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HYPOTHESIS

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Variability in the levels of exosomal miRNAs among human subjects could be explained by differential interactions of exosomes with the endothelium

Kyriacos Felekkis | **Myrtani Pieri** | **Christos Papaneophytou**

Department of Life and Health Sciences, School of Sciences and Engineering, University of Nicosia, Nicosia, Cyprus

Correspondence
Christos Papaneophytou, Department of Life and Health Sciences, School of Sciences and Engineering, University of Nicosia, Nicosia 2417, Cyprus.
Email: papaneophytou.c@unic.ac.cy

Abstract

Exosomes are 30–100 nm endosome-derived membrane vesicles, that contain specific RNA transcripts including mRNAs, and microRNAs (miRNAs) and have been implicated in cell-to-cell communication. Exosomal miRNAs in blood circulation have been attracting major interest as potential diagnostic and prognostic biomarkers in a variety of diseases including stroke, cancer, and inflammatory disorders. Despite the progress made in the utilization of circulating exosomal miRNAs as biomarkers for various human diseases and conditions, there are still difficulties in functionally utilizing such methods in the clinic due to the high variability observed among subjects. Attempts to use miRNA signatures have improved but have not eliminated the problem. Additionally, standardized laboratory practices may partially reduce variability but there is still an unknown biological factor that hinders the proper use of miRNAs as biomarkers. We hypothesize that this variability might be partially attributed to a differential interaction among circulating exosomes carrying those miRNAs with endothelial surface molecules that themselves may vary among individuals due to secondary conditions, for example, inflammation status. This differential interaction could potentially add variability to the level of the examined miRNA that is not directly attributed to the primary condition under study.

KEYWORDS

biomarkers, exomiRs, exosomes, inflammation, miRNAs, normalization

1 | INTRODUCTION

Exosomes, a specific class of EVs, are extracellular phospholipid bilayer vesicles with a diameter of 30–100 nm that can be found in different types of cells in eukaryotes especially in DCs, lymphocytes, ECs, and endothelial cells. Exosomes are released by membrane fusion into various biological fluids, including, plasma, urine, breast milk, and cerebrospinal fluid[1] playing an important role in cell-to-cell communication[2] and affecting physiological changes in recipient cells by transferring their cargo. Recently, they have also been implicated in various diseases including cardiovascular, autoimmune, neurodegenerative disorders, cancer, stroke, and many others.[3] In addition to proteins and lipids, various nucleic acids have

Abbreviations: AGO-2, argonaute 2 protein; CAM, cell adhesion molecules; c-miRNAs, circulating miRNAs; DCs, dendritic cells; ECs, epithelial cells; EVs, extracellular vesicle; HDL, high-density lipoprotein; miRNAs, microRNAs; NPM1, nucleophosmin I.

been identified as the cargo of exosomes including mRNAs and noncoding RNAs.[4,5] Interestingly miRNAs, a specific class of non-coding RNAs, account for more than 50% of all exosomal RNAs[6] while the proportion of miRNAs is higher in exosomes than in their parent cells.[7] During the circulation of exosomes, their cargo (i.e., miRNAs) can be taken up by neighboring and/or distant cells and modulate recipient cell activity. In general, exosomes carry their components including miRNAs of their original cells, and interact with adjacent or distant cells to perform information exchange between different cells under both physiological and pathophysiological conditions.[8]

2 | CIRCULATING miRNAs

MiRNAs are short (~22 nucleotides in length) single-stranded RNAs, that have been implicated in the regulation of gene expression by binding to target mRNAs in eukaryotic cells[9] modulating in this way various cellular functions including cell growth, proliferation, differentiation, and apoptosis.[10] Many miRNAs regulate up to hundreds of mRNAs, while their expression is tissue-specific.[11] Thus, due to their significant biological role as regulators of gene expression, miRNAs play an essential role both in physiological and pathophysiological processes.[12]

