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Review

Plasma MicroRNA as a novel diagnostic

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ABSTRACT

MicroRNAs (miRNAs) are small, single-stranded, endogenous, non-coding RNAs necessary for proper gene expression. Their mechanism of action controls translation by base-pairing with target messenger RNA (mRNAs) thus leading to translation blockage or mRNA degradation. Many studies have shown that miRNAs play pivotal roles in cancer, cardiovascular disease and neurodegenerative disorders. The lack of blood-derived biomarkers and those markers of poor specificity and sensitivity significantly impact the ability to diagnose in general and at early disease stage specifically. As such, new, non-invasive and quantifiable biomarkers are needed. As post-transcriptional regulators of gene expression, miRNAs have been confirmed to be notably stable in cells, tissues and body fluids. These and other advantages make miRNAs ideal candidates as potential biomarkers and early experimental findings support this finding. This review examines the use of miRNAs as biomarkers in cancer, neurodegenerative, cardiovascular and liver disease and viral infection.

1. Introduction

MicroRNAs (miRNAs) are a group of small, endogenous, non-coding RNAs consisting of 21-23 nucleotides. Mature miRNAs act as post-transcriptional regulators of gene expression, by base-pairing with mRNA molecules in the 3′ untranslated region (3′-UTR), controlling the translation process of the target mRNA. Depending on the degree of complementarity of the miRNAs and the target mRNA, this leads to translation blockage, or less frequently, mRNA degradation [1]. Genes encoding miRNAs have their own regulatory sequences and promoters that control their expression, but some miRNAs genes are composed in clusters, and are regulated with other members of the same cluster. MiRNAs genes are mostly distant from the gene they interfere with, which suggests that they are from different, independent transcription units [1,2].

Over the last 20 years, miRNAs has become the object of intense research, due to its potential participation in the development of various diseases [3]. At this time, over 1,900 different miRNAs molecules have been identified in human cells, which all control the expression of different genes [4]. It has been proved that miRNAs can modulate up to 60% of protein-encoding genes affecting the cellular cycle, differentiation, proliferation and apoptosis [3,5].

Biosynthesis of miRNAs is a complex, multi-step process. The first step is a nuclear phase, in which miRNAs are transcribed to long pri-miRNA by RNA polymerases II and III. Pri-miRNA consists of a double-stranded core (~30 base pairs), two flanking unstructured single-

Abbreviations: 3′-UTR, 3′Untranslated Region; *ABC*, Activated B-cell like; *AC*, Adenocarcinoma; *ACE*, Angiotestin-converting Enzyme; *ACLF*, Acute-on-Chronic Liver Failure; *AD*, Alzheimer's Disease; *AIDS*, Acquired Immune Deficiency Syndrome; *ALT*, Alanine Transaminase; *AS*, Aortic Stenosis; *AST*, Aspartate Transaminase; *BACE1*, Beta-side Amyloid Precursor Protein Cleaving Enzyme 1; *B-CLL*, B-Cell Lymphoma 2; *CA19-9*, Carbohydrate Antigen 19-9; *CAD*, Coronary Artery Disease; *CEA*, Carcinoembryonic Antigen; *CHC*, Chronic Hepatitis C; *CK*, Creatinine Kinase; *CK-19*, Cytokeratin 19; *DCM*, Dilated Cardiomyopathy; *DGCR8*, Di George Syndrome Critical Region 8; *DLBCL*, Diffuse Large B Cell Lymphoma; *ds-miRNA*, Double-stranded miRNA; *DVT*, Deep Vein Thrombosis; *GCB*, Germinal Center B cell-like; *HIV*, Human Immunodeficiency Virus; *ICM*, Ischemic Cardiomyopathy; *IL8*, Interleukin 8; *INR*, International Normalized Ratio; *IPA*, Ingenuity Pathway Analysis; *MI*, Myocardial Infarction; *miRNA*, MicroRNA; *MRI*, Magnetic Resonance Imaging; *mRNA*, Messenger RNA; *mTOR*, Mammalian Target of Rapamycin; *NAFLD*, Non-Alcoholic Fatty-Liver Disease; *NGS*, Next-Generation Sequencing; *NSCLC*, Non-Small Cell Lung Cancers; *NSTEMI*, Non-ST-Elevation Myocardial Infarction; *PACT*, Protein Activator of PKR; *PD*, Parkinson's Disease; *PDK1*, Pyruvate Dehydrogenase Kinase 1; *PE*, Pulmonary Embolism; *PET-CT*, Positron Emission Tomography-Computed Tomography; *PSA*, Prostate Specific Antigen; *PT*, Prothrombin Time; *PTEN*, Phosphatase and Tensin Homolog; *qRT-PCR*, Quantitative Real-Time PCR; *RISC*, RNA-Induced Silencing Complex; *SCC*, Squamous Cell Carcinoma; *sncRNA*, Small Non-Coding RNA; *STAT3*, Signal Transducer and Activator of Transcription 3; *STEMI*, ST-Elevation Myocardial Infarction; *TGF-β*, Transforming Growth Factor β; *TRBP*, Trans-Activation Response RNA-Binding Protein; *VTE*, Venous Thromboembolism.

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stranded tails, a terminal loop, and several thousand nucleotides. In the next step, pri-miRNA molecules are cleaved into shorter precursor particles, consisting of ~70 nucleotides, pre-miRNA, that have a characteristic hairpin-shaped structure. Pre-miRNAs are processed by the Microprocessor complex, which includes the RNase III enzyme Drosha, and the Di George syndrome critical region 8 (DGCR8), double stranded-RNA binding protein [5]. After enzymatic treatment the pre-miRNAs have ~70 nucleotides in length, and are ready to be exported to the cytoplasm by the Exportin-5 protein [6]. In the cytoplasm, a complex consisting of the RNase III enzyme Dicer, protein activator of PKR (PACT) and trans-activation response RNA-binding protein (TRBP) process the pre-miRNAs from long to short, double-stranded duplexes. In the next step of the miRNAs' biosynthesis, short, double-stranded miRNAs (ds-miRNAs) inosculate with RNA-induced Silencing Complex (RISC) [5,6]. RISC belongs to a family of heterogeneous complexes that play a major role in gene silencing. Several of its mechanisms (repression of translation, mRNA degradation, heterochromatin formation and DNA elimination), mean that the construction of its complexes can be quite diverse. However, there are two invariable themes in every RISC complex: a core, composed of an AGO protein from the Argonaute family, responsible for binding to the small non-coding RNAs (sncRNAs); and sncRNAs, the function of which is to lead the RISC complex to the target mRNA. Argonaute proteins not only bind the sncRNAs, but also position them into a proper configuration that simplifies target recognition [7]. The way in which the RISC complex interacts with the target mRNA is determined by the AGO proteins, of which four are distinguished in human cells. miRNAs only bind to an Ago2, resulting in cleavage of target mRNA [6]. Furthermore, AGO unwinds the ds-miRNAs, leaving only one strand – a guide strand, which constitutes the mature miRNA molecule [5]. In gene silencing, the extent of the complementarity between the target mRNA and miRNAs determines the end result of the silencing. This will be either inhibition of translation, or mRNA cleavage [6].

