Review Article

Circulating MicroRNAs: Methodological Aspects in Detection of These Biomarkers

(circulating miRNAs / molecular biomarkers / intercellular communication / body fluids)

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Abstract. MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that regulate expression of protein-coding genes involved in important biological processes and (patho)physiological states. Circulating miRNAs are protected against degradation, indicating their relevant biological functions. Many studies have demonstrated an association of the specific profile of circulating miRNAs with a wide range of cancers as well as non-malignant diseases. These findings demonstrate the implication of circulating miRNAs in the pathogenesis of diseases and their potential as non-invasive disease biomarkers. However, methods for measurement of circulating miRNAs have critical technical hotspots, resulting in a discrepancy of the reported results and difficult definition of consensus disease biomarkers that may be implicated in clinical use. Here, we review functions of circulating miRNAs and their aberrant expression in particular diseases. Further, we discuss methodological aspects of their detection and quantification as well as our experience with the methods.

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Abbreviations: CA – carbohydrate antigen, CAGE – cap analysis gene expression, CEA – carcinoembryonic antigen, CNS – central nervous system, CRC – colorectal cancer, DGE – digital gene expression, DLBCL – diffuse large B-cell lymphoma, dPCR – digital PCR, ddPCR – droplet dPCR, HDL – high-density lipoprotein, HUVEC – human umbilical vein endothelial cell, miRNAs – microRNAs, NGS – next-generation sequencing, NHL – non-Hodgkin's lymphoma, PSA – prostate-specific antigen, qRT-PCR – quantitative real-time PCR, SAGE – serial analysis of gene expression, SBS – sequencing-by-synthesis, SCID – severe combined immunodeficiency, TLDA – TaqMan low-density array.

Introduction

MicroRNAs (miRNAs) are short (~ 22 nucleotides) endogenous non-coding RNA molecules that play an essential role in the regulation of gene expression at the post-transcriptional levels. Their sequences are highly conserved throughout various organisms. In the last decade, it has been repeatedly proved that miRNAs play crucial roles in a wide variety of biological processes such as development, differentiation, proliferation, and apoptosis. Since they influence expression of genes involved in fundamental signalling pathways, their deregulation often triggers various pathological processes, such as cardiovascular disease, neurological disease or cancer (Bartel, 2004).

Biogenesis of miRNAs is a multistep process taking place both in the nucleus and cytoplasm (Fig. 1). miRNA genes are transcribed from genomic DNA by RNA polymerase II, resulting in pri-miRNA transcripts that can encode sequences for multiple miRNAs. Within the nucleus, these pri-miRNA transcripts are cleaved by RNase III type endonuclease Drosha, and ~ 70 nucleotide hairpin precursors termed pre-miRNAs are released. Pre-miRNAs are subsequently transported into the cytoplasm. In the cytoplasm, these hairpin precursors are cleaved by Dicer into a dsRNA duplex (miRNA-miRNA* duplex) containing both the mature miRNA strand (miRNA) and its complementary strand (miRNA*). The mature miRNA strand (also referred to as the guide strand) is preferentially loaded into the miRNA-induced silencing complex (miRISC), whereas the complementary strand is excluded and degraded. miRISC mediates post-transcriptional regulation of gene expression by inhibition of translation or triggering mRNA degradation. The type of repression relies on the degree of sequence complementarity between miRNA and target messenger RNA (mRNA): partial complementarity induces inhibition of translation; perfect complementarity causes mRNA decay (He and Hannon, 2004).

According to the current version of the miRBase database (www.mirbase.org, release 20), there are 1,872 precursor miRNAs and 2,578 mature miRNAs known in human. Each miRNA has hundreds of potential target genes and it is generally believed that miRNAs regulate