MiRNAs carry unique properties such as their remarkable stability in plasma, their resistance to harsh conditions like boiling, low and high pH, RNases, long storage conditions as well as their withstanding of repetitive freezing and thawing cycles.[13] This stability means that they are long-lived in various biological fluids[14,15] and are therefore proposed as a promising class of novel biomarkers that may be used to either diagnose life-threatening diseases or to monitor disease progression and even to guide various treatment protocols. In addition to being packed into vesicles such as microvesicles[16] or exosomes,[17] c-miRNAs can be loaded into RNA-binding proteins such as AGO-2,[18] HDL, or NPM1.[19] Specifically, 10% of c-miRNAs are secreted in exosomes (termed exomiRs), while the other 90% form complexes with AGO-2, NPM1, or HDL.[20] It has been proposed that exomiRs are the best source compared to non-exosomal, cell-free, or whole unfractionated samples when studying miRNAs as biomarkers owing to their advantages in terms of quantity, quality, and stability.[21]

ExomiRs have drawn much attention in recent years as it has been suggested that the loading of microRNAs into exosomes is not a random process but rather a well-regulated one.[22] Also, exomiRs can stably exist in the blood, urine, and other bodily fluids of healthy individuals and patients, and can reflect their tissue or cell of origin by the presence of specific surface proteins.[23,24] Interestingly, the amount and composition of exosomal miRNAs differ between healthy individuals and patients.[8] Thus, exomiRs have been demonstrated to play an important role in disease progression, such as stimulating angiogenesis and facilitating metastasis in cancers,[8] being involved in inflammatory diseases, rheumatoid arthritis,[25] and even diagnosing acute stroke of various types.[26]

3 | CHALLENGES IN USING EXOmiRS AS BIOMARKERS

ExomiRs show potential for use as noninvasive biomarkers to indicate disease states as well as to guide therapy.[8] Nonetheless, despite the initial optimistic views of their potential for clinical application and the great enthusiasm on the idea of using these molecules as disease biomarkers, currently, there are no c-miRNA-based diagnostics in use.[20] One of the main reasons is the lack of consistency in findings and the many contradictory publications on the alterations of exosomal c-miRNAs concentrations in various pathological conditions.[20]

One reason for this discrepancy observed between different studies could reside in methodological heterogeneity in sample storage and handling, different extraction methods of exosomes and/or miRNAs as well as differential data normalization.[13] Therefore, several preanalytical and analytical issues need to be addressed and many challenges must be overcome before the accurate utilization of exomiRs as potential biomarkers for various diseases.[13]

Indeed, the technical issues and heterogeneity observed of exosome isolation protocols have been the subject of extensive evaluations and debates.[27] Different methods are employed for the isolation of exosomes such as size exclusion chromatography, ultracentrifugation, density centrifugation, immunoaffinity techniques, and ExoQuick™ Precipitation (System Biosciences)[23] resulting in differential exosomal content, including miRNAs and proteins.[24] To this end, the International Society for Extracellular Vesicles has proposed standard procedures and guidelines for the analysis of EVs and the reporting of the results.[28] These guidelines were last updated in 2018.[29]

Moreover, handling bias can occur during the extraction step and c-miRNAs may be contaminated by cellular miRNAs, while the c-miRNA levels differ among various blood fractions (i.e., whole blood, plasma, and serum) due to the miRNA "trafficking" between cellular components and the extracellular environment (for a review on the topic see Reference 13).