MiRNAs particles play a pivotal role in the regulation of biological processes, which creates the opportunity for their use as predictive markers in different diseases. One of the more general groups of diseases in which miRNA sequences are used to identify and diagnose patients, is cancer. Diversity in miRNAs expression levels in cancer cells shows specific changes, pointing to the idea that miRNAs regulation could be linked with genetic mutations, such as deletion or insertion. It suggests the potential role of microRNA as oncogenes, leading to transformation of normal cells into cancerous cells, or suppressor genes, which are responsible for the genetic stability of the cell and for the inhibition of cell division and tumor development [8].

It is now thought that the miRNA expression profile in tumor cells helps with diagnosis and monitoring of patient responses to the type of therapy used. A study by Iorio et al. showed that the expression profile of just 15 miRNAs allowed for the correct distinguishing of 76 samples of breast cancer from 10 normal tissue samples. Furthermore, varied miRNAs expression was also found to be a marker for important histopathological features, such as metastasis ability, angiogenesis and the stage of cancer [9]. In Rosenfeld et al.'s study, scientists linked 48 miRNAs sequences to 22 types of tumor with an accuracy of 90% over 253 samples [10]. Based only on expression of 3 miRNAs (miR-21, miR-155 and miR-221), it is possible to divide Diffuse Large B Cell Lymphoma (DLBCL) into two classes: germinal center B cell-like (GCB), and activated B cell-like (ABC) [11].

The MiR-15a and miR-16-1 genes are well-known miRNA sequences, located in chromosome 13 (13q14) where deletion often occurs. A lack of these genes or highly reduced gene expression is often observed in patients with B-cell Chronic Lymphocytic Leukemia (B-CLL), because of a disproportion in the level of the B-cell Lymphoma 2 protein (BCL-2), which has antiapoptotic properties and protects tumors from death. In a normal, physiological state, miR-15a and miR-16-1 act as suppressors of tumor development. Low concentrations of miR-15 and miR-16 have been also seen in patients with mantle cell lymphoma, prostate cancer and multiple myeloma [12]. Another example of disproportion in the expression level of microRNAs is miR-21, the overexpression of which has been confirmed in lung, prostate, breast, colon, head and neck, stomach, esophagus and pancreatic tumors. miR-21 leads to augmented proliferation and growth of tumors and a decreased response to apoptosis [13]. Clustered miRNAs sequences also play an important role in cancer development. The miR-17-92 cluster is well known as an oncogene, because of its overexpression in lung cancer, malignant lymphoma and many other types of cancer [14]. This cluster is located in chromosome 13 (13q31), which is known to be amplified. High expression of miR 17-92 contributes to accelerated and more aggressive tumor development, especially malignant tumors [15].

Fast-growing progress in technology and science has allowed scientists to identify 200 miRNAs expressed in cardiomyocytes. Because miRNAs mostly have tissue-specific expression, scientists have decided to use them in diagnosis of tissue-specific diseases. In the case of the heart's miRNAs, many participate in pathogenesis of cardiac hypertrophy, heart failure and ischemia [16]. In a study conducted by Ikeda et al. miRNAs were collected from the ventricular myocardium of 67 donors. Out of 428 miRNAs measured (of which 13 miRNAs sequences had differential expression in aortic stenosis (AS)), 8 miRNAs had differential expressions in ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM). MiR-19a and miR-19b were downregulated 2 to 2.7 times in DCM and AS. Furthermore, miR-214 was 2 to 2.8 times higher in all three disorders (DCM, ICM and AS) [17].

miR-1 and miR-133a, which are responsible for direct cardiomyocyte differentiation from stem cells and cardiac development, have been shown to have a very important role in the development of heart disease. Dysregulation of miR-1 and miR-133a can lead to pathological hypertrophy, and to ventricular septal defects and DCM. Inhibition of miR-1 in adults causes augmented regulation of twinfilin-1, an actin-binding protein that regulates cytoskeleton, inducing hypertrophy [16,18].

2. Plasma microRNA as a potential diagnostic biomarker

Advancing scientific progress has allowed scientists to study miRNAs with greater precision. Nowadays, miRNAs are considered as potentially useful diseases biomarkers. In the past few years, scientists have focused on the profile of miRNAs in plasma, due to its remarkably high stability in body fluids and resistance to endogenous RNase activity, a wide range of pH, multiple freeze-thaw cycles and prolonged storage times. They are also easy to obtain through non-invasive methods, are highly sensitive to early detection and have high specificity to different disease entities [19,20].

It is well known that expression levels of certain miRNAs are altered in many disease states. About 10% of all human miRNAs particles can be found in plasma, and the differences in expression of miRNAs can be observed in various stages of disease development. Many genome-wide associated studies show that miRNAs genes are often located in fragile sites of cancer-associated genomic regions, suggesting its potential roles in tumorigenesis [21].

