Repository of the Max Delbrück Center for Molecular Medicine (MDC) in the Helmholtz Association

The translational potential of microRNAs as biofluid markers of urological tumours

Fendler, A. and Stephan, C. and Yousef, G.M. and Kristiansen, G. and Jung, K.

The translational potential of microRNAs as biofluid markers of urological tumours

Annika Fendler[1,2,3], Carsten Stephan[2,3], George M. Yousef[4], Glen Kristiansen[5] and Klaus Jung[2,3]

[1]Department of Signal Transduction, Invasion and Metastasis of Epithelial Cells, Max Delbrück Center of Molecular Medicine in the Helmholtz Association, Robert-Rössle-Straβe 10, 13092 Berlin, Germany.

[2]Department of Urology, University Hospital Charité, Schumannstraβe 20/21, 10117 Berlin, Germany.

[3]Berlin Institute for Urologic Research, University Hospital Charité, Schumannstraβe 20/21, 10117 Berlin, Germany.

[4]Department of Laboratory Medicine, St. Michael's Hospital, University of Toronto, 30 Bond Street, Toronto M5B 1W8, Canada.

[5]Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Straβe 25, 53127 Bonn, Germany.

Correspondence to K.J. klaus.jung@charite.de

Abstract | MicroRNAs (miRNAs) are secreted by cells in vesicles, bound in a ribonucleoprotein complex or as free molecules. These miRNA secretion pathways are dysregulated in cancer, making miRNAs attractive candidate molecules for liquid biopsies. A number of studies have investigated the regulation of miRNA secretion into blood and urine and suggested that miRNAs are noninvasive diagnostic, prognostic and surveillance markers in urological carcinomas, and research in this area has increased over the past 5 years. However, methodological and analytical pitfalls exist and require addressing to enable future translation of the laboratory findings regarding miRNAs as biomarkers into clinical practice in bladder cancer, kidney cancer, prostate cancer and testicular cancer.

The promise of microRNAs (miRNAs) in the diagnosis, prognosis and therapy for urological tumours has caused much excitement.[1] The particular importance of miRNAs within the field of urology has been reflected in the rising number of annual publications on this topic indexed in the PubMed database, which has increased from 132 in 2010 to an estimated 400 in 2016 (Fig 1). During this period a considerable increase in the number of articles regarding miRNAs occurred in relation to all articles concerning urological neoplasms from 1.06% in 2010 to an estimated 2.63% in 2016. Moreover, ~3.7% of all articles regarding miRNAs refer to urological neoplasms. This increased research effort, enabled by use of novel methodologies, such as more sensitive or single cell sequencing approaches, digital polymerase chain reaction (PCR) or different variations of crosslinking immunoprecipitation methods and state-of-the-art bioinformatic facilities, has distinctly improved our understanding of the molecular processes that occur in urological tumours and also resulted in increased focus on the translational potential of miRNA research into clinical practice.

MiRNAs have great potential as novel cancer biomarkers.[2-6] In this Review we provide an update on the use of miRNAs specifically within the blood and/or urine of patients with urological cancers as diagnostic, prognostic and predictive biomarkers. In particular, we discuss the origin of miRNAs in biofluids, their use as biomarkers and the qualitative and quantitative changes in miRNAs in biofluids of patients with bladder, kidney, prostate and testicular cancer.

[H1] Biofluid miRNAs as biomarkers

Molecular processes associated with tumour characteristics, such as tumour stage, grade, size, aggressiveness and metastasis, result in dysregulated miRNA expression profiles in cancer tissue. This dysregulation was reported in The Cancer Genome Atlas (TCGA) studies, which included most urological malignancies.[7-11]

Potentially, these molecular alterations in tumours are mirrored in biofluids, and can be easily detected in blood and urine[12], providing the rationale for the potential use of miRNAs as noninvasive cancer biomarkers. According to Fuentes-Arderiu[13], a biomarker is defined as a "human or animal biological property whose *in vitro* measurement or identification is useful for the prevention, diagnosis, prognosis, treatment, and follow-up of human or animal diseases, and for their understanding". Finding molecular markers for diagnosis, follow-up monitoring, therapy choice, response prediction and surveillance is of critical importance, especially with regards to personalized medicine.[14] Personalized medicine aims to stratify patients into distinct molecular subtypes, which will allow to decide for the most suitable therapeutic approach to prevent unnecessary treatments with severe side effects.

MiRNAs can be detected in biofluids as free (or naked) circulating miRNAs, bound to ribonucleoprotein complexes (especially Argonaute proteins), high-density lipoproteins or nucleophosmin or in extracellular vesicles, such as exosomes, microvesicles or apoptotic bodies (Fig 2).[15-19] miRNAs that are bound, complexed or contained in extracellular vesicles are protected from degradation and, as a result, are stable in biofluids.[20,21] The most extensively studied miRNA-containing vesicles are exosomes. Packaging of miRNAs into vesicles seems to be selective, as miRNA content in the secreting cells and in vesicles differ from each other.[22] Exosomal secretion of miRNAs increases in metastatic cancer cells, suggesting they have a specific role in tumour progression.[23] The precise function of secreted vesicles is still under discussion, but results show that miRNAs enable cells to communicate with each other.[6,18,19]

Detecting miRNAs in biofluids is challenging and methodological pitfalls have to be taken into account. Factors that could affect miRNA measurements in the preanalytical, analytical and postanalytical phases of detection include the method of

sample collection, processing conditions, storage conditions, RNA isolation technique, quality control, quantification principle and the method of data evaluation (Box 1).[24-28] Furthermore, the development and evaluation of a biomarker assay is a multistage process, including the identification of suitable markers in discovery phase, the development of specific, sensitive and robust assays and their validation in various steps (Box 2). The development of a marker assay depends on the specific clinical question and the distinct aspects for the respective tumour type must be considered. This process goes well beyond the development of pure diagnostic markers. For example, for prostate cancer, factors such as the identification of suitable markers for the surveillance of patients need consideration, to avoid overtreatment with the risk of adverse effects.[5]

All these critical aspects should be considered as the basis for a continuous communication between laboratory and bioinformatics scientists, clinicians and statisticians in order to ensure reliable data for the clinical decision-making process. These characteristics are a useful starting point for the evaluation of the current status and future challenges regarding using miRNAs as biomarkers in biofluids.

[H1] Biofluid miRNAs in bladder cancer

The low diagnostic sensitivity of cytology for detecting low-grade bladder tumours as well as the inconvenience for patients and the cost of cystoscopy diagnosis and follow-up monitoring resulted in the development of numerous noninvasive urine-based tests.[29] These are, for example, FDA approved tests, such as the NMP22 test (based on the measurement of the nuclear mitotic apparatus protein 22 [NMP22]), the bladder tumour antigen (BTA) test (based on the detection of the human complement factor H-related protein) or the UroVysion assay (based on a fluorescence in situ hybridization assay of detecting chromosomal in exfoliated

bladder cancer cells). All these commercially available assays have limited clinical validity mostly due to their low diagnostic specificity with the consequence of high rate of false-positive results under benign urinary tract conditions such as infection, inflammation or haematuria.[29] Thus, the dysregulation of miRNAs in bladder tumour tissue and its possible reflection in urine and in blood have been suggested as promising new biomarkers for bladder cancer.[30] The European Association of Urology (EAU) guidelines on non-muscle-invasive urothelial carcinoma of the bladder define three main purposes in applying new molecular markers for bladder cancer: firstly, screening of the population at risk of bladder cancer; secondly, evaluation of patients with symptoms suspicious for bladder cancer; and thirdly, facilitated surveillance of patients with bladder cancer to reduce the number of cystoscopies.[31] Bladder cancer can be distinguished in to two different types, non-muscle-invasive bladder cancer (NMIBC) is confined to the mucosa or submucosa, in contrast to muscle-invasive bladder cancer (MIBC) that has invaded the muscle. In addition to the diagnosis of cancer, determining the recurrence and the risk of progression of NMIBC to MIBC are of clinical interest. For MIBC, events of progression and metastasis (which can change in response to the different therapy options) are connected to the clinical end points progression-free survival, cancer-specific survival and overall survival. NMIBC and MIBC differ particularly in their molecular characteristics.[32] A legitimate hope exists that an miRNA pattern dependent on the progressive nature of the bladder cancer[33] could be used as a diagnostic, prognostic and predictive marker through its reflection in biofluids.

[H3] miRNAs in urine samples.

In 2010, Hanke *et al.*[34] published the first report on urinary miRNAs as diagnostic tools for bladder cancer. Since then, further studies have been published (Table 1).[34-

[61] In general, retrospective observational studies in the form of case-control studies have been performed, mostly in single centres. Prospective cohort studies have been the exception. Furthermore, despite the urgent need for markers for surveillance of patients with bladder cancer, few studies investigating miRNAs in bladder cancer have addressed this issue.[52]

The diagnostic capacity of miRNAs are promising with regards to the global discrimination criterion of an area under the receiver operating characteristics curve (AUC) >0.75 (Table 1).[42,45,48,50,51] Moreover, some studies reported on the usefulness of miRNAs as prognostic markers, although there is no agreement on the use of specific miRNAs.[41,51,52] The use of miRNA combinations seems to be more advantageous than that of single miRNAs.[42,43,52,54] However, the lack of external validation of all miRNAs proposed as biomarkers prevents general conclusions on the clinical utility of miRNAs at present. In addition, factors such as differing study designs (in the relation to the objective of the study and the clinical heterogeneity of study cohorts — patients with MIBC or NMIBC were both included) and use of different urine test materials for miRNA measurements — urinary miRNAs were determined in native noncentrifuged urine, in sediment, in urine supernatant after first standard centrifugation and in extracellular vesicles like exosomes — make comparison difficult. In addition, the processing conditions for the preparation of test material were often not clearly reported. No consensus exists on what type of sample should be used, although test kits are commercially available for all these sample materials (from companies such as Norgen, Qiagen and Exiqon).[45] Furthermore, the choice of which miRNAs to detect differed between each study. For example, in some studies miRNAs were selected for measurement in urine samples in relation to their differential expression in tumour tissue identified in external or investigators' own previous studies. This approach might not result in the detection of the most

suitable biomarkers. miRNA secretion is a highly regulated process and the composition of miRNAs in biofluids and cells can differ greatly. Other authors decided on the panel of miRNAs according to the detection of released miRNAs in conditioned cell culture medium despite the fact that cell lines frequently differ in their molecular characteristics in comparison to the primary tumour cells. However, genome-wide discovery experiments in a first study phase, using sequencing or array technologies, and subsequent validations with individual RT-qPCR for each miRNA in the biofluid of interest are more meaningful and comprehensive.[50,54,59]

Importantly, in most studies, the evaluation of the diagnostic validity of miRNAs was limited without consideration of the recommendations included in the EAU guidelines. Generally, direct comparison of the performance of miRNAs with conventional cytology or other urine tests was not performed, with the exception of studies by Sapre *et al*.[52] and Eissa and colleagues.[48] Sapre *et al*.[52] calculated that their recommended miRNA monitoring set (diagnostic sensitivity 88%, diagnostic specificity 48% and negative predictive value 75%) could reduce the cystoscopy rate by 30%. Eissa *et al*.[48] used an integrative approach combining cytology results with data regarding the expression of two miRNAs (miR-96 and miR-210) and the long noncoding RNA urothelial cancer associated 1 (*UCA1*) and achieved remarkable discrimination between patients with bladder cancer and those with negative cystoscopy results with an AUC value of 0.933. Thus, in contrast to these two examples, the real benefit of the miRNA measurements remains frequently unclear in several studies, but their effectiveness in comparison to standard tests should be disclosed when reporting study results. The Standards for Reporting Diagnostic Accuracy (STARD) guidelines recommend comparison between the reference standard and the new test for assessing the real benefit of the new marker assay.[62]

Haematuria is a key symptom of bladder cancer.[31] Thus, miRNAs occurring in erythrocytes must be excluded as biomarkers for bladder cancer.[61,63] However, several studies have investigated miRNAs that are obviously affected by haemolysis with regards to their levels, apparently without consideration of the potential for interference (Table 1). Thus, numerous miRNAs that have been suggested to be cancer-specific biomarkers in various studies are likely to reflect haematuria but not the dysregulation of miRNAs in bladder cancer tissue.

[H3] miRNAs in serum and plasma samples.

Current data and understanding regarding miRNAs in blood are similar to those regarding miRNAs in urine. To date, eight studies concerning miRNA levels in serum or plasma have reported (Table 1). Published in 2013, the first studies in this field measured selected miRNAs that had previously been found to be dysregulated in bladder cancer tissue. However, data from two studies published in 2015[54,59], used a genome-wide, array-based profiling discovery phase with subsequent validation phases and corresponding large training and validation cohorts. Their results can be robustly assessed concerning the diagnostic and prognostic potential of circulating miRNAs (Table 1). Du *et al.*[59] achieved a global discrimination between control participants and patients with bladder cancer with an AUC value of 0.711, using the combination of miR-497 and miR-663b expression levels, which had been selected from eight differentially expressed miRNAs that had been identified in the discovery phase. These data were obtained using a two-phase validation method with separate training and validation cohorts. Jiang *et al.*[54] applied a similar approach; however, they identified, based on sequencing data of pooled serum samples in a discovery phase, a robust panel of six miRNAs in a training phase with 120 patients and 120 controls and a validation phase with 110 patients and 110 controls. Using the six-

miRNA diagnostic set, the investigators were able to differentiate between patients with NMIBC and those with MIBC with an AUC value of 0.899. Furthermore, risk stratification of patients with NMIBC regarding tumour recurrence was achieved by using an additional two-miRNA prognostic panel.

[H1] MiRNAs as biomarkers for bladder cancer

Based on these results, miRNAs in urine and serum or plasma should be considered in further studies as potential biomarkers for bladder cancer. However, solving the numerous issues concerning the preanalytical and analytical conditions, as well as data analysis, study design and choice of miRNAs investigated, is essential before well-designed multicentre systematic studies are performed. Currently, the application of a diagnostic or prognostic test validated in external studies is not available for clinical practice.

