Circulating miRNAs - let's not waste the potential

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Abbreviations list

microRNAs (miRNAs)

Cell free miRNAs (cf-miRs)

Quality Control (QC)

MicroRNAs (miRNAs) were first described in 1993 [1-2], formally classified in the early 2000's [3] and have been at the forefront of cell physiology research for the last two decades. In humans, at least 61 tissues express over 1900 miRNAs (https://asia.ensembl.org/Homo_sapiens/Info/Annotation) with some level of tissue-specificity [4]. MiRNAs primarily, [5] but not exclusively [6], act by repressing a target mRNA molecule and the translation of the corresponding protein. Most functional miRNA/mRNA interactions are governed by base complementarity principles, but the scientific community is only starting to appreciate the complexity of miRNA targeting rules [7] and the full extent of their influence on gene expression.

MiRNAs are not only present in tissues. Biological fluids, including whole blood, plasma and serum, as well as breast milk, cerebrospinal fluid, saliva and urine, all contain extracellular, or circulating "cell free" miRNAs (cf-miRs) that are secreted by cells[8]. Cf-miRs were soon identified as potential biomarkers for a range of physiological and pathophysiological conditions and have since received growing attention. The popularity of cf-miRs is two-fold: the reasonable ease of access to most extracellular fluids, and the discovery of high levels of regulation of specific cf-miRs in the presence of diseases [4, 8-10]. These include cancer [11], cardiovascular disease [12] and liver disease [13], but also exercise [14], making cf-miRs promising biomarkers. As such, miRNAs have not only instigated a rare paradigm shift in the way we study gene regulation, but have also allowed access to a novel, non-invasive marker of human health [15]. In April 2020, a PubMed search using the terms "human" and "microRNA" or

"miRNA" returned over 100,000 articles. When refined to add the term "circulating", the same search returned close to 3500 articles, all published in the last decade.

Cf-miRs are found in circulation in two distinct forms, "vesicular" cf-miRs (miRNAs that are contained in extracellular vesicles such as exosomes, microvesicles or apoptotic bodies) or "non-vesicular" cf-miRs (miRNAs that are free in circulation as part of RNA-binding protein complexes). The presence of non-vesicular miRNAs in circulation may result from active export systems and/or passive leakage through the plasma membrane following cell damage or death [16]. However, their biological function and how they are protected from RNase degradation remain poorly characterised [17]. Some evidence suggests that non-vesicular cf-miRs are protected by forming RNA-binding protein complexes, including nucleophosmin-1 (NPM1) *in vitro* [18] and Argonaute2 (AGO2) *in vivo* [16, 19]. The conditions in which tissues selectively secrete vesicular and non-vesicular miRNAs into the circulation and their subsequent uptake by recipient cells are not fully understood. However, a number of recent studies indicate that they are tightly-regulated and highly selective process [20-22] that play a functionally important role in cross-tissue communication [20, 23].

Accessing vesicular cf-miRs relies on complex extraction and sorting techniques [24-26]. In contrast, the first step of the classical RNA isolation process lyses extracellular vesicles, leading to the extraction of both vesicular and non-vesicular cf-miRs. For this reason, the majority of the literature to date has focused on the whole cf-miR pool without attempting to discriminate between the two fractions. There are however significant differences in the miRNA profile of the vesicular and non-vesicular pool [27-28], possibly owing to selective loading of miRNAs into extracellular vesicles [21, 29]. However, early limited evidence suggest that in plasma, most cf-miRs may not be associated with exosomes/microvesicles [19]. Distinguishing between the two different cf-miR forms may therefore be essential to understand their specific physiological function and improve biomarker sensitivity [16].

A series of molecular techniques are used to measure the expression of miRNAs from tissues and fluids (Figure 1). Single miRNA expression is usually assessed using primer-based real time PCR, while miRNA arrays and small RNA sequencing have the potential to profile the expression of hundreds of miRNAs simultaneously. Reflecting the more general trend in cf-miR research, in our field of exercise physiology, only 10 studies to date have focused on vesicular cf-miRs, with the vast majority of studies investigating the whole cf-miR pool. Regrettably, it is not uncommon to observe important discrepancies in cf-miR expression and regulation between these studies [30]. Differences in analytical techniques, research protocols and participant characteristics account for some of these differences. However, a number of methodological factors that are not unique to the exercise field have been broadly overlooked in cf-miR research.

Most studies have been performed in whole blood, serum and plasma, and it is important to acknowledge that blood cells either contain [31] or produce [32] their own miRNAs. Differences in blood