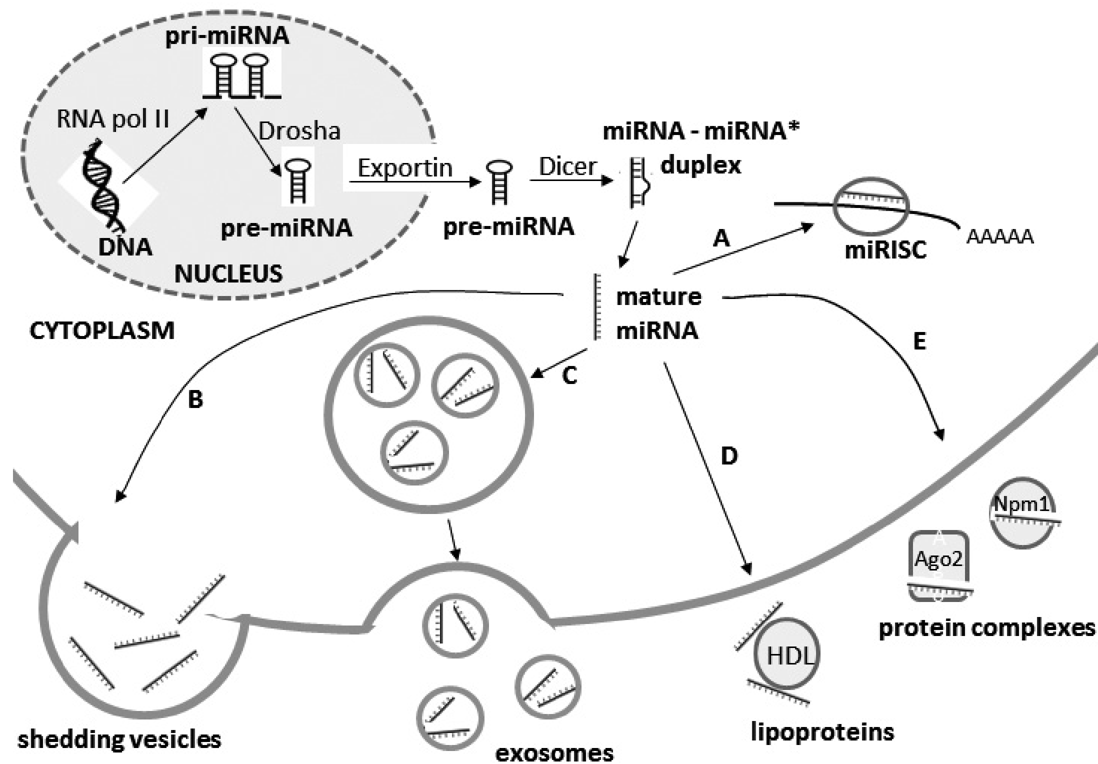

Fig. 1. Biogenesis of miRNAs and different mechanisms of their transport to the extracellular environment
In the nucleus, miRNA genes are transcribed from genomic DNA by RNA polymerase II, resulting in pri-miRNA transcripts that are further cleaved by RNase III into shorter precursors termed pre-miRNAs. Pre-miRNAs are subsequently transported into the cytoplasm, where they are processed by Dicer into a dsRNA miRNA-miRNA* duplex. The mature miRNA strand is loaded into the miRNA-induced silencing complex (miRISC), whereas the complementary strand (miRNA*) is excluded and degraded. miRISC (**A)** mediates post-transcriptional repression of gene expression. The processed miRNAs are exported from the cell to the extracellular environment by various transport mechanisms. Two distinct classes of microvesicles in which miRNAs are packed and excreted from cells have been described. Shedding vesicles (**B**) with encapsulated cytoplasmic components including miRNAs are formed and released into extracellular surroundings by budding and fission of the plasma membrane. Exosomes (**C**) are intracellularly formed vesicles residing within the lumen of multivesicular bodies and are released upon fusion of these bodies with the plasma membrane. miRNAs are also exported from cells through their incorporation into high-density lipoproteins (HDLs) (**D**) or in protein complexes (**E**) with proteins such as Ago2 or Npm1.

more than 30 percent of protein-coding genes in the human genome (Lewis et al., 2005).

Like mRNAs, the majority of miRNAs are expressed in a tissue-specific manner. For example, miR-122 is preferentially expressed in the liver (Lewis and Jopling, 2010), miR-124 in neurological tissues (Sun et al., 2013a), miR-133 is enriched in muscles (Luo et al., 2013), and miR-208a is abundant in the heart (van Rooij et al., 2007). Moreover, it has been demonstrated that changes in the spectrum of tissue miRNAs correlate with various pathophysiological conditions (Lu et al., 2005).

Some of miRNAs, originally found in tissues, have also been identified in body fluids such as plasma (Laterza et al., 2009), serum (Cortez and Calin, 2009), saliva (Park et al., 2009), and urine (Hanke et al., 2010). Since then, many non-cellular miRNAs present in various body fluids have been documented. Examination of 12 human body fluids showed a distinct composition of the miRNA profile in various fluid types (Weber et al., 2010).

In latest years, scientists have extensively been concerned with searching for good blood-based biomarkers as the collection of blood samples is much less invasive than taking a biopsy of a certain tissue. Non-cellular miRNAs circulating in the blood – referred to as circulating miRNAs – are produced not only by blood cells, but also by cells of different body tissues and organs. Moreover, the amount and spectrum of circulating miRNAs seem to vary with changing pathophysiological conditions of the organism (Chen et al., 2008). These facts have brought the idea of using circulating miRNAs as disease biomarkers.

Origin of circulating miRNAs

First extracellular small RNAs were observed in the blood in 2004 (El-Hefnawy et al., 2004). Mitchell et al. (2008) reported that extracellular miRNAs are stable in human plasma/serum and Lawrie et al. (2008) found miRNAs in the serum of patients suffering from lym-

phoma. Since then, many efforts have been devoted to the research of this new phenomenon. Several studies investigated the stability of circulating miRNAs (Mitchell et al., 2008; Ge et al., 2014), showing their high stability under distinct conditions of storage such as room temperature, multiple freeze-thaw cycles (Mitchell et al., 2008), and long-term storage (Ge et al., 2014). The high stability of circulating miRNAs, despite the high levels of RNase activity in the blood degrading exogenously added RNA within seconds (Tsui et al., 2002), indicates that circulating miRNAs are unlikely to exist in unprotected state. Until now a number of different miRNA carriers have been described; membrane-derived vesicles, lipoproteins, and ribonucleoprotein complexes have all been found to transport extracellular miRNAs (Fig. 1).

One of the mechanisms of miRNA export to the extracellular environment is mediated by microvesicles (Hunter et al., 2008), in which miRNAs are packed and excreted from cells. Two distinct classes of microvesicles, differentiated by their biogenesis and secretory mechanisms, have been described: large shedding vesicles (0.1–1 μm) and smaller exosomes (30–100 nm). Shedding vesicles with encapsulated cytoplasmic components including miRNAs are formed and released into extracellular surroundings by budding and fission of the plasma membrane (Cocucci et al., 2009; Muralidharan-Chari et al., 2010). Exosomes are intracellularly formed vesicles residing within the lumen of multivesicular bodies and are released upon fusion of these bodies with the plasma membrane (Muralidharan-Chari et al., 2010; Zomer et al., 2010). Another type of vesicles containing miRNAs are apoptotic bodies, which are released from a cell that undergoes apoptosis (Zernecke et al., 2009).

Another mechanism of miRNA export from the cell is through their incorporation into high-density lipoproteins (HDLs) (Vickers et al., 2011) or formation of complexes with proteins such as Ago2 (Arroyoa et al., 2011), the effector component of the miRNA-induced silencing complex that directly binds miRNAs, or Npm1 (Wang et al., 2010a), which is involved in the biogenesis of ribosomes. Although the dominant model of extracellular miRNA form is still the miRNA encapsulation in membrane-bound vesicles, it has been shown that the majority of circulating miRNAs in human plasma and serum co-fractionate with Ago2 protein, rather than with vesicles (Turchinovich et al., 2011).

Sorting of miRNAs into different types of vesicles seems to be selective. Vickers et al. (2011) discovered that HDL-associated miRNA profiles significantly differed from exosome-associated miRNAs in the spectrum and abundance of miRNAs. Villarroya-Beltri et al. (2013) found that the loading of miRNAs into exosomes is controlled by recognition of specific sequence motifs present in these miRNAs. Another study compared the content of miRNAs in microvesicles and their maternal cells. The results showed a significantly different spectrum of miRNAs between microvesicles and maternal cells, suggesting selective packaging of miRNAs into microvesicles (Diehl et al., 2012).

