguyen, 2013; Brase, 2011; Stuopelyte, 2016; Yfantis, 2008; Costa-Pinheiro, 2015; Haldrup, 2014; Nam, 2018)	↑ (Porzycki, 2018; Nguyen, 2013; Brase, 2011; Cheng, 2013; Haldrup, 2014; Wach, 2015), (Bryant, 2012) - EVs	↑ (Zedan, 2018; Gao, 2016; Endzelinš, 2017; McDonald, 2018), (Huang, 2015) - EVs ↓ (Kachakova, 2014) No diff. PCa vs controls (De Souza, 2017)	↑ (Foj, 2017; Stuopelyte, 2016) No diff. PCa vs controls (Bryant, 2012) No diff. PCa vs BPH (Fredsoe, 2018)
let-7a	↓ (Szczyrba, 2010; Wach, 2012; Kelly, 2015; Porkka, 2007; Tian, 2015) No diff. PCa vs BPH (Pesta, 2010)	↑ (Haldrup, 2014)	↑ (Mello-Grand, 2018) ↓ (Endzelinš, 2017) - EVs	↓ (Fredsoe, 2018)
let-7c	↓ (Szczyrba, 2010; Kurul, 2019)	↓ (Cochetti, 2016)	↓ (Kachakova, 2014; Chen, 2012)	↑ (Fredsoe, 2018), (Foj, 2017) - EVs

Note: Shown data were obtained from clinical and basic research studies based on patient samples and considering meta-analysis by Song *et al.* [10] and Bertoli *et al.* [9]
miRNAs in bold – opposing data published for certain types of specimens.
↑: Upregulated expression; ↓: Downregulated expression
BPH: Benign prostate hyperplasia; EV: Extracellular vesicle; PCa: Prostate cancer.

in PCa, but only some were reported in several studies (Table 2). Diagnostic panels were also proposed, but without significant overlap. It is interesting that certain miRNAs were found to be contradictorily dysregulated by different scientific groups (Table 2). The lack of reproducibility has been attributed to various patient cohorts, differences

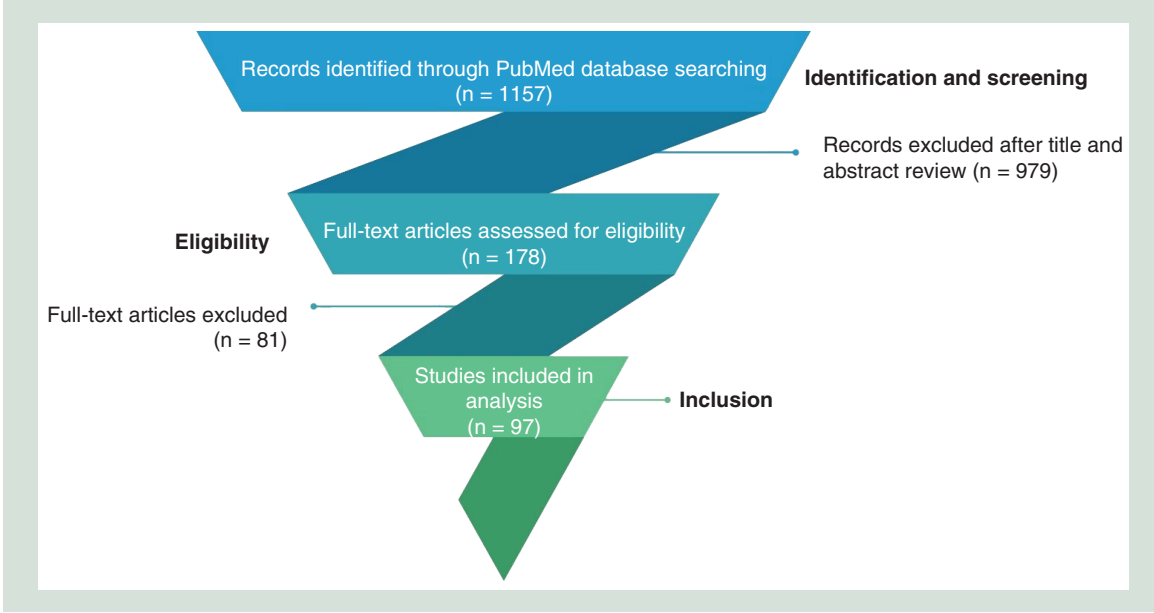

Figure 1. Flowchart of study selection process. Of more than thousands of papers identified, only 8% met the set criteria.

