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Circulating biomarkers in classical Hodgkin lymphoma

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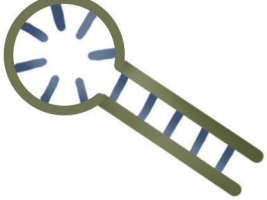

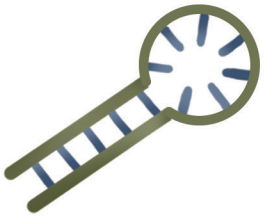

CHAPTER 6

Pre-analytical, analytical and post-analytical challenges in circulating miRNA studies

Wouter J Plattel, Joost Kluiver, Anke van den Berg

Abstract

Circulating microRNAs (miRNAs) derived from whole blood, serum, plasma or extracellular vesicles have the potential to serve as non-invasive biomarkers for a wide spectrum of diseases and have therefore received an increasing amount of interest. However, several pre-analytical, analytical and post-analytical challenges need to be overcome to increase reproducibility of results before clinical application. In this review we summarize these issues from sample collection until data analysis. We discuss main causes of lack of reproducibility in the majority of current reports and place current findings into biological perspective. We conclude that there is an urgent need for standardization of collection procedures, isolation methods and data normalization, comparison of different profiling platforms and accurate description of methods. Only when these challenges will be resolved, circulating miRNAs might redeem their promise as non-invasive disease specific biomarkers.

Introduction

After the discovery of significant amounts of circulating cell free RNA at the end of the last century, extracellular circulating miRNAs were first described in 2007.[1] Subsequent reports showed that these miRNAs are highly stable in serum or plasma despite the presence of abundant ribonucleases.[2] Based on disease specific miRNA profiles in many tissues and their stability in patients' serum or plasma, circulating miRNAs were proposed to be the future's "liquid biopsy" in oncology. In 2008, Lawrie et al. found elevated levels of circulating miRNAs in patients with diffuse large B-cell lymphoma compared to healthy controls.[3] Chim et al. found fetus derived miRNAs in plasma of pregnant women. Since these original publications, there has been a strong increase in the annual number of reports on circulating miRNAs as diagnostic, prognostic or disease response markers.[4]

Of the more than 10,000 reports published since 2008, not a single circulating miRNA has been processed into clinical practice. The main reason for the lack of clinical implementation is the low reproducibility of results or the lack of consistent results between different studies, even when the techniques and sample sources are similar.[5] Most differences can be attributed to pre-analytical, analytical and post-analytical variances that have a dramatic impact on the results. Moreover, there is a marked overlap of the circulating miRNAs between different tumor types and diseases. For example, miRNA-141 was originally identified as one of the circulating miRNAs reflecting pregnancy.[4] Since then, this miRNA has been claimed to be a specific marker for prostate cancer, lung cancer, hepatocellular carcinoma and also of several non-malignant diseases.[4, 6, 7] In addition, the very same miRNA has also been correlated with both favorable and unfavorable prognostic characteristics in these tumor types. This raises the question whether this miRNA specifically reflects disease subtype or is more likely to reflect general disease or physiological states possibly related to immune response or blood cell count.

In this review we summarize pre-analytical, analytical and post-analytical challenges in circulating miRNA research and describe our own data and speculate on future approaches.

Pre-analytical variances

Patient and technical factors

Circulating miRNAs have been described in virtually all body fluids, such as serum, plasma, breast milk and tears. In this review we will focus on miRNAs derived from blood samples, such as whole blood, plasma or serum.

An important factor to keep in mind is that several patient specific characteristics can influence miRNA levels in blood. These characteristics include fasting state, blood cell count, intravascular hemolysis and comorbidities such as inflammatory conditions that may influence the observed miRNA spectrum.[8-13] Next, technical variations during venapuncture can influence miRNA levels such as the use of different needle sizes that cause different amounts of admixture of miRNAs derived from skin tissue and extravascular hemolysis. Irrespective of the sample source chosen, it is advisable to standardize collection methods and exclude hemolytic samples from analysis.