cell number therefore constitute a confounding factor when assessing changes in the cf-miR pool. Using cell-free plasma and serum may bypass this issue, pending that haemolysis is strictly controlled at collection as well as in the final extract [33]. In addition, platelet-derived miRNA contamination is frequent in archived plasma and serum samples, and residual platelets should be thoroughly eliminated prior to [34] or after [35] freezing to ensure accurate results. Another challenge intrinsic to cf-miR detection resides in the low starting RNA amounts, owing to miRNA dilution and limited sample availability. In most cases, RNA concentration is too low to be reliably measured. As a result, most studies use the RNA extracted from an equal amount of fluid without formal quantification. This approach is valid but contingent to the use of strict analytical controls at each stage of the downstream process. For PCR based studies, Nielsen et al. have proposed a stringent workflow including several synthetic spike-in controls during the extraction, reverse-transcription and amplification processes to account for potential differences in starting RNA amounts [33]. Although integral to the reliability and reproducibility of the results, these quality control steps are time consuming, cost generating and have not been systematically implemented [30]. Lastly, while high-throughput technologies come with global normalization strategies, normalization of single cf-miR expression must be carefully considered. There is a lack of reliable housekeeping miRNAs, and single-strand DNA quantification is hardly possible due to low starting RNA concentrations [36]. Software using the most stable miRNAs from a given panel to compute a normalization factor are therefore warranted when no other option is available. Numerous studies, especially recent ones[33, 37-39] , have successfully implemented a combination of these quality control steps. However, the bulk of published studies may still rely on inaccurate approaches that compromise the reliability of the results and risk misinforming the field. A final consideration intrinsic to human physiology research resides in striking the right balance between participant burden and appropriate statistical power. Ethical considerations combined with financial constraints as well as the very nature of exploratory research have led to the publication of numerous underpowered studies. It is important to bear in mind that, because large effect sizes are rare in physiological sciences, significant findings based on small sample sizes are more likely to constitute false-positives[42]. This remains a significant barrier for implementation of fundamental results into clinical settings. There is now sufficient published research in the field to estimate appropriate effect sizes, and researchers should take advantage of the non-invasive nature of cf-miRs research to aim for larger sample sizes. An effort should also be made to interpret new findings in the light of a pragmatic combination of statistical significance, physiological relevance and the existing literature.

In conclusion, cf-miRs are attractive potential biomarkers that are readily available as they do not rely on invasive tissue sampling. The apparent simplicity and specificity of the approach has led to the rapid development of a considerable body of research. Measuring cf-miR expression levels however presents unique challenges that have been frequently overlooked. Unless specifically isolating extracellular vesicles, their cargo becomes part of the cf-miR pool. Vesicular and non-vesicular cf-miRs may

however have different physiological relevance and role in tissue cross-talk. Secondly, a majority of published studies have failed to put the relevant quality controls in place, leading to significant discrepancies and a lack or reproducibility in the field. Finally, our functional knowledge is limited and more research is warranted to understand the biological principles underlying the release and uptake of both vesicular and non-vesicular cf-miRs. It has been suggested early on that cf-miR research might be blinded by its own potential [15]. It is now our role as researchers to safeguard this important research field in order to prevent 'blinded by potential' becoming 'wasted potential'.

Figures

Figure 1. Graphical summary representing non-vesicular and vesicular cell free miRNA (cf-miR) extraction and analysis techniques including critical quality control (QC) steps.

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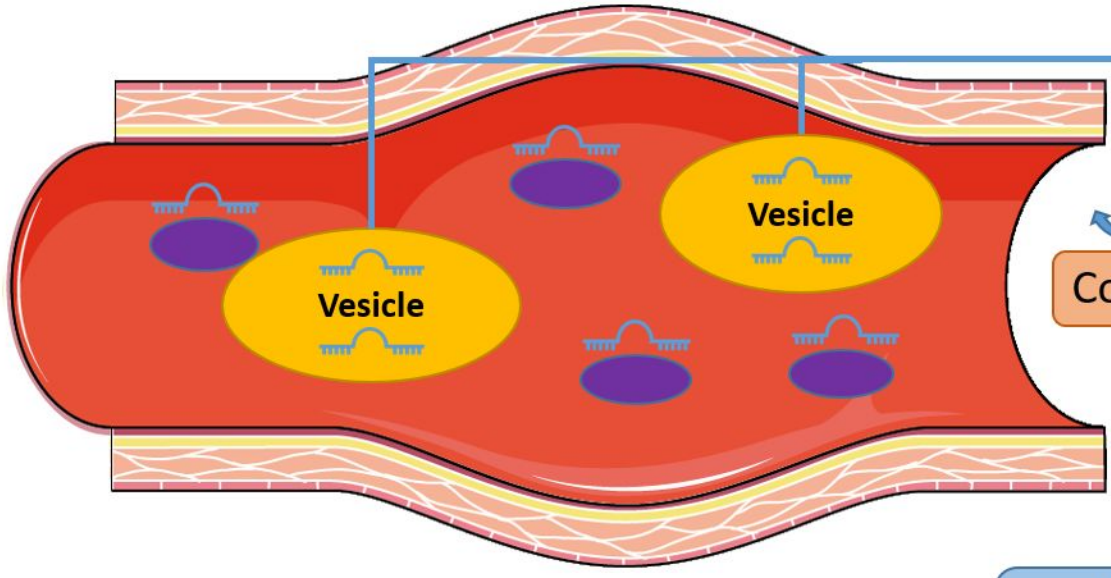

EV isolation, sorting

QC (haemolysis)

Collection

PCR-based Analysis

QC (spike-in)

RNA extraction

QC (spike-in)

Reverse-transcription

QC (spike-in)

PCR

QC

small RNA Seq
miRNA arrays

Global

Normalization factor

Normalization

cf-miRs

RNA-binding protein complex