in sample processing, measurements and data analysis [9,10]. As was pointed out by Jarry *et al.*, lack of concordance together with mixed correlations between tissue-specific and circulating miRNAs highlights the preanalytical and postanalytical challenges of miRNA research [11]. When analyzing published studies to identify specific miRNAs or panels of miRNAs that could be useful in PCa management, it is very challenging to conclude anything with a reasonable level of confidence. Published papers often lack necessary information. Indeed, Song *et al.* in their meta-analysis of studies on miRNA in PCa had to exclude a third of published studies due to insufficient data and absence of key information [10]. In this review, by analyzing and comparing published studies, we have identified challenges in miRNA research of PCa, especially regarding obstacles in study comparison. Here, we present and discuss the current best practice, as well as suggest possible methodological solutions and more suitable research designs. To our knowledge, this is a unique kind of review among the literature regarding miRNA in PCa. Its specificity lies in a multilevel approach used to compare published studies that enables a broad analysis of preanalytical and analytical factors. Indeed, some aspects have already been addressed several years ago, although never specifically in PCa [11–15]. However, in the meantime many more studies have been published and research techniques evolved leading to new, crucial issues and challenges requiring awareness.

Evidence acquisition

In order to analyze and compare current miRNA clinical research on PCa, a PubMed-based literature search was conducted with the last update on 23 May 2019. The following search algorithm was used: '(microRNA OR miRNA OR miR-) AND prostate cancer AND (serum OR plasma OR urine OR tissue OR blood)'. Initial title and abstract screening resulted in 178 articles assessed for eligibility. Inclusion criteria were English papers focused on human miRNA research in clinical PCa samples; reviews, meta-analysis or other types of papers were excluded, as well as papers unrelated to miRNA or PCa. Papers studying the therapy effect on miRNA or basic research papers were excluded. Full text evaluation led to the exclusion of 81 papers due to the following reasons: nonclinical samples, methodology studies, lack of sufficient data, studies on genetic alternations of miRNAs, analysis data obtained by tumor databases and reviews. A flowchart of the study selection process is shown in Figure 1. It should be noted that studies included after a full text evaluation are the ones that will be discussed and compared in the following sections.

Control groups & PCa patients

In this analysis, papers defining control groups as benign prostate hyperplasia (BPH) patients and/or healthy individuals were considered only. While comparing PCa patients with healthy age-matched controls, it is important to consider the fact that 50% of men older than 50 years have BPH [16]. Therefore, criteria for choosing the healthy control group should be more clarified and stringent. Unfortunately, criteria differ, some studies gave detailed explanations and inclusive/exclusive criteria, while others just declared choosing healthy individuals. Aside from routine physical examination, in most studies, PSA testing was chosen as the sole inclusion criterion. However, reported cutoff values varied from those even lower than 2 [17,18], over 4 [8,19–24] to 10 ng/ml [25,26]. In addition to PSA testing, some studies included DRE as an inclusion criterion for healthy controls [8,21,23,25,27,28].

More thorough criteria for determination of healthy controls included one or more negative biopsy results [25,26,29], no history of prostatitis [21,30,31] and prostate hyperplasia exclusion by ultrasound and/or magnetic resonance [19,24,28,30]. When healthy controls are recruited as patients that have been referred to urology departments for clinical issues other than prostate pathology [32], researchers should be cautious since upregulated levels of certain miRNAs are not exclusive for PCa. They are found in bladder, kidney and testicular cancers, as well as various other kidney diseases [33,34]. Surprisingly, a high number of studies did not report which criteria were implemented, rather they just declared control individuals as healthy [32,35–39].

Ethnicity of the populations included in studies also varied when mentioned [22,24,28,36,37,40–45]. While some researchers reported there was no difference in miRNA levels between ethnic groups [42], others reported the opposite [46]. Srivastava *et al.* reported differences in miR-101 levels between African American and Caucasian American populations, while miR-25 did not significantly differ [37]. It is possible that racial differences have an effect on certain miRNAs in some diseases [47,48], while the effect on others is not significant or present at all.

Analyzed studies also differ in staging and grading criteria of the subgroups of PCa patients. Most papers included PCa patients regardless of tumor stage, while others were focused on either localized PCa or metastatic PCa. Since miRNA expression can represent specific pathophysiological processes, this may be one of the reasons why contradicting findings were reported.

Certain number of patients received some type of therapy such as hormonal or radiotherapy before sampling. Indeed, it has been shown that ionizing radiation alters miRNA expression in PCa, including some miRNAs (miR-106b, miR-107, miR-133b, miR-143, miR-145 and miR-379) being tested as potential PCa diagnostic biomarkers [49,50]. The radiotherapy-induced change in miRNA expression in whole blood of PCa patients was confirmed by Templin *et al.* [51]. Though there are no data on how long radiotherapy affects miRNA levels, it seems reasonable to consider possible variations due to therapy. Regarding hormonal therapy, it is known that it causes upregulation (usually) or downregulation of specific miRNAs [52]. The same effect is identified after chemotherapy, leading to suggestions that miRNAs could be used for prediction of chemotherapy response rather than in diagnostic protocols [53]. Certain miRNAs could also be used as radiotherapy-induced damage and toxicity biomarkers [54,55], as well as predictors of radiotherapy resistance in PCa patients [56]. Therefore, measuring specific miRNA levels before therapy or its level changes in body fluids during therapy could fill in the shortage of predictive biomarkers not only in PCa, but in other cancers as well [57–59].

