Title

MicroRNAs as biomarkers in Chronic Kidney Disease

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Abstract

Purpose of review: This review summarises recent data supporting the concept that urinary microRNAs are a useful new class of biomarker. They may improve capacity to stratify patients with CKD according to risk of progression, and may also inform about response to therapy.

Recent findings: MicroRNAs are present, stable and readily quantifiable in tissues and body fluids, including urine, and have widespread importance as regulators in the kidney. Urinary microRNAs are typically released from the nephron or downstream structures, and their abundance may reflect altered microRNA expression in the kidney, or release into the lumen by the cells comprising the different regions of the nephron. As a consequence, abundance of specific microRNAs in the urine may change in various pathological states. Large-scale studies are now needed, to test the capacity of specific microRNAs to inform about risk and response to therapy.

Summary: Urinary microRNAs appear useful sentinels for pathological processes occurring in the kidney and may enable a "personalised medicine" approach to the management and stratification of renal disease.

Introduction

Biological markers of disease have been studied for millennia. Hippocrates, credited with conceiving that diseases had natural rather than supernatural causes, reflected in his writing on indirect measurements of health such as evaluation of the patient's breath or urine [1]. "Biomarker" is a more contemporary term, defined by a National Institutes of Health Working Group in 1998 as, "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". The requirement for objective measurement is key, since it allows comparison of values from different individuals or time points. Some commonly utilized clinical parameters can also be considered as biomarkers, for example blood pressure. Other biomarkers in routine use in the care of patients with Chronic Kidney Disease (CKD) include measurements of excretory kidney function, most commonly in the form of serum creatinine concentration and extrapolated estimates of glomerular filtration rate (eGFR), and measurements of urinary protein excretion. Figure 1 depicts potential uses of biomarkers in the nephrology setting.

There are two key goals for existing and future biomarkers: firstly, to stratify patients according to risk of disease progression or complications (e.g. likely rate of decline in kidney function or risk of major adverse cardiovascular event) and, secondly, to identify surrogates that can be used to measure response to treatment (e.g. reduction in urinary protein excretion following pharmacological blockade of the renin-angiotensin system). In these terms, existing biomarkers

are useful but limited. Existing measures of excretory kidney function have well-documented inaccuracies and, by measuring loss of function retrospectively, do not provide data on the current activity of pathological processes in the kidney. Urinary protein excretion is a valuable marker of glomerular damage and informs about risk of progressive decline in excretory function, but is not specific for these.

Therefore, new biomarkers are needed. Biomarkers are most valuable when they correlate to a specific modifiable outcome, and are not simply a means of labeling patients with a disease. The discovery of the M-type phospholipase A2 receptor as a target antigen in idiopathic membranous GN is an excellent example [2]. In this case, advancement of molecular understanding in this disease has not only improved diagnostic distinction from secondary membranous GN, but also provides serological evidence of disease severity. This helps identify those with greatest potential to benefit from immunosuppression –people who would previously have first undergone a period of potentially deleterious "watchful waiting."[3].

MicroRNAs also show promise as a new class of biomarker in CKD. MicroRNAs were first discovered as a mechanism of post-transcriptional regulation of gene expression in the nematode *C. elegans*, and have since been found to have widespread expression and importance. They are present in all human cells and are now linked to many physiological and pathological processes. A recent excellent review summarises our current knowledge about the role of microRNAs in renal diseases [4**].

MicroRNAs are stable in tissues, and resist degradation in pathological specimens. They can be measured by sensitive molecular techniques that employ nucleic acid amplification approaches, including Quantitative Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) and Next Generation Sequencing. More recently, these observations have been extended to body fluids, including urine. Figure 2 depicts the biogenesis of urinary miRs, which are stabilized either by association with extracellular vesicles (EVs) or specialised proteins [5, 6]. The significance of certain miRs being located in EVs versus protein-bound in urine is not yet known. The origin of urinary miRs is predominantly the nephron and downstream urinary tract [7], with limited reports of freely filtered miRs from the systemic circulation [8]. Crucially, urinary microRNA concentrations have been found to associate with important clinical characteristics, including histopathological diagnosis. Therefore, there is considerable interest in utilising these accessible, non-invasive and informative biomarkers in the context of renal disease.