2.1. Use of plasma microRNAs in diagnosis of cancers

According to data from the World Health Organization, prostate cancer is the most common malignancy among adult men, with almost 1.3 million cases per year. The occurrence of prostate cancer correlates with their age and race [22]. The first-line test in the diagnosis of prostate cancer is measurement of the prostate-specific antigen level (PSA). Because of the lack of specificity for early detection of prostate malignancies, and high rates of excessive treatment and diagnosis linked with PSA testing, there is an urgent clinical need for better diagnostic tools that will help in diagnosis and prediction of prostate cancer. At this time, the gold standard in diagnosis of prostate cancer is

prostate biopsy, but it is burdened with some limitations that can generate false negatives. Randomly collected samples can indicate an abnormal biopsy result and require repeated biopsies with more advanced equipment (i.e. MRI). In recent years, scientists have developed several molecular assays that provide new possibilities for the non-invasive diagnosis of prostate cancer [23]. A study conducted by Matin et al. investigated the plasma levels of cancer-associated miRNAs. Of 372 cancer-associated miRNAs, Matin et al. showed dysregulation of 11 in patient samples in comparison to healthy controls, indicating that 4 have potential meaning: miR-4289; miR-326; miR-152-3p and miR-98-5p. Based on these 4 miRNAs, a diagnostic test was performed in patients diagnosed with prostate cancer in comparison to healthy control. In most cases, the sensitivity and specificity of the testing exceeded the accuracy of the PSA test. The direct mechanism of action of these miRNAs remains unclear, despite the fact that circulating miRNAs are associated with cell-to-cell communication and play an important role in processes such as proliferation, metastasis and angiogenesis [24].

Bai et al. conducted a study in which they investigated plasma miR-19a in patients diagnosed with esophageal squamous cell carcinoma (ESCC), one of the most aggressive malignancies in the world. Imaging exams and biopsies are the gold standard in the diagnosis of esophageal cancer and significantly improve the detection of these malignancies, but are still very invasive or demand radiation. A less dangerous method of patient diagnosis is the use of traditional tumor markers, cytokeratin 19 (CK-19) and Cyfra21-1. However, their sensitivity leaves a lot to be desired, and so the search for less invasive and more sensitive methods of diagnosing esophageal cancer became the basis for Bai et al.'s research. MiR-19a is a member of the miR-17-92 cluster, which is highly expressed in ESCC. The data obtained by Bai et al. showed that in comparison to healthy controls, the plasma concentration of miR-19a in diagnosed patients was significantly augmented ($p < 0.001$). They also investigated the potential role of miR-19a as a diagnostic marker for ESCC. In comparison to Cyfra21-1, miR-19a was more sensitive and specific. The best results were obtained with a combination of miR-19a and Cyfra21-1, which achieved a sensitivity of 92.37%. The most important discovery in the study by Bai et al. was a decrease in the level of plasma miR-19a after surgical removal of cancer ($p < 0.001$). This suggests that miR-19a could be secreted by ESCC cells and could be an important indicator in the evaluation of therapeutic effects [21].

Lymphoid neoplasms are a type of cancer caused by transformation of normal lymphoid cells. Diagnosis is based on biopsy and PET-CT (positron emission tomography-computed tomography) imaging. Due to the high percentage of false-positive diagnoses (over 20%), patients are being unnecessarily exposed to radiation, having unnecessary biopsies and surgical investigations, and are having to settle the high financial costs of their treatment and diagnosis. Thus, as with other cancers, new, less invasive and more sensitive and specific tests are always being sought [25]. Lawrie et al. reported that miR-155, miR-210 and miR-21 had different expression in DLBCL patients in comparison to healthy controls, suggesting the potential role of these 3 miRNAs as biomarkers of lymphoma. Furthermore, they have been found that miR-21 has an anti-proliferative effect on various cancers [26]. Feng et al. additionally reported five plasma miRNAs that were significantly dysregulated in DLBCL, in comparison to healthy volunteers: miR-15a ($p = 0.0001$); miR-16-1 ($p = 0.0003$); miR-21 ($p = 0.0049$); miR-29c ($p = 0.0020$), and miR-155 ($p = 0.0023$) [27]. In a study conducted by Khare et al., 10 dysregulated plasma miRNAs in DLBCL patients were identified. In contrast to Lawrie's and Feng's studies, Khare et al. examined all miRs present in the plasma, not only those that were overexpressed. This method opens the possibility of discovering more significant miRNAs associated with DLBCL. Khare et al. reported increased plasma levels of miR-532-5p and miR-124, and down-regulated levels of miR-141, miR-425, miR-145, miR-345, miR-197, miR-424, miR-122 and miR-128. In their study, Ingenuity Pathway Analysis (IPA), was used to define mRNAs targeted by these selected miRs and to determine in which biological processes they are involved. They found up-regulation in different signaling pathways (STAT3, IL8, p13k/AKT and TGF-B), and potential down-regulation of p53 and PTEN pathways, which are very important in proliferation, differentiation and tumorigenesis. Khare et al. also identified DLBCL patients as having augmented levels of miR-25, miR-26b, miR-30d, miR-182, miR-186, miR-30a, miR-140 and miR-125a in comparison to a control group, and down-regulated levels of miR-23a, miR-93, miR-122 and miR-144. IPA analysis of selected miRNAs in both groups found that the cAMP-mediated pathway and p53 pathway can be downregulated, such as with PTEN expression [25].

Lung cancer is the most common and deadliest type of cancer, causing over 1.7 million deaths worldwide every year [22]. Nearly 90% of lung cancers are non-small cell lung cancers (NSCLC) that consist of squamous cell carcinoma (SCC) and adenocarcinoma (AC). The primary risk factor of NSCLC development is tobacco smoking. Unfortunately, for most patients the prognosis is bleak, resulting in an approximately 14% chance of a 5-year survival rate. This high mortality rate is caused by late diagnosis, when the cancer is already at an advanced stage. Therefore, the development of sensitive, specific biomarkers that can precisely diagnose the early stages of lung cancer are essential. Many studies have looked at the same lung cancer-related miRNAs [28]. For example, Zhang et al. showed upregulation of plasma miR-145, miR-20a, miR-21 and miR-223 in NSCLC patients in comparison to a healthy control group [29]. Gao et al. reported upregulation of miR-324-3p and downregulation of miR-1285 in stage I lung cancer [30]. Zaporozhchenko et al. discovered significant upregulation of miR-19b and miR-21, and downregulation of miR-25 and miR-183 with $p < 0.05$ in all cases. Furthermore, of all the miRs that have been tested, miR-19b showed the most significant diagnostic value in lung cancer patients [31]. Leng et al. investigated a whole plasma miRNAs profile showing dysregulation in the expression level of not only the same miRNAs discovered previously, but also new miRs that had not previously been linked with lung cancer. Leng et al. then developed a diagnostic panel of four-plasma miRNAs (miR-126, miR-145, miR-210 and miR-205-5p), which showed 91.56% sensitivity and 96.23% specificity in a development cohort and 91.18% sensitivity and 96.67% specificity in validation cohorts. The same research team suggested that the role of particular miRs consists in their use as diagnostic panels in tumor development. miR-145 acts as an inhibitor of lung cancer cell invasion and migration by targeting PDK1 on the mTOR signaling pathway. MiR-210 is linked with regulation of the hypoxic response of tumor cells. The expression of miR-126 and miR-205-5p varies in different types of cancer that constitute them as a significant biomarker in the diagnosis and prognosis of various other cancers, not only NSCLC [28].