To summarize, there is an immense lack of data on control group-framing parameters used in published studies, which *a priori* reduces the reliability of knowledge deduced by published data comparison. In addition, when comparing studies with well-defined and described control groups, we found that very different control group designs were used (Figure 2). These control group designs are not always comparable and could lead to different if not conflicting results and conclusions.

In addition to what has been discussed above, it is noteworthy to highlight interindividual variation as an aspect often neglected in designing controls and experimental groups when studying miRNA as potential cancer biomarkers. Factors such as physical activity, cigarette smoking, diet, pharmacological treatment, kidney function *etc.*, all have been shown to affect miRNA levels in biofluids [13]. For example, Marzi *et al.* found that serum miRNAs levels substantially differ between fasting and nonfasting individuals and strongly advised the fasting condition when collecting blood [60]. Although it is not a focus of this review, we strongly encourage authors to address this and similar parameters as well when defining and reporting inclusion and exclusion criteria.

Preanalytical aspects of sample types

Predominant sample types used for miRNA research in PCa are tissue, blood (mostly serum) and urine. The plurality of different specimen types represents another layer in complexity of miRNA published data comparison.

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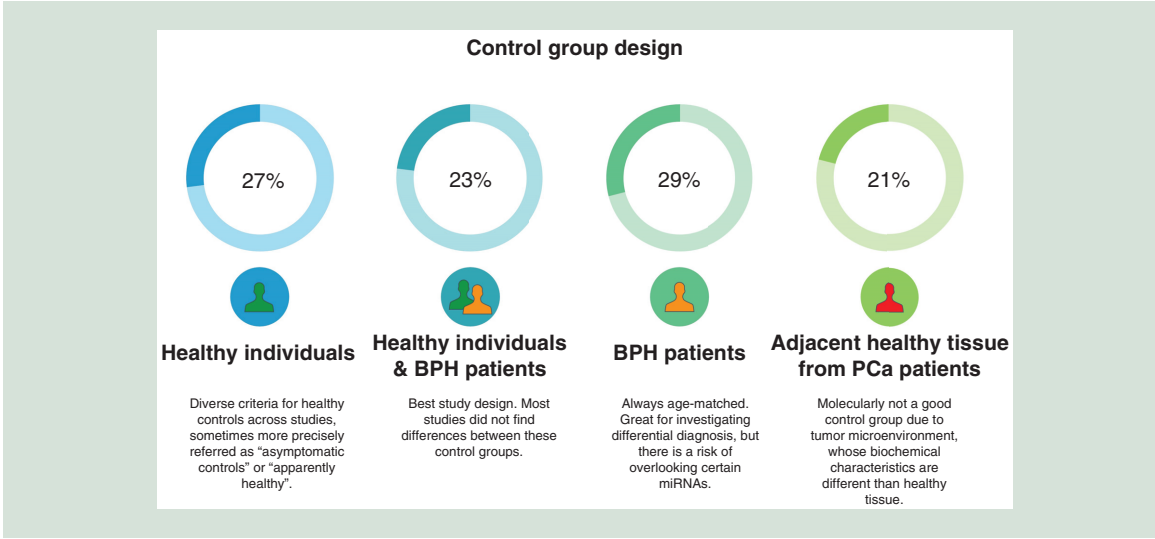

Figure 2. Control group design. Representation of various control groups definitions set in miRNA research in PCa. BPH: Benign prostate hyperplasia; PCa: Prostate cancer.

Liquid samples

Circulating miRNA levels are shown to be affected by a broad spectrum of factors, both intrinsic and extrinsic. Several issues are to be considered when defining liquid sample of choice. Another prominent question should be whether exosome or microvesicles are to be specifically analyzed, and how to approach these differences.

A wide list of technical issues that could alter miRNA levels begin with specimen collection. With respect to plasma, it has been reported that EDTA as an anticoagulant is preferred, while heparin is to be avoided since it inhibits PCR [13]. Still, some authors reported using heparin as an anticoagulant for healthy controls in their research of miRNA in PCa [21].

When blood was analyzed, in most of the studies serum was the sample of choice, a certain few utilized plasma, while whole blood was used rarely. Extreme differences in serum, plasma and whole blood are obvious, so they should not be compared at all. Data obtained on plasma and serum respectively should be compared with caution because there is still no consensus whether this comparison is appropriate. Thus far, opposing data have been published. Some studies reported higher concentration of miRNA in serum than plasma [61], while others showed the opposite [12,62]. High correlation between serum and corresponding plasma levels has been recorded several times as well [8,61,62]. Others report no correlation, rather they have found differential expression in miRNAs between corresponding serum and plasma specimens [11,63,64]. Differences in miRNA concentration between plasma and serum were assigned to miRNAs released from platelets and erythrocytes [12,61]. That release is most probably a result of both coagulation and different processing (centrifugation rate and number of centrifugation steps) [11,62,63,65]. Regarding PCa research, only Mitchell *et al.* published data on comparing plasma and serum, reporting strong correlation for *miR-15b*, *miR-16*, *miR-19b* and *miR-24*. However, that research included only three individuals [8]. Therefore, before concluding anything when comparing a large amount of data on miRNAs from liquid biopsies in PCa, one should be aware of these challenges. To date, none of the blood-related sample is preferred but plasma seems to be a sample of choice in studying circulating miRNAs [61,64].