Next, we consider microRNAs as biomarkers in specific types of kidney disease. To this end, we highlight selected examples of recent studies that have progressed our understanding of miRs in kidney disease and their future use as biomarkers, and we apologise to the many workers in this field whose research we have not been able to highlight due to lack of space.

Diabetic Nephropathy

Diabetic Nephropathy (DN) is the leading cause of end-stage renal failure worldwide. There is an unmet clinical need to improve the molecular understanding of this multifactorial disease, in order to establish new biomarkers and therapeutic targets alike. The consequent focus on DN has led to significant progress in elucidating the roles of miRs in molecular-cellular pathways that result in the histological hallmarks of DN. The complexity of their crosstalk is demonstrated, for example, by the apparently conflicting roles of miR-192 as both anti- and pro-fibrotic mediator in TGFβ-induced fibrosis [9*]. Such detailed mechanistic miR analyses in models of renal pathology provide prime data for biomarker development, as discussed below.

Proteinuria is one of the most widely used biomarkers in CKD, with urinary albuminuria excretion widely adopted in the diagnosis and monitoring of DN. Albuminuria serves a useful purpose in assessing the integrity of the glomerular filtration barrier (GFB) and is easily detectable in urine using inexpensive bedside tests. However, albuminuria has shortcomings, including that it is detectable only once renal damage has ensued, and that it is not specific to DN [10]. Albuminuria is also a weak prognosticating tool in DN- the idiosyncrasies in its relationship to clinical progression are summarised in a recent article by Alicic et al. [11].

Urinary miRs represent biomarkers with potential not only to augment the utility of albuminuria as a surrogate marker of GFB integrity, but may also

predict the progression of DN *before* the onset of GFB breakdown. In a recent report, Mohan et al showed that urinary miR-451-5p was increased in the urinary exosomes of diabetic rats three weeks prior to significant albuminuria and three weeks before histological changes of glomerulosclerosis and tubulointerstitial fibrosis were noted. Interestingly, kidney tissue expression of miR-451-5p fell during the development of DN [12**]. Thus miRs secreted into the urine by the injured nephron in the early stages of DN may be used not simply to denote the current state of damage, but to differentiate those patients likely to progress to DN who would warrant specialist nephrology surveillance. This represents a novel means of stratifying an ever-growing population of diabetic patients.

There is currently an increase in studies utilising urinary miR "biomarker panels"- multiple miRs measured in combination, to optimize diagnostic sensitivity and specificity [13] as well as being more widely informative of disease processes [14]. In recent work, Kato et al describe a megacluster of nearly 40 microRNAs coordinately increased in the glomeruli of mouse models of DN and renal cells treated with diabetogenic stimuli. Using synthetic antisense oligonucleotides, these researchers were able to inhibit the host transcript and decrease expression of this entire cluster of miRNAs, resulting in attenuation of early DN features *in vitro* and in mice [15**]. These data emphasise the coordinate regulation of miR expression and downstream actions, and support the idea that use of multiple miR panels may be an effective means of monitoring a multifactorial condition like DN. However, for use as DN biomarkers, additional barriers to implementation in care pathways that might arise from the

development of more complex diagnostic assays and methods of data analysis for multiple miRs should be borne in mind.

Acute Kidney Injury (AKI)

Given the high prevalence of AKI in at-risk populations and its strong association with mortality, biomarkers capable of stratifying AKI risk in specific patient cohorts are highly desirable. Animal models representing a wide range of AKI aetiologies have recently been used to identify candidate biomarkers.

In a rat model of contrast-induced AKI (CI-AKI), increased miR-188, miR-30a and miR-30e were detected in plasma. Subsequent analysis replicated these findings in 71 patients who developed CI-AKI following coronary angiography or percutaneous coronary intervention. These miRNAs may therefore have potential utility as early CI-AKI biomarkers [17*].

AKI is a frequent complication of major cardiac surgery, and is associated with increased mortality and morbidity. In an analysis of 115 cardiac surgery patients, baseline miR-21 abundance was reduced in the 42 patients that suffered post-operative AKI. Furthermore, those with AKI stage 2/3 had significantly increased mortality over the 2.9 year follow up period. Therefore, Gaede et al propose serum miR-21 as a biomarker to stratify high-risk patients in this context [18*].