Sample source, processing and tube selection

Whole blood, serum and plasma have all extensively been used in miRNA discovery studies and each source has its advantages and disadvantages. Whole blood samples have the advantage of easy processing and no bias in cell lysis. Taking into account the relative abundance of cell derived miRNAs compared to cell-free miRNA in plasma or serum, the main disadvantage of using whole blood is that variation in cell counts might cause important differences that might obscure the more subtle differences caused by the disease of interest.[12] Serum is the most abundantly used source in circulating miRNA studies and has the advantage of being less influenced by cell counts including platelets. Slightly higher RNA yields and higher number of miRNAs can be detected in serum over plasma samples probably caused by release of miRNAs during the coagulation process.[14] This is also the main disadvantage of using serum, because differences in sample processing time and thus clotting time might result in differences in the content and levels of the miRNAs. We tested whether the level of three miRNAs highly abundant in red blood cells and platelets might be influenced by clotting time (45 min up to 24 hours) or might differ between yellow-top serum separating tubes (SST) or serum coagulation tubes (CAT) in 2 healthy controls. No consistent changes were observed between coagulation time or coagulation tube used, although the 8 h SST tube sample showed slightly lower CT values. These data indicate that variation in coagulation time does not result in significant alterations in miRNA levels making serum an easy and reliable sample source (Figure 1).

Plasma is the second most commonly used sample in miRNA discovery studies in the field of oncology and has the potential to be less influenced by cell counts compared to whole blood.[15] However, depending on the method of isolation there is a still high risk of admixture with

platelets that contain high levels of miRNAs.[14] Especially in the early years of studying circulating miRNAs there has been, if described, a huge variation in the methods on plasma isolation. Some researchers use a single-step centrifugation method varying from 800 to 2000 RCF while others actively deplete for platelets using a two-step isolation method. As shown in Figure 2A, resulting platelet counts in plasma samples vary greatly when different centrifugation speeds are used. Levels of three frequently studied circulating miRNAs are highly influenced by presence of platelets, with up to 35-fold differences in levels of selected miRNAs (Figure 2B). Cheng et al. also showed a significant correlation between higher miRNA levels with higher platelet and microparticle counts.[16] Over 70% of the detectable miRNAs were substantially affected by the processing procedure of the plasma samples, which could mainly be attributed to platelet admixture. Cheng et al. showed that platelets including their miRNAs can be efficiently depleted from frozen plasma samples even after multiple years of storage. In contrast, we could confirm effective depletion of platelet derived miRNAs of only 1 out of 3 tested miRNAs, while the other two miRNAs showed similar levels in platelet depleted and platelet rich plasma samples (Figure 2B). A possible explanation for this difference might be that either freeze-thawing or high centrifugation speeds (5000g) as used in our protocol might have resulted in membrane disturbance and the subsequent release of platelet derived miRNAs. Pritchard et al. showed that both red and white blood cell counts also have a major impact on plasma miRNA levels. They showed that over 50% of reported cancer associated miRNAs are likely to originate from blood cells even when samples were not hemolytic.[12] Finally, as both heparin and citrate are known inhibitors of reverse transcriptase and polymerase, enzymes that are used in both qRT-PCR and next-generation sequencing (NGS) techniques, it is advisable to use EDTA as anticoagulant when choosing plasma as sample source.[17-20]