[H1] Biofluid miRNAs in kidney cancer

The clinical management of patients with renal cell carcinoma (RCC) is mainly based on traditional clinicopathological and radiological examinations. However, the diagnostic, prognostic and predictive models that are based on these conventional data alone have limited accuracy and need improving.[64] In contrast to other cancer types, robust noninvasive blood or urine biomarkers for all histological RCC subtypes are still lacking. Owing to these issues, the discovered effect of miRNAs on the initiation and progression of RCC has fuelled the desire to translate these new insights into novel miRNA-based biomarkers in biological fluids, such as blood and urine.

[H3] MiRNAs in serum, plasma and urine samples.

Available studies concerning miRNAs as biomarkers for RCC have striking particularities in their designs, preanalytical conditions and analytical conditions (Table 2).[65-76] Specifically, relatively few studies, including only one multicentre study[67], have been conducted, and most studies did not perform an internal validation with a training and validation set or even an internal validation approach, such as bootstrapping or cross-validation. Moreover, all studies except one used serum as the source of RNA despite plasma being the blood component of choice since the release of miRNAs from blood cells such as the miRNA-enriched platelets into serum occurs during the coagulation process.[77] Furthermore, collection processing was often incompletely described, and only one study[73] controlled for the effect of haemolysis as an interfering factor in the miRNA measurements. In addition, different strategies were applied for normalizing RT-qPCR data and contrasting results regarding the regulation of miR-378, as example, were reported already in three studies.[68,72,74]

All studies conducted to date were primarily focused on the diagnostic objective of discrimination between patients with RCC and healthy control subjects. The study cohorts consisted either of patients with only clear cell RCC (ccRCC)[69-71,74] — the subtype with the highest incidence — or combined with patients of papillary, chromophobe or sarcomatoid RCC.[67,68,72,75] To date, no studies have been performed that investigate the clinical need for differentiating histological RCC subtypes from biofluid samples. Discrimination of RCC subtypes using miRNAs has already been achieved successfully using tissue samples and is important for the risk stratification of patients.[78] The use of more than one miRNA is generally recommended to obtain improved accuracy and robustness of results.[72,74] Some studies compared circulating miRNA data with the simultaneously acquired miRNA

profiling data of matched RCC and normal tissue.[67,69,71] The observed changes in circulating miRNAs were not always concordant with the tissue expression levels[67,79] and association with clinicopathological factors like tumour stage and grade were not observed.[69,71,72,75] Such differences should not generally be interpreted as a contradiction; such distortions between cellular and circulating markers are possible as the molecular releasing processes of the different miRNAs from cells into the extracellular compartment are highly regulated. This phenomenon is well-known for other biomarkers, such as enzymes and cytokines.[80]

All but one of the single-centre studies reported acceptable discriminative abilities of different miRNAs with AUC values >0.75 (Table 2). However, the necessity of validating these results in independent validation cohorts became evident in a multicentre study.[67] miR-1233 was the only miRNA out of seven potential candidates selected after discovery and verification phases that maintained different expression values between patients and control subjects in a validation phase. It had only an AUC value of 0.588 (95%CI 0.505–0.671) for independent study cohorts, which is lower than the AUCs that had been reported in studies without independent study cohorts.[68,70,71]

In two studies, a decline in the concentration of miR-210 and miR-378 was observed after nephrectomy for treatment of RCC[70,72], and both miRNAs were suggested as potential indicators of cancer recurrence in follow-up monitoring of patients with RCC. Only one study demonstrated that miRNA expression could function as an independent prognostic marker, in which an increased expression level of miR-221 was associated with poor overall survival.[75] The predictive ability of a multivariate model that included tumour stage, Fuhrman grade and age ≥60 years was improved if the circulating miR-221 expression level was also included, demonstrated by the increase of the hazard ratio from 4.7 without miR-221

expression to 10.7 with expression included.[75] However, another study investigating the diagnostic and prognostic utility of miR-221 and miR-378 did not verify this predictive ability.[72] Thus, the evaluation of the potential of miRNAs as tools for risk stratification remains an important research goal.

Gamez-Pozo *et al*.[76] performed a particularly interesting study with regard to the use of miRNA signatures for personalizing treatment of metastatic RCC. The investigators examined the miRNA profiles of leucocytes in peripheral blood samples from patients receiving sunitinib for advanced RCC using microarrays. RT-qPCR-validated predictive models were developed using differentially expressed miRNAs identified in the microarray analysis to distinguish patients with likely to be resistance to sunitinib before the treatment was started; these models were also informative with regards to overall survival. This promising approach already published in 2012 has yet to be verified in an independent study.

Surprisingly, only one study using urine samples for miRNA analyses in patients with RCC has been reported. Von Brandenstein *et al*.[65] measured miR-15a levels in whole-urine samples from patients with ccRCC. Distinctly higher concentrations were observed in ccRCC patients than in the urine of patients with benign renal oncocytoma, urinary bladder cancer or other, nonurological tumours. This increase was probably caused by an active release of miRNAs from the tumour cells in exosomes. After nephrectomy, the concentration of miR-15a decreased to nearly zero by the time of hospital discharge. Thus, careful assessment of the potential of miRNAs in urine and its fractions as diagnostic and prognostic markers for RCC is urgently required.

[H1] MiRNAs as biomarkers in kidney cancer

In summary, despite some promising data no immediate prospect exists that miRNAs will be introduced as diagnostic or prognostic biomarkers either alone or in combination with the traditional clinicopathological factors in the routine management of patients with RCC. The lack of translational potential of miRNAs as biofluid biomarkers in RCC is probably a result of insufficient consideration of preanalytical and analytical variables, study heterogeneity and the retrospective nature of the reported studies. Also, studies using reference and target miRNAs that could be affected by haemolysis (such as miR-16, miR-21, miR-210 and miR-451)[61,63] are possibly biased. Prospective studies are necessary and should consider all variables that could influence the detection of miRNAs in biofluids (Box 1).

[H1] Biofluid miRNAs in prostate cancer

The use of serum PSA concentration as a screening marker for prostate cancer has resulted in controversial recommendations and debates.[81] One key point regarding the critical assessment of this marker is its limited ability to discriminate between aggressive tumours that need treatment and clinically insignificant tumours or benign prostatic diseases that do not require intervention but should undergo active surveillance.[81] Consequently, current biomarker research is focused on the translation of the new insights into molecular alterations in prostate cancer[82] into noninvasive biomarkers in biofluids, including miRNAs that would not have the disadvantages of PSA. Other purposes are the development of monitoring assays that reflect response to hormonal therapies, chemotherapeutic or radiotherapeutic options for prostate cancer treatment or improve prognostic information for both clinicians and patients.[3,5]

[H3] miRNAs in blood, serum and plasma samples.

In 2008, Mitchell *et al.*[20] presented the first data showing differing circulating miRNA concentrations in the serum of patients with metastatic prostate cancer in comparison with matched healthy controls. These findings triggered a storm of enthusiasm and resulted in a wave of subsequent studies and numerous reviews. To date, 37 reports investigating miRNAs in whole-blood samples, serum, plasma, peripheral mononuclear cells and exosomes have been published. Also, 10 articles have been published regarding urinary miRNAs in samples from patients with prostate cancer. (Table 3).[20,83-126]

The starting points of these studies for the identification of useful miRNAs (such as a profiling discovery phase, dysregulated tumour tissue expression data, cell culture results or data in the literature) and the subsequent validation phase were different, as has been observed for studies regarding miRNA expression in other urological tumours. This disparity and the heterogeneity and size differences of study cohorts make a comparison of the data between studies difficult. However, an assessment of the numerous studies with regards to the translation of miRNA biomarker research into clinical practice is facilitated by taking into account assessment criteria (Box 2). This comparison is possible by comparing the results regarding the same miRNAs measured in different studies with broadly similar objectives. The most frequently investigated miRNAs were miR-141 (in 15 studies), miR-21 (in 10 studies), miR-375 (in eight studies) and miR-221 (in seven studies). For example, the usefulness of miR-141 as marker for early detection of prostate cancer was only found in two out of six corresponding diagnostic studies.[121,126] In nine out of these 15 miR-141-studies, miR-141 was only evaluated as a marker of advanced or metastatic prostate cancer (Table 3).[20,84,96,99,101,103,113-115] but was frequently, if at all reported, not better than the routine marker PSA.[114,115,118] These data underline the fact that the clinical relevance of a marker can only be truly

assessed if studies with similar questions are compared: a useful diagnostic marker might be a useless prognostic marker and vice versa. Similarly, miR-21 proved to be unsuitable as an early marker in four studies (Table 3).[88,100,102,122] In addition, serum miR-21 expression levels discriminated between localized and metastatic prostate cancer, but its differential diagnostic power assessed by AUC values was surpassed by the traditional prostate cancer marker PSA.[118] By contrast, high levels of miR-21 in peripheral blood mononuclear cells in 75 patients with prostate cancer and 75 controls were strongly associated with prostate cancer presence (AUC = 0.833), recurrence and metastasis, but corresponding data for PSA or the Prostate Health Index were not reported.[123] miR-375 appears to be a specifically useful marker for prostate cancer. Two studies showed that use of miR-375 expression levels provided better discrimination between patients with prostate cancer and control subjects than PSA.[113,120] miR-375 was also reported as a marker for metastatic prostate cancer[96,118] and a prognostic marker for overall survival.[124] However, two other studies did not find that this miRNA could be used as tool to discriminate between patients with a positive biopsy result and those with a negative result or between patients stratified as having low-risk or high-risk prostate cancer.[88,121] These examples illustrate that results regarding the utility of specific miRNAs as biomarkers can be discrepant between various studies. These differences can be caused by differences in sample material and the study objective. Results published since 2015[111-113,120,121,123-126] (Table 3) report more promising diagnostic and prognostic data than earlier studies. One reason for this improvement could be that combinations of different miRNAs[111,121], miRNAs in combination with other markers[113,120] and miRNAs in blood cells[123] or exosomes[124] were investigated, rather than single miRNAs. However, the promising results of these studies are yet to be validated.

[H3] miRNAs in urine samples.

In 2012, the FDA approved the Progensa *PCA3* test that determines the ratio of PSA mRNA to the long noncoding RNA PCA3 in urine after standardized digital manipulation of the prostate for use in the decision-making process for repeat biopsy. Since then, several studies have been published that investigate the use of miRNAs in urine as predictive markers for positive biopsy results or the presence of high-risk cancer. However, results are divergent (Table 3)[83-92] and no firm conclusions can be made regarding the utility of miRNAs in urine as biomarkers for prostate cancer.

[H1] MiRNAs as biomarkers in prostate cancer

Whether the determination of miRNA in blood, serum, plasma and/or urine of patients with prostate cancer could be a useful clinical tool remains an open question. The evaluation of the various studies according to the criteria for the development of a biomarker assay (Box 2) need to be considered. Specifically, the selection of miRNAs that are not affected by haemolysis is not only necessary for the determination of miRNAs in serum and plasma samples but also, in particular, in urine samples; generally, the urine-based studies did not consider that urine samples after digital rectal examination (DRE) might become dipstick-positive for haematuria, as has been observed in 30–40% of samples, depending on the examiner (K. Jung, unpublished work). However, several studies used haemolysis-affected miRNAs as their markers (Table 3) and these study results are probably biased. Moreover, different urine preparations (such as whole urine, sediment or supernatant after centrifugation either after DRE or without DRE) were used. In this situation, contamination of miRNA levels is possible from unspecific bladder cells that interfere with the detected concentration of miRNAs released after DRE from prostate tumour cells as exosomal

and protein-bound miRNAs or miRNAs in cells shed from the tumour.[92] Moreover, most studies investigating the diagnostic and/or prognostic utility of miRNA biomarkers did not consider STARD or Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines.[62,127] These guidelines require that results of the comparator test (the reference standard) for the diagnostic objective and results in relation to the standard prognostic variables for predicting clinical end points should always be reported, in addition to the results regarding the biomarker assay examined. Several studies were not appropriately designed for answering the question of whether miRNA biomarkers enable incremental information beyond the conventional standards for predicting biopsy outcome or disease progression. This inadequacy is evident in urinary and blood miRNA studies as PCA3 in urine and the best serum PSA derivative, the Prostate Health Index, were rarely used as comparator tests.[86,92] Studies that applied multivariate Cox regression analysis for the assessment of clinical usefulness of miRNA concentrations as independent markers for predicting biochemical recurrence after prostatectomy or other outcomes are currently the exceptions.[104,111,124]

[H1] Biofluid microRNAs in testicular cancer

In 2010, the overexpression of the miRNA clusters miR-371–373 and miR-302–367 in all testicular cancers and their association with stem cells, as previously described[128,129], was reaffirmed.[130] Subsequently, the expression levels of these tissue-based signatures were measured in serum.[131-136] These liquid biopsy data confirmed the potential utility of miRNA measurements for discriminating patients with seminoma or those with nonseminoma from healthy control subjects. A selected set of four miRNAs (miR-371a-3p, miR-372, miR-373 and miR-367) present in the two clusters that showed the highest discriminative power for diagnosis and follow-up of

cancer, combined with reference miRNAs (miR-20a and miR-93) and spike-in controls was developed as the 'targeted serum miRNA assay' (TSmiR).[136] This test showed a distinctly higher diagnostic sensitivity of 98% in a cohort of 80 patients with testicular cancer and 47 control subjects from five different groups than the standard markers α-fetoprotein and β-human chorionic gonadotropin, which had sensitivities of only 36% and 57%, respectively. In addition, serum levels of these four miRNAs normalized after orchiectomy and decreased after chemotherapy in patients with metastatic disease, and miR-371a-3p was the most sensitive marker for detecting these changes.[134,136] Two independent studies, confirmed these promising results for miR-371a-3p as a biomarker for testicular cancer; however these reports only included a limited number of participants.[132,133] A simplified assay for analysing miR-371a-3p without endogenous controls was recommended to reduce laboratory work, analytical time and costs.[137] Results of another study confirmed the discriminative ability of serum miR-371a-3p and miR-372 concentrations and also identified numerous novel discriminative serum miRNAs (miR-511, miR-26b, miR-769, miR-23a, miR-106b, miR-365, miR-598, miR-340 and let-7a), which were identified using a high-throughput profiling system, but did not validate the results.[131] Ruf et al.[138] evaluated next-generation sequencing miRNA data obtained from RNA isolated from whole-blood samples from patients with seminoma using support vector machines as classifier method with different combinations of two miRNAs to discriminate between metastasized and nonmetastasized patients. Their approach combined simplification of sample collection with sophisticated technology and data analysis, which are important landmarks for translational research. Overall, 33–35 miRNAs with differential expression were identified and complete discrimination of patients with metastasized seminoma from those with nonmetastasized seminoma was achieved

using numerous combinations of pairs of these miRNAs, for example miR-18a and miR-532, miR-19b-1 and miR-342 or miR-28 and miR-574.