Short-term stability and resistance to freeze–thaw cycles of miRNAs in liquid biopsies was tested by different groups, again with opposing results [8,12,66–68]. Probably due to these confusing data, investigators usually prefer to process samples as soon as possible, according to published methodology. Still, for clinical use, of great importance remains the discussion on how long samples are stable at room temperature as well as at subzero temperature, usually at -80°C. In various studies of miRNA in PCa, samples were stored for several years [20,39,40,44,69–77], some even over 10 years. It seems that the effect of storage conditions on miRNA levels depends on the specimen itself. Still, there is no consistency in data showing the impact of longer sample storage. Grasedieck *et al.* underline that there is a significant decrease in serum miRNA levels six or more years after freezing serum at -80°C, while there is no significant decrease after 2–4 years [67]. The same storage effect was reported by Balzano *et al.* [78].

On the other hand, Rounge *et al.* declared that there was no notable degradation of miRNA after even 40 years storage at just -25°C [79]. Since most investigators seem to agree that miRNA levels in serum or plasma should not significantly change when samples are kept under -20°C for several months to 5 years, it would be best to avoid long storage (>5 years) of serum/plasma samples, at least until there are more widely accepted guidelines on storage conditions [66,67,78,80]. The same cannot be expected for whole-blood samples since storage at -80°C for a few months significantly changes miRNA levels [66]. Indeed, one should exercise caution when choosing whole blood as a sample as it is crucial to ensure stability of the RNA before the isolation step.

In publications where urine-derived miRNAs in PCa patients were studied, samples were usually stored at -20 or -80°C [18,23–26,81]. It has been shown that miRNAs in different urine fractions are stable under various storage conditions [82,83]. However, only the stability of an exosome fraction was studied after a long period of sample storage. Results suggest exosome miRNA stability with a sample stored at -80°C for 12 months with considerable degradation after 24 months [84]. Still, the question of how long certain urine fractions should be stored before miRNA analysis is yet to be addressed.

Another challenge that should be overcome in order to comprehensively compare published results is to answer the question if and how different urine fractions alter obtained results considering miRNA. Some researchers used urine sediments [26,81], while others used whole urine [17,19], cell-free urine fraction [25] or exosomes [23,85]. Other factors such as processing conditions (centrifugation), urine volume, using first morning voided specimen or catheterized urine should be considered. It is important to highlight that in some studies urine was collected after DRE massage [18,26] and that introduces uncertainty into the obtained results. Pellegrini *et al.* have recorded that DRE significantly raises extracellular vesicle (EV) RNA yield in urine [86]. Therefore, collection of post-DRE urine might increase sensitivity for urine-based biomarkers if miRNAs are from EVs, in other words, exosomes or microvesicles, are of interest. To our knowledge, there is no proof that DRE raises the concentration of circulating cell-free miRNA in urine as well. Therefore, studies on urine cell-free miRNAs in PCa where DRE was done before sampling could be incomparable with the ones where it was not. In addition, it seems that profile and expression of miRNAs originating from different fractions are not comparable. Zavesky *et al.* noticed that in urine of patients with gynecological cancers, miRNA expression greatly differs between the exosomal and supernatant fraction [87]. It is reasonable to expect the similar principle in urine of PCa patients. In whole plasma samples of PCa patients, Endzelinš *et al.* have indeed compared EV-incorporated miRNAs and cell-free miRNAs [88]. For the first time, miRNAs from these different sources were compared in PCa patients, and it was confirmed that EV-incorporated miRNAs represent a minor fraction of whole plasma miRNAs. Also, miRNA levels in these two fractions do not correlate, as was shown before [89]. Furthermore, it seems that some miRNAs show better diagnostic performance in plasma, while others in EVs [88]. Therefore, exosomal miRNAs and plasma or serum miRNAs should be considered as two completely distinct entities.

In conclusion, according to most of the published data, exosomal miRNA in serum, plasma or urine of PCa patients seems to have the highest potential of translation in future clinical practice [23,26,74,85,90,91].