Early detection of colorectal cancer gives a very good prognosis for survival. Unfortunately, as with other types of cancer a lack of efficient, practical tools for diagnosis causes colorectal cancer of the deadliest malignancies. Current methods are quite reliable, although unpleasant for patients and cost-ineffective and unprofitable, and reliant on novel biomarkers that can help diagnosing and identifying colorectal cancer. Some studies have shown that miRNAs can be good biomarkers for colorectal cancer [32]. Kanaan et al. developed a diagnostic panel of 8 plasma miRNAs (miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532, miR-532-3p, miR-652) that shows differences in their expression levels in patients with colorectal cancer, in comparison to healthy controls [33]. Wang et al. conducted a study identifying the dysregulation of miR-409-3p, miR-7 and miR-93 in colorectal cancer patients, in comparison to healthy persons [34]. Vychytilova-Faltejskova et al. reported a study in which patients with colon cancer were distinguished from healthy volunteers on the basis of a miRNA-panel consisting of miR-23a-3p, miR-27a-3p, miR-142-5p and miR-376c-3p. In comparison to currently-used diagnostic tests, this miRNA-signature testing has achieved stunning results. In 168 patients diagnosed with colorectal cancer, CEA testing identified 79 colon cancer patients, thus achieving 47% efficiency. In comparison, Ca19-9 testing identified only 46 colon cancer patients, for 27% efficiency. Use of the plasma-miRNA-

based panel then identified 149 cancer cases, thus showing 89% efficiency. The subsequent combination of CEA, CA19-9 and miRs-panel reached the highest diagnostic efficiency, at a level of 96% [35]. Wikberg et al. conducted a study with patients who had donated blood, even as far back as 20 years previously, before receiving a colorectal cancer diagnosis. Comparing the plasma concentration of particular miRNAs in diagnosed patients, healthy controls and pre-diagnostic samples from the same cases they investigated 4 miRs that differed between all groups (miR-21, miR-25, miR-18a and miR-22). A diagnostic 4-plasma-miRNA-based panel achieved 67% sensitivity and 90% specificity, and was selected on the basis of previously reported studies linked with cancer, with the most significant diagnostic value being miR-21 [32,36–40].

2.2. Use of plasma microRNA in diagnosis of cardiovascular disturbances

Coronary Artery Disease (CAD) is caused by an inflammatory process associated with atherosclerosis. Formation of plaques, their growth and acute rupture lead to blockage of blood flow in coronary arteries, causing damage to the heart muscle. The unexpected and sudden onset of the symptoms of CAD make it difficult to predict. That's why new, molecular biomarkers are needed for faster prediction and diagnosis of this disease. Fichtlscherer et al. conducted the very first study showing differences in circulating miRNAs in patients with angiographically confirmed CAD, in comparison to healthy controls. They showed that levels of miR-126, miR-17, miR-92a, miR-155 and miR-145 were significantly lower in CAD patients [41]. Furthermore Diehl et al. and Weber et al. showed that levels of miR-155 in CAD patients are significantly reduced, which could indicate the high affinity of miR-155 to CAD [42,43]. Further analysis showed that patients using angiotensin-converting enzyme (ACE) inhibitors also resulted in downregulation of miR-155, which might explain this reduction [43].

Many studies have focused on the association of dysregulation of miRNAs and development of Myocardial Infarction (MI). In a study conducted by Corsten et al., circulating miR-208b and miR-499 saw 1,500-fold and 90-fold increases respectively, for patients with MI in comparison to a control group with no cardiac disease [44]. In their work, Ai et al. showed significant upregulation of miR-1 in plasma in MI patients, compared to healthy volunteers [45]. Almost the same results were confirmed by D'Alessandra's research team, which found augmented levels of miR-1, miR-133a, miR-133b and miR-499-5p in the plasma of patients with ST-elevation myocardial infarction (STEMI), in comparison to healthy controls [46].

In a study conducted by Gidlof et al. the expression of miR-1 was 300 times greater, miR-133a 70 times greater, miR-208b 3,000 times greater, and miR-499-5p 250 times greater (all with $p < 0.01$) in patients with STEMI, compared to healthy controls [47]. Kuwabara et al. also showed augmented levels of miR-1 and miR-133a in patients with Acute Coronary Syndromes in comparison to controls without any circulatory disorders [48]. In a study by Olivieri et al., miR-499-5p saw an 80-fold increase in patients with Non-ST-elevation Myocardial Infarction (NSTEMI), in comparison to a healthy control group. Additionally, miR-21, miR-133a, miR-1 and miR-423-5p were significantly increased, but not as much as miR-499-5p [49]. Wang et al. demonstrated in a study that miR-1, miR-133a and miR-499 were meaningfully augmented in the plasma of patients after MI incidents, in comparison to a healthy group [50]. As can be seen, many studies shows dysregulation of the same plasma's miRNAs in detection of MI. The large number of repeatable results indicate that miR-1, miR-133a, miR-133b, miR-499, miR-499-5p and miR-208b could be potential biomarkers for MI. Other studies have shown that miR-208b was not always detectable, but apart from this, Wang et al. suggest that miR-208b could be an ideal candidate for an MI biomarker, as it showed the highest sensitivity and specificity, being detectable in over 90% of MI cases [48–50].

Currently, protein-based biomarkers are gold standard in diagnosis of MI. High sensitive troponins and creatinine kinase (together with CK-MB isoenzyme) are main protein-based biomarkers used to detect myocardial necrosis. Troponins are a group of regulatory proteins located in skeletal, cardiac muscle fibers and thin filaments. For myocardial injury, only cardiac troponins T and I are used, because they differ from skeletal form, in comparison to troponin C which is identical with skeletal form [51]. Troponins are released into the bloodstream 4 to 12 hours after myocardial necrosis occur, reaching peak at 12-48 hours from first symptoms onset. Unfortunately, troponin elevation may occur in other conditions, not always linked with myocardial necrosis (i.e. sepsis, acute pulmonary embolism, atrial fibrillation, heart failure) [52]. Creatinine Kinase (CK), together with CK-MB isoenzyme, was firstly presented as a cardiac biomarker in 1979. Because its composition of two subunits M – muscle and B – brain, there are three different isoenzymes: CK-BB – largely presented in brain and internal organs, MB – heart and skeletal specific, widely used for the diagnosis of myocardial necrosis, and CK-MM – skeletal muscle isoform [53]. Beside the fact of usefulness of CK-MB in diagnosis of myocardial necrosis, its specificity is lower to cardiac muscle than troponins, and cannot be used alone, without any other protein-based biomarker. Furthermore, elevation of CK-MB may be present in renal disease or muscular injury [52].