Another recent focus has been the use of mesenchymal stem cells in the repair of renal injury. Zhu and co-workers showed that bone marrow mesenchymal stem cell treatment alleviated renal injury in a cisplatin-induced rat model of AKI. Upregulated expression of miR-146b was seen in kidney tissues from AKI rats in comparison with control animals, and elevated serum miR-146b was detected in early disease. Cisplatin treatment upregulated miR-146b expression in rat kidney epithelial cell line NRK-52E. These cells were protected from drug-induced apoptosis by knockdown of this miR and consequent derepression of ErB4 [18*]. MiR-146b may therefore have utility as an early AKI biomarker, and decreasing the abundance of this miR has possible therapeutic potential.

Glomerulonephritis

It has been suggested that the use of urinary miRs or other biomarkers as a "liquid biopsy" could remove the requirement for renal biopsy in patients with various forms of acute glomerulonephritis (GN). However, it will be hard for a biomarker or biomarker panel to replace the rich histopathological data derived from a tissue biopsy, and we instead envisage the role of miR biomarkers in this context as augmenting the use of invasive biopsy by a) identifying whom to biopsy and b) allowing regular surveillance of disease activity, including response to treatment. In these terms, miRs have recently been proposed as biomarkers in the diagnosis and monitoring of patients with Focal Segmental Glomerulosclerosis [19, 20], with miR-193a particularly well characterized as having a direct role in a mouse model of this disease [21].

In IgA Nephropathy (IgAN), Serino et al recently conducted a retrospective, international multicenter study to determine the sensitivity of miR biomarkers. Combined serum levels of miR-148b and let-7b (regulators of IgA1 O-glycosylation) differentiated between healthy blood donors and patients with IgAN. Moreover, the miR signature was specific for IgAN when tested against other glomerular diseases, and remained an independent predictor of IgAN irrespective of renal function or corticosteroid treatment [22*].

Renal Cell Carcinoma and other malignancies

The first definitive link between miRs and pathology was made by Calin et al in their work on Chronic Lymphocytic Leukaemia [23]. Since then, there has been a strong trajectory in oncology of discoveries about fundamental mechanisms feeding through into greater capacity to stratify patients using the associated biomarkers, including miRs. In Renal Cell Cancer (RCC) specific miRs have been found to function as oncogenes [24*] and tumour suppressor genes [25], and recent studies have linked miR expression and patient outcomes, including prediction of metastatic disease [26, 27], treatment response to sunitinib [28-30] and post-operative recurrence [31*].

In oncology patients, recent reports show that changes in miR expression can help define response to immunosuppressant treatment [32, 33]. Similar studies have not, to our knowledge, been done yet in patients with GN and other non-malignant renal diseases. We predict that such studies might demonstrate potential for miR quantification to predict response to immunosuppressant treatment, thus potentially minimising ineffective exposure of patients to classes

of drugs with a high inherent burden of treatment. Such tools may ultimately assist clinicians in assessing the risk-benefit ratio of immunosuppressant treatment in complex cases of, for example, lupus nephritis or vasculitis.

In patients with RCC, tissue expression of miR-193b at the time of nephrectomy has been found to predict eGFR at 12 months [34]. Most studies in this area have focused exclusively on tissue miR expression, but a recent study by Butz et al demonstrates the potential of urinary miRs in distinguishing between patients with clear cell renal carcinoma (ccRCC) and healthy participants, and a return of aberrant urinary miR expression to normal following successful surgical intervention [35].

Whilst providing promising leads in individualising the management of RCC, these small studies, with a predominant profiling/sequencing focus, lack the large-scale patient recruitment and longitudinal data to derive adequate power to validate these miRs as robust RCC biomarkers.

MicroRNAs as therapeutic targets

MicroRNAs have rapidly progressed to the point of being tested as therapeutic targets, with a number of phase I clinical trials underway, predominantly in oncology. The most well established miR-based therapeutic agent in non-malignant disease is miravirsen, a miR-122 antagonist that blocks the viral replication of the hepatitis C virus, currently in phase II clinical trials [36, 37]. In Nephrology, anti-miR-21 treatment is currently undergoing Phase II testing in patients with Alport syndrome (ClinicalTrials.gov identifier NCT02855268)