Function of circulating miRNAs

Unlike comprehensively described function of cellular miRNAs, the function of miRNAs present in the extracellular environment remains somewhat speculative. Since the discovery of circulating miRNAs, a growing body of evidence has indicated that these molecules are not only mere leftovers of cellular degradation without any specific functions. Instead, it has been suggested that active exchange of miRNAs between cells via exosome vehicles may have a significant function in long-distance cell-to-cell communication. The first report describing this role of circulating miRNAs showed that embryonic stem cell microvesicles could transfer a subset of miRNAs to mouse embryonic fibroblasts *in vitro* (Yuan et al., 2009). Since then, several studies brought evidence of miRNA transport between different types of cells. For instance, Umezu et al. (2013) showed that exosomal transfer of miR-92a derived from leukaemia cell line K562 to co-cultured human umbilical vein endothelial cells (HUVECs) led to significant repression of integrin α5, the target gene of miR-92a.

A recent study of Fonsato et al. (2012) has demonstrated that microvesicle-based delivery of selected miRNAs from stem cells to tumour cells might inhibit tumour growth and stimulate apoptosis. They documented that microvesicles derived from human adult liver stem cells might *in vitro* reprogram HepG2 hepatoma and primary hepatocellular carcinoma cells by inhibiting their growth and survival. *In vivo* intratumour administration of microvesicles induced regression of ectopic tumours developed in severe combined immunodeficiency (SCID) mice. Moreover, the anti-tumour effect of microvesicles from human adult liver stem cells was also observed in tumours other than liver, such as lymphoblastoma and glioblastoma.

Despite this growing evidence of the functionality of cell-free miRNAs, it is still not clear whether circulating miRNAs are real fundamental players in cell-to-cell communication or whether they only help to refine particular specialized processes. Although exosomal miRNAs have been hypothesized to be involved in intercellular communication, most extracellular miRNAs are probably bound in Ago2-miRNA complexes, which may be by-products of dead cells that remain in extracellular space due to the high stability of the Ago2 protein and Ago2-miRNA complex (Turchinovich et al., 2011).

Nevertheless, evidences of active transport of miRNAs between distant cells inspired the hypotheses about new therapeutic approaches using microvesicles or other nanotechnology-based carriers to transfer miRNAs targeting a gene of interest in diseased cells (van den Boorn et al., 2013). Another promising treatment strategy appears to be drug-induced suppression of exosome formation or inhibition of their uptake by recipient cells (Grasedieck et al., 2013). Although promising in the mouse model (Bryniarski et al., 2013), the path to the clinical administration of these concepts in human gene therapy remains complicated. Prior to clinical translation of exo-

some-mediated nanodelivery, this technology requires further development by refinement of isolation, purification, loading, delivery and targeting protocols.

Circulating miRNAs as disease biomarkers

Recently, numerous studies have focused on expression profiling of circulating miRNAs in human diseases (summarized in Table 1). They have aimed to identify new biomarkers for diagnostics, disease subclassification, and monitoring of the progression status. As it is believed that specific disease-related biomarkers, including miRNAs, may be transported from the disease-affected tissue to blood circulation, the measurement of circulating miRNA levels in the blood could be successfully applied in diagnostics, especially in cases where a novel non-invasive or minimally invasive approach is required.

Probably, the biggest scientific effort has been devoted to the development of circulating miRNA tests for the detection of human malignancies with the intention to significantly reduce the worldwide health burden of cancer. Hitherto used blood-based protein biomarkers of cancer such as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA) or carbohydrate antigen (CA) suffer from low sensitivity and specificity, which make their application limited especially in early stages of the disease. The first evidence of the potential applicability of circulating miRNAs as non-invasive diagnostic markers was reported by Lawrie et al. (2008). They measured levels of three miRNAs (miR-155, miR-210 and miR-21) with known tissue-specific expression in the serum from diffuse large B-cell lymphoma (DLBCL) patients, demonstrating higher levels of these miRNAs in patient than control sera and proving miR-21 association with relapse-free survival. Another pioneering work applying circulating miRNAs as blood-based fingerprints for the detection of prostate cancer was published by Mitchell et al. (2008). They showed that miRNAs originating from human prostate cancer xenografts entered the circulation and were readily measured in the plasma, and could robustly distinguish xenografted mice from the controls. This concept was extended to human cancer, where serum levels of miR-141 (miRNA expres-

Table 1. Circulating miRNAs proposed as biomarkers of diseases

Disease	Body fluid	miRNA	References
Epithelial tumours			
prostate cancer	serum	up: miR-141	Mitchell et al. (2008)
lung cancer	serum	up: miR-21	Liu et al. (2012)
	plasma	up: miR-21, miR-155 down: miR-145	Tang et al. (2013)
breast cancer	serum	up: miR-155	Sun et al. (2012)
	plasma	down: miR-30a	Zeng et al. (2013)
colorectal cancer	plasma	up: miR-17-3p, miR-92	Ng et al. (2009)
Haematological malignancies			
diffuse large B-cell lymphoma	serum	up: miR-21	Lawrie et al. (2008)
	serum	up: miR-15a, miR-16-1, miR-29c, miR-155	Fang et al. (2012)
multiple myeloma	serum	up: miR-720, miR-1246 down: miR-1308	Jones et al. (2012)
	plasma	up: miR-148a, miR-181a, miR-20a, miR-221, miR-625, miR-99b	Huang et al. (2012)
	plasma	down: miR-92a	Yoshizawa et al. (2012)
non-Hodgkin's lymphoma	plasma	down: miR-92a	Ohyashiki et al. (2011)
adult T-cell leukaemia	plasma	up: miR-155 down: miR-126	Ishihara et al. (2012)
acute leukaemia	plasma	down: miR-92a	Tanaka et al. (2009) Ohyashiki et al. (2010)
acute myeloid leukaemia	plasma	down: miR-150, miR-342	Fayyad-Kazan et al. (2013)
chronic lymphocytic leukaemia	plasma	up: miR-150, miR-150*, miR-29a, miR-135*, miR-20a	Moussay et al. (2011)
Non-malignant diseases			
acute myocardial infarction	plasma/serum	up: miR-1, miR-133, miR-499, miR-208	Fichtlscherer et al. (2011)
Alzheimer's disease	serum	down: miR-125b	Tan et al. (2014)
Huntington's disease	plasma	up: miR-34b	Gaughwin et al. (2011)
schizophrenia	plasma	up: miR-130b, miR-193a-3p	Wei et al. (2015)

up – up-regulation, down – down-regulation

sed in prostate cancer) could distinguish patients with prostate cancer from healthy controls.