Li et al. conducted a study in which miR-1, -133a, -208b and -499 were compared to troponin T to determine which biomarker is more sensitive and specific. Results showed, that troponin T was more specific and sensitive than panel of four miRNAs [54]. Similar results were obtained from Li et al. where miR-1 was compared to troponin T, also showing lower specificity and sensitivity than troponin T [55]. However, combination of miRNAs and troponins could give higher sensitivity and specificity for myocardial infarction patients.

Stroke is the second most common cause of death, and the third main cause of temporary or permanent disability of patients due to a lack of oxygen supply to brain cells following blockage of the blood flow in the cerebral arteries. Stroke causes not only death and disability but is also a leading cause of depression and dementia [56,57]. In a study conducted by Sepramaniam et al., statistically significant dysregulation in the expression of miRNAs in plasma in stroke cases was found in 105 miRNA particles. In their experiments, they selected 32 miRNAs that can distinguish stroke subtypes during the acute phase of stroke. Subsequently, five plasma miRs (miR-125b-2; miR-27a; miR-422a; miR-488 and miR-627) were found to be potential biomarkers for diagnosis of stroke, because of the consistency of the alteration in blood from stroke patients, independent of age, stroke severity or the presence of metabolic complications [58]. Long et al. reported that miR-30a and miR-126 levels in plasma were significantly down-regulated in patients diagnosed with stroke until 24 weeks. Surprisingly, miRNA levels in plasma returned to normal 48 weeks after the symptoms began [59].

Venous Thromboembolism (VTE) is a common cardiovascular disorder that includes deep vein thrombosis (DVT) and acute pulmonary embolism (APE), and is initiated with formation of a blood clot in a deep vein, usually in the lower leg. It is estimated that 60-70% of patients with symptomatic VTE can go on to develop DVT. Sometimes, the blood clot is dislodged and travels through the blood vessels to the lungs, where it limits blood supply, thus causing APE, third most common cause of death by cardiovascular disorder, after ischemia and stroke [60,61]. Because VTE can have different clinical manifestations, diagnosis can be incorrect and/or nonspecific, especially for DVT that in many cases is silent and do not give any symptoms. Challenging diagnosis and a lack of sensitive and specific testing has led to a high mortality rate and poor prognosis for those who experience VTE [61]. However, VTE and its complications are preventable. Proper administration of antithrombotic drugs can meaningfully reduce the risk of death (from 25% to 1.5%). Despite this, VTE remains very dangerous and is an often overlooked problem. Starikova et al. conducted a study in which, for the very first time, dysregulated miRNAs were selected for their potential role as biomarkers in VTE cases, compared with healthy controls. Of 742 miRNAs, the expression of only 9 were seen to be

meaningfully different between VTE patients and healthy volunteers [60].

Advanced PE shows a high mortality rate, in which 25% of patients with the initial clinical symptoms will die. However, rapidly-spreading knowledge of miRNAs' functions has allowed scientists to determine potential biomarkers for cardiovascular system disturbances. Wang et al. investigated the utility of plasma miR-134 as a potential biomarker for APEs. They found that in comparison to healthy controls, the miR-134 level was significantly higher in patients with diagnosed acute and chronic pulmonary embolism ($p < 0.05$) [61]. Similar research were made by Xiao et al. In their study, the level of miR-134 in plasma was significantly higher in patients with APE in comparison to healthy controls. Despite both studies having small study and control groups, the fact that the same results were obtained by both research teams suggests that miR-134 has great potential as a biomarker of pulmonary embolism (PE) [62]. In a study conducted by Wang et al., the research team showed that levels of miR-27a and miR-27b in plasma were significant in patients with diagnosed APE, in comparison to healthy volunteers [63].

2.3. Use of plasma miRNAs in diagnosis of neurological disorders

Parkinson's Disease (PD) is a neurodegenerative disease that results in difficulties with proper movement, coordination and balance [64]. It is a highly complex and heterogeneous disease of the central nervous system, discovered in 1817 by James Parkinson [65]. The main histopathological hallmark of PD are fibril inclusions called Lewy Bodies, mainly consisting of alpha-synuclein. In genetically determined PD, with mutation in alpha-synuclein gene, incorrectly build protein is stored in nerve cells, forcing cells to enter the apoptosis pathway [66]. In this cell loss, along with the duration of the disease and the advancement of its development, 50-70% of neurons are lost compared to healthy controls [67,68]. The complexity of PD has caused the current standard in diagnosis and prognosis to be based on subjective clinical assessment of motor features, and imaging exams. Finding measurable, specific and sensitive molecular biomarkers would help in early diagnosis of this disease, and thus to extension of the lifetime of, and an augmented quality of life, for sick patients [64]. Khoo et al.'s study identified a diagnostic panel consisting of miR-1826, miR-450b-3p, miR-626 and miR-505, which showed higher dysregulation in PD patients compared to healthy volunteers. The diagnostic panel achieved 91% in sensitivity, 100% in specificity and 100% in positive predicted value [64]. Furthermore, miR-1826 has also been found to be increased in the plasma of multiple sclerosis patients [69]. In a study by Cardo et al., only miR-331-5p was found to be significant in patients' plasma [70]. Later, Li et al. showed the dysregulation of miR-124-3p and miR-137-3p [71]. Ding et al. conducted a study with a larger population, assessing 5 serum miRNAs (miR-195, miR-185, miR-15b, miR-221 and miR-181a) for their use as potential biomarkers in diagnosis of PD, in comparison to healthy controls [72]. Interestingly, all of these studies showed different results, which would require confirmation and further analysis in a much larger population.