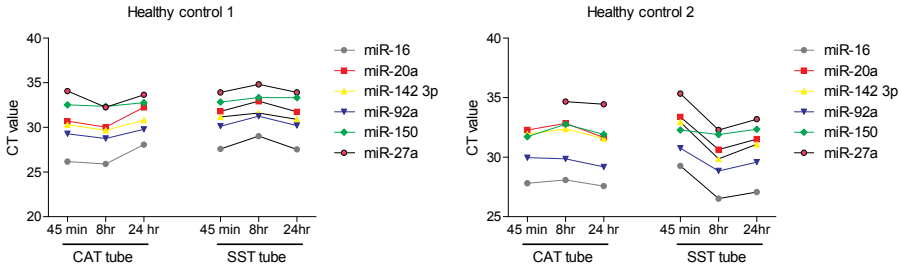

Figure 1. Impact of different serum isolation methods and clotting time on miRNA levels. Raw CT values for 6 miRNAs tested in serum derived from two healthy controls using two different serum collection tubes and three different time points from blood-draw until serum processing. CAT = red-top serum collection tube (BD Vacutainer®); SST = yellow-top serum separator tube (BD Vacutainer®).

In conclusion, many patient factors and especially blood cell admixture and counts have major impact on circulating miRNA studies with fold differences that by far exceed the fold differences reported in many papers. We therefore propose to use either serum samples or platelet depleted plasma samples for analysis of circulating miRNAs.

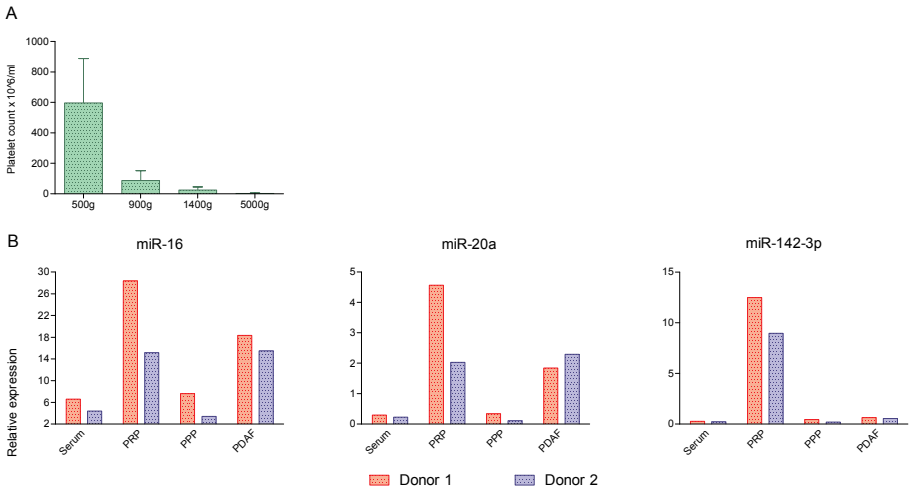

Figure 2. Impact of centrifugation speed on platelet admixture and circulating miRNA levels.
(A) Mean platelet count and standard deviation from 6 plasma samples obtained with different centrifugation speeds. (B) Relative expression of miR-16-5p, miR-20a-5p and miR-142-3p in serum, platelet rich plasma (PRP), platelet poor plasma (PPP) and plasma with platelets depleted after freezing of platelet rich plasma (PDAF) in two healthy donors. PRP was obtained by single step centrifugation of 900xg for 10 min at room temperature. PPP was obtained by applying a second ultracentrifugation step for 10 minutes at 1800xg and PDAF was obtained after thawing a PRP sample and two sequential centrifugations at 1800xg and a third centrifugation at 5000xg to lose all platelets counted by an automatic blood cell counter. Expression levels were related to exogenous spiked in cel-miR-39 before RNA isolation after sample denaturation.