Since then, attention has been paid to the measurement of circulating miRNAs in various types of tumours. Extensive research has been focused on plasma/serum miRNAs in lung cancer. Especially miR-21 and miR-155 were proved by several studies as potential novel non-invasive biomarkers for early detection of lung cancer (Rabinowits et al., 2009; Liu et al., 2012; Tang et al., 2013). Similarly to lung cancer, an elevated serum level of miR-155 was associated with hormone-sensitive breast cancer (Zhu et al., 2009). Moreover, miR-155 and miR-30a have been demonstrated as more accurate than predictive markers CEA and carbohydrate antigen 15-3 (CA 15-3) that are currently used for monitoring treatment in metastatic breast cancer (Sun et al., 2012; Zeng et al., 2013). In colorectal cancer (CRC), two members of polycistronic Oncomir-1 cluster, miR-17-3p and miR-92, were validated as increased in CRC plasma as well as CRC tissue, and plasma levels of these markers were significantly reduced after surgery. Interestingly, miR-92 alone had the potential to discriminate CRC from other diseases, such as gastric cancer and inflammatory bowel disease (Ng et al., 2009).

Unlike epithelial tumours, much less information is available on circulating miRNAs in haematological malignancies. This is surprising given the fact that the diseased cells are in close proximity to the plasma/serum, and thus the miRNA export to these fluids and consequently easier data acquisition should be enabled compared to distant tumours. Besides the above-mentioned pioneering study of Lawrie et al. (2008) and another one (Fang et al., 2012) on DLBCL, the prognostic validity of circulating miRNAs in the plasma/serum was tested e.g. in multiple myeloma (Huang et al., 2012; Jones et al. 2012; Yoshizawa et al., 2012), non-Hodgkin's lymphoma (NHL) (Ohyashiki et al., 2011), T-cell leukaemia (Ishihara et al., 2012), acute leukaemia (Tanaka et al., 2009; Ohyashiki et al., 2010; Fayyad-Kazan et al., 2013) or chronic lymphocytic leukaemia (CLL) (Moussay et al., 2011). However, research of circulating miRNAs in haematological diseases is often focused on TaqMan qRT-PCR measurement of the level of a limited number of miRNAs with previously published deregulated expression in the affected cells (such as Oncomir-1 miRNAs) and comprehensive information on complete miRNA profiles in blood fluids in leukaemia and lymphoma is still incomplete. It is noteworthy that out of the miRNAs extensively tested, low plasma levels of miR-92a from the Oncomir-1 cluster were observed in multiple myeloma (Yoshizawa et al., 2012), NHL (Ohyashiki et al., 2011) and acute leukaemia (Tanaka et al., 2009; Ohyashiki et al., 2010), suggesting that measurement of the plasma miR-92a level could be useful for monitoring the disease status and/or initiation of therapy in haematological malignancies.

It should be highlighted that some particular miRNAs, previously suggested as cancer markers, lack the disease specificity. The most striking examples are probably miR-21 and miR-210, whose deregulation was described in multiple diseases (Table 2). These miRNAs should then not be used as specific markers for particular diseases. Nevertheless, it would be valuable to find such a prevalent miRNA that would be able to cover the widest possible spectrum of tumours; such molecule could finally act as a legendary universal cancer marker.

Circulating miRNAs were also proved as specific and sensitive indicators of various non-malignant diseases. Extensive research has been focused especially on cardiovascular diseases. For instance, cardiac injury as it occurs after acute myocardial infarction increases the circulating levels of several myocardial-derived miRNAs (e.g, miR-1, miR-133, miR-499, miR-208), whereas patients with coronary artery disease or diabetes showed reduced levels of endothelial-enriched miRNAs, such as miR-126 (Fichtlscherer et al., 2011). Circulating miRNAs

Table 2. Deregulation of circulating miR-21 and miR-210 in the plasma/serum in various diseases

miR-21			**miR-210**		
Disease	**Deregulation**	**Reference**	**Disease**	**Deregulation**	**Reference**
aortic stenosis	up	Villar et al. (2013)	breast cancer	up	Jung et al. (2012)
breast cancer	up	Wang and Zhang (2012)	colorectal cancer	up	Wang et al. (2012b)
colorectal cancer	up	Wang and Zhang (2012)	conventional renal cell cancer	up	Zhao et al. (2013)
oesophageal cancer	up	Wang and Zhang (2012)	lung cancer	up	Shen et al. (2011)
gastric cancer	up	Wang and Zhang (2012)	pancreatic cancer	up	Ho et al. (2010)
glioblastoma	up	Ilhan-Mutlu et al. (2012)	preeclampsia	up	Gunel et al. (2011)
hepatocellular carcinoma / chronic hepatitis	up	Xu et al. (2011)	type 1 diabetes	up	Nielsen et al. (2012)
Hodgkin's lymphoma	up	Jones et al. (2014)			
lung cancer	up	Wang and Zhang (2012)			
non-small cell lung cancer	up	Wei et al. (2011)			
paediatric Crohn's disease	up	Zahm et al. (2011)			
prostate cancer	up	Yaman Agaoglu et al. (2011)			

were further studied in central nervous system (CNS) diseases such as Alzheimer's disease, Huntington's disease, multiple sclerosis, schizophrenia, and bipolar disorder (reviewed by Jin et al., 2013). For example, Tan et al. (2014) investigated a potential role of several miRNAs as diagnostic biomarkers for Alzheimer's disease and showed that reduction of the serum miR-125b level might serve as a useful non-invasive biomarker of the disease.

At present, first clinical trials investigating circulating miRNAs as predictive markers are ongoing, such as studies focusing on breast cancer (clinicaltrials.gov identifiers NCT01612871, NCT01722851), paediatric cancer (patients with central nervous system tumours, leukaemia and lymphoma; NCT01541800) or sepsis (NCT00862290). Although it has been widely proved that circulating miRNAs have great perspectives for future applications as specific and sensitive non-invasive biomarkers, several obstacles remain to be solved before their incorporation into clinical practice. The most critical issues that hinder the transition of circulating miRNAs from research into clinical application are discussed in the chapter Methodological aspects of detection of circulating miRNAs.