Alzheimer's Disease (AD) constitutes the most common neurological disorder characterized by memory loss, intellectual disability, psychiatric symptoms and disorientation, caused by neuronal death in the hippocampus. The exact cause of Alzheimer's disease is not fully known and there is non currently available treatments that can stop or reverse its progression [73–75]. This very complex disease is diagnosed on the basis of brain imaging tests, neurological exams and mental status assessments [74]. Geekiyanage et al. conducted a study in which miR-137, miR-181c, miR-9 and miR-29a/b in plasma were downregulated in AD patients, compared to controls [76]. In Kiko et al. work plasma miR-34a and miR-146a were significantly reduced in AD patients [77]. Further, Bhatnagar et al. showed that miR-34c is meaningfully increased in AD patients' plasma, in comparison to a normal elderly control. They also suggest that because of the association of miR-34c with oxidative defense signaling and cellular survival, overexpression of miR-34c can lead to overall weakening of cell survival and stress defense [78]. Sorensen et al. conducted a study in which they detected 308 different miRNAs in the blood, with significant up-regulation of miR-590-5p and miR-142-5p and downregulation of miR-194-5p in AD-diagnosed patients, compared to healthy volunteers [79]. In a study conducted by Nagaraj et al., previously reported miRNAs linked with AD (miR-502-3p, miR-103a-3p, miR-1260a, miR-200a-3p, miR-142-3p, miR-301a-3p [80–84]), as well as 9 novel miRNAs (miR-320a, miR-18a-5p, miR-33a-5p, miR-30b-5p, miR-483-5p, miR-320b, miR-320c, miR-151-5p, miR-486-5p), were identified and suggested as the best candidates for future biomarkers of AD. Results from analyses showed 3 to 4 fold increased plasma's levels of miR-200a-3p in patients diagnosed with AD. Previous experimental work showed the same result in mononuclear cells from blood. This phenomenon might suggest that miR-200a-3p is secreted by mononuclear blood cells. Nagaraj's research team also found that transcripts regulated by miR-200a-3p are located in the 3'UTR region of BACE1, one of the major enzymes in the amyloidogenic proteolysis of amyloid precursor protein. Thus, this miRNA could be associated with the regulation pathway of BACE1 in the brain and blood cells, which may then suggest that miR-200a-3p contributes to development of AD [85].

2.4. Use of plasma miRNA in diagnosis of liver diseases

According to the data from the American Liver Foundation, around 88,000 people (70% men) die of alcoholic liver disease every year in the United States. Furthermore, over 1 million people in the US live with Hepatitis B, with 43,000 new diagnoses every year. It is estimated that 2.7-3.9 million individuals in the United States are infected with hepatitis C, of which 75% are unaware of their condition. Fatty liver disease is the most common liver disorder, and has been diagnosed in 100 million people in the United States alone [86]. Due to the wide range of varying factors affecting the promotion of liver inflammation (such as alcohol abuse, viral infections, bacterial infections, toxins, poor diet), miRNAs play an important role in regulating the process of fibrogenesis [87].

Zhang et al. conducted a study evaluating the potential role of miR-122 as a biomarker, which is highly specific to the liver, is associated with differentiation, development and homeostasis of hepatocytes, and regulates lipid metabolism [88–90]. They found that the miR-122 concentration in patients with chronic HBV infections was significantly higher than in a healthy control group ($p < 0.001$). They also found that the level of miR-122 in plasma correlates with ALT activity (Spearman $r = 0.896$, $p < 0.001$). Furthermore, they suggested that miR-122 can be used as a potential biomarker for liver injury, on the basis of results obtained from a ROC curve analysis (AUC for miR-122 was 0.989) [88]. Bihrer et al. conducted a study in which serum miR-122 was increased in patients diagnosed with Chronic Hepatitis C (CHC) [91]. Bala et al. found that levels of miR-122 and miR-155 are also increased in alcoholic liver disease. MiR-155 is well known to be associated with liver fibrosis, mediates cellular growth, and be involved in inflammatory and immune diseases [92–94]. Their experiment was performed on alcohol-fed mice, and showed a significant increase of miR-122 and miR-155 in serum [92]. In a study conducted by Cermelli et al., four miRNAs were evaluated as a potential biomarker for CHC. The results showed that in 18 patients diagnosed with CHC, miR-122 saw a 10.8-fold increase ($p < 0.0001$) and miR-16 a 3-fold increase ($p = 0.0002$) over the control group. Moreover, miR-34a exceeded the detection threshold in CHC patients, while the level of miR-21 was not significant. The same experiments were performed on an independent group of 35 HCV-infected patients. The results showed that the level of miR-122 was 7.9 times higher ($p < 0.0001$), and miR-16 6.3 times higher ($p < 0.0001$) in comparison to healthy controls. MiR-34 levels also exceeded the detection threshold, while miR-21 remained insignificant. The results that Cermelli et al. obtained from experiments on

CHC patients were very promising, and so the same research team evaluated the potential role of these four miRNAs in 34 patients diagnosed with Non-Alcoholic Fatty-Liver Disease (NAFLD). Cermelli et al. found that the level of miR-122 increased 7.2 times ($p < 0.0001$), and the level of miR-16 5.5 times ($p < 0.0001$) in NAFLD patients, in comparison to healthy volunteers. MiR-34 exceeded the detection threshold and miR-21 was not significant [95]. In order to confirm the results obtained by Cermelli et al., Salvoza et al. conducted a study which showed the upregulation of miR-34a and miR-122 in NAFLD serum patients [96]. Similar results for miR-122 in CHC patients were obtained by Butt et al. The expression level measured on 123 treatment-naïve CHC patients showed significant up-regulation of serum miR-122, compared to healthy volunteers ($p = 1.52*10^{-14}$). Furthermore, miR-122 was positively correlated with ALT, AST, INR, PT, albumin level and necroinflammation, suggesting its potential role as a biomarker for liver issues. Butt et al. also showed that serum miR-122 has great diagnostic potential (AUC of 0.954 under ROC curve) [97]. The positive correlation between miR-122 and ALT/AST, and significant upregulation in CHC patients, were both confirmed by El-Hefny et al. [98] In the case of hepatitis B, Ji et al. conducted a study in which serum miR-122 was significantly higher ($p < 0.001$) than in the control group. Furthermore, the expression of miR-223, which is important in regulation of hematopoietic and embryonic stem cell differentiation and regulation of hepatocyte apoptosis, was also meaningfully increased in hepatitis B patients ($p < 0.01$) [99,100]. Roderburg et al. focused on finding potentially useful circulating miRNAs in patients with liver fibrosis/cirrhosis. The results obtained from their experimental work showed significant downregulation of miR-29 in plasma [101]. Zheng et al. then found that serum miR-21, miR-486-5p, miR-130a, miR-192, miR-148a, miR-143, miR-200a, miR-194 and miR-122 were all upregulated in HBV-related Acute-on-Chronic Liver Failure (ACLF) patients, in comparison to healthy controls ($p < 0.05$) [102].