[H1] MiRNAs as biomarkers in testicular cancer

In summary, the current data considered together and despite the limited sample sizes in the individual studies provide convincing evidence for the use of miRNAs as biomarkers for testicular cancer. Supported by further positive data from an ongoing prospective multicentre study of the German Testicular Cancer Group (AUO No. AH 14/15-MicroRNA, German Association of Urogenital Oncology; http://auo-online.de),the introduction of miRNA measurements is anticipated for the clinical management of patients with testicular germ cell tumours in the near future.

[H1] Future perspectives

The number of studies investigating circulating miRNAs in urological tumours has increased greatly in the past 5 years. Next-generation sequencing has become faster and more affordable enabling rapid systematic sequencing of samples in large-scale, multicentre studies, facilitating biomarker research. Current studies confirm that a noninvasive measurement of circulating miRNA concentrations in urological carcinomas is possible and that dysregulation of miRNAs is specific for patients with cancer. A number of study results show that the expression changes that have been described in tissue are not always reflected in blood or urine, highlighting that regulated secretion pathways exist. Systematic multicentre studies, addressing the specific miRNA expression patterns (as have been performed in tissue samples) are missing in biofluids. Without these unbiased screening studies, some potential markers might still be undiscovered. The comparability and translational potential of the current studies is limited, even though the number of studies is increasing. The

reasons for these discrepancies are the existing preanalytical and analytical differences and the heterogeneity of tested specimens.

The different miRNA secretion pathways influence which samples are most suitable for marker detection. Few studies have addressed which miRNA component in biofluids (such as free miRNA, exosomal miRNA or miRNA bound in ribonucleoprotein complexes) is the most suitable for sensitive and robust marker detection. However, most circulating miRNAs are bound to proteins or packaged in vesicles and free circulating miRNAs only have limited stability, therefore, compartmentalized miRNAs seem to be the most suitable tumour markers.[2]

No consensus exists regarding which miRNA isolation method is most suitable for miRNA detection in serum, plasma or urine samples, and different preparations of blood (such as plasma, serum or whole blood) or urine (such as whole urine, supernatant or sediment taken after or without DRE) have been used. Other important analytical factors include haematuria and haemolysis. Follow-up studies must stringently control miRNA expression to mitigate the effects of haemolysis in order to exclude false-positive results. Analytical issues such as sensitivity, specificity, accuracy and robustness of the detection assays have not been sufficiently studied and need to be addressed. Next-generation sequencing and digital PCR might prove to be more accurate and sensitive than conventional miRNA expression profiling by microarray and RT-qPCR. A comparison of miRNA sequencing, microarray or RT-qPCR profiling indicates that each method has specific weaknesses, highlighting that which method to use must be considered with regards to the specific study design and end point.[139]

Validation of miRNA expression in independent cohorts and multicentre studies is limited. Additional prospective, multicentre studies, which stringently control preanalytical and analytical conditions, are necessary. In these studies, the end

points should be clearly defined and should address the specific clinical needs, such as surveillance markers in bladder cancer to reduce the need of invasive cystoscopy, stratification of aggressive prostate tumours or predictive markers in kidney cancer, which are important clinical issues that have not been sufficiently addressed. These studies would also enable the inclusion of miRNA markers into multivariate models. The resulting models should be compared with current standard markers. Without the existence of these studies, conclusive results on single miRNAs or miRNA combinations as diagnostic, prognostic or predictive markers is not possible.

Conclusions Circulating miRNAs are promising marker candidates for urological tumours. In testicular cancer particularly, a number of studies have good overlap in regard to the specific miRNA markers enabling comparison of the data and speedy translation of miRNAs as diagnostic markers into clinical practice. In bladder, kidney and prostate cancer, further studies are needed to address the methodological differences and large prospective studies concerning the respective clinical questions should be initiated to discover the usefulness of miRNAs as biomarkers in biofluids.

References

1. Schaefer, A., Stephan, C., Busch, J., Yousef, G. M. & Jung, K. Diagnostic, prognostic, and therapeutic implications of miRNAs in urologic tumors. *Nat. Rev. Urol.* **7**, 286-297 (2010).
2. Chen, M., Calin, G. A. & Meng, Q. H. Circulating microRNAs as promising tumor biomarkers. *Adv. Clin. Chem.* **67**, 189-214 (2014).
3. Fabris, L. *et al.* The potential of microRNAs as prostate cancer biomarkers. *Eur. Urol.* **70**, 312-322 (2016).
4. Fedorko, M. *et al.* MicroRNAs in the pathogenesis of renal cell carcinoma and their diagnostic and prognostic utility as cancer biomarkers. *Int. J. Biol. Markers* **31**, e26-e37 (2016).
5. Filella, X. & Foj, L. miRNAs as novel biomarkers in the management of prostate cancer. *Clin. Chem. Lab. Med.* doi:10.1515/cclm-2015-1073 (2016).
6. Junker, K., Heinzelmann, J., Beckham, C., Ochiya, T. & Jenster, G. Extracellular vesicles and their role in urologic malignancies. *Eur. Urol.* **70**, 323-331 (2016).
7. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* **499**, 43-49 (2013).
8. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322 (2014).
9. Cancer Genome Atlas Research Network. The molecular taxonomy of primary prostate cancer. *Cell* **163**, 1011-1025 (2015).
10. Davis, C. F. *et al.* The somatic genomic landscape of chromophobe renal cell carcinoma. *Cancer Cell* **26**, 319-330 (2014).
11. Linehan, W. M. *et al.* Comprehensive molecular characterization of papillary renal-cell carcinoma. *N. Engl. J. Med.* **374**, 135-145 (2016).
12. Weber, J. A. *et al.* The microRNA spectrum in 12 body fluids. *Clin. Chem.* **56**, 1733-1741 (2010).
13. Fuentes-Arderiu, X. What is a biomarker? It's time for a renewed definition. *Clin. Chem. Lab. Med.* **51**, 1689-1690 (2013).
14. Ibrahim, R., Pasic, M. & Yousef, G. M. Omics for personalized medicine: defining the current we swim in. *Expert Rev. Mol. Diagn.* doi:10.1586/14737159.2016.1164601 (2016).
15. Arroyo, J. D. *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U. S. A* **108**, 5003-5008 (2011).
16. Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. & Remaley, A. T. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* **13**, 423-433 (2011).
17. Wang, K., Zhang, S., Weber, J., Baxter, D. & Galas, D. J. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res.* **38**, 7248-7259 (2010).
18. Franzen, C. A., Blackwell, R. H., Foreman, K. E., Kuo, P. C. & Gupta, G. N. Urinary exosomes: the potential for biomarker utility, intercellular signaling and therapeutics in urological malignancy. *J. Urol.* **195**, 1331-1339 (2016).
19. Iftikhar, H. & Carney, G. E. Evidence and potential in vivo functions for biofluid miRNAs: From expression profiling to functional testing: Potential roles of extracellular miRNAs as indicators of physiological change and as agents of intercellular information exchange. *Bioessays* **38**, 367-378 (2016).
20. Mitchell, P. S. *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U. S. A* **105**, 10513-10518 (2008).
21. Mall, C., Rocke, D. M., Durbin-Johnson, B. & Weiss, R. H. Stability of miRNA in human urine supports its biomarker potential. *Biomark. Med.* **7**, 623-631 (2013).
22. Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol* **9**, 654-659 (2007).
23. Ostenfeld, M. S. *et al.* Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic properties. *Cancer Res.* **74**, 5758-5771 (2014).
24. McDonald, J. S., Milosevic, D., Reddi, H. V., Grebe, S. K. & Algeciras-Schimnich, A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin. Chem.* **57**, 833-840 (2011).
25. Kirschner, M. B., van Zandwijk, N. & Reid, G. Cell-free microRNAs: potential biomarkers in need of standardized reporting. *Front. Genet.* **4**, 56 (2013).

26. Farina, N. H. *et al.* Standardizing analysis of circulating microRNA: clinical and biological relevance. *J. Cell. Biochem.* **115**, 805-811 (2014).
27. Mlcochova, H., Hezova, R., Stanik, M. & Slaby, O. Urine microRNAs as potential noninvasive biomarkers in urologic cancers. *Urol. Oncol.* **32**, 41-49 (2014).
28. Ralla, B. *et al.* Nucleic acid-based biomarkers in body fluids of patients with urologic malignancies. *Crit. Rev. Clin. Lab. Sci.* **51**, 200-231 (2014).
29. Xylinas, E. *et al.* Urine markers for detection and surveillance of bladder cancer. *Urol. Oncol.* **32**, 222-229 (2014).
30. Netto, G. J. Molecular biomarkers in urothelial carcinoma of the bladder: are we there yet? *Nat. Rev. Urol.* **9**, 41-51 (2012).
31. Babjuk, M. *et al.* EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. *Eur. Urol.* **59**, 997-1008 (2011).
32. Knowles, M. A. & Hurst, C. D. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nat. Rev. Cancer* **15**, 25-41 (2015).
33. Rosenberg, E. *et al.* Predicting progression of bladder urothelial carcinoma using microRNA expression. *BJU Int.* **112**, 1027-1034 (2013).
34. Hanke, M. *et al.* A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *Urol. Oncol.* **28**, 655-661 (2010).
35. Yamada, Y. *et al.* MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology. *Cancer Sci.* **102**, 522-529 (2011).
36. Miah, S. *et al.* An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer. *Br. J. Cancer* **107**, 123-128 (2012).
37. Puerta-Gil, P. *et al.* miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. *Am. J. Pathol.* **180**, 1808-1815 (2012).
38. Snowdon, J., Boag, S., Feilotter, H., Izard, J. & Siemens, D. R. A pilot study of urinary microRNA as a biomarker for urothelial cancer. *Can. Urol. Assoc. J.* **15**, 1-5 (2012).
39. Wang, G. *et al.* Expression of microRNAs in the urine of patients with bladder cancer. *Clin. Genitourin. Cancer* **10**, 106-113 (2012).
40. Yun, S. J. *et al.* Cell-free microRNAs in urine as diagnostic and prognostic biomarkers of bladder cancer. *Int. J. Oncol.* **41**, 1871-1878 (2012).
41. Kim, S. M. *et al.* Cell-Free microRNA-214 from urine as a biomarker for non-muscle-invasive bladder cancer. *Korean J. Urol.* **54**, 791-796 (2013).
42. Mengual, L. *et al.* Using microRNA profiling in urine samples to develop a non-invasive test for bladder cancer. *Int. J. Cancer* **133**, 2631-2641 (2013).
43. Shimizu, T. *et al.* Methylation of a panel of microRNA genes is a novel biomarker for detection of bladder cancer. *Eur. Urol.* **63**, 1091-1100 (2013).
44. Tölle, A. *et al.* Identification of microRNAs in blood and urine as tumour markers for the detection of urinary bladder cancer. *Oncol. Rep.* **30**, 1949-1956 (2013).
45. Zhang, D. Z. *et al.* Cell-free urinary microRNA-99a and microRNA-125b are diagnostic markers for the non-invasive screening of bladder cancer. *PLoS One* **9**, e100793 (2014).
46. Zhou, X. *et al.* Urinary cell-free microRNA-106b as a novel biomarker for detection of bladder cancer. *Med. Oncol.* **31**, 197 (2014).
47. Eissa, S., Habib, H., Ali, E. & Kotb, Y. Evaluation of urinary miRNA-96 as a potential biomarker for bladder cancer diagnosis. *Med. Oncol.* **32**, 413 (2015).
48. Eissa, S., Matboli, M., Essawy, N. O. & Kotb, Y. M. Integrative functional genetic-epigenetic approach for selecting genes as urine biomarkers for bladder cancer diagnosis. *Tumour Biol.* **36**, 9545-9552 (2015).
49. Liu, W. *et al.* MiRNA-141 and miRNA-200b are closely related to invasive ability and considered as decision-making biomarkers for the extent of PLND during cystectomy. *BMC Cancer* **15**, 92 (2015).
50. Long, J. D. *et al.* A non-invasive miRNA based assay to detect bladder cancer in cell-free urine. *Am. J. Transl. Res.* **7**, 2500-2509 (2015).
51. Wang, J. *et al.* Downregulation of urinary cell-free microRNA-214 as a diagnostic and prognostic biomarker in bladder cancer. *J. Surg. Oncol.* **111**, 992-999 (2015).
52. Sapre, N. *et al.* A urinary microRNA signature can predict the presence of bladder urothelial carcinoma in patients undergoing surveillance. *Br. J. Cancer* **114**, 454-462 (2016).
53. Scheffer, A. R. *et al.* Circulating microRNAs in serum: novel biomarkers for patients with bladder cancer? *World J. Urol.* **32**, 353-358 (2014).
54. Jiang, X. *et al.* Serum microRNA expression signatures identified from genome-wide microRNA profiling serve as novel noninvasive biomarkers for diagnosis and recurrence of bladder cancer. *Int. J. Cancer* **136**, 854-862 (2015).

55. Kriebel, S. *et al.* Analysis of tissue and serum microRNA expression in patients with upper urinary tract urothelial cancer. *PLoS One* **10**, e0117284 (2015).

56. Adam, L. *et al.* Plasma microRNA profiles for bladder cancer detection. *Urol. Oncol.* **31**, 1701-1708 (2013).

57. Feng, Y. *et al.* microRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer. *BMC Urol.* **14**, 50 (2014).

58. Feng, Y. *et al.* miR-19a acts as an oncogenic microRNA and is up-regulated in bladder cancer. *J. Exp. Clin. Cancer Res.* **33**, 67 (2014).

59. Du, M. *et al.* Circulating miR-497 and miR-663b in plasma are potential novel biomarkers for bladder cancer. *Sci. Rep.* **5**, 10437 (2015).

60. Armstrong, D. A., Green, B. B., Seigne, J. D., Schned, A. R. & Marsit, C. J. MicroRNA molecular profiling from matched tumor and bio-fluids in bladder cancer. *Mol. Cancer* **14**, 194 (2015).

61. MacLellan, S. A., MacAulay, C., Lam, S. & Garnis, C. Pre-profiling factors influencing serum microRNA levels. *BMC Clin. Pathol.* **14**, 27 (2014).