[38*], based upon the antifibrotic effects of anti-miR-21 seen in alpha3-chain Type IV collagen knockout mice [39]. MiR-21 has a well-characterised association with organ fibrosis via repression of PPARα-regulated signaling pathways in Fatty Acid oxidation, mitochondrial biogenesis, and anti-inflammatory signaling [39]. It is noteworthy that the antifibrotic action of anti-miR-21 is not specific to Alport syndrome, but may be applicable to many CKD aetiologies. This emphasises the importance of understanding the processes that change as a consequence of altered miR expression, and not just seeing miRs as markers of disease. In this review, we have presented the advances in miR biomarkers by clinical diagnosis, but it may be equally appropriate to describe the role of miRs in precise pathophysiological pathways, many of which are common across several renal diagnoses that come under the umbrella of "CKD"-diseases dominated by proteinuria, immune complex deposition, tubular dysfunction etc.

Common to the above examples of clinical trials of anti-miRs is the prior elucidation of precisely what the miR is doing, through thorough molecular profiling of the affected pathway, known miR targets and the downstream effects of target repression. We propose that the same approach will be useful in investigating miRs as biomarkers. The rapid advancement of sequencing technology means that it is now possible to generate vast amounts of data from profiling even single cells- but taking this approach to selecting potential miR biomarkers in isolation, without also understanding their role in disease mechanisms, will be challenging to translate into a validated test suitable for use in clinical practice. Characterization of miR actions at the molecular-cellular

level, including identification of targets and downstream effects of target repression, is also important.

Conclusion

MicroRNAs have significant potential as biomarkers in renal disease. The challenge is to translate this to clinical use. In figure 3, we outline the steps required in the development of clinically usable miR biomarkers. Momentum is building in technological development [40, 41*], spurred on by the growing body of evidence that suggests miR biomarkers can play a part in the era of personalised medicine.

Key points

- miRs are released from the nephron/urinary tract and may be detected in patient urine at a time-point before significant renal damage has occurred
- We predict miR biomarkers will enable stratification of renal diseases in a purposeful way that is amenable to treatment modification ("personalised medicine" approach)
- Translation of miR biomarkers into clinical use is currently limited by miR detection methods and the need for large-scale validation studies
- Understanding the role of miRs in renal pathophysiology is a key component of their development as biomarkers, which in turn evolves into new therapeutic target opportunities

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Figure Legends

Figure 1. Potential Uses of miR Biomarkers in Nephrology. MiR biomarkers may augment our current ability to diagnose and serially monitor renal conditions. Their most useful application may be in stratifying patients according to modifiable outcomes (e.g. risk of developing AKI or CVD) or providing a personalised signature of disease that may inform treatment decisions (such as risk of renal allograft failure or likelihood of response to chemotherapy agents in renal cancers). Additionally, miRs may be critical determinants of specific mechanistic pathways in the kidney. CVD=cardiovascular disease RRT=renal replacement therapy RAAS= renin-angiotensin-aldosterone system.

Figure 2. The biogenesis of urinary microRNAs. MiRs are released by cells of the nephron and downstream in the urinary tract. They may be associated with membrane-bound extracellular vesicles such as microvesicles, formed by outward budding of the plasma membrane, and exosomes, secreted from multivesicular endosomes formed in the endocytic tract. MiRs can also exist bound to Argonaute proteins [5] and other proteins including high-density lipoproteins (HDL) [6].

Figure 3. Phases of miR Biomarker Development. Initial discovery experiments often utilise profiling/sequencing techniques to establish differentially expressed microRNAs in samples of interest. Clinical validation involves large retrospective and prospective cohort analysis from heterogeneous