2.5. Use of plasma miRNA in HIV infected patients

The human immunodeficiency virus (HIV) is part of the Lentivirus group (of the family *Retroviridae*), which is characterized by long incubation periods. The ongoing weakening of the organism results in the creation of favorable conditions for various infections and cancer diseases [103]. The highest modern standard for assessing the development of AIDS is the number of circulating CD4+ T cells in peripheral blood and viral loads, but these predictors are not always highly detectable. As such, discovering new biomarkers for AIDS is necessary for the early detection, monitoring and therapy of the disease [104].

Munshi et al. conducted a study in which the plasma levels of miR-150 and miR-146b-5p were evaluated for their potential use as biomarkers of HIV infection. The results showed that plasma miR-150 and miR-146b-5p were significantly augmented in patients, compared to healthy controls. Interestingly, the expression of miR-150 and miR-146b-5p in peripheral blood mononuclear cells was significantly downregulated (0.51 +/- 0.08 ($p < 0.05$) [104]. Qi et al. conducted a study in which they created a diagnostic panel of miRNAs. Their results showed that the combination of miR-29a, miR-233, miR-27a, miR-151-3p and miR-19b demonstrates high sensitivity and specificity in the detection of HIV-1 infection. Moreover, the same miRNAs were meaningfully associated with CD4+ T cell count, suggesting their potential role in progression monitoring [105]. In a study conducted by Reynoso et al., the levels of two miRNAs were altered in HIV patients and healthy controls. Expression of plasma miR-29b-3p and miR-33a-5p were then seen to be significantly downregulated ($p < 0.05$) [106].

3. Application of miRNA profiling techniques in clinical practice

Plasma miRNAs are very interesting candidates for clinical laboratories, because of their remarkabely high stability in body fluids and easy-to-use in clinical routine. On the other hand, miRNA tests has some serious limitations that hinder their introduction into clinical practice. The origin of circulating miRNAs is not fully understood, but it is likely that it is derived from all body tissues, including white blood cells and blood platelets. The highest changes in expression patterns of particular miRNAs are usually observed in body tissues. In circulating miRNAs expression profiles, changes may be subtle. That is why, the knowledge about the expression profiles of miRNAs in various tissues is essential for developing tests based on plasma or serum patterns [107]. What is more, in comparison to protein-based biomarkers, quantification of miRNAs need appropriate standardization, which is actually the biggest limitation. At this moment, there is no one optimal strategy about normalization. In many studies exogenous oligonucleotides (added in established concentration) are used as reference gene i.e. cel-miRNA-39, -54 and -268 obtained from *Caenorhabditis elegans*. In other studies, authors use the mean of all the expressed miRNAs or stable small RNAs (i.e. RNU24, RNU6B) for normalization. Unfortunately, there is no one accepted normalization strategy use for quantification of miRNA [108].

For many clinical laboratories, detection technology is a serious limitation. Microarrays were one of the first methods used for miRNAs gene expression profiling. Based on nucleic acid hybridization, microarrays provide quantification of large numbers of miRNAs simultaneously. In contrast to other techniques, microarrays are best used for comparative analysis between two groups (i.e. patients vs. healthy controls) [109]. Big advantage of microarray technique is large number of data obtained from single experiments. On the other hand, there is a possibility of cross-hybrydization during microarray, resulting in different expression profiles. That is why, results from microarrays can differ from other miRNA profiling techniques [110].

Quantitative Real-Time PCR (QRT-PCR) is one of the main technique used for diagnostic tests like viral load testing, or gene expression profiling. Because of a small amount of miRNAs, the amplification process during PCR reaction results in the precision and sensitivity of QRT-PCR, wchich shows even subtle changes in gene expression. Furthermore, existing commercial kits are very simple, fast and ready-to-use. Besides the fact that QRT-PCR is excellent technique used for measurement of particular miRNAs showing the most blanced specificity, reproducibility, sensitivity and accuracy, it is not proper for discovery of novel miRNAs patterns [110].

Next-generation Sequencing, also known as NGS, is modern technique that provides opportunities to quantify known sequences of genes and discover potentially novel genes or transcripts simultaneously. Furthermore, NGS allows to analyze different samples in a single experiment. Those advantages shows that NGS could be a gold standard in clinical practice for profiling miRNA gene expression. Unfortunately, in comparison to qRT-PCR, NGS is pricy platform, which consume a lot of time to perform it properly [110].

Rapidly evolving field in research technology provide many measurement platforms to determine miRNAs gene expression. Any of available platforms show different sensitivity, reproducibility, specificity an accuracy, causing difficulty in choosing the right method to perform the experiment. Mestdagh et al. conducted a miRNA quality control study, in which all common platforms are compared to each other. To evaluate the accuracy of the studied platforms, authors compared the expression of samples expressed in two various samples, A – 100% Universal Human miRNA Reference RNA, and B – 100% Human Brain RNA mixed in the 4:1 ratio (sample C) and 1:4 (sample D). Expression of particular miRNAs should be exactly 3-fold higher (in C sample) or lower (in D sample). The highest accuracy was shown in platform that presented the lowest fold change difference between observed expression. Reproducibility was assessed by means of duplicated A-D samples. The highest reproducibility was shown by microarray. Detection rate and sensitivity was evaluated on the basis of detected replicates, in which qRT-PCR showed the best results. Further, authors determine the specificity of analyzed platform by measuring gene expression of miRNAs showing differences in nucleotide sequence (vary from one to four nucleotides). On the basis of signal intensity, the

Table 1
Summary of changes in miRNA expression profile in various disease entities.