62. Bossuyt, P. M. *et al.* Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Standards for Reporting of Diagnostic Accuracy. *Clin. Chem.* **49**, 1-6 (2003).

63. Kirschner, M. B. *et al.* The impact of hemolysis on cell-free microRNA biomarkers. *Front. Genet.* **4**, 94 (2013).

64. Sun, M. *et al.* Prognostic factors and predictive models in renal cell carcinoma: a contemporary review. *Eur. Urol.* **60**, 644-661 (2011).

65. von Brandenstein, M. *et al.* MicroRNA 15a, inversely correlated to PKCalpha, is a potential marker to differentiate between benign and malignant renal tumors in biopsy and urine samples. *Am. J Pathol.* **180**, 1787-1797 (2012).

66. Schmitt, J. *et al.* Treatment-independent miRNA signature in blood of Wilms tumor patients. *BMC Genomics* **13**, 379 (2012).

67. Wulfken, L. M. *et al.* MicroRNAs in renal cell carcinoma: diagnostic implications of serum miR-1233 levels. *PLoS One* **6**, e25787 (2011).

68. Redova, M. *et al.* Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma. *J. Transl. Med.* **10**, 55 (2012).

69. Cheng, T. *et al.* Differential microRNA expression in renal cell carcinoma. *Oncol. Lett.* **6**, 769-776 (2013).

70. Zhao, A., Li, G., Peoc'h, M., Genin, C. & Gigante, M. Serum miR-210 as a novel biomarker for molecular diagnosis of clear cell renal cell carcinoma. *Exp. Mol. Pathol.* **94**, 115-120 (2013).

71. Iwamoto, H. *et al.* Serum miR-210 as a potential biomarker of early clear cell renal cell carcinoma. *Int. J. Oncol.* **44**, 53-58 (2014).

72. Fedorko, M. *et al.* Combination of miR-378 and miR-210 serum levels enables sensitive detection of renal cell carcinoma. *Int. J. Mol. Sci.* **16**, 23382-23389 (2015).

73. Ludwig, N. *et al.* Circulating serum miRNAs as potential biomarkers for nephroblastoma. *Pediatr. Blood Cancer* **62**, 1360-1367 (2015).

74. Wang, C. *et al.* A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma. *Sci. Rep.* **5**, 7610 (2015).

75. Teixeira, A. L. *et al.* Higher circulating expression levels of miR-221 associated with poor overall survival in renal cell carcinoma patients. *Tumour Biol.* **35**, 4057-4066 (2014).

76. Gamez-Pozo, A. *et al.* MicroRNA expression profiling of peripheral blood samples predicts resistance to first-line sunitinib in advanced renal cell carcinoma patients. *Neoplasia* **14**, 1144-1152 (2012).

77. Wang, K. *et al.* Comparing the microRNA spectrum between serum and plasma. *PLoS One* **7**, e41561 (2012).

78. Youssef, Y. M. *et al.* Accurate molecular classification of kidney cancer subtypes using microRNA signature. *Eur. Urol.* **59**, 721-730 (2011).

79. Hauser, S. *et al.* Analysis of serum microRNAs (miR-26a-2*, miR-191, miR-337-3p and miR-378) as potential biomarkers in renal cell carcinoma. *Cancer Epidemiol.* **36**, 391-394 (2012).

80. Friedel, R., Diederichs,F. & Lindena,J. Release and extracellular turnover of cellular enzymes in *Advances in Clinical Enzymology* (eds. Schmidt, E., Schmidt,F.W., Trautschold,I. & Friedel,R.) 70-105 (S. Karger AG, Basel, 1979).

81. Qaseem, A., Barry, M. J., Denberg, T. D., Owens, D. K. & Shekelle, P. Screening for prostate cancer: a guidance statement from the Clinical Guidelines Committee of the American College of Physicians. *Ann. Intern. Med* **158**, 761-769 (2013).

82. Dietrich, D. *et al.* Nucleic acid-based tissue biomarkers of urologic malignancies. *Crit. Rev. Clin. Lab. Sci.* **51**, 173-199 (2014).

83. Ahumada-Tamayo, S. MicroRNA determination in urine for prostate cancer detection in Mexican Patients at the Hospital General "Dr. Manuel Gea Gonzalez". *Rev. Mex. Urol.* **71**, 213-217 (2011).
84. Bryant, R. J. *et al.* Changes in circulating microRNA levels associated with prostate cancer. *Br. J. Cancer* **106**, 768-774 (2012).
85. Srivastava, A. *et al.* MicroRNA profiling in prostate cancer - the diagnostic potential of urinary miR-205 and miR-214. *PLoS One* **8**, e76994 (2013).
86. Casanova-Salas, I. *et al.* Identification of miR-187 and miR-182 as biomarkers of early diagnosis and prognosis in patients with prostate cancer treated with radical prostatectomy. *J. Urol.* **192**, 252-259 (2014).
87. Haj-Ahmad, T. A., Abdalla, M. A. & Haj-Ahmad, Y. Potential urinary miRNA biomarker candidates for the accurate detection of prostate cancer among benign prostatic hyperplasia patients. *J. Cancer* **5**, 182-191 (2014).
88. Sapre, N. *et al.* Curated microRNAs in urine and blood fail to validate as predictive biomarkers for high-risk prostate cancer. *PLoS One* **9**, e91729 (2014).
89. Yun, S. J. *et al.* Urinary microRNAs of prostate cancer: virus-encoded hsv1-miRH18 and hsv2-miR-H9-5p could be valuable diagnostic markers. *Int. Neurourol. J.* **19**, 74-84 (2015).
90. Egidi, M. G. *et al.* Characterization of kallikreins and microRNAs in urine sediment for the discrimination of prostate cancer from benign prostatic hyperplasia. *J. Cancer Sci. Ther.* **7**, 130-136 (2015).
91. Korzeniewski, N. *et al.* Identification of cell-free microRNAs in the urine of patients with prostate cancer. *Urol. Oncol.* **33**, 16-22 (2015).
92. Stephan, C., Jung, M., Rabenhorst, S., Kilic, E. & Jung, K. Urinary miR-183 and miR-205 do not surpass PCA3 in urine as predictive markers for prostate biopsy outcome despite their highly dysregulated expression in prostate cancer tissue. *Clin. Chem. Lab. Med.* **53**, 1109-1118 (2015).
93. Santos, J. I. *et al.* Influence of peripheral whole-blood microRNA-7 and microRNA-221 high expression levels on the acquisition of castration-resistant prostate cancer: evidences from in vitro and in vivo studies. *Tumour Biol.* **35**, 7105-7113 (2014).
94. Leidinger, P. *et al.* Differential blood-based diagnosis between benign prostatic hyperplasia and prostate cancer: miRNA as source for biomarkers independent of PSA level, Gleason score, or TNM status. *Tumour Biol.* **37**, 10177-10185 (2016).
95. Lodes, M. J. *et al.* Detection of cancer with serum miRNAs on an oligonucleotide microarray. *PLoS One* **4**, e6229 (2009).
96. Brase, J. C. *et al.* Circulating miRNAs are correlated with tumor progression in prostate cancer. *Int. J. Cancer* **128**, 608-616 (2011).
97. Mahn, R. *et al.* Circulating microRNAs (miRNA) in serum of patients with prostate cancer. *Urology* **77**, 1265.e9-1265.e16 (2011).
98. Moltzahn, F. *et al.* Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res.* **71**, 550-560 (2011).
99. Selth, L. A. *et al.* Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease. *Int. J. Cancer* **131**, 652-661 (2011).
100. Zhang, H. L. *et al.* Serum miRNA-21: elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy. *Prostate* **71**, 326-331 (2011).
101. Cheng, H. H. *et al.* Circulating microRNA profiling identifies a subset of metastatic prostate cancer patients with evidence of cancer-associated hypoxia. *PLoS One* **8**, e69239 (2013).
102. Egidi, M. G. *et al.* Circulating microRNAs and kallikreins before and after radical prostatectomy: are they really prostate cancer markers? *Biomed. Res. Int.* **2013**, 241780 (2013).
103. Nguyen, H. C. *et al.* Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer. *Prostate* **73**, 346-354 (2013).
104. Selth, L. A. *et al.* Circulating microRNAs predict biochemical recurrence in prostate cancer patients. *Br. J. Cancer* **109**, 641-650 (2013).
105. Zhang, H. L. *et al.* An elevated serum miR-141 level in patients with bone-metastatic prostate cancer is correlated with more bone lesions. *Asian J. Androl.* **15**, 231-235 (2013).
106. Haldrup, C. *et al.* Profiling of circulating microRNAs for prostate cancer biomarker discovery. *Drug Deliv. Transl. Res.* **4**, 19-30 (2014).
107. Kotb, S. *et al.* Circulating miRNAs 21 and 221 as biomarkers for early diagnosis of prostate cancer. *Tumour Biol.* **35**, 12613-12617 (2014).
108. Singh, P. K. *et al.* Serum microRNA expression patterns that predict early treatment failure in prostate cancer patients. *Oncotarget* **5**, 824-840 (2014).

109. Wang, S. Y. *et al.* miR-19, miR-345, miR-519c-5p serum levels predict adverse pathology in prostate cancer patients eligible for active surveillance. *PLoS One* **9**, e98597 (2014).

110. Westermann, A. M. *et al.* Serum microRNAs as biomarkers in patients undergoing prostate biopsy: results from a prospective multi-center study. *Anticancer Res.* **34**, 665-669 (2014).

111. Mihelich, B. L., Maranville, J. C., Nolley, R., Peehl, D. M. & Nonn, L. Elevated serum microRNA levels associate with absence of high-grade prostate cancer in a retrospective cohort. *PLoS One* **10**, e0124245 (2015).

112. Sun, X. *et al.* Prognostic implications of tissue and serum levels of microRNA-128 in human prostate cancer. *Int. J. Clin. Exp. Pathol.* **8**, 8394-8401 (2015).

113. Wach, S. *et al.* The combined serum levels of miR-375 and urokinase plasminogen activator receptor are suggested as diagnostic and prognostic biomarkers in prostate cancer. *Int. J. Cancer* **137**, 1406-1416 (2015).

114. Agaoglu, F. Y. *et al.* Investigation of miR-21, miR-141, and miR-221 in blood circulation of patients with prostate cancer. *Tumour Biol.* **32**, 583-588 (2011).

115. Gonzales, J. C. *et al.* Comparison of circulating microRNA 141 to circulating tumor cells, lactate dehydrogenase, and prostate-specific antigen for determining treatment response in patients with metastatic prostate cancer. *Clin. Genitourin. Cancer* **9**, 39-45 (2011).

116. Chen, Z. H. *et al.* A panel of five circulating microRNAs as potential biomarkers for prostate cancer. *Prostate* **72**, 1443-1452 (2012).

117. Shen, J. *et al.* Dysregulation of circulating microRNAs and prediction of aggressive prostate cancer. *Prostate* **72**, 1469-1477 (2012).

118. Watahiki, A. *et al.* Plasma miRNAs as biomarkers to identify patients with castration-resistant metastatic prostate cancer. *Int. J. Mol. Sci.* **14**, 7757-7770 (2013).

119. Medina-Villaamil, V. *et al.* Circulating MicroRNAs in blood of patients with prostate cancer. *Actas Urol. Esp.* **38**, 633-639 (2014).

120. Kachakova, D. *et al.* Combinations of serum prostate-specific antigen and plasma expression levels of let-7c, miR-30c, miR-141, and miR-375 as potential better diagnostic biomarkers for prostate cancer. *DNA Cell Biol.* **34**, 189-200 (2015).

121. Kelly, B. D. *et al.* A circulating microRNA signature as a biomarker for prostate cancer in a high risk group. *J. Clin. Med.* **4**, 1369-1379 (2015).

122. Chen, H. *et al.* Evaluation of plasma miR-21 and miR-152 as diagnostic biomarkers for common types of human cancers. *J. Cancer* **7**, 490-499 (2016).

123. Huang, W., Kang, X. L., Cen, S., Wang, Y. & Chen, X. High-level expression of microRNA-21 in peripheral blood mononuclear cells is a diagnostic and prognostic marker in prostate cancer. *Genet. Test. Mol. Biomarkers* **19**, 469-475 (2015).

124. Huang, X. *et al.* Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. *Eur. Urol.* **67**, 33-41 (2015).

125. Xu, S. *et al.* Downregulation of miR-129 in peripheral blood mononuclear cells is a diagnostic and prognostic biomarker in prostate cancer. *Int. J. Clin. Exp. Pathol.* **8**, 14335-14344 (2015).

126. Li, Z. *et al.* Exosomal microRNA-141 is upregulated in the serum of prostate cancer patients. *Onco. Targets. Ther.* **9**, 139-148 (2016).

127. Altman, D. G., McShane, L. M., Sauerbrei, W. & Taube, S. E. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. *PLoS Med.* **9**, e1001216 (2012).

128. Voorhoeve, P. M. *et al.* A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124**, 1169-1181 (2006).

129. Gillis, A. J. *et al.* High-throughput microRNAome analysis in human germ cell tumours. *J. Pathol.* **213**, 319-328 (2007).

130. Palmer, R. D. *et al.* Malignant germ cell tumors display common microRNA profiles resulting in global changes in expression of messenger RNA targets. *Cancer Res.* **70**, 2911-2923 (2010).

131. Rijlaarsdam, M. A. *et al.* Identification of known and novel germ cell cancer-specific (embryonic) miRs in serum by high-throughput profiling. *Andrology* **3**, 85-91 (2015).

132. Spiekermann, M. *et al.* MicroRNA miR-371a-3p in serum of patients with germ cell tumours: evaluations for establishing a serum biomarker. *Andrology* **3**, 78-84 (2015).

133. Syring, I. *et al.* Circulating serum miRNA (miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p) as biomarkers in patients with testicular germ cell cancer. *J. Urol.* **193**, 331-337 (2015).

134. Dieckmann, K. P. *et al.* MicroRNAs miR-371-3 in serum as diagnostic tools in the management of testicular germ cell tumours. *Br. J. Cancer* **107**, 1754-1760 (2012).

135. Murray, M. J. *et al.* Identification of microRNAs From the miR-371~373 and miR-302 clusters as potential serum biomarkers of malignant germ cell tumors. *Am. J. Clin. Pathol.* **135**, 119-125 (2011).