Circulating miRNAs in healthy individuals

The majority of studies of differential expression are based on comparison of miRNA profiles of patients to those of healthy individuals. Many studies attempted to characterize a panel of circulating miRNAs in healthy individuals that might represent a baseline for comparative analyses. Chen et al. (2008) performed Solexa sequencing of miRNAs in the serum of 30 healthy subjects and found 100 and 91 miRNAs in male and female subjects, respectively. These miRNAs showed similar levels and range of expression; thus, their altered expression might indicate pathological status in patient samples. A similar number of "normal" miRNAs was identified by others later on; 91 miRNAs in the plasma (6 controls) using Sanger sequencing (Mitchell et al., 2008), 148 miRNAs in the plasma (7 controls) using Agilent microarrays (Tanaka et al., 2009), 170 miRNAs in the plasma (4 controls) using Agilent microarrays (Wang et al., 2010b), 106 miRNAs in the plasma and 118 miRNAs in the serum using TaqMan qPCR arrays (Wang et al., 2012b), and 105 miRNAs in microvesicles from blood cells using TaqMan qPCR arrays (Hunter et al., 2008). In our experiments, we detected 191 miRNAs in the plasma derived from seven healthy subjects using Agilent microarrays. The most abundant miRNAs were miR-4454, miR-6089, miR-223-3p, miR-451a, and miR-6090. The list of the top 20 most abundant miRNAs in our data set is shown in Table 3 (unpublished data).

In terms of inter-individual variability, differential expression of circulating miRNAs between females and males was not found either in the serum (Chen, 2008) or in microvesicles (Hunter et al., 2008). Hunter et al. (2008) also compared miRNA profiles from microvesicles between age groups and did not detect any differences. In contrast, Noren Hooten et al. (2013) performed comparative analysis of serum miRNAs between middle-aged and elderly individuals and determined age-related miRNAs (decreased expression of miR-151a-5p, miR-181a-5p and miR-1248 in older subjects). Interestingly, these age-related miRNAs are involved in the regulation of inflammatory pathways and expression of miR-181a negatively correlated with pro-inflammatory cytokines IL-6 and TNFα.

Table 3. The top 20 most abundant miRNAs in human plasma of healthy individuals

No.	miRNA
1	miR-4454
2	miR-6089
3	miR-223-3p
4	miR-451a
5	miR-6090
6	miR-6125
7	miR-3960
8	miR-21-5p
9	miR-4516
10	miR-16-5p
11	miR-5100
12	let-7b-5p
13	miR-2861
14	let-7a-5p
15	miR-6087
16	miR-1273g-3p
17	miR-4459
18	miR-23a-3p
19	let-7f-5p
20	miR-638

The data were measured by Agilent Human miRNA Microarrays (Sure Print G3 Unrestricted miRNA 8x60K, Release 19.0) and the miRNAs are arranged in the descending order with the highest expression as the top value. The whole list of miRNAs detected in the data set comprised 191 miRNAs (unpublished data).

Studies by Ortega et al. (2013) and Prats-Puig et al. (2013) showed that obesity in adults and children was associated with changes in the plasma levels of specific circulating miRNAs. Consistently, both studies demonstrated down-regulation of miR-221 and up-regulation of miR-142-3p in adult and prepubertal obese patients. Notably, miR-423-5p showed an opposite trend of deregulation, down-regulation in childhood and up-regulation in adult obesity. The results suggest that even non-pathological obesity (various grades of obesity) may contribute to the variability of circulating miRNAs within the population.

Only few studies focused on the differences in expression profiles of circulating miRNAs within individuals under normal physiological conditions that change during time (e.g. age, cell count, hormonal changes associated with menstrual cycle or pregnancy). Rekker et al. (2013) attempted to address changes in the plasma miRNA profiles in healthy women (N = 9) at four time-

points of the menstrual cycle. They concluded that the levels of circulating miRNAs in the women were not significantly influenced by the processes occurring during the menstrual cycle. In contrast to the menstrual cycle, pregnancy alters miRNA levels in maternal plasma. Chim et al. (2008) reported increased expression of miR-141 in the plasma of pregnant women at the third trimester. Similar up-regulation of miR-141 at the third trimester was reported by others (Gilad et al., 2008; Miura et al., 2010; Mouillet et al., 2010). These studies determined other miRNAs associated with pregnancy (e.g. up-regulation of miR-526a, miR-527, miR-149, miR-299-5p, miR-135b, miR-424, miR-517a, miR-518b, miR-518e, and miR-524); however, they measured the miRNA levels at different weeks of gestational age in the first and the third trimester. A comprehensive study of circulating miRNA levels in all trimesters was performed by Li et al. (2012). They showed that the content of circulating miRNAs in maternal plasma dynamically changed along the pregnancy progress and showed the specific pattern in each trimester.

Several studies demonstrated an association of specific circulating miRNAs with preeclampsia (e.g. up-regulation of miR-210, Gunel et al., 2011 and Xu et al., 2014; up-regulation of miR-1233, Ura et al., 2014; up-regulation of miR-141, miR-29a and down-regulation of miR-144, Li et al., 2013), congenital heart defects (up-regulation of miR-19b, miR-22, miR-29c and miR-375, Zhu et al., 2013), neural tube defects (up-regulation of miR-142-3p, miR-144, miR-720, miR-575, miR-765, and miR-1182, Gu et al., 2012), and foetal growth restriction (up-regulation of miR-27a, miR-30d, miR-141, miR-200c, miR- 205, miR-424, and miR-451, Mouillet et al., 2010). Thus, altered levels of circulating miRNAs may serve as biomarkers of pregnancy-related diseases and congenital defects.

Methodological aspects of detection of circulating miRNAs

Circulating miRNAs represent promising disease biomarkers; however, methods for their isolation, measurement, and quantification have specific limitations and critical technical hotspots (sample processing, inefficient isolation, haemolysis in blood samples, variable efficiency of reverse transcription and PCR, inconsistency in reference genes, and various wide-genome platforms), resulting in a discrepancy of the reported results. The inconsistent results obtained by various methods make it difficult to define consensus disease biomarkers that may be implicated in clinical use. Thus, the methods are being under development in order to improve their sensitivity, specificity, and reproducibility.