Tissue

Regarding miRNA stability in stored formalin-fixed, paraffin-embedded (FFPE) tissues, again, conflicting data were published. Some studies did not find significant change in miRNA number or level depending on FFPE sample age [92,93], while others declared a notable decrease in detected miRNA depending on sample storage time [94–96]. Peskoe *et al.* studied the effect of long-term storage (>10 years) on FFPE prostate tissues and demonstrated significant loss of miRNA stability, with some miRNAs being more stable compared with others [94]. It seems important to consider their suggestion to use sample block age rather than RNA quality when adjusting data [94,97]. It is disturbing that most studies on PCa FFPE tissue did not report how long their samples were stored. Adjustment of data according to storage time is not even to be expected, so standardization remains a challenge yet to be addressed in the future. Many studies analyzed fresh-frozen PCa tissues as well. Nothing here needs to be discussed since fresh-frozen tissue corresponds to living tissue at best. The right question seems to be whether data based on fresh-frozen tissue analysis could be at all comparable to those based on FFPE samples. Generally, a high correlation in specific miRNA between matched frozen and FFPE samples analyzed by RT-qPCR [93,98] and long nucleic acid-based miRNA array [92] was found. However, it has been proposed that data obtained by deep sequencing of FFPE and fresh frozen tissue cannot be compared [99]. As far as we know, only Leite *et al.* and Nonn *et al.* have compared more than one pair of FFPE and fresh frozen PCa tissue samples by RT-qPCR and reported a very good or good correlation for 14 different miRNAs [100,101]. In short, it seems valuable indeed to report how long

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samples were stored prior to analysis in order to critically interpret and compare data. It is also advisable to consider adjusting data when analyzing miRNAs in FFPE samples stored for more than a decade.

Many studies compared PCa tissue with adjacent normal tissue [40,44,77,102–108] or noncancerous tissue from radical prostatectomy of PCa patients [81]. These are very valuable data, but that study design should not be compared with data obtained by studies where miRNA expression was compared with BPH or healthy prostate tissue. Walter *et al.* demonstrated that different profiles of miRNAs are expressed in prostate tumor tissue, normal epithelium and adjacent stroma in the same individuals [102]. Differential expression of miRNA in adjacent tumor tissue was also suggested as a potential predictive biomarker for specific cancers [109,110]. Moreover, although adjacent microenvironment appears morphologically normal, it has a potential integral role with tumor tissue and it may be biologically altered [111].

Analytics

Variations in the analytical phase between studies are maybe the most obvious. Sample preparation and RNA isolation alone are responsible for more than 50% of intra-assay variation [12,60,65]. Regarding commercial kits for isolation, their performance with RNA yield and quality were compared several times [12,61,112,113]. Kits for isolation of RNA from biofluids that are most frequently used for miRNA analysis are liquid–liquid extraction TRIzol LS (Invitrogen, Life Sciences, CA, USA) and variety of column-based kits like *mir*Vana PARIS Kit (Ambion Inc., Life Sciences, TX, USA), miRCURY RNA kit (Exiqon, Vedbaek, Denmark), miRNeasy serum/plasma kit (Qiagen GmbH, Hilden, Germany) and miRNA purification kit (Norgen Biotek Corp., Thorold, Canada). For RNA isolation from serum and plasma of PCa patients, miRNeasy and *mir*Vana PARIS kit are most frequently used. Sourvinou *et al.* declared that column-based protocols have a better analytical performance than TRIzol LS, but that was argued by McDonald *et al.* Among the kits, *mir*Vana was reported to be the best-performing column-based kit by McDonald *et al.* and Sourvinou *et al.* [12,114]. However, Marzi *et al.* and Kroh *et al.* declared that Qiagen's miRNeasy kit has better performance than *mir*Vana [60,115]. In the end, Brunet-Vega *et al.* declared that most available kits gave comparable results in terms of cycle quantification (Cq) values [112].

As in other studies researching miRNAs in cancer, microarray and single RT-qPCR are most widely used in PCa research. Low consistency and strong differences in the limits of detection between screening platforms were repeatedly shown [116,117]. Furthermore, no correlation between TaqMan Low Density Array (TLDA) and single TaqMan assays (Applied Biosystems, CA, USA) was reported even though the same methodology was used in these assays [65]. It was suggested that it could be due to the preamplification step in TLDA. That may be the reason why many authors could not validate their findings from TLDA-analysis using single qPCR. Binderup *et al.* and Campomenosi *et al.* showed good correlation between qPCR and digital droplet PCR when normalization was to cel-miR-39 [65,118]. However, to date, very few studies have used digital droplet PCR in PCa research [119]. Normalization is a crucial step in the analysis of qPCR data, which serves to remove experimentally induced variation and to differentiate biologically significant changes. There are several normalization approaches for relative quantification. They include endogenous miRNAs, synthetic spike-ins and data-driven methods that include algorithms (NormFinder, geNorm), mean or median expression value, quantile normalization and many other strategies suitable for arrays [120]. There is no single recommendation across published studies. Jarry *et al.* analyzed the frequency of miRNA expression normalization methods and showed there is still no preferred one [11]. They reported that in the discovery phase similar amount of studies did not report using normalizators as frequently as ones using endogenous miR-16 or small nucleolar miRNAs. In the validation phase, small nucleolar miRNAs were preferred, followed by spike-ins (cel-miR-39) and miR-16 [11]. In PCa research, an extremely wide palette of normalizators have been used. The cel-miR-39 and endogenous RNU6 were used slightly more often than others, according to our analysis of published papers. It is not rare to find algorithms NormFinder and geNorm being used, but other methods and specific endogenous miRNAs are mostly used in one, and no more than two different studies. It is a significant setback that should be resolved in order to compare published results and deduce a comprehensive conclusion with a reasonable chance of being translated in clinical practice. Unfortunately, there is a strong disagreement in recommending which normalizing strategy should be used, especially in analyzing liquid biopsy samples.