136. Gillis, A. J. *et al.* Targeted serum miRNA (TSmiR) test for diagnosis and follow-up of (testicular) germ cell cancer patients: A proof of principle. *Mol. Oncol* **7**, 1083-1092 (2013).

137. Spiekermann, M., Dieckmann, K. P., Balks, T., Bullerdiek, J. & Belge, G. Is relative quantification dispensable for the measurement of microRNAs as serum biomarkers in germ cell tumors? *Anticancer Res.* **35**, 117-121 (2015).

138. Ruf, C. G. *et al.* Small RNAs in the peripheral blood discriminate metastasized from non-metastasized seminoma. *Mol. Cancer* **13**, 47 (2014).

139. Mestdagh, P. *et al.* Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. *Nat. Methods* **11**, 809-815 (2014).

Review criteria

A PubMed search was performed for original articles recorded in the database from 2010 to February 2016. The MeSH term "MicroRNAs" combined with the search string ["microRNAs" OR "microRNA" OR "micro-RNA" OR "micro-RNAs" OR miRNAs"] both alone and in combination with the MeSH terms "prostatic neoplasms", "kidney neoplasms", "urinary bladder neoplasms" and "testicular neoplasms". The urological MeSH terms were always linked with the corresponding keywords "prostate cancer", "renal carcinoma", "bladder cancer" or "testicular cancer" using the Boolean operator "OR" to improve the search strategies. In addition, reference lists of the identified articles and corresponding reviews were searched to ascertain further relevant publications.

Key points

- Several hundred miRNAs occur in biofluids as free molecules or are secreted in vesicles and bound in a ribonucleoprotein complex. The secretion of miRNAs into biofluids is dysregulated in cancer making miRNAs potential noninvasive tumour biomarkers

- Studies performed on samples from patients with urological carcinomas suggest that miRNAs in blood, serum, plasma and urine can be applied as novel diagnostic, prognostic and surveillance biomarkers

- Inconsistent and contradictory results from studies in bladder, kidney and prostate cancer impede translation of miRNA measurements into routine clinical practice, but a clinically useful signature confirmed in several studies has been developed for testicular cancer

- Preanalytical, analytical and postanalytical differences as well as insufficient power and the heterogeneity of studies are the main factors in shortcomings in this research field

- Prospective, multicentre studies that consider all these deficiencies in their design are necessary to assess the real clinical benefit of miRNA measurements in the biofluids of patients with urological carcinomas

ABOUT THE AUTHORS

ANNIKA FENDLER, PhD is a postdoctoral fellow at the Department of Signal Transduction, Invasion and Metastasis of Epithelial Cells, Max Delbrück Center of Molecular Medicine in the Helmholtz Association, Berlin-Buch, Germany.
She studied biotechnology at the University of Applied Sciences, Darmstadt, Germany. During her PhD at the Free University Berlin and the Berlin Institute for Urologic Research, she started her research on the regulation of microRNAs in prostate cancer, their utilization as molecular tumour markers and their functional impact and continued this research focused on kidney cancer. Her present research interests are cancer stem cells and non-invasive tumour markers in kidney cancer.

CARSTEN STEPHAN, MD, PhD is Professor at the Department of Urology, University Hospital Charité, Berlin, Germany.
He studied human medicine in Berlin (Charité) and Baltimore (Johns Hopkins Hospital). Since 1999 he works as MD at the Department of Urology, Charité, Berlin and completed a postdoctoral fellowship at the Mount Sinai Hospital, Toronto, Canada. In 2011, he became a MBA (health care) and since 2014 he is an APL professor. He was awarded to various scientific prices for his research on prostate cancer and he is author/coauthor of about 230 publications.

GEORGE M. YOUSEF, MD, PhD is Professor at the University of Toronto; Canada and

Head of the Division of Molecular Diagnostics at St. Michael's Hospital, Toronto.

He is an anatomical pathologist and his research laboratory is one of the leading laboratories focusing on the cancer biomarkers in renal cell carcinoma and prostate cancer using an integrated genomic, quantitative proteomic, transcriptomic and miRNA approaches with extensive bioinformatics analysis. His laboratory is also internationally recognized in miRNA research. He has over 200 publications, serves as a reviewer for over 40 journals and is the Editor-in-Chief of the Canadian Journal of Pathology.

GLEN KRISTIANSEN, MD, PhD is Full Professor and Chairman of the Institute of Pathology, University of Bonn, Germany.
His special interest is genito-urinary pathology with a focus on prostate cancer and molecular pathology of solid tumours (>270 original papers). He is the european representative in the council of the International Society of Urological Pathology (ISUP), chair of the European Network of Pathology (ENUP) and in charge of the reference pathology of several national prostate cancer trials in Germany.

KLAUS JUNG, MD, PhD is Professor and since his retirement in charge of the scientific direction of the Berlin Institute for Urologic Research of the Foundation Urologic Research, Berlin in cooperation with the Department of Urology, University Hospital Charite, Berlin, Germany.
His research interests focus on molecular urooncology, tumour markers and general aspects in laboratory medicine. He is author/coauthor of more then 600 scientific publications and book chapters.

Box 1 | Variables influencing the determination miRNAs in blood samples[‡]

Preanalytical phase
    Sample collection
● Method of blood collection (aspiration or vacuum extraction systems)
● Type of collection devices for serum (with or without enhanced coagulation using silicagel or kaolin) or plasma (using citrate, EDTA or heparin as anticoagulants) in different tubes (glass or plastic tubes, with gel separation)
● Haemolysis effect (inadequate blood sampling, sample mixing)
● Presence of endogenous inhibitors

    Processing conditions
● Time span between venipuncture and centrifugation
● Centrifugation conditions (forces, time, repeat centrifugation)
● Separation of serum or plasma from blood clot

    Storage conditions
● Temperature (long-term storage at -80 °C preferred)

Analytical phase
    RNA isolation protocols
● Use of different sample volumes
● Type of RNA isolation procedure with manufacturer's specifics (such as Trizol, column-based or bead-based methods with or without genomic DNA removal, carrier addition, and enrichment of small RNA fraction)
● Elution with different solutions and volumes

    RNA quality control
● Absorption ratios 260:280 nm and 230:260 nm; RNA integrity number or RNA quality indicator

    Quantification principles
● Different methods for analysing with different sensitivity and/or specificity (different array platforms, different sequencing methods and different RT-qPCR principles [such as Life Technologies, Qiagen, Exiqon and Nanostring] based on different complementary DNA synthesis conditions with or without preamplification and quantitative PCR methods [DNA-binding dyes or probes])
    Digital PCR
● Different instruments associated with various software

Postanalytical phase
    Data evaluation
● Different normalization strategies (endogenous reference genes, spike-in controls, absolute quantification, relation to starting sample volume, global mean or median approach)

[‡] Modified from Ralla, B. *et al. Crit. Rev. Clin. Lab. Sci.* **51,** 200-231 (2014) . EDTA, ethylenediaminetetraacetic acid; RT-qPCR, reverse-transcription quantitative PCR.

Box 2 | Development phases of biomarker assays for clinical practice

Discovery and selection of potential biomarkers:
 Identification and selection of differentially expressed miRNAs according to the objective based on various principles:
● Genome-wide profiling in the corresponding sample material on array or sequencing basis
● Selected differentially expressed miRNAs identified in the cancer tissue
● Released miRNAs from corresponding cancer cell cultures into conditioned medium
● Data from the literature

Assay setup and performance control:
 All working steps (preanalytical, analytical, postanalytical) of the determination of the selected biomarker should be established for a robust, accurate and reproducible assay or at least a fit-for-purpose assay

Validation by clinical assessment
 Confirmation of utility as a screening, diagnostic, prognostic and/or predictive marker in retrospective and/or single-centre studies with internal validation

Validation by clinical usability
 Confirmation in prospective, multicentre studies as advantageous or at least equivalent in comparison with standard procedures in the decision making process of the clinical management of patients

Legends to the figures

Figure 1 | Total annual microRNA (miRNA) publications indexed in the PubMed database relating to urological tumours. The literature search was performed for the period from 2010 to February 2016.

Figure 2 | The origin of the circulating microRNAs (miRNAs). MiRNAs are secreted by cells via specific regulated pathways. Exosomes are secreted when multivesicular bodies fuse with the plasma membrane. Microvesicles are formed by outward bulging of the cell membrane. Apoptotic bodies are formed at the late stage of apoptosis via membrane blebbing and contain cytoplasmic and nuclear components of the cells. miRNA can also be secreted bound to ribonucleoprotein complexes. The main protein binding partners are Argonaute proteins[15], but they can also bind to other proteins such as high-density lipoproteins[16] and nucleophosmin.[17] Freely circulating miRNAs can be detected, which are either directly released by cancer cells or by vesicles, for example apoptotic bodies.

Table 1 | Studies regarding miRNAs in urine and blood as potential biomarkers in bladder cancer

Reference (year)	Study with marker development phases, number of patients and controls [*]	Sample	Method; reference method [‡]	Significant miRNA expression[§]	Clinically relevant findings[∥]
Urine					
Hanke *et al.* (2010)[34]	Discovery: 27 patients (nine G1, nine G2/3, nine urinary tract infections), nine controls Validation: 29 patients (11 G1, 18 G2/3, 9 infections), 11 controls	Whole-urine	Discovery: TaqMan array (Early access kit, 157 miRNAs) Validation: RT-qPCR (TaqMan); miR-152	**miR-126** ↑ **miR-182** ↑	Diagnosis of cancer with miR-126: miR-182 ratio as best indicator: (AUC = 0.768, DS = 72%, DSp = 82%)
Yamada *et al.* (2011)[35]	100 patients 74 controls (healthy adults, some with urinary tract infection)	Urine sediment	RT-qPCR (TaqMan); absolute quantification	**miR-96** ↑ **miR-183** ↑	Diagnosis of cancer with miR-96 (AUC = 0.831, DS = 71%, DSp = 79%) and miR-183 (AUC = 0.817, DS = 74%, DSp = 77%)
Miah *et al.* (2012)[36]	68 patients (with new or recurrent cancer) 53 age-matched controls	Urine sediment	RT-qPCR (TaqMan); RNU44 and RNU48	**miR-15b** ↑ miR-135b ↓ miR-1224-3p↑	Diagnosis of cancer using the three miRNAs: (AUC = 0.86, DS = 94.1, DSp = 51%)
Puerta-Gil *et al.* (2012)[37]	37 patients 57 controls (healthy adults, benign urological diseases)	Urine (fraction not defined)	RT-qPCR (TaqMan); miR-16	**miR-143** ↓ **miR-222** ↑ miR-452 ↑	Diagnosis of cancer with miR-222 (AUC = 0.718) and miR-452 (AUC = 0.848)
Snowdon *et al.* (2012)[38]	Eight patients Five controls	Whole-urine	RT-qPCR (TaqMan); RNU6B	miR-125b ↓ **miR-126** ↑	Without AUC data
Wang *et al.* (2012)[39]	51 patients 24 subjects with negative cystoscopy results	Urine sediment and supernatant	RT-qPCR (TaqMan); RNU48	miR-200 family miR-141 ↓ miR-200a ↓ miR-200b ↓ miR-200c ↓ miR-429 ↓	Differentiation between study groups only with sediment data: AUC = 0.706–0.804 and for miR-200a DS = 100% and DSp = 53%

Yun et al. (2012)[40]	207 patients (138 with NMIBC and 69 with MIBC) 144 controls	Urine supernatant	RT-qPCR (Qiagen); RNU6	miR-145 ↓ miR-200a ↓	Diagnosis of NMIBC and MIBC with miR-145: AUC = 0.729 and 0.790, DS = 78% and 84%, DSp = 61% and 61% Prognostic marker: miR-200a as an independent marker for NMIBC recurrence
Kim et al. (2013)[41]	138 NMIBC 144 controls	Urine supernatant	RT-qPCR (Qiagen); RNU6	miR-214 ↑	Diagnosis of cancer and prognostic marker for NMIBC recurrence
Mengual et al. (2013)[42]	Discovery: 30 patients and 10 controls Validation: 151 patients and 121 patients with twothirds as a training set and onethird as a test set	Urine sediment (from 50-100 ml urine)	Discovery: TaqMan array Validation: RT-qPCR (Exiqon); miR-30c and miR-103	Panel 1: **miR-18a*** ↑ **miR-25** ↑ miR-187 ↑ **miR-140-5p** ↓ miR-142-3p ↓ miR-204 ↓ Panel 2: **miR-125b** ↓ **miR-92a** ↑	Diagnosis of cancer with panel 1: (AUC = 0.92, DS = 85%, DSp = 87%) Discrimination between low-grade and high-grade tumours with panel 2: AUC = 0.83, DS = 85%, DSp = 74%
Shimizu et al. (2013)[43]	Discovery: 86 patients, 20 individuals without cancer Validation: training set with 86 patients and test set with 34 patients	Urine sediment	Methylation-specific PCR, bisulfite sequencing and bisulfite pyrosequen-cing	Methylated miRs miR-9-3 ↓ miR-124-2 ↓ miR-124-3 ↓ miR-137 ↓	Diagnosis of cancer using the combined miRNAs: AUC = 0.91 Detection of Ta stage and low grades G1 and G2: AUC = 0.862, DS = 68%, DSp = 89%
Tölle et al. (2013)[44]	Discovery: Eight patients and four healthy controls Validation: 36 patients and 19 controls	Whole-urine	Discovery: TaqMan array Validation: RT-qPCR (Qiagen); absolute quantification	miR-520e ↑ miR-618 ↑ miR-1225-5p ↑	Diagnosis of cancer: AUC = 0.679–0.764
Zhang et al. (2014)[45]	Discovery: Six patients and three controls Validation: 50 patients and 21 controls	Urine supernatant	Discovery: Agilent array, Validation: RT-qPCR (Exiqon); RNU6B	miR-99a ↓ miR-125b ↓	Diagnosis of cancer with both miRNAs in a logistic model: AUC = 0.876, DS = 87%, DSp = 82% Discrimination between G1 and G≥2 tumours : AUC = 0.831, DS = 79%, DSp = 88%
Zhou et al. (2014)[46]	112 patients 78 controls	Urine supernatant	RT-qPCR (Qiagen); RNU6	**miR-106b** ↑	Diagnosis of cancer: AUC = 0.802, DS = 76.8%, DSp = 72.4% Suggested as tumour recurrence marker after surgery