populations, often achieved using (costly) international multicentre studies. MiR detection assays and analysis platforms must be analytically validated to confirm reliability and reproducibility of test results across hospital settings. Clinical utility is confirmed using pre-defined patient/treatment outcomes, but for complete integration into clinical practice, the test must also be user-friendly and cost-effective. This complex development pathway requires collaboration between academia, industry and health care services; the predominant partner at each stage is indicated above.
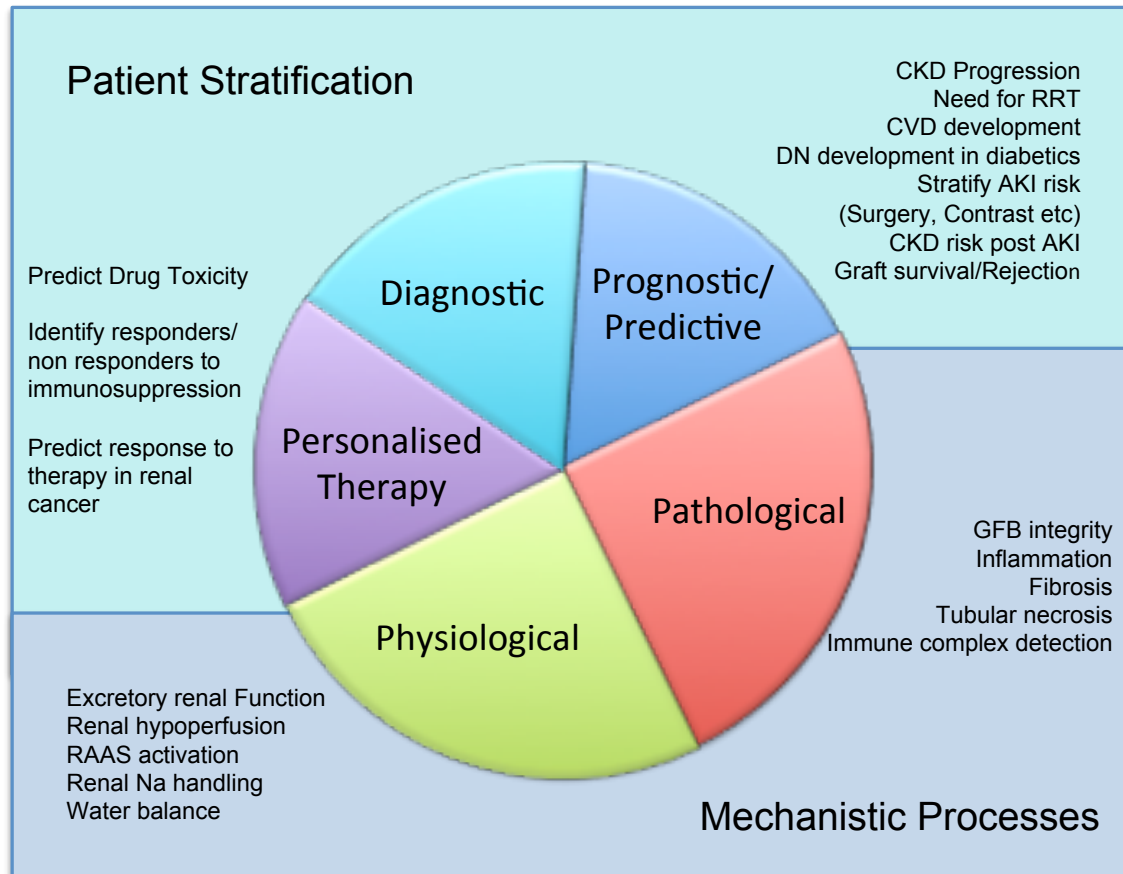