A significant number of authors agreed on endogenous small nucleolar controls (RNU44, RNU 48 and U6) not being suitable for plasma samples [61,120,121]. However, in some studies, the combination of endogenous (miR-16) and exogenous (cel-miR-39) miRNAs were used and suggested for normalization [65,114,122]. Sourvinou *et al.*

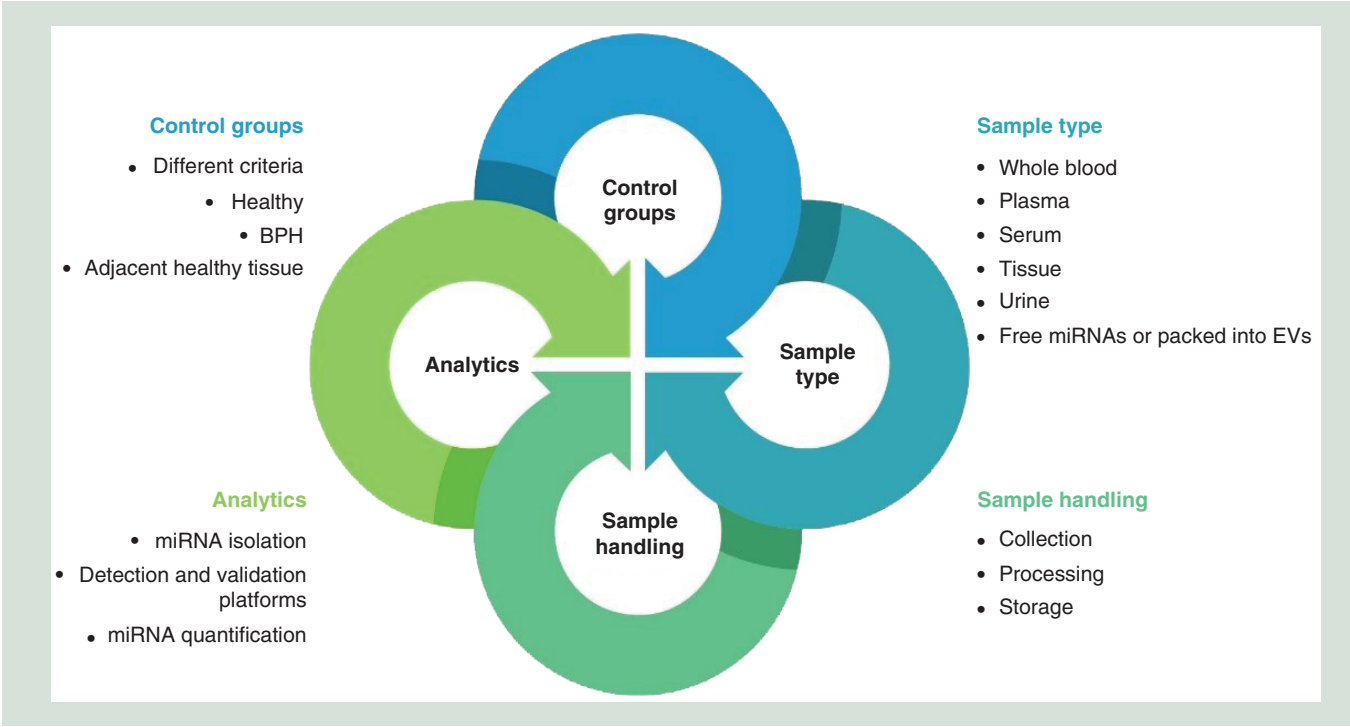

Figure 3. Factors that influence inability of comparison between studies of miRNAs in prostate cancer.
BPH: Benign prostate hyperplasia; EVs: Extracellular vesicle.

explained that such an approach ensures compensation of both sample quality and differences in recovery rates between different samples [114].