Eissa *et al.* (2015)[47]	94 patients 90 controls (including 30 with benign bladder lesions)	Urine sediment	RT-qPCR (Qiagen); RNU6	**miR-96 ↑**	Diagnosis of cancer: AUC = 0.822, DS = 72.3%, DSp = 88.9%
Eissa *et al.* (2015)[48]	Prospective study combined with other tests: 94 patients, 56 with negative cystoscopy results with benign urinary conditions and 60 healthy controls	Urine sediment	RT-qPCR (Qiagen); RNU6	miR-210 ↑ **miR-96 ↑**	Diagnosis of cancer with miRNAs combined with cytology and lncRNA-UCA1: AUC = 0.933, DS = 100%, DSp = 89.5% Reduction of false-negative urine cytology results from 49% to 0%
Liu *et al.* (2015)[49]	78 patients who underwent lymph node dissection (23 positive nodes, 54 negative nodes)	Urine sediment	RT-qPCR (TaqMan); RNU6	miR-141 ↓ miR-200b ↓	Prediction of positive lymph nodes by combined use of miRNAs and cancer stage: AUC = 0.749, better than CT staging: AUC = 0.679
Long *et al.* (2015)[50]	Prospective study Discovery: pooled samples from 3 groups of 85 patients versus 2 groups of 45 controls Validation: 85 patients, 45 controls	Urine supernatant, exosomes	Discovery: Exiqon array Validation: RT-qPCR (Exiqon); absolute quantification	**let-7b ↑** **miR-15a ↑** **miR-21 ↑** **miR-26a ↑** **miR-93 ↑** **miR-191 ↑** miR-200c ↑ miR-940 ↑	Diagnosis with the panel of miR-26a, miR-93, miR-191, miR-940: AUC = 0.858, SD = 70%, DSp = 84%, partly subgroup differentiation Different miRNA expression in exosomes versus urine, clinical information not reported
Wang *et al.* (2015)[51]	Multicentre study 292 patients (148 with NMIBC and 144 with MIBC) and 169 controls	Urine supernatant	RT-qPCR (Takara); RNU6 and RNU48	miR-214 ↓	Diagnosis of cancer: AUC = 0.838, DS = 90.5%, DSp = 65.6% Diagnosis of NMIBC and MBIC: AUC = 0.657and 0.927, DS = 58% and 90.5%, DSp = 71% and 83% Prognostic marker for overall and recurrence free survival, but not independent

Sapre *et al.* (2016)[52]	Discovery: 30 patients with nonrecurrent disease , 30 with recurrent disease and 21 controls Validation: 25 patients with cancer and 25 patients without cancer in a surveillance study	Whole-urine	RT-qPCR (TaqMan); osmolarity adjusted	miR-16 ↑ miR-21 ↑ miR-34a ↑ miR-200c ↑ miR-205 ↑ miR-221 ↑	Monitoring marker set: prediction of recurrence with AUC = 0.74, DS = 88%, DSp = 48%Reduced cystoscopy rate by 30%
Whole-blood					
Tölle *et al.* (2013)[44]	Discovery: Eight patients, four healthy controls Validation: 38 patients, 20 controls	Whole-blood (PAXgene)	Discovery: TaqMan array Validation: RT-qPCR (Qiagen); absolute quantification	miR-26b-5p ↑ miR-144-5p ↑ miR-374-5p ↑	Diagnosis of BC: AUC = 0.774–0.824
Serum					
Scheffer *et al.* (2014)[53]	Discovery: 11 patients with NMIBC, 11 patients with MIBC and 10 controls Validation: 65 patients with NMIBC, 61 patients with MIBC and 105 controls (with nonmalignant urological disease)	Serum	RT-qPCR (Qiagen); cel-miR-39	miR-141 miR-639	No different levels between study groups
Jiang *et al.* (2015)[54]	Discovery: pooled samples from 10 patients with NMIBC, 10 patients with MIBC, 10 healthy controls Validation: Training set;120 patients and 120 controls and validation set; 110 patients and 110 controls	Serum	Discovery: Sequencing (Illumina) Validation: RT-qPCR (Takara); miR-16-5p and miR-193-5p	miR-152 ↑ miR-1486-3p ↑ miR-3187-3p ↓ miR-15b-5p ↓ miR-27a-3p ↓ miR-30a-5p ↓	Diagnosis of BC with all miRNAs: AUC = 0.899, DS = 80%, DSp = 89%; differentiation of NIMBC versus MIBC: AUC = 0.841, DS = 90%, DSp: 66.4% Prognostic marker set miR-152 and miR-3187-3p: recurrence free survival for NIMBC, not for MIBC

Kriebel *et al.* (2015)[55]	34 patients with upper urinary tract urothelial cancer 34 controls	Serum	RT-qPCR (Qiagen); RNU1-4 and SNORD43	miR-141 ↑ from 10 miRNAs studied	Diagnosis of cancer: AUC = 0.726, DS = 70.5%, DSp = 73.5%
Plasma					
Adam *et al.* (2013)[56]	20 patients 18 controls	EDTA-plasma	RT-qPCR (TaqMan); RNU6	No individual miRNA different between patients and controls	Differentiation only with machine learning procedure
Feng *et al.* (2014)[57]	50 patients 50 controls	EDTA-plasma	RT-qPCR (TaqMan); RNU6	miR-99a ↓	Diagnostic marker: no information concerning diagnostic accuracy
Feng *et al.* (2014)[58]	50 patients 50 controls	EDTA-plasma	RT-qPCR (TaqMan); RNU6	**miR-19a** ↑	Diagnostic marker: no information concerning diagnostic accuracy
Du *et al.* (2015)[59]	Discovery: Pools of 10 patients and 10 controls Validation: Training set with 56 patients and 60 controls and test set with 109 patients and 115 controls	EDTA-plasma	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); miR-16	miR-497 ↓ miR-663b ↑	Diagnosis of cancer with the two combined miRNAs: AUC = 0.711, DS = 69.7%, DSp = 69.6%
Other					
Armstrong *et al.* (2015)[60]	214 NMIBC Two MIBC	Matched tissue Plasma White blood cells Urine exosomes	Discovery: NanoString profiling Validation: RT-qPCR (TaqMan); digital PCR	25 different upregulated miRNAs	ifferent miRNA relationships between matched samples; six miRNAs (miR-21-5p, miR-29b-3p, miR-200c-3p, miR-200b-3p, miR-205-5p and miR-4454) commonly upregulated in cancer tissue and urine exosomes

*Development phases refer to definitions given in Table 1. [‡]Manufacturer's name of the assay is given in brackets. [§]Significant ↑, ↓, upregulated and downregulated miRNAs in patients in comparison to controls or the comparison cohort. [||]Relationship between dysregulated miRNAs and clinical question. **miRNAs** highlighted in **bold** are haemolysis-affected according to MacLellan *et al.*[61] AUC, area under the receiver operating characteristics curve; DS, diagnostic sensitivity; DSp, diagnostic specificity. EDTA, ethylenediaminetetraacetic acid; miRNA, microRNA; MIBC, muscle-invasive bladder cancer, NMIBC, non-muscle-invasive bladder cancer; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

Table 2 | Studies regarding miRNAs in blood and urine and blood samples as potential biomarkers renal cell carcinoma

Reference (year)	Study with marker development phases, number of patients and controls[*]	Sample	Method; reference method[‡]	Significant miRNA expression[§]	Clinically relevant findings[‖]
Urine					
von Branden-stein et al. (2012)[65]	18 patients (7 ccRCC, 6 pRCC and 5 chRCC) 25 controls (oncocytoma, urological diseases, other tumours)	Whole-urine	RT-qPCR (TaqMan); 5S rRNA	**miR-15a** ↑	Significantly increased versus controls, but no further diagnostic data reported
Whole-blood					
Schmitt et al. (2012)[66]	43 patients (Wilms tumour) 19 controls (healthy adults)	Whole-blood (PAXgene)	RT-qPCR (Qiagen); RNU6B	miR-520d-3p ↑ miR-197 ↑ miR-224 ↑ **miR-20a** ↓ **miR-126** ↓ **miR-144*** ↓	Diagnosis of RCC using the six-miRNA signature: AUC = 0.97, DS = 99.1%, DSp = 94.8%
Serum					
Wulfken et al. (2011)[67]	Multicentre study. Discovery: six ccRCC patients, six controls Validation: 84 patients (69 ccRCC, 10 pRCC, three chRCC, two sRCC), 93 controls	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); cel-miR-39	miR-1233 ↑	Diagnosis of RCC: AUC = 0.588, DS = 77.4%, DSp = 37.6%
Redova et al. (2012)[68]	Discovery: 15 ccRCC, 12 controls Validation: 90 patients (73 ccRCC, eight pRCC, nine chRCC), 35 controls (blood donors)	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); miR-16	**miR-378** ↑ **miR-451** ↓ miR-150; ↓	Diagnosis of RCC using the two-miRNA signature miR-378 and miR-451: AUC = 0.86, DS = 81%, DSp = 83%
Cheng et al. (2013)[69]	12 ccRCC 12 controls (benign kidney lesions)	Serum	RT-qPCR (Takara); RNU6	**miR-21** ↑ miR-141 ↓ miR-224 ↑	Significant changes compared with controls, but no further diagnostic data reported
Zhao et al. (2013)[70]	68 ccRCC 42 healthy controls	Serum	RT-qPCR (Qiagen); 5S rRNA	**miR-210** ↑	Diagnosis of RCC: AUC = 0.874, DS = 81%, DSp = 79.4%
Iwamoto et al. (2014)[71]	34 ccRCC 23 healthy controls	Serum	RT-qPCR (TaqMan); miR-16	**miR-210** ↑	Diagnosis of RCC: AUC = 0.77, DS = 65%, DSp = 83%
Fedorko et al. (2015)[72]	195 patients (157 ccRCC, 26 pRCC, 12 chRCC) 100 controls (blood donors)	Serum	RT-qPCR (TaqMan); absolute quantification	**miR-378** ↑ **miR-210** ↑	Diagnosis of RCC: AUC = 0.85, DS = 80%, DSp = 78% Suggested as a monitoring marker
Ludwig et al. (2015)[73]	43 patients (Wilms tumour) 13 controls	Serum (haemoly-sis control performed)	RT-qPCR (Qiagen); cel-miR-39	miR-100-5p ↑ **miR-130b-3p** ↑	Diagnosis of cancer using the two-miRNA signature: AUC = 0.796, DS = 69.2%; DSp = 90%

Wang *et al.* (2015)[74]	Discovery: 25 ccRCC, 25 controls Validation: 107 ccRCC, 107 controls (training set: 28 of each; test set: 79 of each)	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); let-7d/g/i	miR-193-3p↑ miR-362 ↑ miR-572 ↑ miR-28-5p ↓ **miR-378 ↓**	Diagnosis of ccRCC: AUC = 0.807, DS = 80%, DSp = 71%
Plasma					
Teixeira et al. (2014)[75]	43 RCC 34 controls	Plasma	RT-qPCR (TaqMan); RNU44	**miR-221 ↑**	Diagnosis of RCC: AUC = 0.696, DS = 72.5%, DSp = 33.3% Prognosis of overall survival: independent marker; increase of C-index for TNM stage, Fuhrman grade and age from 0.800 to 0.961
Other					
Gamez-Pozo *et al.* (2012)[76]	Prospective cohort study: 38 carcinoma patients (advanced stage) treated with sunitinib; discovery and validation phases	Leuco-cytes (Leuko-LOCK)	Discovery: Agilent array Validation: RT-qPCR (Exiqon); mean of miR-103 and miR-191	miR-192 miR-193a-3p miR-410 miR-424* miR-501-3p miR-1181	Predictive models of response to sunitinib treatment: for poor response with miR-192, miR-193a-3p, miR-501-3p; for prolonged response with miR-410, miR-424*, miR-1181

[*]Development phases refer to definitions given in Box 2. [‡]Manufacturer's name of the assay is given in brackets. [§]Significant ↑, ↓, upregulated and downregulated miRNAs in patients in comparison to controls or the comparison cohort. [||]Relationship between dysregulated miRNAs and clinical question. **miRNAs** highlighted in **bold** letters are haemolysis-affected according to MacLellan et al.[61] AUC, area under the receiver operating characteristics curve; cc, clear cell; ch, chromophobe; EDTA, ethylenediaminetetraacetic acid; DS, diagnostic sensitivity; DSp, diagnostic specificity; miRNA, microRNA; p, papillary; RCC, renal cell cancer; RT-qPCR, quantitative real-time reverse-transcription polymerase chain reaction; s, sarcomatoid.