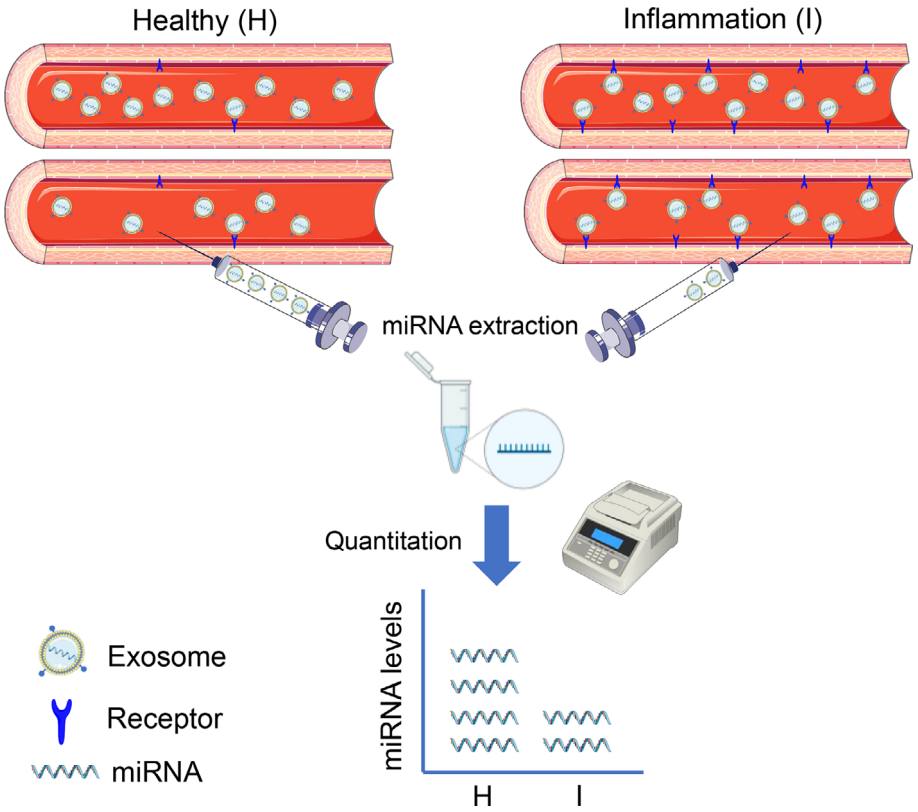

FIGURE 1 Graphical representation of proposed hypothesis. The variability of exosomal miRNA levels in patients' samples compared to healthy individuals may be partly explained by the interactions of exosomes with endothelial receptors that are overexpressed in pathological conditions such as inflammation

4 | HYPOTHESIS

Herein, we hypothesize that variability among different studies and individuals may also be explained by a differential interaction among circulating exosomes carrying miRNAs with endothelial surface (adhesion) molecules (Figure 1).

The endothelium expresses different receptors (Table 1) depending upon their organ of origin and their functional status.[32] This phenomenon has been nicknamed endothelial ZIP codes.[33] Likewise, EVs including exosomes express various CAM (Table 1), while it has been demonstrated that EV attachment to endothelial cells is dependent upon their exposed PSGL-1 attaching to the P-selectin expressed from Weibel–Palade bodies and platelet alpha-granules, and upon the tethering of the exposed surface phosphatidylserine to Tim4, lactadherin/MFG-E8 and probably other CAM.[32] Alternatively, exosomes can also bind to immune cells in the blood, expressing the same adhesion markers as endothelial cells (e.g., PECAM1, VCAM1, etc.), thus contributing to variability of miRNA levels. However, the latter can be determined and corrected experimentally following sample extraction by modifying lysis protocols and normalization strategies. As such, in this report, we will focus on the endothelial binding mechanism that can ultimately lead to the highest degree of miRNA variability among subjects.

An increase in the expression of endothelial markers and CAM after the application of reactive oxygen species or proinflammatory stimuli has been reported.[34] Exosomes are released in response to various pathophysiologic stimuli including shear stress, complement activation, sepsis, hypoxia, and inflammation.[35] Exosomes have surface proteins that have been shown to interact with integrins and other surface markers on endothelial cells.[36] Typically, exosomes are highly enriched in proteins with various functions, such as tetraspanins (CD9, CD63, CD81, CD82), which take part in cell penetration, invasion, and fusion events; heat shock proteins (HSP70, HSP90), as part of the stress response that is involved in antigen binding and presentation; MVB formation proteins that are involved in exosome release (Alix, TSG101); as well as proteins responsible for membrane transport and fusion.[37] Depending on their origin, exosomes contain specific sets of known proteins, including tetraspanins, heat shock proteins (Hsps), annexins, and