The first step of the experimental workflow is to obtain a representative population of circulating miRNAs from the target body fluid. Sources of circulating miRNAs are usually the plasma/serum, urine, and saliva (Weber et al., 2010); less common are specific populations of microvesicles or exosomes. Preparation of microvesicles or exosomes requires a multistep protocol including immunopurification and ultracentrifugation procedures (Simpson et al., 2009; Yuana et al., 2014). Although more complicated, this approach provides information about miRNA distribution in specific populations of miRNA carriers (Hunter et. al., 2008).

Separation of plasma or serum is easy; on the other hand, these fluids have a high content of blood proteins. Moreover, the serum and plasma show different spectra of miRNAs. Wang et al. (2012a) reported that the serum contained higher concentration of miRNAs compared to the plasma from the same individuals, suggesting release of additional RNAs (likely from platelets) during the coagulation process. As well, Cheng et al. (2013) demonstrated that platelet contamination persists after standard protocols, influencing the plasma levels of circulating miRNAs, and additional steps are necessary to limit its effect. Generally, the serum is suggested to provide more consistent miRNA measurements because it is free of anti-coagulants such as heparin that may inhibit downstream PCR applications (Yokota et al., 1999). Thus, archived plasma samples are not recommended for biomarker identification.

Haemolysis occurring in blood samples may affect the plasma levels of particular miRNAs. Kirschner et al. (2011) showed increased levels of miR-16 (often used as a reference gene) and miR-451 (highly expressed in red blood cells) in the plasma obtained from haemolysed blood samples. A significant increase in miRNA levels from haemolysed plasma specimens was also found by others (Pritchard et al., 2012). Haemolysis can be monitored spectrophotometrically by measurement of free haemoglobins (oxyhaemoglobin absorbance at 414 nm) (Blondal et al., 2013). In case that samples are no longer available, the levels of miRNAs enriched in erythrocytes (miR-451 or miR-144) (Rasmussen et al., 2010; Blondal et al., 2013) can be measured and compared to non-haemolysed samples.

Circulating miRNAs are present in body fluids in low concentration and isolation of sufficient amounts of miRNAs may be difficult and needs modifications in standard protocols. There are many commercial kits for isolation of total RNA and a few kits focused on the enrichment of miRNAs; however, some studies reported differential efficiency of commonly used isolation kits. Monleau et al. (2014) compared three commercial extraction kits (Qiagen miRNeasy kit, Norgen Biotek Total RNA Purification kit, and Macherey-Nagel NucleoSpin miRNAs kit) and found that although these kits had equal performances in extracting miRNAs from peripheral blood mononuclear cells, the Macherey-Nagel kit provided higher yields, better RNA purity and more detectable miRNAs when isolating miRNAs from the serum. Moreover, the Macherey-Nagel kit does not employ the phenol/chloroform step. Further, Kroh et al. (2010) reported significantly higher yields of circulating miRNAs using Qiagen miRNeasy kit compared to Ambion MiRvana PARIS. The same kits plus Norgen Biotek total RNA isolation kit were tested by Li and

Kowdley (2012), who again showed variability between the kits in terms of RNA quality and quantity and concluded that the Qiagen miRNeasy Kit recovered more miRNAs than the other kits. In our studies, we compared Qiagen miRNeasy kit with a modified phenol-chloroform extraction method using Trizol LS reagent according to the procedure of Filkova et al. (2014). The phenol-chloroform method showed consistently higher yields (approx. 400 ng of RNA from 500 µl of plasma) of plasma miRNAs compared to the commercial kit (approx. 50 ng of RNA from 200 µl of plasma), and was therefore more useful for obtaining higher concentrations of plasma miRNAs necessary for microarray profiling. Taken together, these findings demonstrate variable efficiency of different isolation procedures, recommending that samples within one study have to be processed by the same extraction method to avoid technical artefacts.

As miRNAs are less abundant in body fluids compared to their cellular levels, quantification of RNA yield is another issue that has to be addressed. RNA extracted from the plasma/serum is almost undetectable by using the NanoDrop spectrophotometer (Moret et al., 2013). Moreover, it should be noted that the miRNA content in blood circulation is not stable and may change under various pathophysiological conditions. In this context, O'Driscoll (2007) showed that overall concentrations of extracellular nucleic acids in the circulation were generally higher in cancers than in normal tissues. Therefore, using an equal volume of input material rather than the same amount of RNA is probably more accurate for quantification of circulating miRNAs.

Various techniques have been applied to quantification of circulating miRNAs. These days, high-throughput technologies for miRNA profiling (TaqMan cards, microarrays, deep sequencing) prevail over those for low-throughput analyses (northern blotting, cloning). For quantitative analysis of a particular circulating miRNA, quantitative real-time PCR (qRT-PCR) is the most common method. Generally, two quantification strategies are used to determine the levels of expressed miRNAs by qRT-PCR: relative or absolute quantification. Relative quantification measures the relative change in miRNA expression levels and it is based on comparison of the expression levels of a target miRNA and a reference gene (housekeeping or control gene). Data of relative quantification are usually expressed as raw Ct, ΔCt, 2 – ΔCt or 2 – ΔΔCt and are comparable across multiple experiments. There is a long list of used reference genes and their selection is one of the major methodological issues. The most reported reference miRNA is miR-16, which is expressed at a stable level in most tissues (Liang et al., 2007; Kirschner et al., 2011; Xiang et al., 2014). On the other hand, some studies demonstrated inconsistent expression of miR-16 in plasma/serum samples (e.g. Chen et al., 2008; Filkova et al., 2014). Other reported reference genes for cell-free circulating miRNAs are miR-142-3p, miR-638, RNU44, RNU48, RNU66, U6 and 18S, etc. (e.g. Resnick et al., 2009; Tanaka et al., 2009; Han et al., 2014). A combination of several consistent reference genes across all (control and disease) samples selected as a reference for the normalization may be statistically superior to one commonly used reference gene. This approach was reported by Chen et al. (2013), who confirmed a combination of let-7d, let-7g and let-7i as the most stable reference for normalization of serum miRNAs.