Table 3 | Studies regarding urine and blood as potential biomarkers in prostate cancer

Reference (year)	Study with marker development phases, number of patients and controls[*]	Sample	Method; reference method[‡]	Significant miRNA expression[§]	Clinically relevant findings[‖]C
Urine					
Ahumada-Tamayo et al. (2011)[83]	Nine patients with prostate cancer Nine patients with BPH	Urine sediment after prostate massage	TaqMan array	21 differentially expressed miRNAs	Diagnosis of prostate cancer: 19 miRNAs were twofold higher and two miRNAs twofold lower in BPH than in prostate cancer; no further diagnostic data were given
Bryant et al. (2012)[84]	70 patients with localized and 40 advanced prostate cancer, 17 controls (not defined)	Urine sediment after DRE	RT-qPCR (TaqMan); mean of RNU44 and RNU48	**miR-107 ↑** **miR-574-3p ↑**	Diagnosis of prostate cancer and/or prediction of prostate biopsy result: both miRNAs discriminate cancer better than PCA3 (AUCs of 0.74 and 0.66 versus 0.61)
Srivastava et al. (2013)[85]	36 patients with prostate cancer 12 healthy men	Whole-urine (collection not described)	RT-qPCR (TaqMan); RNU48	miR-205 ↓ miR-214 ↓	Diagnosis of prostate cancer and/or of positive prostate biopsy result: combined use: DS = 89%, DSp = 80%; no comparator test was given
Casanova-Salas et al. (2014)[86]	45 patients with cancer-positive biopsy 47 patients with negative biopsy	Urine sediment after DRE	RT-qPCR (TaqMan); mean of RNU44 and RNU48	**miR-182 ↑** miR-187 ↓	Diagnosis of cancer and/or prediction of positive biopsy: combination of miR-187, PSA and PCA3 (AUC = 0.711) better than PSA alone (AUC = 0.615)
Haj-Ahmad et al. (2014)[87]	Discovery: pooled samples from eight patients with prostate cancer, 12 with BPH and 10 healthy men Validation: selected miRNAs in all individual samples	Whole-urine	Discovery: LC Science Paraflo array Validation: RT-qPCR (TaqMan); 5S rRNA	**miR-484** miR-1825 ↑	Diagnosis of prostate cancer and/or prediction of positive biopsy result: combination of both miRNAs: DS = 45%, DSp = 75%, not better than PSA
Sapre et al. (2014)[88]	Discovery: 17 patients with low-risk and 16 with high-risk cancer Validation: 22 patients with high-risk and 14 with indolent prostate cancer	Whole-urine after DRE	TaqMan array and RT-qPCR; global mean approach	**miR-16 ↑** **miR-20a ↑** **miR21 ↑** miR-34a ↑ **miR-106b ↑** miR-145 ↑ **miR-182 ↑** miR-205 ↑ **miR-221 ↑** miR-**222 ↑** **miR-331 ↑** miR-375 ↑ miR-218 ↓ (selected miRs as published at least in two studies)	Prediction of lethal from indolent prostate cancer at radical prostatectomy: discrepant data, optimal combination miR-16, miR-21 and miR-222 in the discovery phase with AUC = 0.73, but only 0.35 in the validation phase

Yun *et al.* (2014)[89]	Discovery: 14 patients with prostate cancer and five with BPH Validation: 463 patients with prostate cancer and 302 with BPH in three validation cohorts and a biopsy cohort of 150 patients	First morning urine supernatant	Discovery: Agilent array Validation: RT-qPCR (Qiagen); total RNA input	miR-615-3p ↑ miR-4316 ↑ hsv1-miR-H18 ↑ hsv2-miR-H9-5p ↑	Diagnosis of prostate cancer and/or prediction of positive biopsy result: both virus encoded miRNAs had higher AUCs (0.761 and 0.738) than PSA (0.613) within the PSA gray zone (3–10 ng/ml) Suggested combination: PSA and hsv2-miR-H9-5p
Egidi *et al.* (2015)[90]	41 patients with localized prostate cancer 38 patients with BPH with negative biopsies	Urine sediment after DRE	RT-qPCR (Qiagen); miR-191	miR-9-3p ↓ **miR-19a-3p ↓**	Diagnosis of prostate cancer and/or prediction of positive prostate biopsy result: both miRs with AUCs of 0.769 and 0.723, respectively, but urine PSA mRNA was better (0.837); best combination urine PSA mRNA plus miR-19a-3p with an AUC of 0.880
Korzneniewski *et al.* (2015)[91]	71 patients with prostate cancer 18 men with prostate negative biopsy	Urine supernatant, without DRE	RT-qPCR (TaqMan); cel-miR-39	miR-483-5p ↑ miR-1275 miR-1290	Diagnosis of prostate cancer and/or prediction of positive biopsy result: only miR-483-5p discriminated (AUC = 0.694), but serum PSA (AUC = 0.81) was better; no improvement using combination of PSA and miR-483-5p
Stephan *et al.* (2015)[92]	38 patients with cancer-positive biopsy patients 38 patients with negative biopsy	Urine sediment after DRE	RT-qPCR (TaqMan); total RNA input, PSA mRNA, miR-130b	miR-183 miR-205	Diagnosis of prostate cancer and/or prediction of positive biopsy result: no differentiation between both cohorts, but discrimination with Progensa and own PCA3 tests
Whole-blood					
Santos *et al.* (2014)[93]	45 patients with CRPC	Whole-blood and isolation of white fraction	RT-qPCR (TaqMan); RNU44	**miR-7 ↑** **miR-221 ↑**	Prediction of patients with early castration resistance, response to therapy and overall survival
Leidinger *et al.* (2016)[94]	Discovery: 115 patients with prostate cancer and 39 with BPH Validation: 15 patients with prostate cancer and 15 with BPH	Whole-blood (PAXgene)	Discovery: Geniom Biochip array, Validation: RT-qPCR (Qiagen); RNU48	**miR-659 ↑** **miR-675 ↑** **miR-1180 ↑** **miR-1225 ↑** **miR-221* ↓** **miR-518-5p ↓** **miR-708* ↓**	Differences were reported without conclusive data of diagnostic accuracy and preoperative risk
Serum					
Mitchell *et al.* (2008)[20]	25 patients with metastatic prostate cancer 25 healthy men	Serum	RT-qPCR (TaqMan); cel-miR-39, miR-54 and miR-238	miR-100 ↑ miR-125 ↑ miR-141 ↑ **miR-143 ↑** miR-205 ↑ **miR-296 ↑**	First report on circulating miRNAs, their stability in blood and difference between patients and healthy men: best discrimination by miR-141 ↑ (AUC = 0.907)

Lodes et al. (2009)[95]	Six patients with prostate cancer Eight controls	Serum	In-house microarray	15 miRNAs	Diagnostic data not reported for patients
Brase et al. (2011)[96]	Discovery: Seven patients with metastatic prostate cancer, 14 with localized prostate cancer Validation: two cohorts patients with prostate cancer of different risk status (n = 45 and 71)	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); cel-miR-9, miR-54 and miR-238	miR-141 ↑ miR-375 ↑	Prognosis: prediction of patients with high-risk cancer. Increased miRNAs corresponded to cliniocpathological end points and were helpful in prediction of high risk patients
Mahn et al. (2011)[97]	45 patients with prostate cancer, 18 with BPH and 20 healthy men	Serum	RT-qPCR (TaqMan); cel-miR-39	**let-7i ↑** **miR-26a ↑** **miR-32 ↑** **miR-195 ↑**	Combined use of all four miRs: AUC = 0.758, but not better than PSA (AUC = 0.834)
Moltzahn et al. (2011)[98]	Three groups of 12 patients with prostate cancer with different risk statuses 12 healthy blood donors	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); global median approach	**miR-20b ↑** **miR-93 ↑** **miR-106a ↑** miR-874 ↑ miR-1207-5p ↑ miR-1274a ↑ **miR-24 ↓** **miR-26b ↓** miR-30c ↓ **miR-223 ↓**	Discrimination of prostate cancer and controls: miRNAs ↓ and miRNA ↑ with AUC = 0.78–0.93. Prognostic ability: miR-93, miR-106, miR-242 for patients with high-risk cancer
Selth et al. (2011)[99]	25 patients with metastatic prostate cancer 25 healthy men (selected miRNAs from profiling in a mouse model)	Serum	RT-qPCR (TaqMan); cel-miR-39	miR-141 ↑ miR-298 ↑ miR-346 ↑ miR-375 ↑	miR-141, miR-298, miR-346, miR-375 were increased in patients with metastatic prostate cancer; but no diagnostic data were presented
Zhang et al. (2011)[100]	56 patients with prostate cancer and six with BPH	Serum	RT-qPCR (TaqMan); RNU6	**miR-21 ↑**	miR-21 ↑ in metastatic, but not in localized prostate cancer
Cheng et al. (2013)[101]	Discovery: pools from 25 patients with metastatic CRPC and 25 healthy men Validation: 21 patients with prostate cancer and 20 healthy men	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); absolute quantification	miR-141 ↑ miR-200a ↑ miR-200c ↑ **miR-210 ↑** miR-375 ↑	Discrimination between healthy men and metastatic patients by the five miRNAs: AUC = 0.638–0.899); no comparison to standard factors were given; miR-210 like PSA in relation to androgen deprivation therapy
Egidi et al. (2013)[102]	38 patients with prostate cancer before and after radical prostatectomy 40 healthy controls	Serum	RT-qPCR (Exiqon); miR-93	**miR-21 ↔** miR-141 ↓	miR-21: no difference between prostate cancer and controls; miR-141: lower levels in patients than in controls

|					
Nguyen *et al.* (2013)[103]	28 patients with low-risk prostate cancer, 30 with high-risk prostate cancer and 26 with metastatic CRPC	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan); RRNU6	miR-141 ↑ miR-375 ↑ **miR-378** ↑ miR-409-3p ↓	Prediction of low-risk and metastatic cancer: with different levels of all four miRNAs. Data as real prognostic markers were not given
Selth *et al.* (2013)[104]	Discovery: eight patients with and without biochemical relapse Validation: 31 versus 39 patients with and without relapse	Serum	Discovery: TaqMan array Validation: RT-qPCR (TaqMan) cel-mir-39	miR-141 ↑ **miR-146b-3p** ↑ **miR-194** ↑ miR-375 ↑	Prediction of biochemical relapse: miR-146b-3p and miR-194 predicted recurrence in univariate, miR-146b-3p in multivariate analysis with standard clinicopathological factors
Zhang *et al.* (2013)[105]	20 patients with localized prostate cancer, 30 patients with bone-metastatic prostate cancer and six with BPH	Serum	RT-qPCR (TaqMan); RNU6	miR-141 ↑	No different miR-141 levels between BPH and localized cancer, but increased by bone metastasis
Haldrup *et al.* (2014)[106]	31 patients with localized or metastatic prostate cancer and 13 with BPH	Serum	Exiqon panels I+II, without RT-qPCR; miR-320a	11 miRNAs	Diagnosis of prostate cancer and prediction of risk groups: only miR-103-2* as early diagnostic marker (BPH versus localized prostate cancer); other combinations to differentiate between BPH, localized and metastatic prostate cancer
Kotb *et al.* (2014)[107]	10 patients with positive and 10 with negative biopsy	Serum	RT-qPCR (TaqMan); SNORD47	**miR-21** ↑ **miR-221** ↑	Predictors of positive prostate biopsy
Singh *et al.* (2014)[108]	Discovery: identification of prostate-cancer-differentiating miRNAs in cell models Validation: 62 patients after prostatectomy with and without biochemical recurrence (>4.5 years)	Serum	RT-qPCR (Exiqon); overall mean	**miR-103** ↑ miR-125b ↑ **miR-222** ↑	Prognosis of radical prostatectomy: prediction of biochemical relapse; combination of miR-103 and PSA as best model
Wang *et al.* (2014)[109]	Discovery: 48 patients with low-risk (Gleason score <7), 48 with higher-risk prostate cancer (Gleason score≥7) Validation: 35 patient with low-risk and 25 with higher-risk prostate cancer	Serum	Fluidigm array; global median approach	miR-19 ↑ **miR-345** ↓ miR-519c-5p ↓	Surveillance biomarker: presurgical serum levels of miRNAs can identify patients with adverse Gleason score in postsurgical specimens independent of age, PSA, biopsy data (AUC = 0.94 versus 0.77 for PSA)

|					
Westermann *et al.* (2014)[110]	Multicentre study, prospective collection of samples. 54 patients with positive biopsy and 79 with negative biopsy	Serum	RT-qPCR (Qiagen); SNORD43 and RNU1-4	**miR-26a-1** miR-141	No difference in miRNA levels between patients with positive and negative biopsies; miR-141 dependent on Gleason score
Mihelich *et al.* (2015)[111]	50 patients with surgical 100% Gleason grade 3 and 30-90% Gleason grade 4+5, with follow-up monitoring after surgery 50 with BPH with negative biopsy	Serum	RT-qPCR (Qiagen); cel-miR-39 and total RNA input	21 miRNAs (selected miRNAs based on literature data)	Prediction of Gleason grade: miR Score 1 and 2 with 14 miRNAs for predicting low-grade from high-grade prostate cancer, and BPH and low-grade cancer from high-grade cancer. Prediction of recurrence-free interval after prostatectomy: miR Risk Score with seven miRNAs
Sun *et al.* (2015)[112]	128 patients with prostate cancer in follow-up data after prostatectomy 100 healthy controls	Serum	RT-qPCR (Takara); RNU6	miR-128 ↓	Prediction of biochemical relapse in univariate and multivariate analyses
Wach *et al.* (2015)[113]	Integrated approach together with other biomarkers. 146 patients with prostate cancer with follow-up data after radical prostatectomy, 35 with BPH and 18 healthy men	Serum	RT-qPCR (Exiqon); miR16 and RNU6b	miR-375 ↑	Diagnosis of prostate cancer and/or prediction of positive biopsy result: miR-375 alone (AUC = 0.720) or together with urokinase plasminogen activator receptor (AUC = 0.755) discriminated better than PSA (AUC = 0.603) Prognosis of overall survival: high levels of both parameters showed poor overall survival
Plasma					
Agaoglu *et al.* (2011)[114]	26 patients with localized prostate cancer, 25 with metastatic prostate cancer and 20 healthy men	Plasma	RT-qPCR (Qiagen); RNU1A	**miR-21** ↑ miR-141 ↑ **miR-221** ↑	Discrimination of patients and healthy men using miR-21 (AUC = 0.88); between metastatic and localized prostate cancer using miR-141 (AUC = 0.755), but PSA with AUC = 0.865
Gonzales *et al.* (2011)[115]	21 patients with metastatic proste cancer in response to therapy (retrospective study)	Plasma	RT-qPCR (TaqMan)	miR-141 ↑	Predictor of clinical outcome (progression) in response to therapy: concordant with PSA or circulating tumour cells