Using miR-16 as an endogenous normalizer was questioned several times, due to its high levels in platelet-poor plasma [65] and its susceptibility to hemolysis [122,123]. Endogenous miR-103-3a was suggested as a suitable normalizer for solid cancerous tissue [124], but was not found to be suitable for analysis of serum samples in PCa and BPH patients, since dysregulated expression in PCa was reported [21,76]. However, it is not clear whether endogenous miRNAs can be used for normalization of biological variability since factors that influence it are still not well studied [115]. Endogenous miRNAs could be used for quality controls since they reflect pre-extraction steps, unlike exogenous miRNAs [112]. More importantly, standardization with spiked-in exogenous miRNA such as the cel-miR-39 is often confused with normalization, and sometimes used for both purposes [11]. Exogenous miRNAs were suggested by Jarry *et al.* and Brunet-Vega *et al.* to be used only as a quality control to check and correct analytical and technical factors like RNA recovery and qPCR efficiency [11,112]. Marzi *et al.* proposed using multiple endogenous miRNAs for normalization, which is like using algorithms that detect miRNAs with the smallest variation between samples [60]. Another solution was proposed by Marabita *et al.* suggesting a focus on adopting standardized methods coupled with a selection of case-specific normalizers for serum miRNA research, rather than finding universal normalizers [125].

Another analytical challenge worth of attention are the differences in data analysis and statistics. The lack of power analysis and reproducibility, in addition to not reporting all the findings but only the ones that authors consider the most significant was critiqued by Jarry *et al.* Moreover, we noticed that the cutoff value differs between studies. Most researchers used a twofold deregulation in samples from PCa patients compared with BPH or healthy ones, while others report using a 1.5-, three- or fourfold change. So, lack of consistency in choosing a cutoff value contributes further to inconsistent results among studies. Finally, in order to be able to reproduce the analysis and compare the results, data reporting in miRNA analysis should be extensive and statistical analysis thoroughly explained [126,127]. More detailed view on variabilities influenced by statistical analysis and data processing, along with valuable recommendations were given by Jarry *et al.* and Witwer [11,126].