Stability and extracellular vesicles

Since the beginning of circulating miRNA reports, miRNA levels have been reported as being very stable in plasma or serum samples.[9, 21-23] Indeed, miRNAs have been detected in serum samples left at room temperature up to 10 days or after multiple freeze/thawing events, although cycle threshold values were higher compared to samples directly stored at -20°C.[22]

There are two main mechanisms by which miRNAs are protected from degradation. First, circulating miRNAs can be bound to proteins, mainly AGO2 and NPM-1.[21] Second, miRNAs can be found in cell derived vesicles or lipoprotein bodies that protect its contents from degradation.[1, 2, 11, 24] Those extracellular vesicles (EV) are created by exocytosis of intraluminal vesicles and

are released in the extracellular space after fusion with the plasma membrane. Depending on their size or cellular origin, these EV are also referred to as exosomes, microparticles or microvesicles. These EV deserve extra attention since there is evidence that extracellular vesicles can selectively be produced and shed upon different physiological conditions or disease states.[25] Moreover, they can be taken up by other cells and thereby represent a novel method of intercellular communication. EVs retain membrane proteins like CD63, CD44 and EPCAM derived from their cell of origin and may play a role in recognition by recipient cells. However, the exact mechanisms and regulation of this exiting form of intercellular communication is largely unknown and subject of investigation by several collaborative groups.[26-28]

To increase the specificity for tumor cell-derived miRNA detection, one might focus on collecting miRNAs from EVs. However, it is good to realize that EVs can be derived from practically all cells including blood cells and platelets. Moreover, there is no gold standard for isolation of EVs and many different methods, such as differential ultracentrifugation, density gradient centrifugation, ultrafiltration, chromatography, immunoaffinity capture using antibody coated magnetic beads, polymeric precipitation and microfluidic devices.[29] Again, all methods have their own advantages and disadvantages in terms of the amount of sample needed, purity, yield, integrity of isolated EV, processing time and the need for specialized equipment.[30, 31] One study compared miRNA profiles of EVs isolated by ultracentrifugation with a quick and easy polymeric precipitation method (ExoQuick™, System Biosciences) and observed comparable results with both methods, although unsupervised clustering showed a clear separation per method. This suggests that the isolation methods resulted in different purities and/or presence of inhibitory factors. Based on these data one of the recommendations made by the International Society of Extracellular Vesicles (ISEV)) is to check for purity of the EV enrichment.[27] In addition, they advise to use platelet depleted plasma samples over serum as starting source for analysis on EV since platelet activation during coagulation of serum results in release of EV from platelets. On the other hand, serum should be chosen for research questions where platelet derived EVs are the subject of the study.[30] Despite advantages of isolating EV, discarding protein bound circulating miRNAs might result in loss of potential biomarkers since the majority of circulating miRNAs were co-purified with protein.[21]

A further improvement of using EV derived miRNAs as tumor biomarkers may be achieved by specific enrichment for tumor derived EV. EVs probably contain cell type specific proteins, mRNAs and miRNAs and efforts are made to select EVs based on the cell type of origin. For example, EVs can be selected by flowcytometrical analysis for specific tumor cell markers. However, this technique requires large amounts of plasma or serum. A recently published promising technique based on capture of EVs using a set of array bound disease specific proteins was effective starting with only 10ul of sample input.[32, 33]

Analytical variances

RNA-isolation

Methods that have traditionally been designed for isolation of RNA from cells or tissue have been widely applied to serum or plasma samples. These are mainly phenol-chloroform extraction methods or column-based methods. Two groups compared the impact of different RNA isolations methods on a selection of miRNAs by qPCR.[9,23] Both studies showed that the miRVana PARIS kit (Ambion inc.) and the miRNeasy mini kit (Qiagen inc.) were the best with respect to lowest CT values or percentage recovered miRNAs compared to an exogenous spike-in. The miRVana PARIS kit and the miRNeasy mini kit are indeed the most commonly used kits either following the manufacturers' protocol or with an adaption using different phenol / chloroform ratios or different amounts of sample input. Also other kits like the miRCURY RNA isolation kit from Exiqon, the MicroRNA Extraction kit, the miRPremier microRNA Isolation kit and the MasterPURe RNA Purification kit are used. Eldh et al. compared different RNA isolation kits for specific RNA isolation from exosomes and found that the miRCURY RNA isolation kit resulted in the highest yield.[34] McDonald et al. analyzed the inter-assay variance for the process of RNA isolation and qRT-PCR and found that the RNA isolation step was responsible for most analytical variance, underlining the need for an exogenous spike-in for normalization of differences of RNA isolation between samples.[9]