Chen et al. (2012)[116]	Discovery: 25 patients with prostate cancer and 17 with BPH Validation: 80 patients with prostate cancer, 44 with BPH and 54 healthy men	EDTA-plasma	Discovery: Illumina miRNA array Validation: RT-qPCR (Qiagen); RNU6	**let-7c** ↓ let-7e ↓ **miR-30c** ↓ miR-622 ↑ miR-1285 ↑	Diagnosis of prostate cancer and/or prediction of positive biopsy result: discrimination between prostate cancer and BPH using upregulated and downregulated miRNAs: AUC = 0.644–0.805; combination of miR-30c or let-7e, with PSA: AUC = 0.886–0.969
Shen et al. (2012)[117]	82 patients with prostate cancer of different risk	EDTA-plasma	RT-qPCR (TaqMan); absolute quantification	**miR-20a** ↑ **miR-21** ↑ miR-145 ↑ **miR-221** ↑	Prediction of high-risk and low-risk cancer with the set of four miRNAs: AUC = 0.763, DS = 29.4%, DSp = 97.4%
Watahiki et al. (2013)[118]	Discovery: pools from 25 patients with localized prostate cancer and 25 with metastatic CRPC patients Validation: RT-qPCR with individual samples	EDTA-plasma	Discovery: Exiqon array Validation: RT-qPCR (Exiqon); miR-30e	**miR-21** ↑ **miR-126** ↑ miR-141 ↑ **miR-151-3p** ↑ **miR-152** ↑ miR-200c ↑ miR-375 ↑ **miR-423-3p** ↑ **miR-16** ↓, miR-205 ↓	miRNAs discriminated between localized and metastatic CRPC (AUC = 0.75–0.88); but not better than PSA alone (AUC = 0.96); association of different miRNAs to prostate cancer progression,
Medina-Villaamil et al. (2014)[119]	10 patients with low-risk, nine with intermediate-risk, 11 with high-risk prostate cancer and 10 healthy controls	EDTA-plasma	Discovery: Hexiqon array with 92 miRNAs Validation: RT-PCR (not specfied)	miR-10a miR-15b miR-124 miR128 miR-187 **miR-188-5p** **miR-196b** miR-199b-5p miR-200b miR-218 miR-330-3p miR-337-3p miR-339-3p	Prediction of risk groups: all miRNAs were different between the risk groups; miR-187, miR-188-5p, miR-196b together with clinical variables as best predictors of risk groups; detailed data were not given
Sapre et al. (2014)[88]	33 patients with low-risk and 37 with high-risk prostate cancer (selected 12 miRNAs published at least in 2 studies)	Plasma	RT-qPCR (TaqMan); RNU48	miR-16	Prediction of low-risk and high-risk cancer: only miR-16 out of 12 miRNAs differentiated risk groups, but with low AUC of 0.62
Kachakova et al. (2015)[120]	59 patients with prostate cancer, 16 with BPH and 11 young healthy men	EDTA-plasma	RT-qPCR (Qiagen); RNU6B	let-7c ↓ **miR-30c** ↓ miR-141 ↓ miR-375 ↓	Diagnosis of prostate cancer and/or prediction of positive biopsy result: miR-375 as best higher discriminator between prostate cancer and controls: AUC = 0.809, DS = 81.3%, DSp = 72.9%); better than PSA (AUC = 0.710, DS = 76.8%, DSp = 53.3%), both combined increased AUC to 0.833

Kelly *et al.* (2015)[121]	75 patients with prostate cancer and 27 patients with negative biopsy	EDTA-plasma	RT-qPCR (TaqMan); miR-16 and miR-425	**let-7a** **miR-16** **miR-21** miR-34a miR-141 **miR-143** miR-145 miR-125b miR-155 **miR-221** miR-375 **miR-425**	Diagnosis of prostate cancer and/or prediction of positive biopsy: combined use of miR-141, miR-145, miR-155, let-7a with AUC of 0.783 and DS = 97%; correlation with D'Amico risk stratification
Chen *et al.* (2016)[122]	65 patients with prostate cancer, 51 with BPH patients and 74 matched healthy men	EDTA-plasma	RT-qPCR (TaqMan); cel-miR-39	**miR-21** miR-152	Diagnosis of prostate cancer: no differences between the groups; unsuitable markers for early screening
Other					
Bryant *et al.* (2012)[84]	Discovery: 78 patients with prostate cancer and 28 healthy men Validation: individual RT-qPCRs, additional 47 patients with and 72 without recurrence after prostatectomy	Circulating microvesicles from plasma or serum	Discovery: Exiqon array Validation: RT-qPCR (TaqMan); cel-miR-39	**miR-107** ↑ miR-141 ↑ miR-200b ↑ miR-375 ↑ **miR-574-3p** ↑	Different miRNA combinations with discrimination between controls and nonmetastatic prostate cancer: not better than PSA. Metastatic and nonmetastatic cancer can be differentiated by miR-141 plus miR-375 (metastatic signature)
Huang *et al.* (2015)[123]	Prospective cohort study: 75 patients with prostate cancer and follow-up data after surgery (25 with recurrence or metastasis, 50 without) And 75 healthy controls	Peripheral blood mononuclear cells	RT-qPCR (TaqMan); RNU6	miR-21	Diagnosis of prostate cancer and/or prediction of positive biopsy result: AUC = 0.833, DS = 87.5%, DSp = 85.7% Marker in follow-up monitoring after surgery: high miRNA values corresponded with recurrence and metastasis
Huang *et al.* (2015)[124]	Discovery: 23 patients with CRPC Validation: 100 patients with CRPC for evaluation of overall survival	Exosomes (EDTA-plasma)	Discovery: sequencing (Illumina) Validation: RT-qPCR (Qiagen); miR-30a-5p and miR-30e-5p	miR-375 ↑ miR-1290 ↑	Prognosis of overall survival: increased values of both miRNAs corresponded with a mortality of 80% at the 20-month follow-up point; significantly improvement in clinical variable-based prediction model by the inclusion of both miRNAs
Xu *et al.* (2015)[125]	98 patients with prostate cancer and follow-up data after radical prostatectomy and 56 healthy men	Peripheral blood mononuclear cells	RT-qPCR (Takara); RNU6	miR-129 ↓	Diagnosis of prostate cancer and/or prediction of positive biopsy result: AUC = 0.846, DS = 88.9%, DSp=66.7% Prognosis: high miRNA values corresponded with poor recurrence-free interval

Li et al. (2016)[126]	51 patients with prostate cancer (31 with nonmetastatic, and 20 with metastatic disease) and 40 healthy controls	Exosomes (serum) and Serum	RT-qPCR (Takara); cel-miR-39	miR-141 ↑	Diagnosis of prostate cancer and/or prediction of positive biopsy result: higher levels in prostate cancer than in BPH both in serum and in exosomes; differentiation between metastatic and localized cancer with AUC of 0.869 better than with PSA (AUC = 0.776)

[*]Development phases refer to definitions given in Box 2. [‡]Manufacturer's name of the assay is given in brackets. [§]Significant ↑, ↓, upregulated and downregulated miRNAs in patients in comparison to controls or the comparison cohort. [||]Relationship between dysregulated miRNAs and clinical question. **miRNAs** highlighted in **bold** letters are haemolysis-affected according to MacLellan et al.[61] AUC, area under the receiver operating characteristics curve; BPH, benign prostatic hyperplasia; CRPC, castration-resistant prostate cancer; DRE, digital-rectal examination; DS, diagnostic sensitivity; DSp, diagnostic specificity; EDTA, ethylenediaminetetraacetic acid; RT-qPCR, reverse-transcription quantitative PCR.
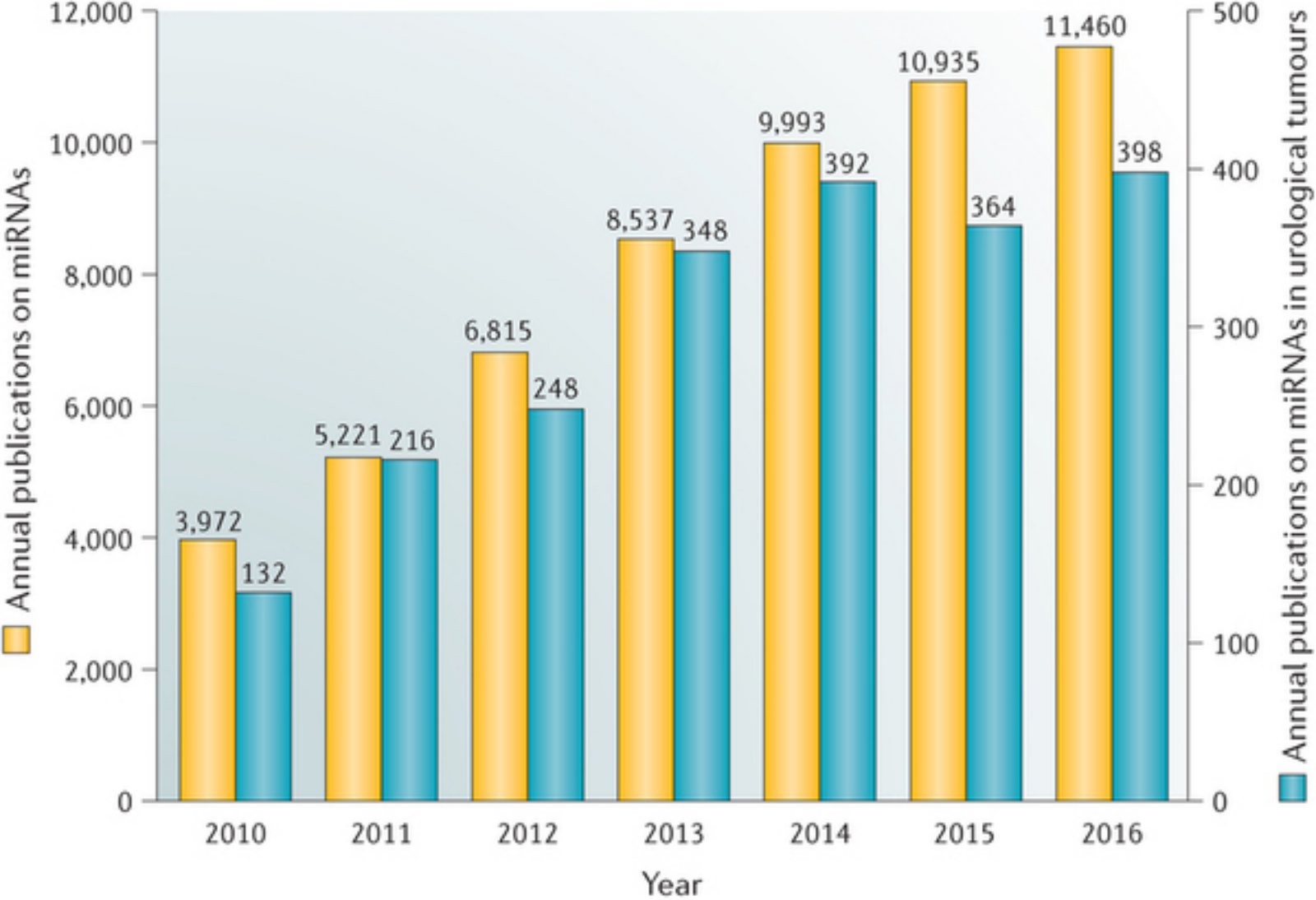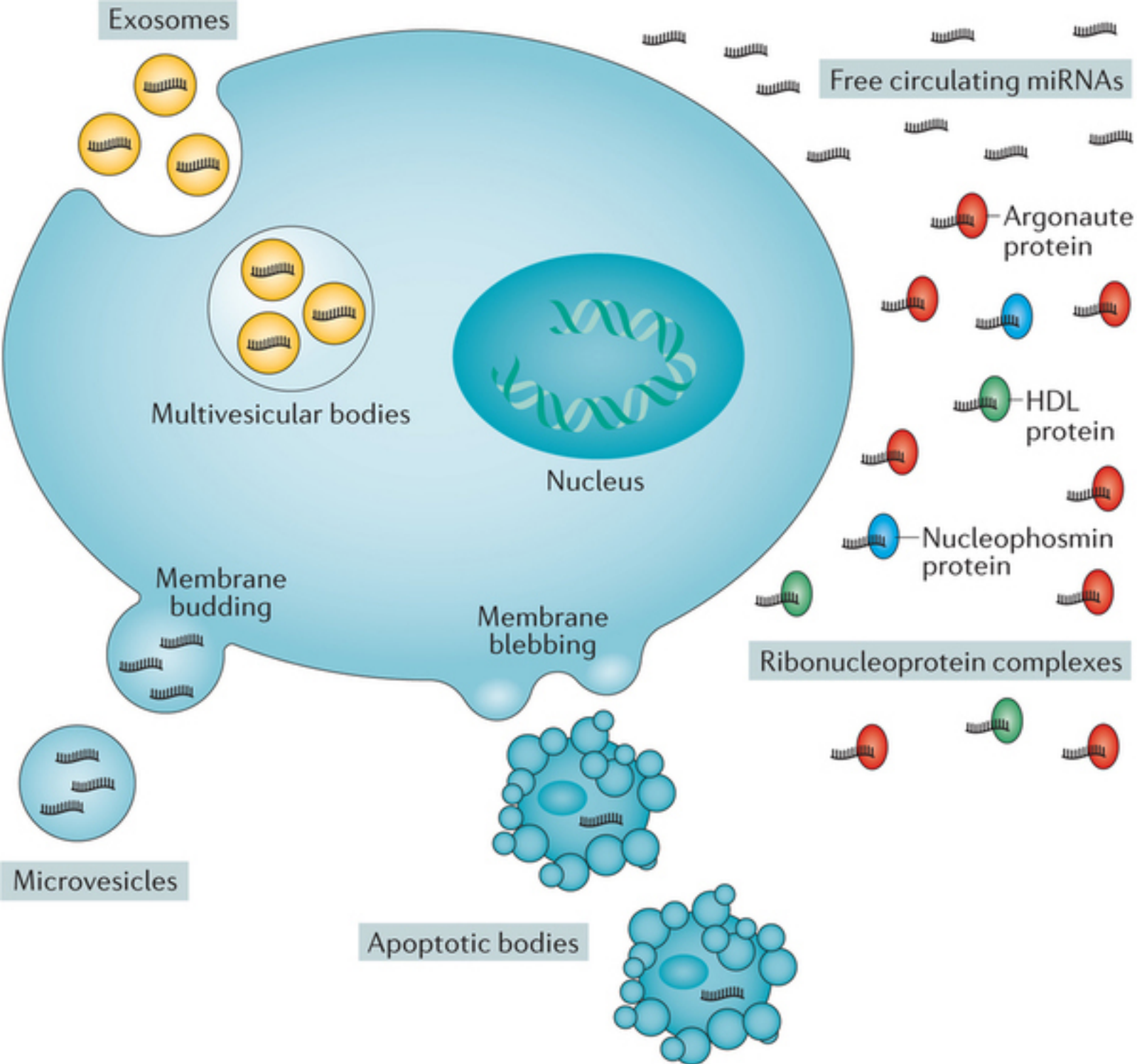
Exosomes

Free circulating miRNAs

Argonaute protein

Multivesicular bodies

Nucleus

HDL protein

Membrane budding

Membrane blebbing

Nucleophosmin protein

Ribonucleoprotein complexes

Microvesicles

Apoptotic bodies