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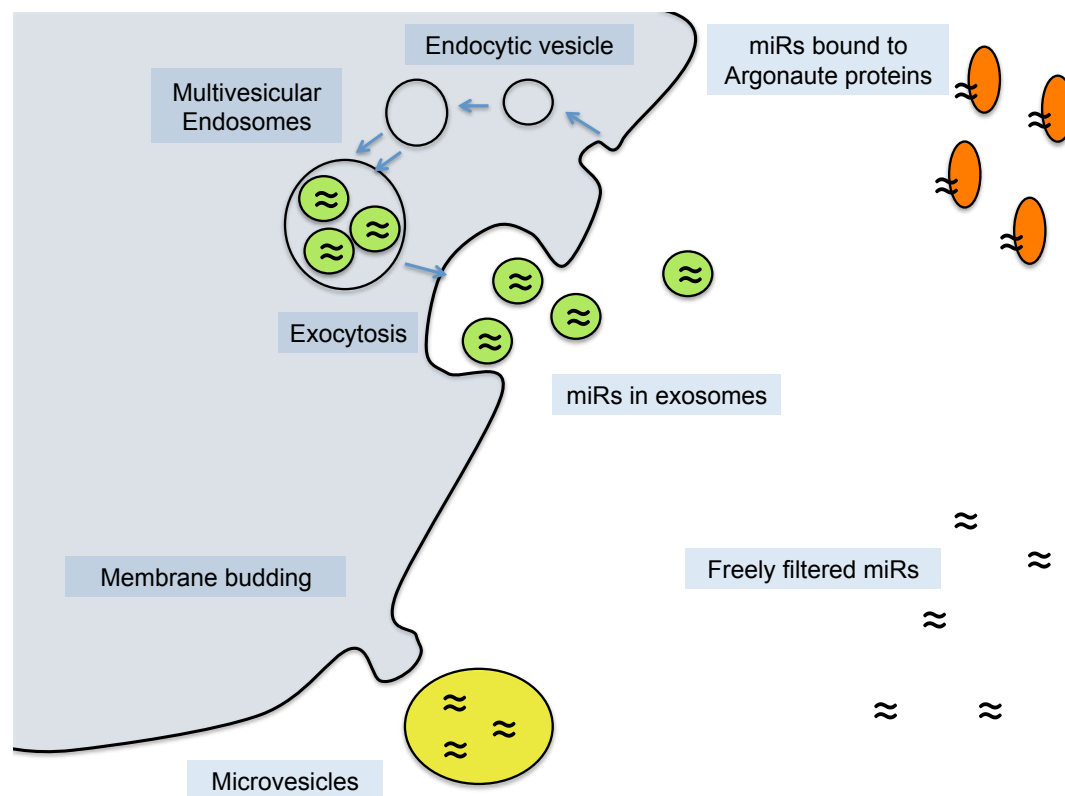
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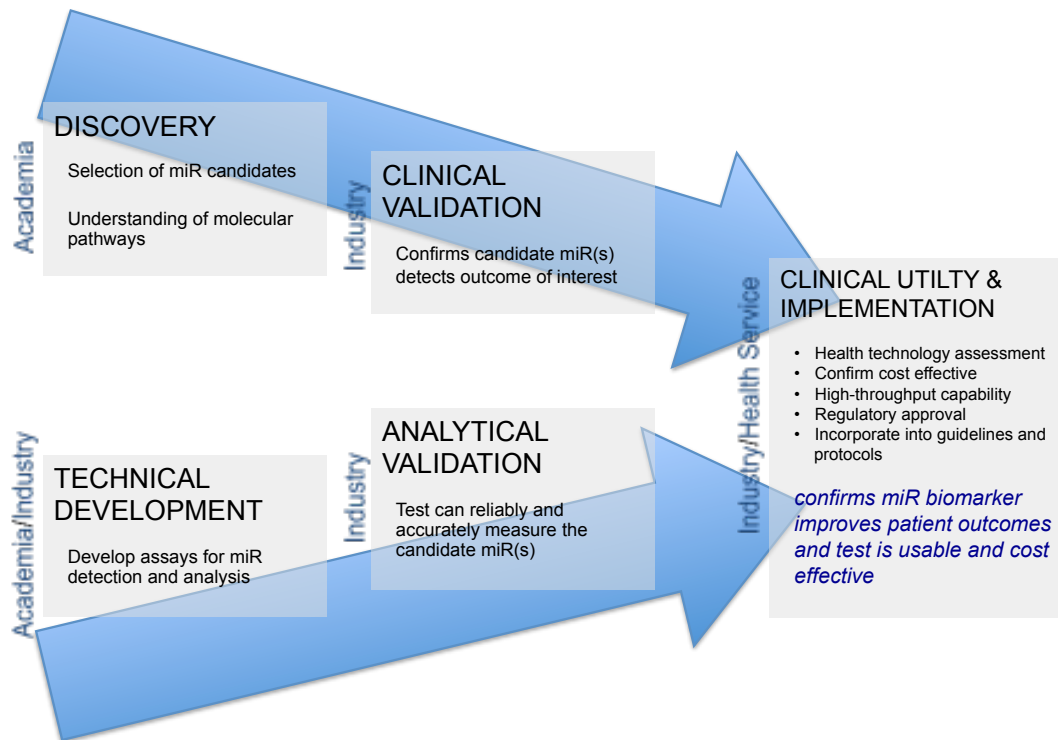
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- must be provided for all figures and should be included in the main body of the text following the references.
- Figures and tables should not be embedded within the text but should be submitted as separate files.

- Figures should be in JPEG, TIFF, EPS, PPT or WORD formats and should have a resolution of at least 300 dpi to be suitable for printing