RNA quantification

Quantification of isolated RNA from serum or plasma has always been problematic, since the total RNA yield from cell free serum or platelet free plasma is generally low and lacks proper ribosomal RNAs that are frequently used to assess quantity. Sample impurity further prevents reliable detection using commonly applied spectrophotometric methods.[2,8,9] Over the last years it has become more popular to perform qRT-PCR-based studies using fixed sample volumes. This approach also prevents introduction of a bias caused by differences in RNA yield between samples from patients and controls. Although most recent studies use this approach, it is frequently not well described how much of the original sample is actually used in each step preventing a proper comparison of the results. Using a fixed volume might have the disadvantage of differences in efficiency of downstream molecular techniques because of the different amounts of RNA and a possible difference of admixture of contaminating factors based on their presence in serum and plasma. We therefore tested two endogenous and one exogenous miRNA in three different volumes of eluate by qRT-PCR. We observed a linear increase in levels of studied miRNAs indicating that using higher volumes did not affect qRT-PCR efficiency for the tested miRNAs (Figure 3).

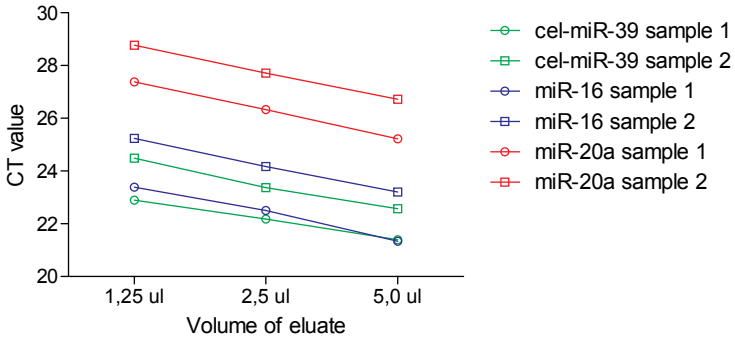

Figure 3. Impact of different input volumes on qRT-PCR efficiency. CT values of two endogenous and one exogenous miRNA in three different volumes of eluate in two healthy controls.

Platforms used to quantify or profile circulating miRNAs

During the last years, different methods have been applied to quantify circulating miRNA or to generate circulating miRNA profiles. These methods included qRT-PCR platforms (TaqMan/SYBR Green based), hybridization based arrays (Agilent/Afimetrix/Exiqon), direct quantification methods (Nanostring/Firefly) or next-generation sequencing (Illumina, ABI SOLiD, Roche GS). Most of these techniques have been extensively reviewed elsewhere.[12,19,35] The main advantages and disadvantages of each technique are summarized in Table 1. It is important to realize that the use of different techniques with differences in sensitivity and specificity can have major influence on the reproducibility between platforms.[36] Indeed, a comparison of three different techniques showed important differences in detection limits.[37] In addition, poor consistency was obtained between Life Technologies miRNA Taqman® assay and miRCURY locked nucleic acid (Exiqon, SYBR green based) qRT-PCR profiling with the Taqman assay resulting in an average 6.7 lower mean CT value, probably because of the pre-amplification step used in the Taqman protocol.