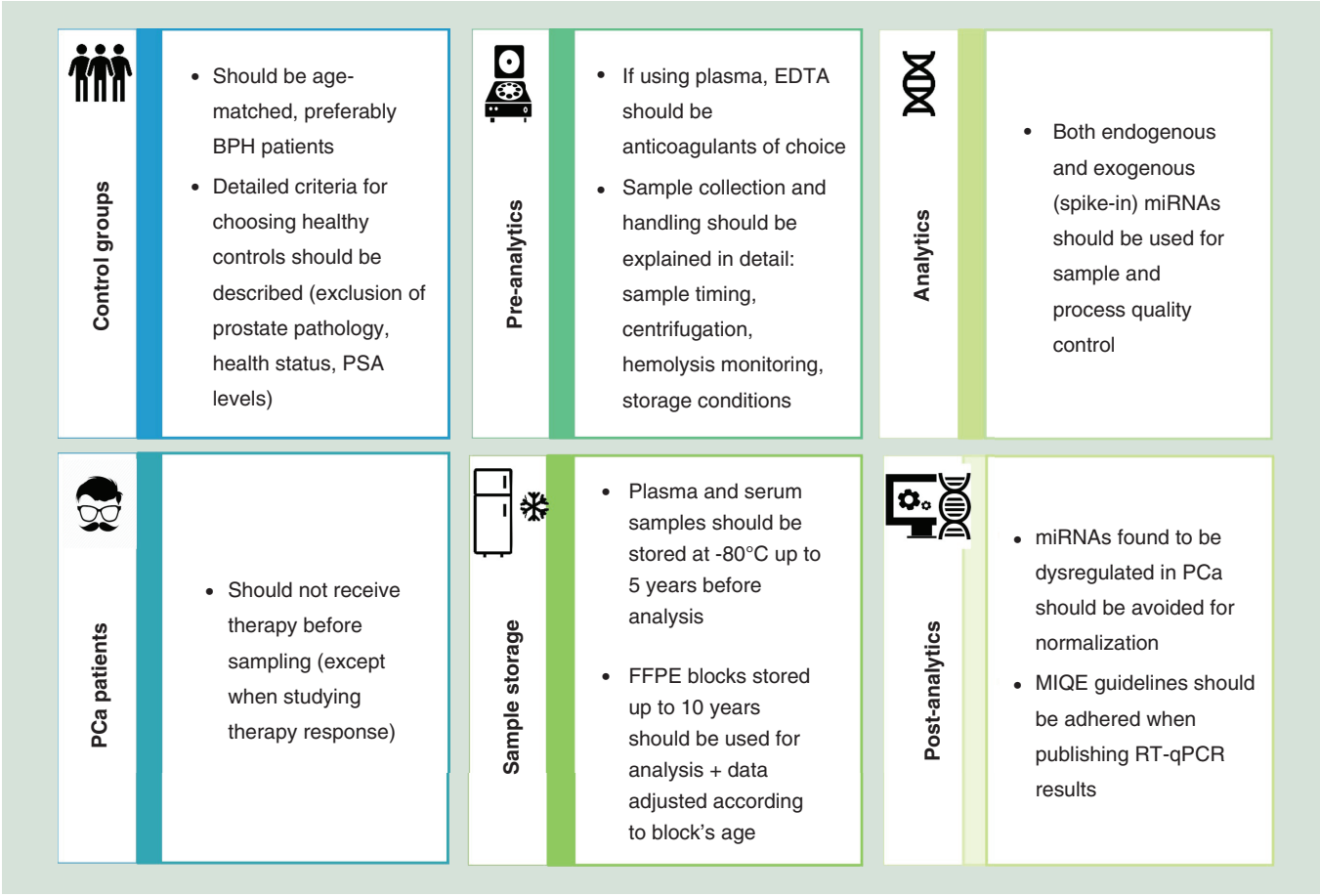

Figure 4. Recommendations for studying miRNAs in prostate cancer. Presented recommendations are based on to date literature. BPH: Benign prostate hyperplasia; EDTA: Ethylenediaminetetraacetic acid (anticoagulant); FFPE: Formalin-fixed paraffin-embedded; MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiment; PCa: Prostate cancer; PSA: Prostate-specific antigen; RT-qPCR: Real-time quantitative reverse transcription PCR.

Conclusion

miRNAs represent a potentially specific, sensitive and reliable biomarkers for PCa, especially in biofluids. Studying miRNAs in PCa is a dynamic field with a growing amount of data being published. However, there is a lack of consistency and reproducibility. Experimental design, methodology and experimental protocols are largely responsible for that fact (Figure 3). In order to overcome this challenge, it seems necessary to establish widely accepted guidelines in the near future, which will determine best blood-related and urine-related specimen, specimen sampling and processing, sample storage, miRNA isolation and quantification, quality control and data analysis. In this review, we propose recommendations for future research that could help in coming a step closer to miRNA translation into clinical practice (Figure 4).

Future perspective

With growing body of research on miRNA as potential biomarkers in PCa, some questions are being answered, while many new ones are being asked. There is still no clear vision whether there is a vivid future for miRNA translation into clinical practice. The ability to compare studies would strongly promote highly expected transition of miRNA into clinical practice. Therefore, additional research on specific preanalytical, analytical and postanalytical aspects is expected to ensure an unambiguous conclusion and broadly accepted agreement on how to achieve comparable and most reliable data on miRNA as a PCa biomarker. Only after this stage, intra- and inter-individual variability of miRNAs in biofluids as well as sensitivity and specificity of miRNA as a disease biomarker are expected to be systematically and comprehensively addressed.

Executive summary

Prostate cancer & miRNAs

- Prostate cancer (PCa) is the most commonly diagnosed neoplasm among men, which lacks better diagnostic and prognostic tools.
- miRNAs, small noncoding RNAs, have emerged as potential biomarkers that could allow distinguishing between PCa and benign prostate hyperplasia, as well as between aggressive and nonaggressive PCa.
- Conflicting data was reported for certain miRNAs, which could be ascribed to (pre)analytical factors.

Control groups & PCa patients

- Control groups include benign prostate hyperplasia and/or healthy patients, but inclusion criteria for healthy controls vastly differ between studies or are not even explained, complicating study comparisons.
- When well-defined and stated in manuscripts, different designs of control groups are used in miRNA research, which could lead to different if not conflicting results and conclusions.
- Analyzed studies also differ in terms of subgroups of PCa patients included: from tumor stage to received radiotherapy or chemotherapy that are recognized to alter miRNA levels.

Preanalytical aspects of sample types

- Predominant sample types used for miRNA research in PCa are tissue, blood (mostly serum) and urine.
- Data obtained on miRNAs in plasma and serum should be compared with caution because there is still no consensus whether this comparison is appropriate, since opposing data have been published.
- It would be best to avoid long storage (>5 years) of serum/plasma samples, at least until there are more widely accepted guidelines on storage conditions.
- Exosomal miRNAs and plasma or serum miRNAs should be considered as two distinct entities.
- Different urine-derived specimens have been used (whole urine, sediment, cell-free fraction and exosomes), without preferred fraction being determined.
- Formalin-fixed, paraffin-embedded storage time represents a factor that highly differs between studies, when mentioned. With significant loss of miRNA stability with formalin-fixed, paraffin-embedded block's age, it has been suggested to use sample block age rather than RNA quality when adjusting data.

Analytics

- Sample preparation and RNA isolation are responsible for more than 50% of intra-assay variation.
- Lack of consistency in choosing a normalization method and cutoff value contributes further to inconsistent results among studies.

Acknowledgments

The authors are very grateful to Anje Kim for improving the use of English in the manuscript.

Financial & competing interests disclosure

This work has been supported in part by Croatian Science Foundation under the project UIP-2017-05-8138, Scientific Center of Excellence for Reproductive and Regenerative Medicine, Republic of Croatia and by the European Union through the European Regional Development Fund, under grant agreement no. KK.01.1.1.01.0008, project 'Reproductive and Regenerative Medicine – Exploring New Platforms and Potentials', and School of Medicine University of Zagreb. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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