In many studies, targeted search was performed to determine miRNAs that could serve as a reliable endogenous control in the specific samples (e.g. Zheng et al., 2013; Liu et al., 2014). The expression stability of the candidate reference gene can be determined with programs such as geNorm, NormFinder, or BestKeeper. To investigate the stability of miRNAs in the plasma, Mitchel et al. (2008) firstly used synthetic *C. elegans* miRNAs (cel-miR-39, cel-miR-54, and cel-miR-238, so called spiked-in-controls) that were added into the isolation process (after addition of a denaturing solution) and this approach (or its modification) was applied by others for normalization to avoid the problem with inconsistent level of endogenous controls. Mitchel et al. (2008) and Kroh et al. (2010) used a combination of several synthetic miRNAs, which is a more reliable approach to normalization than using only one spike-in-control (e.g. Ho et al., 2010).

Absolute quantification relates the PCR signal to the input copy number using a calibration curve. The calibration curves are generated from known concentrations of DNA standard molecules, e.g., RT-PCR product, recombinant plasmid DNA, or synthetic oligonucleotide, etc. Results of absolute quantification are expressed as a defined unit of interest, e.g., copies per ng of total RNA or copies per µl of serum/plasma. There are many comments on the units because miRNA concentration may vary both between individuals and between patients and controls. Therefore, data conversion into copies per µl of serum/plasma seems to be more representative and better reflects the differences associated with a disease state.

Digital PCR (dPCR) technology is a novel alternative for absolute quantification of miRNAs circulating in human biofluids. Compared to the qRT-PCR method, dPCR provides the advantages of absolute quantification without a standard curve and higher robustness to variations in PCR efficiency across different samples and assays. Droplet dPCR (ddPCR) is based on partitioning the reaction mixture into thousands of oil-dispersed, nanoliter-sized microdroplets, and the PCR reaction is carried out in each partition individually. After the PCR, droplets from each sample are analysed individually in a droplet reader. PCR-positive and PCR-negative droplets are counted and converted to absolute counts of copies of the measured target. Recently, ddPCR has also been applied to quantification of miRNAs circulating in the blood. Hindson et al. (2013) found that to quantify circulating miRNAs, ddPCR was superior to qRT-PCR carried out with TaqMan miRNA assays. The study of Miotto et al. (2014) demonstrated that two commercial miRNA assays based on different detection

chemistries (TaqMan assay from Life Technologies and miRCURY LNA assay from Exiqon) could be successfully used to quantify specific miRNAs by ddPCR in human fluids and provided comparable results using the QX200 ddPCR system from Bio-Rad.

For expression profiling of circulating miRNAs, TaqMan Low density array (TLDA, Life Technologies) based on qRT-PCR is probably the most popular platform (e.g. Cuk et al., 2013; Zearo et al., 2014; Ge et al., 2015). To date, there are several available versions of TLDAs with different numbers of tested miRNAs (from 377 to 754 miRNAs). Moreover, technique developments have allowed sample processing in 384-well formats that liken this method to cost-effective high-throughput applications (Schmittgen et al., 2008; Benes and Castoldi, 2010). The TLDA technique is a fast method that takes as little as several hours. Reverse transcription is performed using megaplex pools of RT primers (up to 380 stem-looped RT primers per pool). The main advantage of qRT-PCR-based platforms is their high sensitivity. These platforms require a low input of RNA that ranges from 1–500 ng and the dynamic range of detection (difference between the minimum and maximum miRNA concentrations that can be measured by the detection system) is six (or more) orders of magnitude. Moreover, pre-amplification of cDNA can be done in the case of low amount of starting RNA (http://www.lifetechnologies.com). Although the pre-amplification step significantly reduces the amount of input RNA, its application has the potential to introduce bias due to nonlinear amplification of particular miRNAs from the starting cDNA.

Microarrays, profiling platforms based on hybridization, have the advantages of being high-throughput and cost-effective, relatively quick, and simple to use. Compared to qRT-PCR, microarrays are less sensitive and have a narrower dynamic detection range resulting in saturation of signal intensities of highly expressed miRNAs or no detection of rare transcripts. Further, microarrays may suffer from background and cross-hybridization problems that make it difficult to distinguish between mature and immature miRNA forms. Generally, RNA input ranges from 100 ng to 1 μg. With regard to the lower concentration of cell-free miRNAs in body fluids, the requirement for higher RNA input is the major limitation of microarray application for profiling of circulating miRNAs. Major vendors of microarrays for miRNA detection are Agilent (http://agilent.com) and Affymetrix (http://www.affymetrix.com). Agilent microarrays have a glass slide format and nowadays, they enable analysis of up to 2,549 human miRNAs (Human miRNA Microarray Slide, Release 21.0) with the dynamic range of over 5 logs, ensuring detection of low-abundance miRNAs. The Agilent protocol starts with only 100 ng of total RNA.

Recently, Agilent microarrays have been successfully used for comprehensive analyses of circulating miRNAs in various diseases (e.g. Steudemann et al., 2013; Sun et al., 2013b). Also in our hands, Agilent microarrays were satisfactory for miRNA profiling in the plasma of myelodysplastic syndromes. As an input, we used 300 ng of plasma RNA isolated by the phenol-chloroform method, which resulted in the detection of 207 miRNAs (unpublished data). Affymetrix offers miRNA arrays in three formats; single arrays (cartridges), array strips, and array plates. The arrays (the latest version 4.1) contain 30,424 probe sets for all mature miRNA sequences in the latest miRBase Release 20. The probes for human, mouse, and rat miRNAs are incorporated in the same array and the software allows filtering data by selecting either the human, mouse, or rat analysis options. The Affymetrix array protocol requires as little as 130 ng total RNA and the dynamic range of detection is 4 logs. Many studies have demonstrated Affymetrix arrays as a powerful tool for studying the role of circulating miRNAs in a broad spectrum of developmental and physiological processes (e.g. Duttagupta et al., 2011; Godfrey et al., 2013; Blenkiron et al., 2014).