A very recent comparative paper studied accuracy and sensitivity of hybridization based, qRT-PCR based and sequencing based platforms to study miRNA levels in cells, tissue or serum.[38] They found that the Agilent hybridization based platform has very low accuracy and sensitivity when using low RNA input samples like RNA isolated from serum or plasma. They also showed that platforms based on sequencing have lower accuracy and sensitivity compared to qRT-PCR based platforms when using low input RNA samples. Recently, combining digital PCR with nanoliter-sized droplet technology (droplet digital PCR) has shown potential for reducing variability and improving reproducibility of circulating miRNA results compared to qRT-PCR.[39,40]

Table 1. Short overview of available platforms for circulating miRNA profiling

Platform	Advantages	Disadvantages
qRT-PCR	Sensitive Specific Absolute quantification Low input Low costs	Data normalization No detection of new miRNAs
Hybridization based arrays	High throughput	High and quantified input Less sensitive Less flexibility No gold standard for data normalization No detection of new miRNAs
Direct quantification methods (Nanostring / Firefly)	Sensitive High throughput No RNA isolation step (Firefly)	High and quantified input Data normalization Specialized equipment needed No detection of new miRNAs
Next-Generation Sequencing	Sensitive Specific Flexible Low input Detection of new miRNAs / isomiRs	High costs Difficult data analysis / normalization Specialized equipment needed Possibly less sensitive compared to qRT-PCR

Post-analytical variances

Data normalization

The most important post analytical challenge is the normalization strategy. For the relative quantification of mRNA levels using qRT-PCR, housekeeping genes like GAPDH or HPRT have been generally accepted as an accurate way to quantify data. For miRNA normalization there are only few miRNAs that are described as being potentially stable in tissues and cells but none for body fluids. More often, small nucleolar RNAs (snoRNAs) with comparable sizes are used for normalization in tissue although these have also been correlated with pathologic conditions including cancer.[41,42] For circulating miRNAs, normalization procedures are less straight forward as housekeeping genes used to normalize mRNA or miRNA expression in tissues are not or variably present in serum, plasma or other body fluids.[43,44] Moreover, in some cases circulating levels of snoRNAs like RNU6B and RNU48 have been reported to be disregulated in disease states, similar to what has been found in tissue samples.[45,46] Nevertheless, many groups have used endogenous miRNAs like let-7a, miR-16, miR-223 and miR-1228 or snoRNAs as normalization control.[47] As mentioned earlier, let-7a and miR-16 levels correlate to a high extend with blood cell counts and hemolysis, while others found these miRNAs to be biomarkers for malignancy.[48-54] Thus, the use of endogenous miRNAs is far from optimal.

The use of spiked-in synthetic oligonucleotides that lack homology to known human miRNA sequences, like cel-miR-39, cel-miR-54 or cel-miR-238, directly after denaturation of the serum or plasma sample seems to be a much better alternative.[2,8,9] These exogenous spike-ins can be used to correct for differences in RNA extraction and qRT-PCR efficiency. Some studies use a single spike-in, whereas others use a combination of spike-in controls or combine a spike-in with an endogenous miRNA for which no variance was observed between the groups. A disadvantage of normalization to an exogenous miRNA is that it assumes similar RT and qPCR efficiency for both exogenous and endogenous miRNAs, which might not be the case. Some researchers argue that spiking-in fixed amounts of exogenous miRNAs is less suitable for normalization of samples with different RNA concentration. This might be the case when using fixed volumes of serum or plasma as qRT-PCR input. Since it is hard to exactly quantify the total RNA content together with the known variability in the ratio between ribosomal, mRNA and miRNA levels, spiking-in exogenous miRNA in fixed volumes of RNA eluate is the most optimal available method and is currently widely used for qRT-PCR normalization.[54,55] When performing expression arrays or small RNA sequencing before qRT-PCR validation, another strategy is to search for an invariant or set of invariant miRNAs in the data set that can subsequently be used for normalization during qRT-PCR.

When using qRT-PCR profiling platforms or expression arrays the method of quantile, mean or median normalization is commonly used for tissue samples and cell cultures. There are no studies that determined the most optimal normalization strategy for circulating miRNAs.

Expression profiles from plasma or serum are significantly different from tissue or cell cultures since the number of detected miRNAs is much lower and relative variability in the number of detected miRNAs between the samples is higher. Therefore care should be taken for samples that have signals below the detection limit for a majority of the miRNAs.