TABLE 1 CAM present in exosomes and endothelial cells

Exosomes[30]	Endothelium[31]
• Integrins • Lactadherin • Intercellular adhesion molecule 1	• Intercellular adhesion molecule-1 (ICAM-1) • Platelet-endothelial cell adhesion molecule-1 (PECAM-1) • Vascular cell adhesion molecule 1 (VCAM-1) • P- and E-selectins

membrane-bound mucins; however, the role of the individual components of exosomes is still unknown.[38]

The significance of such interactions between exosome surface proteins and the endothelium is largely unknown and can also remain undetectable. Additionally, these events might be dynamic as they can be modified or altered according to the physiological or pathological state of the endothelium. Various pathological conditions can affect endothelial cells such as altered protein trafficking and polarization in a way that the surface marker repertoire is distorted. As such, it might be expected that this may also transform the interactome of the endothelium with circulating molecules such as exosomes. Attachment of exosomes to the endothelial surface will reduce the percentage of circulating exosomes and thus reduce the percentage of their content. Differential attachment between patients will ultimately result in a differential percentage of exosomal miRNAs in the bloodstream introducing an unexplained variability. Evaluation of signature miRNAs rather than isolated single miRNAs might reduce such variability but even then, it cannot be eliminated.

It may be instructive to determine if EVs from different source cells adhere to different organ-sourced endothelial cells with different densities and with differing endothelial cell responses. It is unknown whether there is a steady state of microparticle attachment to endothelial surfaces in normal circumstances. To verify this hypothesis specific questions should be answered: Are there differences of any such density of adherence depending upon the site of origin of the endothelium? Is there an accelerated adherence of exosomes in disease states that affects endothelial function and integrity?[32]

It is well known that inflammatory cells are very important in the response to injury in all tissues. The recruitment of inflammatory cells facilitates the generation of cytokines, growth factors, and degradative enzymes.[39] Moreover, the expression of molecules on the apical side of ECs that facilitate leukocyte adhesion to the endothelium, such as E-selectin, P-selectin, ICAM-1, and VCAM-1, is generally elevated in short term acute inflammation.[40] The EC barrier allows regulated and selective passage of appropriate solutes and immune cells during resting and inflammatory conditions. This vital function is mediated by interactions between ECs through junctional molecules, such as VE-Cadherin, JAMs, and PECAM-1. Remodeling of the EC membrane during inflammation includes a reorganization of junctional molecules, a response that is pivotal for the regulation of vascular permeability and leukocyte extravasation. Changes in expression levels of junctional molecules can also be temporally and spatially regulated by inflammatory mediators and leukocyte TEM. These mechanisms include cell surface redistribution and internalization of key cell border structures, the recycling of intracellular pools of molecules, and their enzymatic cleavage.[40]

5 | IMPLEMENTATION AND SUGGESTIONS

Below we propose some experimental approaches for verification of the hypothesis presented:

1. Normalization using the total exosome concentration or total exosomal miRNAs.
2. Attachment assay of the labeled exosomal fraction to endothelial monolayer overexpressing different surface integrins.
3. Measurement of medium exosomal level between normal and integrin-overexpressed endothelial monolayer cultures.
4. Upon in vitro verification, animal work can examine the principle. Systemic inflammation can be induced in C57BL/6N mice with lipopolysaccharide treatment, peritoneal contamination, and infection, or cecal ligation and puncture. Exosomal vesicles loaded with specific miRNAs not found in mice (e.g., *Caenorhabditis elegans* specific miRNA) can be injected at different time points and compare the levels of that specific miRNA between normal and inflammation-induced animals.

6 | CONCLUSION

The hypothesis proposed herein, of a putative interaction among circulating exosomes carrying miRNAs with endothelial surface molecules, if verified, could explain partly the variability observed in miRNA concentrations observed in the literature on the use of miRNAs as biomarkers. Pursuing this hypothesis experimentally in the ways proposed here, or other experimental approaches, could shed light on this interaction and hopefully ways to eliminate it during studies. This will greatly pave the way for various clinical applications such as c-miRNA-based diagnostics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Kyriacos Felekkis https://orcid.org/0000-0002-0919-0037
Myrtani Pieri https://orcid.org/0000-0002-8883-4434
Christos Papaneophytou https://orcid.org/0000-0002-4045-8839

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