After introduction of the next-generation sequencing (NGS) technology, deep sequencing of miRNAs (miRNA-seq) was developed to identify novel miRNAs or other small RNA species and assess their expression (Creighton et al., 2009; Wang et al., 2009). Deep sequencing measures the absolute abundance (over a wider dynamic range than is possible with microarrays) and allows overcoming the limitations of array-based analysis, which is restricted to miRNA molecules provided by databases. Compared to qPCR and microarrays, miRNA-seq is more expensive and time-consuming, presents substantial technical demands and generally requires a larger amount of input RNA (500 ng to 5 μg). However, adapted protocols have been recently proposed, giving the opportunity to obtain miRNA-seq data from as little as 5 ng of RNA extracted from blood fluid samples (Williams et al., 2013). Solexa sequencing technology by Illumina has become the dominant NGS technology in deep sequencing of circulating miRNAs (e.g. Wang et al., 2013; Zhi et al., 2013). This technology employs solid-phase bridge amplification to clonally amplify the fragments that are then sequenced by sequencing-by-synthesis (SBS) chemistry. Illumina offers a broad portfolio of NGS platforms from the desktop-sized MiSeq to the large HiSeq X Ten system. A number of other next-generation sequencing technologies are currently in use, such as 454 (Roche) and SOLiD (Life Technologies). Roche 454 Genome Sequencer Systems are based on pyrosequencing. This technology uses emulsion PCR to clonally amplify the fragments that are then sequenced via SBS. The SOLiD sequencing platform also employs emulsion PCR, but sequencing is performed based on ligation. As NGS generates a huge amount of data, there are available public tools for miRNA discovery from deep sequencing, e.g. miRDeep, miRanalyzer, and SSCprofiler (Friedländer et al., 2008; Hackenberg et al., 2009; Oulas et al., 2009; Gomes et al., 2013).

Digital gene expression (DGE) profiling is increasingly popular for miRNA analyses. The method of DGE combines the serial analysis of gene expression (SAGE)

principle with the robust parallel sequencing technology and allows for the discovery of novel miRNAs along with quantitative expression analysis. The DGE workflow includes generation of a specific tag for each transcript through efficient enzyme digestion. Then millions of tags are sequenced using the NGS technology. Compared to RNA-Seq libraries, DGE libraries are less complex because each transcript contains only a single tag and provides useful results with fewer reads per sample. The DGE technology generates such extensive sequencing depth-of-coverage that single-copy resolution of gene expression quantification is possible. The SuperSAGE method improves the original DGE methods through generation of larger tags, allowing for more precise alignment to the transcriptome (Matsumura et al., 2010). For example, Life Technologies offers SAGE kits on its SOLiD platform that generates a longer 27-base pair tag and has a dynamic range of 5 to 6 logs. Cap analysis gene expression (CAGE) is a modified method of tag profiling and is useful for determining the precise 5′ ends of the transcripts (de Hoon and Hayashizaki, 2008).

The choice of the most suitable platform for circulating miRNA profiling depends on the purpose and conditions of the project. If a low amount of RNA input is available, then qRT-PCR-based TLDA is probably the best technology. If you search for novel miRNAs or different isoforms and the RNA amount is not a limiting issue, then NGS technology should be applied. If you want to perform an initial screening of differently expressed miRNAs in a large set of samples, then microarrays should provide you with sufficient data at low cost. Many studies focused on comparison of various technologies/platforms for miRNA profiling and evaluated them from various aspects – sensitivity, specificity, RNA input, time of processing, technical requirements, costs, etc. (e.g. Baker, 2010; Git et al., 2010; Wang et al., 2011) (Table 4). Further, they correlated data obtained from various platforms and notably showed better data correlation between different technologies (qRT-PCR, microarrays, NGS) rather than between platforms based on the same technology (microarrays) (Pradervand et al., 2010; Callari et al., 2012).

The information on miRNA expression profiles is gradually growing, especially with generation of huge amounts of data from the novel high-throughput platforms. Various miRNA databases and specific tools have been introduced: general purpose and sequence databases (e.g. MiRBase, http://www.mirbase.org/), databases of miRNA targets and functions that use various prediction tools (e.g. MiRWalk, http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html), and miRNA expression databases that include data about miRNA involvement in biological and pathological processes (e.g. PhenomiR, http://mips.helmholtz-muenchen.de/phenomir/index.gsp). However, the first database that provides information about all kinds of extracellular miRNAs is miRandola database (Russo et al., 2012) (http://atlas.dmi.unict.it/mirandola/index.html). miRandola provides comprehensive information on extracellular/circulating miRNAs that are classified into four categories based on their extracellular form: miRNA-Ago2, miRNA-exosome, miRNA-HDL and miRNA-circulating (the last is used when authors have not determined the form). The database provides users with various kinds of information including associated diseases, samples, methods used for isolation of miRNAs and description of the experiment. Information about miRNA targets and their annotations are provided through links to miRò (http://ferrolab.dmi.unict.it/miro), which integrates data from different sources to allow identification of associations between genes, processes, functions and diseases at the miRNA level through their predicted and validated targets. Another database that provides information about extracellular miRNAs is ExcellmiRDB (Barupal et al., 2015) (http://www.excellmirdb.brfjaisalmer.com).

Conclusion and future directions

Although circulating miRNAs have been discovered only recently, it is already obvious that they have a real potential to become important diagnostic tools. Before that, however, a number of technological issues must be resolved. Standardized protocols for the isolation, quan-

Table 4. Comparison of the most used technologies and platforms for miRNA profiling

Technology	qPCR arrays	Microarrays	Next-generation sequencing
Vendors of the used platforms	Life Technologies Exiqon SA Biosciences/Qiagen Fluidigm	Agilent Affymetrix Exiqon Ambion Combimatrix Invitrogen	Illumina (Solexa) Roche (454) Life Technologies (SOLiD)
Technical demands	low	moderate	substantial
Time per assay	~ 1 day	~ 2–3 days	~ 1–2 weeks
RNA input	1–500 ng	100 ng – 1 μg	500 ng – 5 μg
Dynamic range	6 logs	3–5 logs	5 logs or more
Cost per sample	400–600 USD	200–300 USD	600–1,000 USD
Highlights	fast and sensitive	large-scale screening and low cost	novel miRNAs and isoforms

tification, and computation as well as high sensitivity and robustness of the methods are essentially needed before they can be fully introduced into clinical practice.

A fundamental, yet fully unanswered question that arises from the extensive research of circulating miRNAs is to what extent miRNA trafficking influences the behaviour of distant cells. It is still unknown whether circulating miRNAs are universal players in intercellular communication or whether they are only involved in refining a limited number of specialized processes. Nevertheless, it is already clear that the recent discovery of circulating miRNAs with their fascinating functions shows us that the world of molecules still definitely conceals many unexpected phenomena waiting to be discovered.

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