Information on appropriate methods for normalization of miRNA sequencing data is scarce and no normalization method is uniformly accepted even for tissue derived miRNAs. One study tested seven commonly used normalization methods and found significant differences depending on the method used. Lowess normalization and quantile normalization performed the best while trimmed mean normalization method was not recommended, although the latter turned out to be applied incorrectly.[1,2]

Validation

Validation of circulating miRNAs is often not properly performed and frequently limited to the same set of samples using a different technique (e.g. qRT-PCR after array discovery). If performed, the size of the independent validation set was in many cancer studies on circulating miRNAs not justified by appropriate power analysis as reviewed by Jarry et al..[15]

Researchers frequently fail to test whether the circulating miRNAs are indeed related to or caused by differences in the affected tissue type. In some cases this is not possible, but in many cases this can easily be done. In cancer patients, only half of the reports studied miRNA profiles both in the circulation and in tissue and only 7% of the reports showed accurate correlation in the same direction.[15] The study of Pritchard et al. underlines the importance of this fact since they showed that 58% of frequently reported cancer associated circulating miRNAs are present at high levels in blood cells and that differences in levels of these miRNAs are mainly dependent on blood cell count.[12] These miRNAs should be left out of data analysis or at least be interpreted with high caution and tested for a possible correlation with blood cell count. When searching for true disease specific circulating miRNAs one should focus on miRNAs that are not or only at low levels expressed in blood cells.

Clinical implications

An important clinical application of altered circulating miRNA levels could be screening of high risk individuals to allow early detection of certain disease states and cancer or to establish treatment response or as predictive markers for treatment response. However, can a limited tumor load cause a detectable upregulation in the circulation? Especially for small tumors the upregulation in the tumor cells must be 10 fold or more compared to normal tissue studies to induce a significant increase of the miRNA levels in the circulation. Moreover, one would expect a correlation of circulating miRNA levels with tumor load. To our knowledge this correlation has not been found or made to date.

An interesting question that needs to be answered is what are the functional implications of higher or lower levels of specific miRNAs in the circulation and how these altered levels contribute to disease pathogenesis. A good explanation for miRNAs showing reduced levels in serum or plasma of cancer patients is still missing. It seems unlikely that tumor cells can influence miRNA expression of other cells, catch away specific circulating miRNAs, or influence stability of specific miRNAs in the circulation. It is more likely that higher or lower levels of circulating miRNAs are not actually caused by tumor-specific miRNAs but might result as a general consequence of neoplastic disease such as inflammatory responses that influence blood cell counts.

Conclusions and future perspectives

The presence of circulating cell-free RNA including miRNAs is an intriguing finding that warrants future exploration. The promise of circulating miRNAs as "the liquid biopsy" is, however, still far away due to many pre-analytical, analytical and post-analytical variances that are summarized in this review. The most critical variables are the pre-analytical variables like sample source and admixture with red blood cells, white blood cells or platelets may alter miRNA levels up to 30-fold. Analytical and post-analytical variables, like data normalization, can lead to marked differences between studies. Current literature is overwhelmed by articles that lack detailed description of methods, like sample source description, raw and normalized data. Moreover, for most of the currently reported circulating miRNA biomarkers proper validation studies are lacking. As the annual number of papers in this field of research is still increasing, it might be anticipated that many of the drawbacks will be solved in the next couple of years. Important steps to be made are implementation of standardized collection protocols, extensive comparison of different platforms and properly powered validation of miRNAs. The extracellular vesicles are the most promising source for clinical applicable biomarkers, as this has the potential to focus on tumor cell derived vesicles. However also in this field, there are still several challenges to be made regarding standardization of isolation protocols and analysis.

Another closely related field of research is to identify the biological function of the circulating miRNAs and exploring their potential functional relevance in disease states. The discrepancies found in circulating miRNAs in relation to miRNA signatures in tissue samples remains intruiging, at least for those that cannot be attributed to changes in circulating blood cell counts.

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