Disease	miRNA	Fold change ($2^{-\Delta\Delta Ct}$)	Direction of change	P value	Ref.
Prostate cancer	miR-4289	2.15[a]	Upregulation	< 0.0004	[24]
	miR-326	2.3[a]		< 0.0004	
	miR-98-5p	2.8[a]		0.0012	
	miR-152-3p	3.5[a]		0.0016	
Diffuse large B-cell lymphoma	miR-155	5.24	Upregulation	0.009	[26]
	miR-210	4.15		0.02	
	miR-21	2.56		0.04	
Non-small cell lung cancer	miR-145	21.67	Upregulation	0.0002	[29]
	miR-20a	13.39		0.00009	
	miR-21	6.15		0.00037	
	miR-223	2.64		0.00148	
Lung cancer	miR-21	1.36	Upregulation	0.0441	[31]
	miR-19b	2.38		< 0.0001	
Colorectal adenomas	miR-15b	242	Upregulation	0.0009	[33]
	miR-17	16		0.0252	
	miR-142-3p	66		0.0031	
	miR-195	18		0.0173	
	miR-331	33		0.0172	
	miR-532	50		0.0151	
	miR-532-3p	21		0.0173	
	miR-625	84		0.0172	
Colorectal cancer	miR-409-3p	4.5	Upregulation	0.0024	[34]
	miR-7	0.2	Downregulation	< 0.0001	[34]
	miR-93	0.4		0.00022	
	miR-23a-3p	2.05	Upregulation	< 0.0001	[35]
	miR-27a-3p	1.69		< 0.0001	
	miR-142-5p	2.4		< 0.0001	
	miR-376c-3p	1.95		< 0.0001	
Acute pulmonary embolism	miR-134	25.39	Upregulation	0.047	[58]
Stroke	miR-125b-2	1.795	Upregulation	0.007	[58]
	miR-27a	3.745		0.002	
	miR-422a	1.755		0.002	
	miR-488	2.124		0.006	
	miR-627	3.992		0.003	
Coronary artery disease	miR-126	0.37[a]	Downregulation	< 0.001	[41]
	miR-17	0.27[a]		< 0.001	
	miR-92a	0.46[a]		< 0.001	
	miR-155	0.48		< 0.001	
	miR-145	0.49[a]		< 0.001	
Cardiovascular diseases	miR-208b	1500	Upregulation	< 0.005	[44]
	miR-499	90		< 0.001	
Myocardial infarction	miR-1	15[a]	Upregulation	< 0.01	[46]
	miR-133a	140[a]		< 0.01	
	miR-133b	57[a]		< 0.01	
	miR-499-5p	105[a]		< 0.01	
	miR-1	300	Upregulation	< 0.01	[47]
	miR-133a	70		< 0.01	
	miR-208b	3000		< 0.001	
	miR-499-5p	250		< 0.01	
	miR-499-5p	80	Upregulation	< 0.05	[49]
Parkinson's disease	miR-331-5p	21[a]	Upregulation	0.001	[70]
	miR-195	1.65	Upregulation	0.000261	[72]
	miR-185	0.41	Downregulation	< 0.0001	
	miR-15b	0.27		< 0.0001	
	miR-221	0.34		< 0.0001	
	miR-181a	0.35		< 0.0001	
Hepatitis C	miR-122 (1st set)	10.8	Upregulation	< 0.0001	[95]
	miR-16 (1st set)	3		0.0002	
	miR-122 (2nd set)	7.9		< 0.0001	
	miR-16 (2nd set)	6.3		< 0.0001	
HIV	miR-29a	4[a]	Upregulation	< 0.01	[105]
	miR-27a	16[a]		< 0.01	
	miR-151-3p	5.65[a]		< 0.01	
	miR-19b	11.31[a]		< 0.01	

[a] Estimated value based on the figures, and relative expression data, given by the studies.

specificity was evaluated. The best results were achieved by qRT-PCR [111].

The choice of the right platform should depend on the effect that we expect after the experiment. Many factors (i.e. RNA concentration) may affect the obtained result. In our opinion, qRT-PCR should be used in clinical laboratories, because it shows the highest sensitivity, detection rate and specificity. Microarrays and NGS should be use in experimental laboratories that focus on finding differences in miRNA expression in various diseases.

4. Conclusion

Many studies from last decade have shown that miRNAs - small, non-coding RNAs that are able to regulate gene expression, cell growth, tissue differentiation, cell proliferation or apoptosis, are differentially expressed in various disease entities. Furthermore, it has been shown that many miRNAs are secreted from cells to body fluids changing the expression patterns in response to disease state. Also, high stability of circulating miRNAs to harmful conditions means that miRNAs are considered to be a potential biomarkers. All studies cited above, shows very promising result for plasma miRNAs as a biomarker (Table 1). Unfortunately, replication of similar results obtained for particular miRNA in various diseases is a common phenomenon, affecting negatively on the specificity of future tests based on those miRNAs. Various studies have shown that miR-27a is differentially expressed in many diseases entities, including APE, Stroke, HIV and Colorectal Cancer. MiR-21 is differentially expressed in DLBCL, NSCLC, CAD and ACLF. Furthermore, miR-19b has also been significant in the diagnosis of Lung Cancer and HIV. The lack of specificity to a particular disease could suggest its use in many other diagnostic panels, with a wide spectrum of activity. Besides this, many studies have shown expression of various miRNAs in the same disease, while more and more experiments, large-scale validation studies and clinical trials are bringing us closer to finding the ideal biomarker and changing the actual state-of-the-art in diagnostics. At this moment, development of an suitable test requires finding several miRNAs, preferably showing differential expression in this particular disease (or related to it) and constructing appropriate diagnostic panel. Very promising studies have been performed by Vychytilova-Faltejskova et al. in which combinations of specific biomarkers in a miRs-panel reached the highest diagnostic efficiency – 96% in patients with Colorectal Cancer ($p < 0.0001$). Promising results were also obtained by Ding et al. in which four downregulated miRNAs were very significant in diagnosis of Parkinson's Disease ($p < 0.0001$). In one study, Cermelli et al. performed two independent experiments on different populations, which showed repeatable results in diagnosis of Hepatitis C ($p < 0.001$).

All of the works described above have shown how great an impact miRNAs will have in future diagnosis. Finding new, better biomarkers that could easily replace the current, not highly-specific nor sensitive biomarkers is a rapidly evolving field in molecular biology. Many studies have already shown very promising results, highlighting the potential of plasma miRNAs in the diagnosis of various diseases entities. On the other hand, methodological and technical limitations, the high cost of experiments and low populations are major impediments to many scientists. In spite of this, we have a hope that in the next few decades, scientists from all over the world will overcome these limitations to perform large-scale studies.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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